

Neuropsychological and neuroimaging findings in traumatic brain injury and post-traumatic stress disorder

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Advances in imaging technology, coupled with military personnel returning home from Iraq and Afghanistan with traumatic brain injury (TBI) and/or post-traumatic stress disorder (PTSD), have increased interest in the neuropsychology and neurobiology of these two conditions. There has been a particular focus on differential diagnosis. This paper provides an overview of findings regarding the neuropsychological and neurobiological underpinnings of TBI and/or PTSD. A specific focus is on assessment using neuropsychological measures and imaging techniques. Challenges associated with the assessment of individuals with one or both conditions are also discussed. Although use of neuropsychological and neuroimaging test results may assist with diagnosis and treatment planning, further work is needed to identify objective biomarkers for each condition. Such advances would be expected to facilitate differential diagnosis and implementation of best treatment practices.

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The goal of this publication is to briefly summarize neuropsychological and neuroimaging findings among adults with traumatic brain injury (TBI) and/or post-traumatic stress disorder (PTSD), and highlight current thinking in the field. Tables have been used to consolidate evidence. The existing data is vast, and complete discussion is outside the purview of this paper. Readers are encouraged to review publications noted for further discussion of specific areas of interest.

Traumatic brain injury (TBI)

Diagnostically, to have suffered a TBI one must have experienced an event (eg, motor vehicle accident, fall) which resulted in a structural injury to the brain or a physiological disruption of brain function (eg, alteration of consciousness [AOC], loss of consciousness [LOC]). TBI severity is classified according to the extent of injury to the brain or altered consciousness post-injury, not to the severity of sequelae reported or observed. See *Table 1* for further information regarding classification of TBI severity. Secondary to a cascade of cellular and molecular events, primary neurological injury associated with a traumatic event can also cause progressive tissue atrophy and related neurological dysfunction. Ultimately, such processes can result in neuronal cell death (secondary brain damage).¹ Cellular mechanisms that modulate pathophysiological and neuroprotective processes appear to contribute to the nature and extent of damage post-injury.² Diffuse axonal injury (DAI), preferential multifo-

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Selected abbreviations and acronyms

ACC	<i>anterior cingulate cortex</i>
AOC	<i>alteration of consciousness</i>
LOC	<i>loss of consciousness</i>
MRI	<i>magnetic resonance imaging</i>
OEF	<i>Operation Enduring Freedom</i>
OIF	<i>Operation Iraqi Freedom</i>
PCS	<i>postconcussive symptoms</i>
PTS	<i>post-traumatic symptoms</i>
PTSD	<i>post-traumatic stress disorder</i>
TBI	<i>traumatic brain injury</i>

cal involvement of myelinated tracks, often occurs and can be related to the primary injury or secondary brain damage. As the severity of the injury increases, so do findings noted on imaging and neuropsychological measures.³ According to the Centers for Disease Control and Prevention, approximately 1.7 million people per year in the United States sustain a TBI.⁴ Most injuries incurred by civilians and military personnel are mild in nature.^{4,5} That is, the associated AOC immediately following the injury is limited (eg, LOC less than 30 minutes). Individuals serving in Iraq and Afghanistan are sustaining TBIs secondary to blast exposure.⁵ Reported estimates of TBI vary between 8% and 23%.^{5,6} Blast exposure can result in TBI via multiple mechanisms including: (i) primary blast—injury caused by the overpressurization wave; (ii) secondary blast—injury secondary to object being thrown by the blast towards the person; and (iii) tertiary blast—when individuals are thrown and strike objects. Additionally, some explosions are accompanied by electromagnetic perturbations which result in “small” and “brief” radiofrequency impulses.⁷ The physiological implications of these impulses is unclear.⁷ In terms of the overpressurized wave and brain injury, the primary means by which blast energy transduction occurs

remains a topic of debate. Potential hypotheses include: (i) transcranial propagation; (ii) the vascular system; and (iii) cerebrospinal fluid entering the foramen magnum.⁷ Injuries noted post-blast exposure include edema, contusion, DAI, hematoma, and hemorrhage.^{7,8} Clear evidence exists regarding the relationship between injury severity, impairment (eg, cognitive) and functional status.^{9,10} In comparing postinjury neuropsychological test performance among individuals with moderate and severe TBI, Bercaw et al¹¹ identified a pattern of performance which suggested that scores at 1 year post-rehabilitation predicted functional outcomes at year 2. Whereas most individuals with a mild TBI return to baseline functioning within one year, between 7% and 33% report persistent symptoms.¹² Regardless of injury severity, one of the most frequently reported symptoms post-TBI is cognitive dysfunction (eg, memory problems).^{9,10} Particularly among those with mild TBI and persistent post-acute symptoms, there is often a disconnect between subjective (eg, self-report) and objective markers (eg, neuropsychological test performance) of such dysfunction. Nevertheless, among those with mild to severe TBI, observed cognitive disturbances have been linked to poorer psychosocial functioning (eg, return to work).¹³

Post-traumatic stress disorder (PTSD)

Postdeployment, military personnel are also reporting post-traumatic symptoms.¹⁴ To meet criteria for PTSD an individual must be exposed to a psychologically traumatic event which facilitates the onset of persistent symptoms. These symptoms must also cause significant distress or impact functioning. The *Diagnostic and Statistical Manual of Mental Disorders, Fourth edition (DSM-IV)*¹⁵ defines a “traumatic event” as one in which “(i) the per-

Criteria	TBI severity		
	Mild	Moderate	Severe
Structural imaging	Normal	Normal or abnormal	Normal or abnormal
Loss of consciousness	0-30 minutes	> 30 minutes and < 24 hours	> 24 hours
Alteration of consciousness/mental state**	A moment up to 24 hours	> 24 hours; severity based on other criteria	
Post-traumatic amnesia	0-1 day	>1 day and < 7 days	> 7 days
Glasgow Coma Score (best available score in first 24 hours)	13 to 15	9 to 12	<9

Table 1. Departments of Defense and Veterans Affairs consensus-based classification of closed traumatic brain injury (TBI) severity.⁶¹ **Alteration of mental status must be immediately related to the trauma to the head. Typical symptoms would be looking and feeling dazed and uncertain of what is happening, confusion, difficulty thinking clearly or responding appropriately to mental status questions, and being unable to describe events immediately before or after the trauma event.

son experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury or a threat to the physical integrity of self or others; and (ii) the person's response involved intense fear, helplessness, or horror” (p 467). PTSD symptoms are clustered into three categories including re-experiencing of the traumatic event, avoidance of trauma-related stimuli and/or emotional numbing, and hyperarousal. PTSD has been conceptualized as a disorder of fear in which the individual has exaggerated fear responses or the inability to control fear responses.¹⁶ It has also been described as a disorder of memory, in which individuals suffering from PTSD seem to “relive their trauma in the form of involuntary recollection,” (p 271).¹⁷ In addition to demonstrating enhanced recall for traumatic memories, distressing recollections for those with PTSD are often “vivid” and “long-lasting.”¹⁸

It is in part these “reliving” experiences that take the form of nightmares, intrusive thoughts, and/or flashbacks, coupled with observed cognitive disturbances that have fostered interest regarding the neurobiological and neuropsychological underpinning of this condition. Despite knowledge that genetic variability, gender, and developmental history appear to impact neurobiological systems and responses to traumatic stimuli,¹⁹ PTSD symptoms are believed to be related to an individual’s dysregulated biological response to stress.²⁰ *Table II* shows brain regions and neurochemical dysfunction often discussed in association with PTSD symptoms. During traumatically stressful situations, neurotransmitter systems and neuroendocrine axes are activated.²⁰ According to Langeland and Olff²⁰ research has primarily focused the hypothalamus-pituitary-adrenal (HPA) axis. The sympathetic-adrenomedullary (SAM) system

Hallmark PTSD symptom	(Over)activation	(Under)activation
Re-experiencing		
Brain region	Amygdala	Prefrontal cortex Anterior cingulate cortex Inferior frontal cortex
Neurochemical	Insula Cortisol Glutamate Norepinephrine	
Hyperarousal		
Brain region	Amygdala Thalamus	Prefrontal cortex
Neurochemical	Cortisol Dopamine Epinephrine Norepinephrine	Serotonin
Avoidance/Numbing/Dissociation		
Brain region	Prefrontal cortex Superior temporal cortex	Hippocampus Insula Prefrontal cortex Anterior cingulate cortex Superior temporal cortex Inferior frontal cortex
Neurochemical	Beta-endorphins Cortisol Dopamine Glutamate	

Table II. Brain regions and neurochemical dysfunction often discussed in association with post-traumatic stress disorder (PTSD) symptoms. Adapted from information presented in ref 66: Hopper JW, Frewen PA, van der Kolk BA, et al. Neural correlates of reexperiencing, avoidance, and dissociation in PTSD: Symptom dimensions and emotion dysregulation in responses to script-driven trauma injury. *J Trauma Stress.* 2007;20:713-725; Copyright © Wiley, 2007; ref 67: Weiss SJ. Neurobiological alterations associated with traumatic stress. *Perspect Psychiatric Care.* 2007; 43:114-122. Copyright © Wiley, 2007

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has also been implicated in that it releases epinephrine which facilitates the flight/fight response.²¹ On the contrary, the contribution of the HPA axis, glucocorticoids, take time to produce. As such their impact, which is primarily on the brain, develops and continues over a longer period.²¹ The SAM and HPA systems are regulated by "limbic brain circuits that involve the amygdala, hippocampus and orbital/medial prefrontal cortex" (p 150).²¹ Neurobiological activation is thought to impact brain functioning and hypothesized to alter the structure of brain regions including the amygdala, hippocampus, locus coeruleus, dorsal raphe nucleus, and prefrontal cortex.^{22,23} Although activation of these systems supports functioning, chronic activation seems to be problematic in terms of psychological and physical health.

At the same time, it has been suggested that neurobiological findings (eg, reduced hippocampal volumes) are instead premorbid characteristics that contribute to the development of PTSD.²⁴ For example, van Zuiden²⁵ and colleagues found that predeployment glucocorticoid receptor numbers were elevated in soldiers reporting higher PTSD symptoms postdeployment; thereby, highlighting the question of whether such biological differences are pre-existing characteristics, the result of the

PTSD, or a combination of the two. Much the same discussion has been had in terms of cognitive dysfunction often noted in those with PTSD.²² A specific focus has been on whether lower intellectual functioning is a precursor of PTSD or a sequela of the condition.^{26,27}

TBI and PTSD co-occurring

Historically, some controversy has existed regarding whether PTSD and TBI can coexist; however, more recent work in this area suggests that they can. If the injury and psychiatrically traumatic event are co-occurring, those with a less severe AOC seem to be at greater risk for developing PTSD. As noted above, complaints are frequently shared between those with TBI and/or PTSD (eg, poor attention); thereby complicating differential diagnosis. This particularly true for those with mild TBI, and/or repeated exposure to trauma (physical, psychological). For example, work by Brenner et al,²⁸ suggested that in retuning soldiers with histories of physical injury, mild TBI and PTSD were independently associated with self-reported memory problems. Moreover, a combination of the conditions was found to be more strongly associated with memory problems than either

Brain Region	Function	PTSD and/or TBI
Amygdala	Generation and maintenance of emotional responses ⁵⁶	PTSD ⁴² ; TBI ¹
Cerebellum	Movement and motor coordination; processing fear memories ⁴⁹	PTSD ⁴⁸ ; Chronic mild TBI ⁵¹
Corona radiata	Attentional processes ⁵⁶	Chronic mild TBI ⁵⁵
Corpus collosum	Intrahemispheric communication ⁴⁸	Acute and chronic mTBI ³⁷ ; Moderate to severe TBI ⁵⁰ ; TBI ¹
Hippocampus	Explicit and declarative memory, working memory, episodic/autobiographical memory, contextual learning ^{37,50} ; control of stress responses and contextual aspects of fear conditioning ¹⁹	PTSD ⁴⁸ ; TBI ¹
Insula	Core affect, associated consciousness of subjective feelings, developing and updating motivational states, autobiographical memory, cognitive control, affective processing, pain, and conveyance of homeostatic information ³⁰	PTSD ⁴²
Internal capsule	Motor and sensory communication	Acute and chronic mTBI ³⁷
Medial temporal lobe	Declarative memory	Chronic mild TBI ⁵¹ ; TBI ¹
Parietal cortex	Volitional and avolitional allocation of attentional resources during the retrieval of episodic memories ⁶²	PTSD ⁶²
Prefrontal cortex	Manipulation of emotions and memories ⁶² ; extinguishing conditioned fear ³² ; inhibitory action on the amygdala ¹⁶	PTSD ^{32,42,47,51} ; TBI ^{1,69}
Anterior cingulate cortex	Processing of cognitive and emotional interactions ⁵⁰ including interference from emotional stimuli and performance monitoring, response selection, error detection, and decision making ⁶⁴ ; conflict monitoring, attention and pain ⁵⁰	PTSD ^{32,41,48}
Uncinate fasciculus	Working memory ³⁷	Chronic mild TBI ⁵⁵

Table III. Brain regions and functions often discussed in relationship to post-traumatic stress disorder (PTSD) and/or traumatic brain injury (TBI). **Acute mild, moderate, and severe

condition alone. In looking at post-traumatic symptoms (PTS) and postconcussive symptoms (PCS) (eg, slowed thinking, poor concentration) among returned Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) Veterans, Benge and colleagues²⁹ found that PTS and PTC were not independent variables, thereby suggesting that incorrect attribution of PCS to history of TBI may preclude referral to appropriate treatment.

Challenges associated with symptom attribution are at least in part related to the fact that common areas of the brain are implicated in both conditions (*Table III* shows brain regions and functions often discussed in relationship to PTSD and/or TBI). Whereas neuroimaging and neuropsychological findings have contributed to the understanding of each of these conditions, and are frequently employed in clinical practice, guidance regarding how to best use these diagnostics tools to inform practice with these populations is limited. Moreover, contextual and/or person-specific factors such as deployment to a combat zone, effort (eg, fatigue, distraction secondary to psychiatric condition) and potential secondary gains (eg, monetary compensation related to legal proceedings) impact performance on diagnostic tools in ways that further complicate interpretation. For example, among returning OIF Soldiers, Vasterling and colleagues⁶ found increased reaction time, poor concentration, and short-term memory problems. Similarly, higher levels of combat intensity have been shown to be related to more efficient reaction time even 1 year post-deployment.³⁰

Further complicating interpretation, for individuals with TBI and/or PTSD deficits in primary areas of cognitive functioning (eg, attention, processing speed) may undermine more complex processes (eg, executive functioning). For example, Nelson and colleagues³¹ found that among OEF/OIF Veterans with TBI, processing speed contributed significantly to performance on measures of executive functioning. Challenges also exist in terms of using experimental findings to guide clinical practice. Research studies frequently discuss significant differences in test scores among those with and without PTSD; however, lower scores do not equal impairment (a score that is two standard deviations below the mean of the general population). McNally³² highlights this point by suggesting that above-average intelligence be considered a protective factor against PTSD versus lower IQ being a risk factor for developing the disorder. A clinician evaluating an individual's performance on objective mea-

asures of functioning must note whether scores are actually impaired, or simply below personal expectations or previous levels of functioning. Making this determination can be particularly difficult if the premorbid data available for review is limited and/or anecdotal in nature.

Cognitive functioning

Cognitive deficits associated with TBI, particularly mild TBI, generally diminish over time. Alternately, PTSD has been associated with enduring cognitive disturbances. Although the etiology of deficits differs between individuals with each of these conditions, significant areas of overlap exist both in terms of subjective complaints and objective findings (eg, attention). Below, the reader will be provided with summarized information regarding neuropsychological findings, clinical and experimental, among those with TBI (mild/moderate and severe) and PTSD. To augment this material readers are encouraged to review *Table IV*, the neuropsychological findings often discussed among those with TBI or PTSD.

TBI (mild)

Although there appears to be general consensus regarding the presence of acute cognitive dysfunction in those with mild TBI,^{33,34} findings regarding the overall effect of mild TBI on long-term neuropsychological test performance have been mixed. Frencham and colleagues³⁵ published a meta-analysis of neuropsychological studies post-mild TBI and found that measures of processing speed, working memory, attention, memory, and executive functioning were most impacted immediately postinjury.³⁵ Overall, their findings indicated that the effect of mild TBI on neuropsychological test performance was small, and that problems decreased as time since injury increased.³⁶ This assertion is supported by a recent study by Brenner and colleagues,²⁸ in which 45 soldiers post-mild TBI completed neuropsychological measures. Twenty-seven had enduring PCS, including cognitive complaints, and 18 did not. Mean time since injury was approximately 41 weeks. Presence of mild TBI symptoms did not impact test performance, and mean participant scores were overwhelmingly unimpaired. Alternately, it may be that neuropsychological measures frequently used in practice are not sophisticated enough to identify subtle postinjury impairments. Imaging studies may increase our understanding regard-

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ing neuropsychological test performance in those with mild TBI. For example, Van Boven and colleagues³⁷ suggested that those with mild TBI may require larger areas of cortex to complete tasks. In addition, the impact of injury on performance may grow as lifetime injury burden increases. This assertion is supported by the work of Belanger and colleagues³⁸ who found that a history of multiple self-reported TBI was associated with poorer performance on tests of delayed memory and executive functioning.

TBI (moderate and severe)

Widespread and enduring cognitive deficits are often noted in those with moderate to severe TBI. Senthani-Raja and colleagues¹⁰ compared the neuropsychological test performance of 112 individuals with complicated mild to severe injuries with matched controls and identified deficits in attention, processing speed, visual and verbal memory, executive functioning, and working

memory. These significantly worse scores were noted long postinjury. The performance of older individuals and long-term survivors was worse. Among a cohort that had been referred for rehabilitation, Draper and Ponsford³⁹ evaluated neuropsychological performance 10 years post-injury and found persisting deficits in processing speed, learning, and executive functioning. Level of impairment was associated with injury severity. Finally, Mathias and Wheaton⁴⁰ conducted a meta-analytic review regarding attention and information processing speed deficits post-severe TBI. Findings suggested large and significant deficits in the areas of information processing speed, attention span, focused/selective attention, sustained attention, and supervisory attentional control. In reviewing the literature on functioning post-severe TBI, Van Boven and colleagues³⁷ suggested that deficits such as those noted above may be related to difficulty adequately recruiting the cortical resources necessary to complete complex cognitive tasks.

Cognitive domain	Traumatic brain injury		Moderate to severe Publication	Post-traumatic stress disorder Publication
	Mild Acute/chronic	Publication		
Attention	Acute/chronic	Frencham et al ³⁵ ; Peskind et al ⁵⁰	Mathias and Wheaton ³⁹ ; Senathi-Raja et al ¹⁰	Aupperle et al ⁴² ; Golier et al ¹¹ ; Samuelson et al ⁴⁵
Sustained attention	Chronic	Kraus et al ³	Mathias and Wheaton ³⁹	Vasterling et al ²⁷ ; Vasterling et al ²⁷
Emotional processing				Halligan et al ¹⁷ ; Milad et al ⁴⁸ ; McNally ¹⁷ ; McNally ³²
Executive dysfunction	Acute/chronic	Frencham et al ³⁵ ; Peskind et al ⁵⁰	Mathias and Wheaton ³⁹ ; Draper and Ponsford ³⁹ ; Senathi-Raja et al ¹⁰	Aupperle et al ⁴² ; Vasterling et al ²⁷
Working memory	Acute/chronic	Frencham et al ³⁵ ; Peskind et al ⁵⁰	Senathi-Raja et al ¹⁰	Aupperle et al ⁴² ; Moores et al ⁴⁶ ; McNally ³² ; Samuelson et al ⁴⁵ ; Vasterling et al ²⁷
Intelligence				Gilbertson et al ²⁶ ; Vasterling et al ²⁷
Language and communication			Levin and Chapman ⁷³	McNally ³²
Learning	Acute	Frencham et al ³⁵	Draper and Ponsford ³⁹ ; Vanderploeg et al ⁷⁴	Samuelson et al ⁴⁵ ; Vasterling et al ²⁷ ; Vasterling et al ²⁷
Processing speed	Acute/chronic	Frencham et al ³⁵ ; Niogi et al ⁵⁶ ; Peskind et al ³⁹	Draper and Ponsford ³⁹ ; Mathias and Wheaton ²⁹ ; Senathi-Raja et al ¹⁰ ; Willmott et al ⁷⁰	Nelson et al ³¹ ; Samuelson et al ⁴⁵
Verbal memory	Acute/chronic	Frencham et al ³⁵	Senathi-Raja et al ¹⁰ ; Lezak et al ⁶⁸	Golier; McNally ³² ; van Pragg
Visual memory	Acute	Frencham et al ³⁵	Senathi-Raja et al ¹⁰	Marx et al ⁴¹

Table IV. Neuropsychological findings often discussed among those with traumatic brain injury or post-traumatic stress disorder.

PTSD

In studying Vietnam combat veterans and their nonexposed identical twin brothers, Gilbertson and colleagues²⁶ found that performance on cognitive tasks (ie, intellectual, verbal memory, attention, executive functioning, and visuospatial skills) was more strongly associated with familial factors than PTSD. Patterns of vulnerability in terms of verbal memory and executive functioning were identified among both exposed and unexposed members of the twin pairs. Further study regarding learning, processing speed, intelligence, and visual recall have supported the theory that pretrauma performance on neuropsychological measures is related to PTSD symptom development.^{41,42} In a recent publication, Aupperle and colleagues⁴² summarized investigations regarding executive function and PTSD, and identified subtle impairments in response inhibition and attention regulation among those with PTSD. The authors described these areas of impairment as potentially pre-dating PTSD, thereby acting as risk factors for the disorder. At the same time, they noted that impairments may be exacerbated by trauma exposure.⁴² This is supported by the work of Vasterling and colleagues²⁷ which suggested that neurocognitive and intellectual performance deficits are independently associated with PTSD. Pretrauma deficits may exacerbate responses to trauma exposure thereby causing subtle impairments “to morph into significant symptoms” which are identifiable on neuropsychological measures and impact day-to-day functioning.⁴¹ Although patterns of cognitive deficits have varied between cohorts with PTSD^{27,43} difficulties in the areas of attention, learning, and memory, particularly verbal, have consistently been identified.^{27,41,44,45} The impact of stress on neuropsychological functioning may in part be time-dependent. For example, in comparing performance on measures of sustained attention between Gulf War and Vietnam Veterans, Vasterling and colleagues²⁷ hypothesized that PTSD-related arousal dysregulation may change over time from a pattern of hyperarousal to disordered arousal. Moreover, recent work suggests that although absolute performance among those with PTSD may be normal, use of neuroimaging techniques allows for the exploration of systems and compensatory recruitment. This is evidenced by the work of Moores and colleagues⁴⁶ who found that individuals with PTSD must recruit larger areas of cortex to complete working memory tasks.

An additional focus has been on whether those with PTSD encode, process, experience, and/or express trauma-related information differently than individuals without this disorder. McNally¹⁷ noted that those with PTSD selectively process trauma-relevant material. Emotional Stroop tasks in which individuals are asked to respond to emotionally loaded content are frequently used to assess such processing. Studies using the Stroop have consistently shown that those with PTSD take longer to name trauma-laden content. Halligan et al⁴⁷ conducted a study regarding assault victims and found that trauma memories were more disorganized in those with PTSD symptoms, and that the magnitude of disorganization predicted PTSD symptom severity. In addition, it has been demonstrated that those with war-related PTSD fail to retain extinction from learned fear.⁴⁸ This deficit was not identified in subject’s co-twins; thereby suggesting that it is acquired and related to PTSD versus a pre-existing vulnerability. Finally, Banich et al¹⁸ discussed how attentional biases for threat in those with PTSD may be moderated by an individual’s tendency to dissociate. Dissociation appears to impact aspects of attention and cognitive control. Alterations in these cognitive control mechanisms can influence memories retrieved.

Neuroimaging

To improve diagnosis and treatment of TBI and/or PTSD, identification of objective biomarkers is of significant clinical import. As evidenced by current advances, neuroimaging in certainly is a key tool in this process. *Table V* shows magnetic resonance imaging (MRI) neuroimaging techniques. Nevertheless, significant challenges exist in terms of summarizing existing findings and translating data to improve clinical practice. Studies often involve diverse cohorts (eg, mild TBI, combat veterans), and employ different paradigms (symptom provocation, cognitive activation) and modalities (eg, diffusion tensor imaging [DTI], functional magnetic resonance imaging [fMRI], single photon emission computed tomography [SPECT]).⁵⁰ As such, findings have varied. Peskind and colleagues noted that fluorodeoxyglucose positron emission tomography (FDG-PET) abnormalities in those with PTSD versus those without this disorder have been “limited and conflicting” (p 5).⁵¹ In terms of validation, experiments supporting newer functional imaging techniques often rely on neuropsychological paradigms. For example, in response to

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findings regarding the positive relationship between DTI results and neuropsychological test performance among those with mild, moderate, and severe injuries, Kraus et al³ suggested that white matter load may be a “useful index.” Much work is being conducted to support these new imaging techniques, and findings are increasing our knowledge regarding those with TBI and/or PTSD.

TBI

Although newer techniques have begun to allow clinicians to explore questions regarding pathogenesis, natural history, neuroplasticity, and treatment response,⁵² historically, neuroimaging has been used to identify and manage acute moderate-to-severe TBI. Less sophisticated structural imaging techniques such as computed tomography (CT) or MRI have been useful in identifying skull fractures or more severe injuries (eg contusion, intraparenchymal hemorrhage); however, they generally fail to adequately detect DAI or brain volume loss. Moreover, in combat or deployment settings these generally common diagnostic tools may not be available to the clinician.⁵³ Research among both Veteran and civilian populations suggests that use of CT and MRI has limited utility in confirming acute or post-acute mild TBI.⁵⁴⁻⁵⁶ In looking at MRI results of veterans long post-TBI, Brenner and colleagues⁵⁵ found that those with moderate to severe TBI were significantly more likely

to have trauma-related findings (physical) than those with mild TBI. In specific, 11 out of 16 veterans with moderate to severe TBI versus 0 out of 16 with mild TBI had MRI findings.

Research regarding newer functional imaging techniques (eg, FDG-PET, DTI, SPECT) suggests that in the future they may be of significant clinical utility, particularly in the context of mild TBI and/or post-acute injuries. For example, DTI findings can be used to create maps of regional connectivity within the brain, otherwise known as tractography, and as such may be a useful tool for highlighting white matter damage post-injury.⁶ Recent studies using such techniques include work by Matthews et al⁵⁷ who used DTI and fMRI to examine the structural and functional neural correlates of major depressive disorder (MDD) in OEF/OIF war veterans with self-reported histories of mild TBI. Those with MDD showed greater activation in the amygdala and other emotional processing structures, lower activation in emotional control structures, and lower fractional anisotropy in several white matter tracts. Using FDG-PET and neuropsychological testing, Peskind and colleagues⁵¹ compared results from 12 OIF veterans with mild TBI and/or PTSD to community volunteers. A decreased cerebral metabolic rate of glucose in the cerebellum, vermis, pons, and medial temporal lobe as well as subtle cognitive impairments (eg, verbal fluency, cognitive processing speed) were noted in the

Technique	What it measures	Applications
BOLD fMRI	Indirect measure of blood flow, BOLD signal changes originate in venules. BOLD fMRI takes advantage of susceptibility differences between oxygenated and deoxygenated blood.	Evaluate regional brain activity related to particular cognitive tasks or sensory/motor stimulation. Evaluate brain networks related to cognitive states. Evaluate brain “resting state” or “default” networks.
PW-MRI	Direct measure of blood flow, allows quantification of blood perfusion.	Assess brain perfusion or resting cerebral blood flow. Evaluate brain function in manner similar to fMRI.
DTI	Indirectly measures diffusion of water molecules. Mean diffusion, diffusion direction, and anisotropy white matter tracts.	Use diffusion anisotropy measures as marker of disease. Improved visualization of edema. Evaluate structural “connectivity” between brain regions.
MRS	Proton (¹ H) MRI spectra typically contain signals from the metabolites N-acetylaspartate, creatine, Choline, glutamate/glutamine, and myo-inositol.	Evaluate changes in brain metabolites related to myelination, neuronal density, edema, etc.
SWI	MRI sequences that are especially sensitive to changes in magnetic susceptibility, in particular blood	Improved detection of hemorrhages. Improved imaging of blood vessels.

Table V. Magnetic resonance imaging (MRI) neuroimaging techniques. BOLD, blood oxygen level dependent; DTI, diffusion tensor imaging, fMRI, functional MRI; MRS, magnetic resonance spectroscopy; PW-MRI, perfusion weighted MRI; SWI, susceptibility-weighted imaging. Reproduced with permission from ref 37: Van Boven RW, Harrington GS, Hackney DB, et al. Advances in neuroimaging of traumatic brain injury and posttraumatic stress disorder. *J Rehabil Res Dev.* 2009;46:717-757. Copyright © Dept of Veterans’ Affairs 2009

veteran sample. Study limitations as described by the authors included the control group being 21 years older than the veteran group, and 10 out of the 12 veterans having a history of co-occurring PTSD. Readers are encouraged to review the following for more through discussions of functional imaging techniques and TBI: Belanger et al,⁵⁴ Niogi and Mukherjee,⁵⁷ Wortzel et al,⁵⁹ and Van Borgen et al.³⁶

Newer techniques such as those described above are frequently unavailable to practitioners. Moreover, based upon the current state of knowledge regarding these measures, significant controversy exists regarding whether they can appropriately be used in clinical settings.⁵⁹ In a recent Letter to the Editor, Adinoff and Devous⁶⁰ suggested that at present there is an absence of empirical evidence to support using SPECT to diagnose and treat psychiatric illnesses. This assertion is consistent with opinions expressed by Niogi and Mukherjee⁵⁷ who stated that "because of substantial overlap in the range of DTI metrics between age-, gender-, and education matched controls and mild TBI patients, diagnostic interpretation in the individual patient relying solely on DTI results remains problematic" (p 251).

PTSD

Garfield and Liberzon⁵⁰ elegantly summarize neuroimaging studies among those with PTSD, by highlighting the convergence of findings regarding the amygdala, anterior cingulate cortex (ACC), medial prefrontal cortex, insula, and hippocampus. The authors note that that findings "lend tentative support to a neurocircuitry model that emphasizes the role of dysregulation in threat-related processing" (p 379). A selection of specific structural and functional findings in support of this model are provided below.

In terms of structural imaging, findings suggest that PTSD is related to reduced hippocampal and ACC volumes.⁵⁰ Reported bilateral reductions in hippocampal volume have ranged from between 5% and 26%.⁶² Gilbertson and colleagues²⁴ suggested that hippocampal volumes may represent a pre-existing, familial vulnerability to PTSD. Equivocal evidence in support of reduced bilateral amygdala volume, and limited findings regarding the insula have also been reported.⁵⁰ Recent work by Eckart and colleagues⁶² noted reduced volume in the prefrontal and parietal regions of refugees with PTSD, and suggested that such disturbances along with

previously reported findings regarding the medial temporal region may highlight memory "disturbances" associated with PTSD.

Functional imaging studies in those with PTSD generally utilize symptom provocation or cognitive activation paradigms.⁵⁰ Symptom provocation entails the participant relating autobiographical information regarding their trauma history.⁵⁰ "Generally evocative" material may be also be used to elicit symptoms.⁵⁰ Cognitive activation paradigms are designed to assess dysfunction in "neuronal processes associated with PTSD" utilizing neuropsychological or neuroscience tasks (p 327).⁵⁰ Garfield and Liberzon⁵⁰ discuss the second strategy as being advantageous in that in that it generates a larger number of general or non-trauma-related responses without eliciting symptoms. Findings among those with PTSD demonstrated an exaggerated amygdala response, deficient prefrontal functioning, and decreased hippocampal activation.⁵⁰ The ACC and insula have been areas of focus, with repeated findings regarding reduced ACC activation among those with PTSD and emerging data regarding hyperactivation of the insula among anxious individuals.⁵⁰ Increased awareness of the interconnected nature of brain processes and the important role of receptors have further supported the use of functional imaging techniques among those with PTSD. Readers are encouraged to review the following publications for a more complete discussion of imaging and PTSD: Garfinkel and Liberzon,⁵⁰ Heim and Nemeroff,¹⁹ Van Boven et al.³⁷

Co-occurring TBI and PTSD

As demonstrated above, TBI and PTSD are each individually complex conditions whose sequelae are contingent on a wide range of individual and systemic factors. Moreover, currently knowledge regarding the two conditions when they are co-occurring is limited. Recent studies suggest that the relationship between TBI and PTSD is complicated. In addition, to the above-noted challenges associated with differential diagnosis, there is mounting evidence that a history of TBI increases risk for developing PTSD.⁶² Bryant and colleagues suggested that damage to the frontal regions of the brain may compromise neural networks which are required to regulate emotional experiences and as such predispose such patients to increased anxiety and depression.⁶² Using functional imaging techniques Matthews and col-

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leagues⁵⁷ identified differences among OEF/OIF combat veterans with mild TBI and with and without MDD. The authors noted that significantly more subjects with MDD reported LOC, and suggested that this alteration in consciousness may uniquely contribute to the development of mental health conditions post-injury by exacerbating pre-existing vulnerabilities or independently increasing the probability of developing a mental health disorder such as depression or PTSD. Work conducted regarding cognitive processing during psychological trauma, such as the development of disorganized traumatic memories and PTSD, may be of use in increasing understanding regarding the increased rate of PTSD among those with TBI.⁴⁷ That is, alterations in consciousness associated with TBI may contribute to the development of disorganized traumatic memories and a subsequent increased risk for PTSD. Co-occurrence may also exacerbate existing symptoms. For example, frank neurological insult such as a TBI may exacerbate PTSD symptoms by creating an inability to self-regulate and inhibit behavioral responses.³¹ Further study regarding the relationship between these two conditions is necessary to facilitate increased understanding and ultimately develop assessment and treatment strategies for those with co-occurring disorders.

Conclusions and implications for clinical practice

Among those with TBI and/or PTSD neuropsychological measures in the context of a comprehensive evaluation may help clarify an individual's strengths and weaknesses. However, the overlap of cognitive disruption noted by those with PTSD and/or TBI suggests that such measures are unlikely to assist in differential diagnosis. This is certainly in part related to the "the complex interplay of neurological, psychological, and physical factors in veterans with [mild] TBI" and/or PTSD, and highlights the need for "specialized evaluation" and management (p 271).²⁹ This stance is supported by best practices outlined in the Departments of Veterans Affairs and Defense updated mild TBI clinical practice guidelines.⁶⁴ The fact that brain regions of interest (eg, hippocampus) are involved in complex cognitive processes such as learning and memory, and as such require a high degree of plasticity, are capable of "life-long neurogenesis," and are vulnerable to physical and emotional insult have created significant challenges for those studying or working

with individuals who have PTSD and/or TBI.³⁷ To assist, resources are being deployed to develop biomarkers for both conditions. Identification of such laboratory biomarkers may assist in the early identification of each of these conditions, and as such facilitate timely intervention. However, until such biomarkers are identified, clinicians will be required to rely upon data (eg, clinical history, neuropsychological testing results, neuroimaging findings) which may or may not result in a definitive diagnosis.

The lack of definitive biomarkers can also place clinicians in the challenging position of determining how and when to use existing experimental data and/or employ newer imaging techniques in clinical practice. In terms of imaging, experts in the field would suggest that caution is warranted until our understanding and ability to integrate findings catches up with our ability to produce such data.⁵⁸⁻⁶⁰ As our knowledge regarding individual risk factors, neural networks, genotypes, and gene expression patterns grows, so may our ability to effectively use both neuroimaging and neuropsychological findings in clinical practice. In the aim of benefiting those with TBI and/or PTSD, experts in the field (eg, clinicians and researchers) should be encouraged to work together to identify means of translating experimental findings to clinical practice. For those with PTSD, understanding may also be enhanced by continued exploration of the neurobiology and neuropsychology of specific symptoms or symptoms clusters versus PTSD on whole.⁶⁵ This focus may also allow for more individualized treatment approaches.

While awaiting the above-described advances, clinicians should be encouraged to include measures of functioning (eg, cognitive, psychosocial) when assessing the impact of a condition. Such measures are frequently employed in the TBI community, and may be of use when evaluating those with co-occurring psychiatric conditions or PTSD. Further study regarding such measures among those with mild TBI and/or PTSD is warranted. Clinicians are also encouraged to contact family members and friends to obtain collateral information regarding their clients' everyday functioning.

In summary, the recent advances in neuroimaging, coupled with the high number of United States military personnel returning from Iraq and Afghanistan with TBI and/or PTSD, have resulted in an increased focus on the neurobiological and neuropsychological underpinning of these two conditions. As data becomes available, so

must guidance regarding how to employ new findings in clinical practice. At present, use of neuroimaging and neuropsychological/psychological test results can certainly assist with diagnosis and treatment planning, par-

ticularly for those moderate to severe TBI. Nevertheless, further work is needed to identify objective biomarkers to facilitate this process among those with one or both of these conditions. □

Hallazgos neuropsicológicos y de neuroimágenes en el daño cerebral traumático y el trastorno por estrés postraumático

El aumento del interés en la neuropsicología y la neurobiología del daño cerebral traumático (DCT) y del trastorno por estrés postraumático (TEPT) se ha facilitado por los progresos en la tecnología de las imágenes y el retorno del personal militar, desde Irak y Afganistán, con una o ambas de estas condiciones. El diagnóstico diferencial ha constituido un foco de especial interés. Este artículo entrega una panorámica de los hallazgos relacionados con las bases neuropsicológicas y neurobiológicas del DCT y/o del TEPT. Se hace mención específica a la evaluación que emplea mediciones neuropsicológicas y técnicas de imágenes. También se discuten los desafíos asociados con la evaluación de sujetos con una o ambas condiciones. Aunque el empleo de resultados de las pruebas neuropsicológicas y de las neuroimágenes pueden ayudar con el diagnóstico y la planificación del tratamiento, se requiere de trabajos adicionales para identificar biomarcadores objetivos para cada patología. Es de esperar que tales avances faciliten el diagnóstico diferencial y la implementación de las mejores prácticas terapéuticas.

Neuro-imagerie et neuropsychologie des lésions cérébrales traumatiques et du syndrome de stress post-traumatique

Les avancées en imagerie, associées au retour des militaires d'Irak et d'Afghanistan atteints de lésions cérébrales traumatiques (LCT) et/ou d'un état de stress post-traumatique (ESPT), ont entraîné un regain d'intérêt pour l'étude de la neuropsychologie et la neurobiologie de ces deux pathologies. Le diagnostic différentiel a été l'objet d'une attention particulière. Cet article propose une revue des connaissances actuelles concernant les anomalies neuropsychologiques et neurobiologiques sous-tendant les LCT et/ou les ESPT et en particulier de l'évaluation au moyen de mesures neuropsychologiques et de techniques d'imagerie. Nous analysons également les difficultés associées à l'évaluation des individus atteints d'une ou des deux pathologies. Bien que l'utilisation des résultats des tests de neuropsychologie et de neuro-imagerie puisse aider au diagnostic et à la mise en place du traitement, il faut encore travailler pour identifier des biomarqueurs objectifs de chaque pathologie. De tels progrès sont attendus pour permettre un diagnostic différentiel et la mise en œuvre de meilleurs traitements.

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Structural alterations in lateral prefrontal, parietal and posterior midline regions of men with chronic posttraumatic stress disorder

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Background: So far, the neural network associated with posttraumatic stress disorder (PTSD) has been suggested to mainly involve the amygdala, hippocampus and medial prefrontal cortex. However, increasing evidence indicates that cortical regions extending beyond this network might also be implicated in the pathophysiology of PTSD. We aimed to investigate PTSD-related structural alterations in some of these regions. **Methods:** We enrolled highly traumatized refugees with and without (traumatized controls) PTSD and non-traumatized controls in the study. To increase the validity of our results, we combined an automatic cortical parcellation technique and voxel-based morphometry. **Results:** In all, 39 refugees (20 with and 19 without PTSD) and 13 controls participated in the study. Participants were middle-aged men who were free of psychoactive substances and consumed little to no alcohol. Patients with PTSD (and to a lesser extent traumatized controls) showed reduced volumes in the right inferior parietal cortex, the left rostral middle frontal cortex, the bilateral lateral orbitofrontal cortex and the bilateral isthmus of the cingulate. An influence of cumulative traumatic stress on the isthmus of the cingulate and the lateral orbitofrontal cortex indicated that, at least in these regions, structural alterations might be associated with repeated stress experiences. Voxel-based morphometry analyses produced largely consistent results, but because of a poorer signal-to-noise ratio, conventional statistics did not reach significance. **Limitations:** Although we controlled for several important confounding variables (e.g., sex, alcohol abuse) with our particular sample, this might limit the generalizability of our data. Moreover, high comorbidity of PTSD and major depression hinders a definite separation of these conditions in our findings. Finally, the results concerning the lateral orbitofrontal cortex should be interpreted with caution, as magnetic resonance imaging acquisition in this region is affected by a general signal loss. **Conclusion:** Our results indicate that lateral prefrontal, parietal and posterior midline structures are implicated in the pathophysiology of PTSD. As these regions are particularly involved in episodic memory, emotional processing and executive control, this might have important implications for the understanding of PTSD symptoms.

Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric condition that may emerge in reaction to a severe threat to life or bodily integrity. On a neuronal level, its symptom development has so far mainly been attributed to disturbed function-

ing of a network located in medial prefrontal and medial temporal lobe structures.¹⁻³ Indeed, reports of PTSD-associated structural alterations within these regions have been numerous. Reduced volumes were reported for the hippocampus,⁴ amygdala⁴ and anterior cingulate cortex (ACC).^{5,6-8} Furthermore, a thinner prefrontal cortex has been

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shown in war veterans with chronic PTSD.^{9,10} Notwithstanding, it has repeatedly been highlighted that the network introduced above cannot satisfactorily account for the complex symptom pattern associated with the disease.¹¹

Research in healthy individuals has revealed that the neuronal network mediating episodic memory and/or emotional processing (functions that are thought to be disturbed in PTSD) is widespread. Based on functional neuroimaging and brain lesion studies, the role of parietal,^{12,13} lateral prefrontal¹⁴⁻¹⁸ and posterior midline structures¹⁹ was particularly emphasized in this context. On a functional level, there is some evidence that these regions might be disturbed in PTSD. During trauma-related, script-driven imagery, increased neuronal activity was reported in retrosplenial and/or posterior cingulate,²⁰ lateral prefrontal²¹ and parietal cortices.^{20,21} Furthermore, patients with PTSD showed an increased resting cerebral blood flow in posterior cingulate and parietal sections.²²

On a structural level, PTSD-associated alterations in these cortical regions have received little attention so far. This might be partly owing to methodological problems with the evaluation of broader cortical regions. Manual segmentations are very time-consuming and not practicable for major sections. The alternative, classical automatic procedures would, on the other hand, not be accurate and sensitive enough to reveal the subtle structural alterations more typical for psychiatric conditions.^{23,24} Moreover, brain structural research on PTSD is impeded by the long-term pharmacological treatment and/or alcohol or substance abuse that is frequently associated with chronic PTSD.²⁵ In particular, enduring and excessive alcohol consumption has repeatedly been shown to have a strong effect on brain structures and may thus distort findings.^{6,26}

We aimed to investigate PTSD-related, structural alterations in cortical regions extending beyond the conventional psychobiological model of this disease. In doing so, we chose specific regions of interest (ROIs) in prefrontal, parietal and posterior midline regions that have previously been associated with episodic memory^{12,14,19} and/or emotional processing,¹⁶⁻¹⁸ and we predicted that patients with PTSD should show reduced volumes in these structures. Furthermore, we speculated a "building-block effect" of traumatization, with greater cumulative exposure to traumatic stress leading to smaller brain volumes. As the currently most popular method of structural brain research, voxel-based morphometry (VBM), has recently come into question,^{23,24,27} we used 2 independent methods (a cortical parcellation technique and VBM) to improve the validity of our results. By choosing a study population that took no regular psychiatric medication and barely consumed alcohol, we controlled for confounding variables that often have hampered PTSD-related brain research.

Methods

Participants

We recruited participants from local shelters for asylum-seekers and Kurdish recreational facilities. Participants were included if they were healthy refugees between the ages of 18

and 55 years. Exclusion criteria were lifetime or current abuse of substances (particularly alcohol), neurologic diseases, any contraindication for magnetic resonance imaging (MRI) and psychiatric conditions other than PTSD or major depression.

The objective of the study was to investigate the effects of traumatization and PTSD on brain morphology. Accordingly, we explicitly screened participants for PTSD having developed as the primary disease in reaction to traumatic stress. In all participants, major depression had developed as a secondary, comorbid disease, and some fulfilled criteria for major depression according to DSM-IV.²⁸ As sex influences on the results of morphometric analyses are well-documented,^{29,30} we selected a male sample to minimize the level of variability not owing to traumatization and/or PTSD. Our final sample comprised participants who currently had PTSD, participants who did not have PTSD but who had repeatedly experienced traumatic stress (traumatized controls) and nontraumatized controls who had not experienced severe traumatic stressors.

We conducted the investigation in 2 stages. At the first meeting, the purpose and the course of the investigation were explained in detail, informed consent was acquired, and diagnostic procedures took place. Magnetic resonance imaging measurements were obtained on a separate day (the time interval never exceeding 2 weeks) at the university hospital of Magdeburg, Germany. Participants received compensation of 70 euros. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Konstanz, Germany.

Diagnostic interviews

Interviews were structured and administered in the maternal language of the participants with the aid of trained interpreters. Initially, sociodemographic information was obtained, and participants were questioned about their health status and smoking habits. Subsequent diagnostic procedures proceeded as follows.

vivo Checklist of war, detention and torture events

We evaluated exposure to traumatic stressors with a shortened version of the vivo Checklist of war, detention and torture events.³¹ The shortened scale is based on the unweighted sum of 28 imprisonment- and nonimprisonment-related traumatic event types (e.g., being beaten or receiving electrical shocks as imprisonment-related items, witnessing the murder of a relative or experiencing bombings as nonimprisonment-related items).

Clinician Administered PTSD Scale

We assessed current and lifetime PTSD symptoms with the Clinician Administered PTSD Scale (CAPS³²). This 30-item, structured interview corresponds to PTSD criteria according to DSM-IV²⁸ and allows a quantification of the 3 clusters of PTSD symptoms (intrusions, avoidance and hyperarousal).

Mini-International Neuropsychiatric Interview

The diagnosis of major depression, suicidal ideations and alcohol or substance dependency or abuse according to

DSM-IV²⁶ was based on the corresponding sections of the Mini-International Neuropsychiatric Interview (MINI).²⁵

MRI acquisition and data analyses

High-resolution, whole-brain, 3-dimensional (3-D) structural MRI scans were acquired on a 3 T Siemens MAGNETOM Trio scanner with an 8-channel phased-array head coil using a T_1 -weighted 3D-magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence in sagittal orientation (echo time [TE] 4.77 ms, repetition time [TR] 2500 ms, TI 1100 ms, flip angle 7°, bandwidth 140 Hz/pixel, matrix 256 × 256 × 192, field of view [FOV] 256 mm, isometric voxel size 1.0 mm³).

FreeSurfer cortical parcellation and volume measurements

We performed cortical reconstruction and volumetric segmentation with the FreeSurfer software package (<http://surfer.nmr.mgh.harvard.edu/>). The precise technical details of these procedures are described elsewhere.^{34,35} In short, each scan is registered into Talairach space, intensity-corrected and skull-stripped. Images are then segmented to identify the boundary between grey and white matter and to create a surface representation of the cortical white matter. Finally, the cerebral cortex is parcellated into units based on its gyral and sulcal structure.³⁶ According to probabilistic information estimated from a reference atlas, a neuroanatomical label is assigned to each vertex of the surface model, and the corresponding information (i.e., volume) is calculated for each section. All procedures with FreeSurfer are conducted in native space.

The quality of the skull-stripping and the accuracy of the grey/white matter boundary as well as the pial surface were reviewed by an anatomically skilled operator, who was blind to any group membership. If necessary, results of the surface reconstruction were edited manually. The following regions that have previously been associated with episodic memory^{12,14,19} and/or emotional processing^{16–18} were chosen for further analysis:

- prefrontal cortex (superior frontal cortex, rostral middle frontal cortex, inferior frontal cortex, orbitofrontal cortex and ACC),
- posterior midline structures (posterior cingulate cortex, isthmus of the cingulate, precuneus), and
- lateral parietal cortex (superior parietal cortex, inferior parietal cortex and supramarginal cortex).

Voxel-based morphometry

As specific preprocessing steps may enhance the accuracy of VBM,³⁷ MRI scans were skull-stripped with BET2³⁸ and bias-corrected³⁹ before analyses. Subsequent VBM analyses were performed using SPM5 (Wellcome Department of Cognitive Neurology, Institute of Neurology, London) running in MATLAB R2006a (Mathworks). Magnetic resonance images were spatially normalized and then segmented based on their intensity distribution and spatial information derived from prior probability maps.⁴⁰ To keep our analysis comparable to previous VBM in patients with PTSD,^{5–7} we smoothed the images with a 12-mm full-width at half-maximum isotropic Gaussian kernel. As the VBM analysis further aimed to repli-

cate the previous cortical parcellation analysis, we focused on the ROIs that have been included in the cortical parcellation analysis. Bilateral ROIs were created based on an average participant (the so-called Bert) provided by FreeSurfer and were then normalized in Montreal Neurological Institute (MNI) space and smoothed with the identical parameters as the participants' MRI scans. Subsequent statistical VBM analyses were masked for the ROIs under investigation.

Statistical analysis

Sample characteristics

We compared sample characteristics and clinical parameters using analyses of variance (ANOVAs). All data were tested for normality with the Shapiro–Wilk test.⁴¹ If the normality assumption was not fulfilled, we calculated nonparametric alternatives (Kruskal–Wallis rank sum tests). For post-hoc comparisons, we used pair-wise t tests and, as a nonparametric alternative, pair-wise Wilcoxon rank sum tests. Post-hoc tests were corrected for multiple comparisons according to Hommel.⁴² We analyzed count data using Fisher exact tests.

Cortical parcellation

As age and intracranial volume (ICV) are potential confounds for volumetric measures of brain structures,²⁸ we considered these 2 parameters as covariates in all structural analyses. Volumetric group differences were analyzed with linear mixed-effects models, in which hemisphere was included as a within-group factor. Specific group differences were clarified by inspection of the corresponding parameter estimates in the linear mixed-effects models. If a significant group × hemisphere interaction (indicating a lateralized group effect) was revealed, each hemisphere was considered separately in a linear model. To control for an effect of lifetime PTSD on volumetric variables, analyses were repeated under exclusion of participants with a diagnosis of lifetime PTSD.

Voxel-based morphometry

We initially explored group differences in SPM5, applying a full factorial model with age and intracranial volume as covariates. Directional t contrasts were defined between groups. The corresponding SPM(t) values were transformed to the normal distribution (SPM(z)) and thresholded at $p < 0.005$ (uncorrected) with a minimum cluster size of 25 voxels. We extracted mean intensity values in the encountered clusters using MarsBaR.⁴³ Intensity values for each cluster were then directly compared in linear models, again including age and intracranial volume as covariates.

Effects of cumulative exposure to traumatic stress

We investigated a putative dosage effect of multiple traumatic event types on the probability of PTSD diagnosis for traumatized participants using a logistic regression model. The effect of the number of different traumatic events on PTSD symptom severity was explored using a bivariate regression model. To reveal a possible relation between the severity of trauma exposure and parcellation results/mean intensity values, these variables were included in a linear

regression model and corrected for age and intracranial volume as covariates. Models were then compared with likelihood ratio tests. We considered the number of traumatic stress types experienced to be influential if the model including trauma exposure was favoured.

We performed all analyses (except the exploration of VBM group differences in SPM5) using the statistical program R (version 2.7.1⁴⁴) with the additional package *nls* (version 3.1–90⁴⁵).

Results

Participants

Fifty-two refugees were included in the study: 20 currently had PTSD, 19 did not have PTSD but had repeatedly experienced traumatic stress (traumatized controls) and 13 nontraumatized controls had not experienced severe traumatic stressors. In 3 of the traumatized controls, an earlier episode of PTSD had remitted.

The main population characteristics of the sample are summarized in Table 1. Participants' mean age was 36 years in the PTSD group (standard deviation [SD] 7.7, range 23–55 yr), 34 years in traumatized controls (SD 9.9, range 21–53 yr) and 29 years in nontraumatized controls (SD 7.2, range 18–48 yr). The group difference regarding age reached

significance: Kruskal–Wallis $\chi^2_2 = 7.35$, $p = 0.025$. Post-hoc tests revealed that nontraumatized controls were younger than participants with PTSD (Wilcoxon rank sum test, $p = 0.010$). However, nontraumatized controls did not differ significantly from traumatized controls, and traumatized controls did not differ from patients with PTSD. In an attempt to control for this confound, age was considered as covariate in every subsequent analysis. Groups tended to differ regarding the years of formal education: Kruskal–Wallis $\chi^2_2 = 5.06$, $p = 0.08$. Post-hoc tests revealed that traumatized controls tended to have had more years of formal education than patients with PTSD (Wilcoxon rank sum test, $p = 0.06$). Participants were mainly of Kurdish ($n = 48$) race. The 4 remaining participants were Albanian, Serbian, Romanian and Turkish, respectively. Forty-nine participants were right-handed, and 3 participants (1 in each control group and 1 patient with PTSD) were left-handed. One participant in the PTSD group had taken antidepressant medication on an irregular basis (maximally once a week). Twenty-nine participants were smokers: 9 nontraumatized controls (mean 18.00, SD 6.40 cigarettes/d), 10 traumatized controls (mean 22.18, SD 13.66 cigarettes/d) and 10 patients with PTSD (mean 23.10, SD 16.04 cigarettes/d). Group differences in the number of smokers or cigarettes smoked per day were nonsignificant. Other than that, none of the participants consumed any psychoactive drugs or medication.

Table 1: Population characteristics of refugees who underwent magnetic resonance imaging to assess the effects of traumatization and PTSD on brain morphology

Characteristic	Group; mean (SD)*			Kruskal–Wallis χ^2_\dagger	p value†
	Nontraumatized controls, $n = 13$	Traumatized controls, $n = 19$	PTSD, $n = 20$		
Age, yr	29.0 (7.2)	34.1 (9.9)	36.2 (7.7)	7.4	0.025
Years of formal education	8.5 (6.0)	10.7 (4.4)	7.6 (4.0)	5.1	0.08
Cigarettes smoked, no./d	12.5 (10.1)	12.8 (15.2)	11.6 (16.2)		0.75
Age at first traumatic experience	—	15.5 (6.8)	16.4 (6.8)		0.86
No. smokers	9	11	9		0.38
No. participants fulfilling criteria for major depression	1	1	15		< 0.001

PTSD = posttraumatic stress disorder; SD = standard deviation.

*Unless otherwise indicated.
†All test results were 2-tailed.

Table 2: Traumatization and symptoms of posttraumatic stress disorder

Measure	Group; mean (SD)		Kruskal–Wallis χ^2_\dagger *	p value*
	Traumatized controls, $n = 19$	PTSD, $n = 20$		
Checklist	7.68 (4.66)	14.80 (5.63)	13.26	< 0.001
CAPS score				
Sum of event list	4.68 (2.24)	6.60 (2.19)	5.53	< 0.001
Intrusion subscale	7.05 (5.19)	22.70 (6.14)	25.66	< 0.001
Avoidance subscale	3.16 (4.95)	26.10 (6.10)	27.42	< 0.001
Hyperarousal subscale	2.84 (4.22)	20.10 (5.99)	25.42	< 0.001
Sum	13.05 (11.98)	68.90 (15.46)	27.04	< 0.001

CAPS = Clinician Administered PTSD Scale;³² Checklist = shortened version of the vivo Checklist of war, detention and torture events;³¹ PTSD = posttraumatic stress disorder; SD = standard deviation.

*All tests were 2-tailed, with $p < 0.01$ indicating a highly significant group difference.

Most of the traumatized participants were exposed to severe traumatic stress more than a decade ago: 44% reported their first traumatic event 10–20 years ago; 41% reported that traumatic experiences had started even more than 20 years ago. Participants were between 5 and 35 years old when they experienced their first traumatic event (mean age 15.8, SD 6.6 yr). Patients with PTSD and traumatized controls did not differ regarding their age at first traumatic experience. As expected, patients with PTSD reported experiencing a greater number of different types of traumatic events (see Table 2 for means and SDs of clinical instruments in traumatized participants). Seventeen participants (1 in each control group and 15 in the PTSD group) fulfilled criteria for major depression according to DSM-IV.²⁸ Eleven participants showed either low

($n = 10$) or high ($n = 1$) suicidality, with higher suicidality in participants with PTSD: Pearson $\chi^2 = 11.26, p = 0.024$.

Of the 52 participants, 3 (1 in each group) were excluded from further analysis because their MRI data were of extremely bad quality owing to movement artifacts.

Group differences in cortical volume and cerebral grey matter

See Figure 1 for a graphic depiction of cortical parcellation results and Table 3 for a succinct summary of corresponding statistical models. No significant group differences were found regarding the cortex as a whole ($F_{2,44} = 0.53, p = 0.59$) or total grey matter ($F_{2,44} = 0.42, p = 0.66$). However, groups differed in the bilateral isthmus of the cingulate ($F_{2,44} = 3.98$,

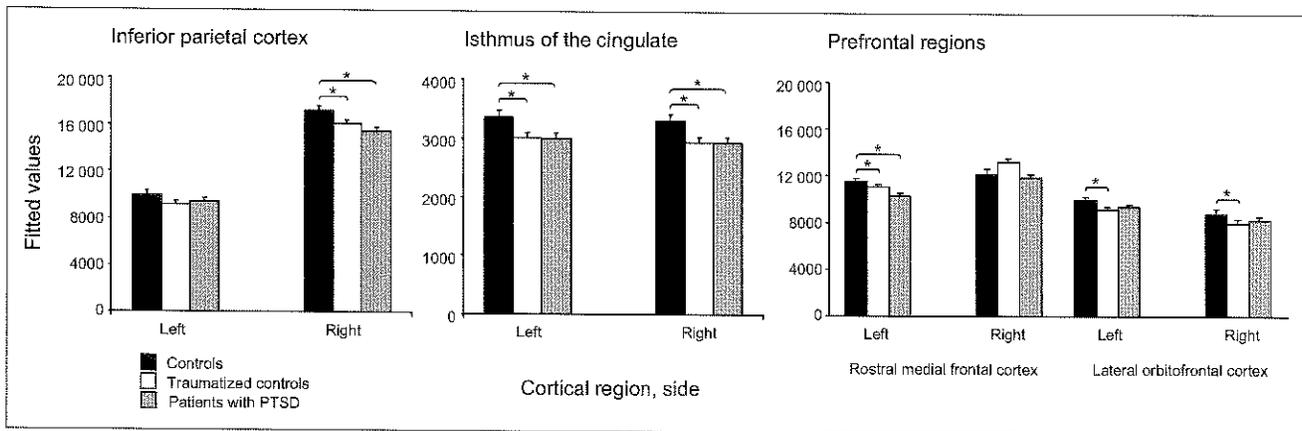


Fig. 1: Graphic depiction of group differences in cortical regions associated with episodic/autobiographical memory. Depicted are the fitted values (predicted group means with the covariates kept constant at the mean of the whole population) and standard errors (original uncorrected volumes were given in millimetres). Significant group differences were found in the bilateral isthmus of the cingulate, the left rostral middle frontal cortex and the right inferior parietal cortex. The bilateral lateral orbitofrontal cortex showed a trend toward group differences. Age and intracranial volume were considered as covariates in all analyses. Precise statistical parameters are presented within the main text. MFC = medial frontal cortex; OFC = orbitofrontal cortex; PTSD = posttraumatic stress disorder.

Table 3: Summary of group differences in cortical volume and cerebral grey matter

Imaging; brain region	Parameter statistics*		Covariate statistics*													
			NTC v. PTSD						Age		ICV		Hemisphere			
			$F_{2,44}$	p value	t_{44}	p value	t_{44}	p value	t_{44}	p value	$F_{1,44}$	p value	$F_{1,44}$	p value	$F_{1,44}$	p value
Parcellation																
Bilateral isthmus of the cingulate	3.98	0.025	-2.59	0.013	-2.48	0.017	-0.04	0.97	14.66	<0.001	41.65	<0.001	1.12	0.30		
Bilateral lateral orbitofrontal cortex	2.38	0.10	-1.49	0.14	-2.17	0.035	0.84	0.41	9.02	0.004	13.57	<0.001	123.95	<0.001		
Left rostral middle frontal cortex	4.12	0.022	-2.68	0.010	-0.84	0.40	-2.03	0.048	8.33	0.006	6.82	0.012				
Right inferior parietal cortex	4.57	0.016	-3.02	0.004	-1.90	0.06	-1.20	0.24	4.92	0.031	28.22	<0.001				
VBM grey matter volumes (extracted with MarsBaR)																
Left isthmus of the cingulate	5.45	0.008	-3.26	0.002	-2.41	0.020	-0.87	0.39	3.69	0.06	16.46	<0.001				
Right inferior parietal cortex	6.69	0.003	-3.65	<0.001	-2.03	0.049	-1.75	0.09	12.93	<0.001	9.62	0.003				
Left rostral anterior cingulate cortex	4.75	0.013	-3.03	0.004	-2.30	0.026	-0.75	0.46	7.34	0.010	10.59	0.002				
Right rostral anterior cingulate cortex	6.01	0.005	-3.24	0.002	-2.96	0.005	-0.22	0.83	3.29	0.08	12.20	0.001				

ICV = intracranial volume; NTC = nontraumatized control group; PTSD = posttraumatic stress disorder; TC = traumatized control group; VBM = voxel-based morphometry. *All tests were 2-tailed, with $p < 0.001$ indicating a significant group difference.

$p = 0.026$). Compared with the nontraumatized controls, the PTSD group ($t_{44} = -2.59, p = 0.013$) and the traumatized controls ($t_{44} = -2.48, p = 0.017$) showed lower volumes in this section. Traumatized controls and patients with PTSD did not differ significantly ($t_{44} = -0.04, p = 0.97$). Furthermore, there was a trend toward a bilateral group difference in the lateral orbitofrontal cortex ($F_{2,44} = 2.38, p = 0.10$). Traumatized controls showed less volume than nontraumatized controls ($t_{44} = -2.17, p = 0.035$). However, the difference between nontraumatized controls and patients with PTSD (with less volume in the PTSD group) did not reach statistical significance ($t_{44} = -1.49, p = 0.14$). Again, traumatized controls and the PTSD group did not differ ($t_{44} = 0.84, p = 0.41$).

We found significant group \times hemisphere interactions in the rostral middle frontal cortex ($F_{2,46} = 4.59, p = 0.015$) and inferior parietal cortex ($F_{2,46} = 4.39, p = 0.018$). Therefore, volumes were compared separately for each hemisphere in these regions. In the rostral middle frontal cortex, we found a significant group difference in the left hemisphere ($F_{2,44} = 4.12, p = 0.023$). Participants with PTSD showed lower volumes

than both control groups (nontraumatized controls v. PTSD, $t_{44} = -2.68, p = 0.010$; traumatized controls v. PTSD, $t_{44} = -2.03, p = 0.048$; nontraumatized v. traumatized controls, $t_{44} = -0.84, p = 0.40$). In the inferior parietal cortex, there was a significant right-hemispheric difference ($F_{2,44} = 4.57, p = 0.015$). In this case, patients with PTSD as well as traumatized controls showed lower volumes than nontraumatized controls (nontraumatized controls v. PTSD, $t_{44} = -3.02, p = 0.004$; traumatized controls v. PTSD, $t_{44} = -1.20, p = 0.24$; nontraumatized v. traumatized controls, $t_{44} = -1.90, p = 0.06$). Excluding traumatized controls who fulfilled the criteria of a lifetime PTSD or left-handed persons did not affect the results.

Voxel-based morphometry grey matter volume

See Figure 2 for a graphic depiction of VBM results and Table 3 for a summary of corresponding statistical models. At the uncorrected significance threshold of $p < 0.005$ (minimum cluster size [k] of 25 voxels), clusters with lower grey matter volumes in patients with PTSD than nontraumatized controls

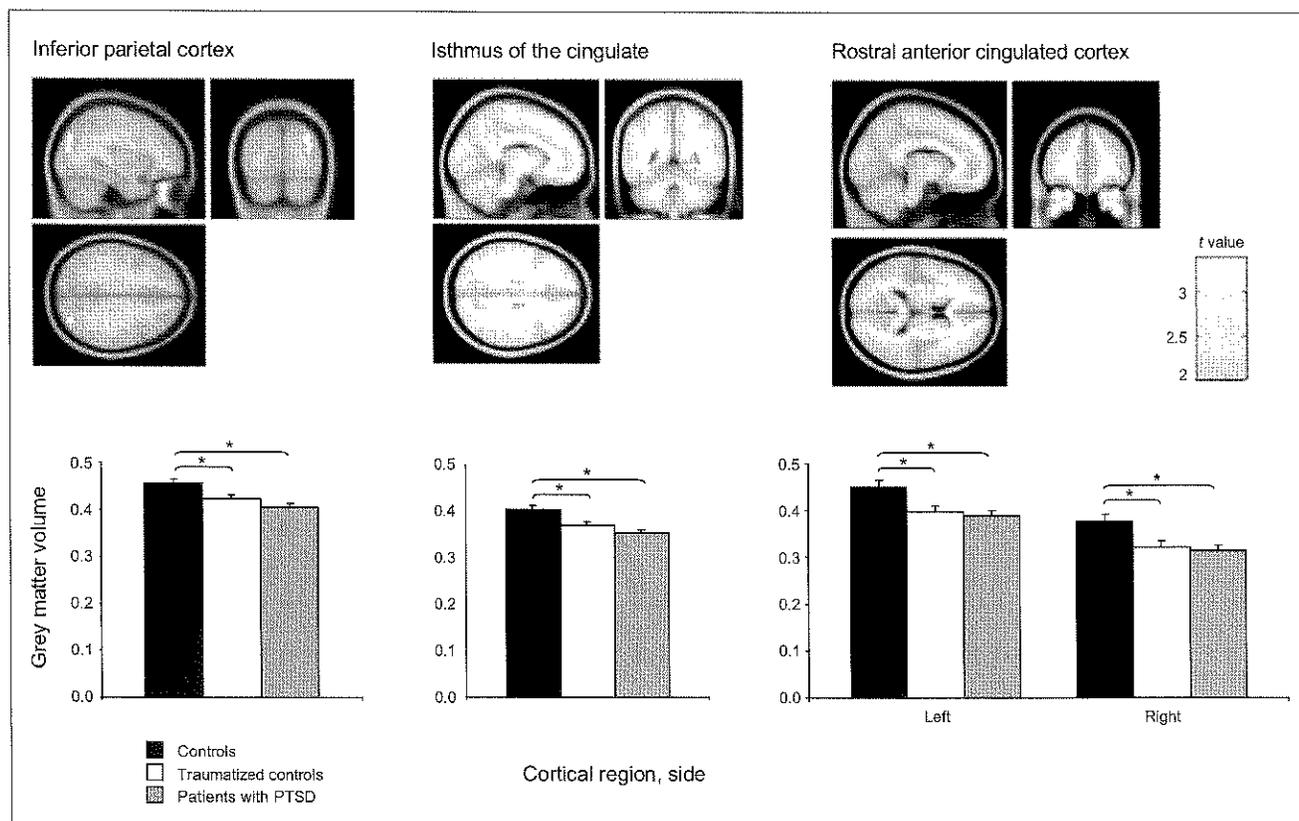


Fig. 2: Brain regions showing less grey matter volume in patients with posttraumatic stress disorder (PTSD) than in nontraumatized controls (at a threshold of $p < 0.005$, uncorrected). Results of the voxel-based morphometry (VBM) analysis did not reach significance within a classic voxel-wise comparison. Bar graphs depict the fitted values (predicted group means with the covariates kept constant at the mean of the whole population) and standard errors of extracted mean volume levels in the respective clusters. After extraction of mean volume levels, significant group differences were found in the inferior parietal cortex (patients with PTSD and traumatized controls showed significantly lower grey matter volume than nontraumatized controls and, as a trend, patients with PTSD showed lower grey matter volumes than traumatized controls), isthmus of the cingulate (patients with PTSD and traumatized controls showed significantly less grey matter volume than nontraumatized controls) and bilateral anterior cingulate cortex (patients with PTSD and traumatized controls showed significantly lower grey matter volume than nontraumatized controls). Precise statistical parameters are presented within the main text.

were found in the vicinity of the left isthmus of the cingulate (peak coordinates x, y, z mm = -10, -48, 28; $k = 111, t = 3.35$), the right inferior parietal cortex (peak coordinates x, y, z mm = 30, -80, 32 and 34, -80, 20; $k = 175, t = 3.43$ and 3.08) and the bilateral rostral ACC (peak coordinates x, y, z mm = -14, 44, 14; $k = 57, t = 3.38$ in the left hemisphere and 16, 40, 16; $k = 36, t = 3.08$ in the right hemisphere). No significant differences were observed comparing healthy and traumatized controls or traumatized controls and patients with PTSD.

In a direct comparison of the mean volumes extracted with MarsBaR, group differences reached significance in all SPM clusters: in the vicinity of the left isthmus of the cingulate, patients with PTSD and traumatized controls showed less grey matter volume than nontraumatized controls ($F_{2,44} = 5.45, p = 0.008$; nontraumatized controls v. PTSD, $t_{44} = -3.26, p = 0.002$; traumatized controls v. PTSD, $t_{44} = -0.87, p = 0.39$; nontraumatized v. traumatized controls, $t_{44} = -2.41, p = 0.020$). In the right inferior parietal cortex, traumatized participants showed less grey matter volume than nontraumatized controls ($F_{2,44} = 6.69, p = 0.003$; nontraumatized controls v. PTSD, $t_{44} = -3.65, p < 0.001$; nontraumatized v. traumatized controls, $t_{44} = -2.03, p = 0.049$). Furthermore, there was a trend with traumatized controls showing less grey matter volume than patients with PTSD ($t_{44} = -1.75, p = 0.09$). In the bilateral rostral ACC, patients with PTSD and traumatized controls showed lower grey matter volumes than nontraumatized controls (left hemisphere: $F_{2,44} = 4.75, p = 0.014$; nontraumatized controls v. PTSD, $t_{44} = -3.03, p = 0.004$; traumatized controls v. PTSD, $t_{44} = -0.75, p = 0.46$; nontraumatized v. traumatized controls, $t_{44} = -2.30, p = 0.026$; right hemisphere: $F_{2,44} = 6.01, p = 0.005$; nontraumatized controls v. PTSD, $t_{44} = -3.24, p = 0.002$; traumatized controls v. PTSD, $t_{44} = -0.22, p = 0.83$; nontraumatized v. traumatized controls, $t_{44} = -2.96, p = 0.005$). See Figure 3 for a graphic depiction of the VBM results and the underlying, smoothed ROIs generated based on the FreeSurfer parcellation.

Building-block effect

We found a strong positive relation between the number of traumatic event types experienced by a participant and the incidence of PTSD ($[\log P(\text{PTSD}) \div P(1-\text{PTSD})] = -3.10 + 0.28 \times \text{vivo Checklist}$; $R^2_{\text{adj}} = 15.81, p < 0.001$). Furthermore, a linear regression analysis showed a significant relation between cumulative exposure to traumatic stress and current symptom severity of PTSD ($\text{CAPS sum } 4.35 + 3.22 \times \text{vivo Checklist}$; $R^2_{\text{adj}} = 0.37, \text{ANOVA } F_{1,35} = 21.91, p < 0.001$).

Likelihood ratio tests supported a significant influence of the sum score of traumatization in the isthmus of the cingulate ($\chi^2_2 = 5.92, p = 0.05$). Furthermore, an influence was revealed in the lateral orbitofrontal cortex ($\chi^2_2 = 8.09, p = 0.018$). In both cases, this effect was mediated by intracranial volume (isthmus of the cingulate: $\text{ICV} \times \text{vivo Checklist}$: $t_{32} = -2.35, p = 0.026$; lateral orbitofrontal cortex: $\text{ICV} \times \text{vivo Checklist}$: $t_{32} = -2.73, p = 0.010$). See Figure 4 for a graphic depiction of the relation between the extent of traumatization and brain volumes and Table 4 for the model equations and respective parameter statistics. No influence of traumatization could be shown for the left rostral middle frontal cortex and the right inferior parietal

cortex. The influence of the sum score of traumatization on parcellation variables could not be replicated for mean volume levels in the respective clusters of the VBM.

Discussion

The scope of the present study was to investigate the influence of traumatization and PTSD on cortical grey matter volumes. To increase the validity of our findings, we implemented 2 independent methods: an automated cortical parcellation analysis and VBM. According to the cortical parcellation, patients with PTSD (and to a lesser extent traumatized controls) showed reduced brain volumes within several lateral prefrontal regions, the right inferior parietal cortex and the bilateral isthmus of the cingulate. Subsequent regression analysis revealed that this volume loss correlated with the extent of traumatization at least in lateral orbitofrontal cortices and the isthmus of the cingulate. These results were partially confirmed by the VBM analysis, showing a PTSD-related decrease of grey matter volumes in the right parietal cortex, left posterior midline regions and, beyond the parcellation findings, in the bilateral rostral ACC. However, VBM results did not survive conventional correction for multiple comparisons and should therefore generally be interpreted with caution.

So far, etiological concepts of PTSD considered its symptom pattern to be mainly associated with alterations in medial prefrontal and medial temporal lobe regions.¹⁻³ Support for this notion came from numerous studies reporting reduced volumes in the hippocampus,⁴ amygdala,⁴ prefrontal cortex^{9,10} and ACC.⁵⁻⁸ However, it has repeatedly been stated that these structures cannot account for all symptoms and deficits observed.¹¹ By demonstrating respective volume reductions within lateral prefrontal, parietal and posterior midline structures the present results provide evidence that these areas might be indeed implicated in PTSD and/or traumatization.

Reports of PTSD- and/or stress-related structural and functional alterations in prefrontal regions are numerous. Besides the previously mentioned volume reductions in the ACC⁵⁻⁸ and lateral prefrontal cortex,^{9,10} patients with PTSD showed altered brain functions in reaction to trauma-related memories in both regions.^{20,21,46} Moreover, a disturbed ability

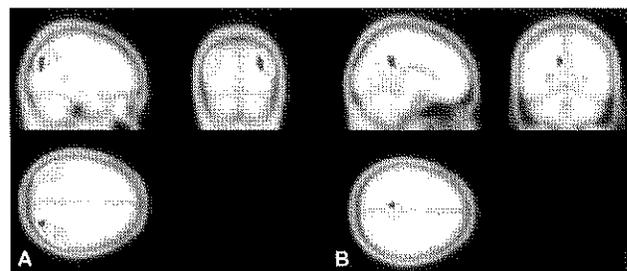


Fig. 3: Graphic depiction of the overlap between the 2 analysis methods in (A) the inferior parietal cortex and (B) the isthmus of the cingulate. Brain regions showing lower grey matter volumes in patients with posttraumatic stress disorder than in nontraumatized controls (at a threshold of $p < 0.005$, uncorrected) are depicted in red. The underlying, smoothed regions of interest generated based on the FreeSurfer parcellation are depicted in yellow.

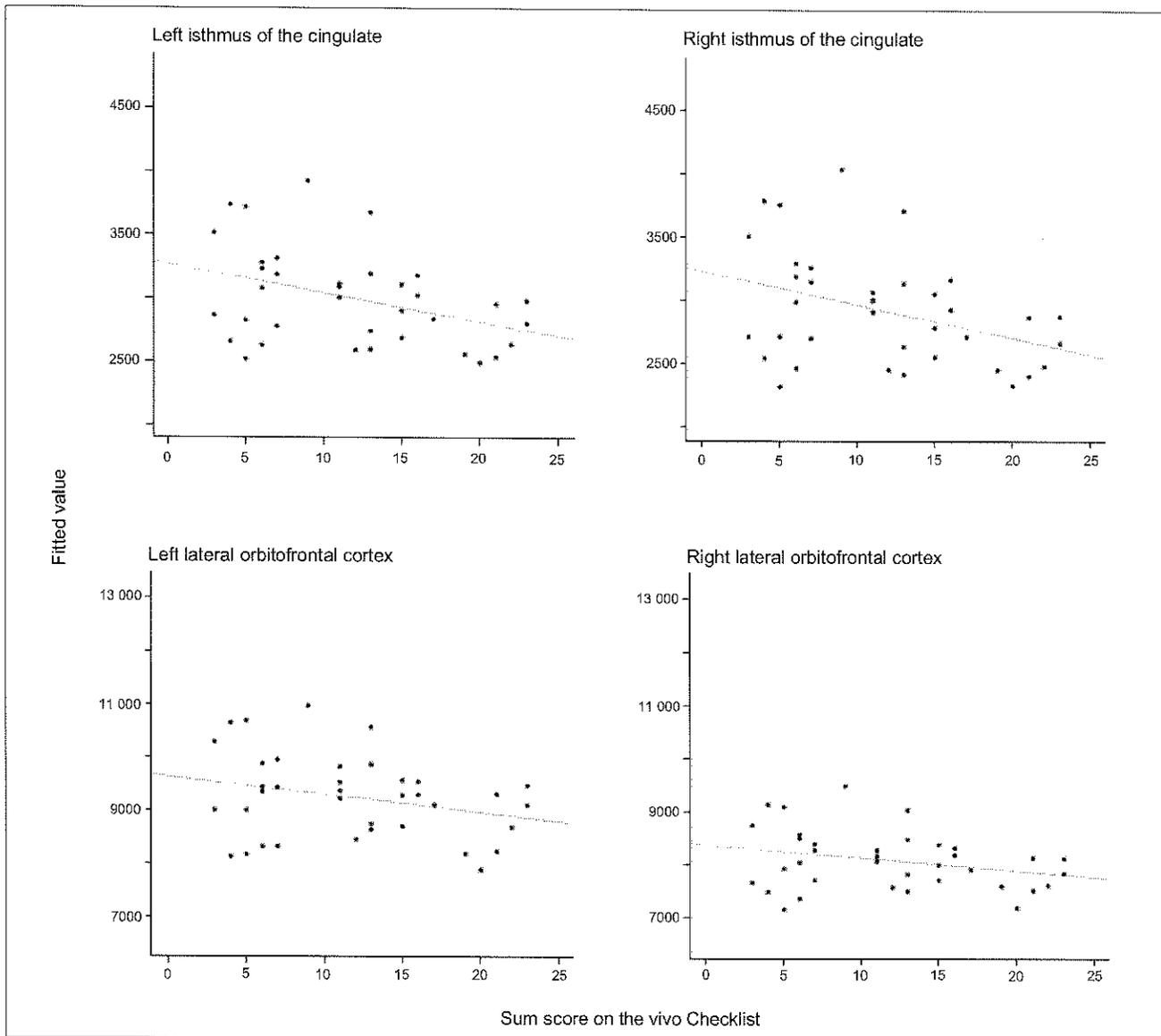


Fig. 4: Correlation between the extent of traumatization and brain volumes. There was a significant relation between the extent of traumatization, the bilateral isthmus of the cingulate and the bilateral lateral orbitofrontal cortex. Scatter plots depict fitted values (predicted group means with the covariates kept constant at the mean of the whole population) of brain volumes. Precise statistical parameters are presented within the main text.

Table 4: Influence of traumatization on cortical volumes

Brain region	Parameter statistics											
	Age		Hemisphere		ICV		Group		vivo Checklist		ICV × vivo Checklist	
	t_{31}	<i>p</i> value	t_{30}	<i>p</i> value	t_{31}	<i>p</i> value	t_{31}	<i>p</i> value	t_{31}	<i>p</i> value	t_{31}	<i>p</i> value
Bilateral isthmus of the cingulate*	-2.18	0.037	1.12	0.27	4.92	< 0.001	0.64	0.53	2.38	0.024	-2.35	0.026
Bilateral lateral orbitofrontal cortex†	-1.81	0.08	9.91	< 0.001	4.66	< 0.001	-0.78	0.44	2.67	0.012	-2.68	0.012

ICV = intracranial volume; vivo Checklist = shortened version of the vivo Checklist of war, detention and torture events.²¹

*Model equation: Isthmus of the cingulate = $-2988 - 13.69 \times \text{age} + 83.95 \times \text{hemisphere} + 0.003 \times \text{ICV} + 87.29 \times \text{group} + 272.12 \times \text{vivo Checklist} - 0.0001 \times \text{ICV} \times \text{vivo Checklist}$.

†Model equation: Lateral orbitofrontal cortex = $-4677 - 26.71 \times \text{age} + 1162.27 \times \text{hemisphere} + 0.007 \times \text{ICV} - 248.09 \times \text{group} + 716.75 \times \text{vivo Checklist} - 0.0001 \times \text{ICV} \times \text{vivo Checklist}$.

of traumatized individuals to down-regulate negative emotional responses was directly associated with reduced brain activity in the lateral prefrontal cortex.⁴⁷ Respective alterations might emerge very early in reaction to traumatic stress, as survivors of a severe earthquake showed an increased resting-state activity in the left lateral prefrontal cortex shortly after having experienced of this traumatic event.⁴⁸

It has recently been highlighted that periods of repeated (psychosocial) stress might alter the activity in the human prefrontal cortex.⁴⁹ In light of corresponding findings of stress-induced dendritic atrophy in rodents,⁵⁰ these processes might manifest themselves in detectable structural alterations when extreme and/or repeated traumatic stress is experienced. Support for this notion might come from the association between the extent of traumatization and the volumes of the lateral orbitofrontal cortex that has been revealed in our data. Substantial volume loss in the lateral orbitofrontal cortex was already reported in war veterans with chronic PTSD.¹⁰ We were able to replicate this finding and add (in line with insights from research in rodents⁵⁰ and reports of the consequences of severe psychosocial⁴⁹ and traumatic⁴⁸ stress on prefrontal brain functions in humans) that this volume loss might be interpreted as a consequence of repeated traumatic stress.

As a potential contribution of parietal and/or posterior midline structures has received relatively little attention in trauma and/or PTSD-related brain research so far, data concerning this topic are still scarce. However, there is some evidence in the literature that supports our suggestion that these structures might play some role in the development of PTSD symptoms as well. During trauma-related, script-driven imagery, an increased neuronal activity was reported in retrosplenial and/or posterior cingulate²⁰ and parietal cortices^{20,21} of patients with PTSD. Furthermore, patients with PTSD showed an increased resting cerebral blood flow in posterior cingulate and parietal sections.²²

Taken together, our results and those in the literature point out that lateral prefrontal, parietal and posterior midline regions might be involved in the pathophysiological model of PTSD. A congruency of the cortical parcellation and VBM analysis, at least in some of the regions, provides further support for the validity of this finding. A previous combination of these 2 methods⁵¹ in traumatized participants revealed highly consistent results between the FreeSurfer parcellation and VBM. These authors implemented the cortical parcellation method to validate their whole-brain VBM analysis.⁵¹ However, we chose to apply the methods in an opposing order. Apart from its popularity in clinical research, there has been emerging concern about some limitations of VBM. Criticism mainly concentrated on a potential distortion of results owing to spatial normalization,²⁷ a bias toward group differences that are spatially well confined²⁹ and statistical procedures that may generally be too strict to reveal subtle morphological alterations.²³ FreeSurfer procedures are, on the other hand, performed in native space, thus avoiding spatial normalization steps that might distort findings. Intersubject and/or template registrations are performed by projecting them onto spherical representations. This approach has been shown to result in a good matching of homologous cortical

regions and should thus be more sensitive than classic VBM.

In line with these preceding considerations, our VBM results generally did not survive conventional correction for multiple comparisons, even though they tended to indicate atrophies in similar regions as the cortical parcellation. Moreover, the results differed between methods for some other brain regions, for example, in the lateral prefrontal cortex where VBM failed to replicate a volume loss that has been revealed with the parcellation method. However, as mentioned previously, it has been suggested that VBM findings might be distorted by normalization steps.^{24,27} As these nuisance effects might be especially pronounced at the edges of the brain, this might help to explain some of these inconsistencies. Moreover, VBM revealed PTSD-related structural alterations in the rostral ACC that were not observed with the parcellation procedure. This parallels previous reports in the literature⁷ and emphasizes the notion that VBM is not sufficiently able to differentiate between factual volume loss and alterations in shape and/or location of brain structures.²⁴ To summarize, our data indicate that FreeSurfer and VBM are both suitable for the investigation of cerebral atrophies. However, our results still support some of the concerns mentioned above^{23,24,27} and imply that VBM should be combined with other methods to increase its informative value.⁵²

Limitations

Some major limitations should be considered when interpreting the present results. Our study population consisted of mainly Kurdish, male refugees exposed to similar severe traumatic experiences in their home countries. As this specific population took no regular psychiatric medication and barely consumed alcohol, we controlled for confounding variables that frequently have hampered PTSD-related brain research. Nevertheless, this sample leads to a limited generalizability of our findings, as conclusions about potential sex differences or the impact of different kinds of traumatization (e.g., childhood abuse) cannot be drawn. As most of our participants had comorbid major depression, we furthermore cannot definitely distinguish how PTSD and depression symptoms contributed to our results. However, in light of the high prevalence of comorbid major depression in patients with PTSD, it has already been suggested that major depression and PTSD symptoms might emerge simultaneously as 2 facets of a general posttraumatic psychopathology.^{5,53} Accordingly, the strict division between these 2 conditions might be artificial and not representative of the factual clinical reality in chronic PTSD.

Another line of concern affects general methodological issues. We had to calculate 12 independent statistical models to investigate the effects of PTSD and/or traumatization on our hypothesized ROIs. However, as we did not directly correct for multiple comparisons within this procedure, we cannot definitely rule out the possibility of false-positive results. However, given the mentioned 12 tests covering our parcellation ROIs, we would expect at most 1 random deviation on a 0.05 significance level. Our finding of 4 regions differing between groups thus largely exceeds the expectations of mere chance. Moreover, our a priori hypotheses were 1-sided, which would allow us to divide the respective *p* values

by 2, thus further strengthening the group differences revealed in our data. Finally, the validity of our findings was further increased by the implementation of 2 independent methods providing an overlapping pattern of results.

Another methodological concern that might limit the interpretation of our results is linked to general constraints of MRI acquisition. We revealed a significant volume loss in the lateral orbitofrontal cortex that was associated with the extent of traumatization. However, MRI acquisition is generally plagued by signal loss in this region. It is hard to quantify or control for the influence of this nuisance factor on our results. Notwithstanding, acquisition parameters have been identical for all participants and individuals have been scanned in an interleaved manner. Accordingly, the measurement error in the lateral orbitofrontal cortex should be constant for the whole population and should not have a systematically bigger effect in one group than the other. Further support for this line of argument comes from the literature. Similar volumetric differences in the lateral orbitofrontal cortex have already been shown in a relatively large sample of former Vietnam veterans,¹⁰ and it seems highly improbable that MRI nuisance artifacts affected 2 completely independent populations in the same direction.

Conclusion

Apart from the concerns mentioned, our findings on PTSD-and/or trauma-related structural alterations in lateral prefrontal, parietal and posterior midline regions might have important implications for the understanding of PTSD symptoms and some associated memory disturbances. These regions are part of a network that is particularly involved in episodic memory, emotional processing and executive control. Prefrontal regions play a particular role in the deliberate manipulation of emotions^{16,17,47} and memories^{14,15,54} and might thus be particularly important for the regulation of highly emotional memories in the aftermath of traumatic experiences.⁵⁵ The parietal cortex, on the other hand, has been suggested to play an important role in the volitional and unvolitional allocation of attentional resources^{12,13} during the retrieval of episodic memories. Accordingly, the successful manipulation of emotional memories seems not only to rely on the interplay between medial temporal and prefrontal cortices but also on an intact functioning of parietal areas. Integrity of the posterior midline structures might finally be particularly important for an unobstructed communication between these structures, as this region is known to serve as a major route of information flow between them.⁵⁶ Disturbances in this hypothesized network, as they are indicated by our data, might help to explain some of the memory disturbances associated with PTSD, such as the fragmentation of traumatic memories,^{1,2} the generally less detailed retrieval of autobiographical memories⁵⁷ or the high occurrence of recurrent, intrusive recollection of traumatic memories. It must be emphasized, however, that this interpretation remains largely speculative. Even though we presented clear evidence that lateral prefrontal, parietal and posterior midline structures might be implicated in the pathophysiology of PTSD, the factual significance of these regions in PTSD symptom development still remains to be clarified.

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Contributors: All authors contributed to study design and approved the article's publication. Dr. Eckart recruited and assessed the participants. Drs. Eckart, Kaufmann and Tempelmann gathered MRI data. Drs. Eckart, Stoppel, Kaufmann and Tempelmann analyzed the data. Drs. Eckart and Stoppel wrote the initial draft of the manuscript, which was critically reviewed by Drs. Elbert and Kolassa. All authors discussed the results and implications and commented on the manuscript at all stages.

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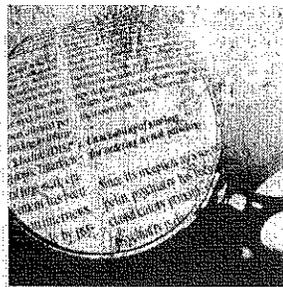
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Post-traumatic stress disorder: the neurobiological impact of psychological trauma

Jonathan E. Sherin, MD, PhD; Charles B. Nemeroff, MD, PhD



The classic fight-or-flight response to perceived threat is a reflexive nervous phenomenon that has obvious survival advantages in evolutionary terms. However, the systems that organize the constellation of reflexive survival behaviors following exposure to perceived threat can under some circumstances become dysregulated in the process. Chronic dysregulation of these systems can lead to functional impairment in certain individuals who become "psychologically traumatized" and suffer from post-traumatic stress disorder (PTSD). A body of data accumulated over several decades has demonstrated neurobiological abnormalities in PTSD patients. Some of these findings offer insight into the pathophysiology of PTSD as well as the biological vulnerability of certain populations to develop PTSD. Several pathological features found in PTSD patients overlap with features found in patients with traumatic brain injury, paralleling the shared signs and symptoms of these clinical syndromes.

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Keywords: stress; psychological trauma; traumatic brain injury; PTSD; biological markers; psychopathology; pathophysiology

Overview of psychological trauma,
post-traumatic stress disorder,
and biological markers

P psychological trauma can result from witnessing an event that is perceived to be life-threatening or to pose the potential of serious bodily injury to self or others. Such experiences, which are often accompanied by intense fear, horror, and helplessness, can lead to the development of, and are required for the diagnosis of, post-traumatic stress disorder (PTSD).¹ It was originally thought that PTSD represented a normative response, at the extreme end of a response continuum, the severity of which related primarily to trauma/stressor intensity. However, it has become clear over time that the response of an individual to trauma depends not only on stressor characteristics, but also on factors specific to the individual.² For the vast majority of the population, the psychological trauma brought about by the experience of profound threat is limited to an acute, transient disturbance. Though transient, such reactions can be quite unpleasant and are typically characterized by phenomena that can be grouped for the most part into three primary domains: (i) reminders of the exposure (including

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Selected abbreviations and acronyms

5HT	<i>serotonin</i>
CRH	<i>corticotropin-releasing hormone</i>
DA	<i>dopamine</i>
GABA	<i>γ-aminobutyric acid</i>
HPA	<i>hypothalamic-pituitary-adrenal</i>
NE	<i>norepinephrine</i>
NPY	<i>neuropeptide Y</i>
PTSD	<i>post-traumatic stress disorder</i>

flashbacks, intrusive thoughts, nightmares); (ii) activation (including hyperarousal, insomnia, agitation, irritability, impulsivity and anger); and (iii) deactivation (including numbing, avoidance, withdrawal, confusion, derealization, dissociation, and depression). As these reactions are self-limiting by definition, in general they provoke minimal functional impairment over time. On the other hand, for a significant minority of the population, the psychological trauma brought about by the experience of profound threat leads to a longer-term syndrome that has been defined, validated, and termed PTSD in the clinical literature. PTSD is often accompanied by devastating functional impairment.

PTSD is characterized by the presence of signs and symptoms in the three primary domains described above for a period extending beyond 1 month (such periods can in some cases occur long after the original, precipitating traumatic exposure). The signs and symptoms of PTSD, therefore, appear to reflect a persistent, abnormal adaptation of neurobiological systems to the stress of witnessed trauma. The neurobiological systems that regulate stress responses include certain endocrine and neurotransmitter pathways as well as a network of brain regions known to regulate fear behavior at both conscious and unconscious levels. Not surprisingly, much research has consequently focused on exploring these systems in more detail as well as attempting to elucidate the pathological changes that occur in patients who develop PTSD. More specifically, there have been and continue to be ongoing efforts to link neurobiological changes identified in patients who suffer from PTSD to the specific clinical features that constitute PTSD, including altered learning/extinction, heightened arousal, and intermittent dissociative behavior as examples relevant to each of the three primary domains. Efforts to identify neurobiological markers for PTSD originally presumed that abnormalities were acquired “downstream” from an exposure, as a consequence of traumatic experience. It

could be, however, that certain abnormalities in the patient with PTSD simply represent pre-existing or “upstream” pathology that is functionally dormant until released by trauma exposure and detected thereafter upon investigation. Along these lines, recent interest has focused on factors that seem to modulate outcome variation in neurobiological systems following trauma exposure including genetic susceptibility factors, female gender, prior trauma, early developmental stage at the time of traumatic exposure, and physical injury (including traumatic brain injury—TBI) at the time of psychological trauma; these parameters likely contribute to vulnerability for, versus resilience against, developing PTSD. Although the biological, psychological, and social ramifications of PTSD have been under scientific scrutiny for some time now, and treatment has improved dramatically, much remains unknown about this condition and controversy persists in both the neuroscientific as well as the clinical/treatment literature. In this text, we review the neurobiological impact of psychological trauma from the perspective that genetic, developmental, and experiential factors predispose certain individuals to the development of PTSD. More specifically, we review the current database as pertains to biological markers of PTSD and the possibility that some biological markers may not be acquired but, rather, may in fact predate trauma until functionally “unmasked” by stress. Where relevant, we also make note of similarities between PTSD and TBI, which extend beyond well-known signs and symptoms (such as irritability and social withdrawal) to include abnormalities in the same neurobiological systems. Lastly, the article includes a short section on basic considerations for future direction. Ideas put forth in this communication are done so in the interest of developing a consistent model for conceptual purposes. It is recognized at the outset that numerous inconsistencies can be found in the literature that highlight the multifactorial and complex nature of this field.

The biology of PTSD

There are a number of factors that must be considered in contemplating the interplay between adverse environmental stimulation, stress responses/reactions, and pathology. In this section, basic findings are reviewed from endocrinology, neurochemistry, and brain circuitry research conducted on patients with a diagnosis of PTSD (*Table I*).

Endocrine factors

Core endocrine features of PTSD include abnormal regulation of cortisol and thyroid hormones, though there is some disagreement about these findings in the literature. Of note, endocrine dysregulation is also found in patients diagnosed with TBI as a result of damage to the pituitary stalk.

The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is the central coordinator of the mammalian neuroendocrine stress response systems, and as such, it has been a major focus of scrutiny in patients with PTSD (Figure 1). In short, the HPA axis is made up of endocrine hypothalamic components, including the anterior pituitary, as well as an effector organ, the adrenal glands. Upon exposure to stress, neurons in the hypothalamic paraventricular

nucleus (PVN) secrete corticotropin-releasing hormone (CRH) from nerve terminals in the median eminence into the hypothalamo-hypophyseal portal circulation, which stimulates the production and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticoids from the adrenal cortex. Glucocorticoids modulate metabolism as well as immune and brain function, thereby orchestrating physiological and organismal behavior to manage stressors. At the same time, several brain pathways modulate HPA axis activity. In particular, the hippocampus and prefrontal cortex (PFC) inhibit, whereas the amygdala and aminergic brain stem neurons stimulate, CRH neurons in the PVN. In addition, glucocorticoids exert negative feedback control of the HPA axis by regulating hippocampal and PVN neurons. Sustained glucocorticoid exposure has adverse effects on hippocampal neurons, including reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis.^{3,5}

Feature	Change	Effect
A. Neuroendocrine		
Hypothalamic-pituitary-adrenal axis	Hypocortisolism	Disinhibits CRH/NE and upregulates response to stress Drives abnormal stress encoding and fear processing
	Sustained, increased level of CRH	Blunts ACTH response to CRH stimulation Promotes hippocampal atrophy
Hypothalamic-pituitary-thyroid axis	Abnormal T3:T4 ratio	Increases subjective anxiety
B. Neurochemical		
Catecholamines	Increased dopamine levels	Interferes with fear conditioning by mesolimbic system
	Increased norepinephrine levels/activity	Increases arousal, startle response, encoding of fear memories Increases pulse, blood pressure, and response to memories
Serotonin	Decreased concentrations of 5HT in:	
	Dorsal raphe	Disturbs dynamic between amygdala and hippocampus
	Median raphe	Compromises anxiolytic effects
Amino acids	Dorsal/median raphe	Increases vigilance, startle, impulsivity, and memory intrusions
	Decreased GABA activity	Compromises anxiolytic effects
Peptides	Increased glutamate	Fosters derealization and dissociation
	Decreased plasma NPY concentrations	Leaves CRH/NE unopposed and upregulates response to stress
	Increased CSF β -endorphin levels	Fosters numbing, stress-induced analgesia, and dissociation
C. Neuroanatomic		
Hippocampus	Reduced volume and activity	Alters stress responses and extinction
Amygdala	Increased activity	Promotes hypervigilance and impairs discrimination of threat
Cortex	Reduced prefrontal volume	Dysregulates executive functions
	Reduced anterior cingulate volume	Impairs the extinction of fear responses
	Decreased medial prefrontal activation	Unclear

Table 1. Summary of neurobiological features with identified abnormalities and functional implications in patients with post-traumatic stress disorder. CRH, corticotropin-releasing hormone; 5HT, serotonin; GABA, γ -aminobutyric acid; NPY, neuropeptide Y; ACTH, adrenocorticotropin; NE, norepinephrine; CSF, cerebrospinal fluid

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Although stressors as a general rule activate the HPA axis, studies in combat veterans with PTSD demonstrate decreases in cortisol concentrations, as detected in urine or blood, compared with healthy controls and other com-

parator groups. This surprising finding, though replicated in PTSD patients from other populations including Holocaust survivors, refugees, and abused persons, is not consistent across all studies.⁶ It has been suggested that

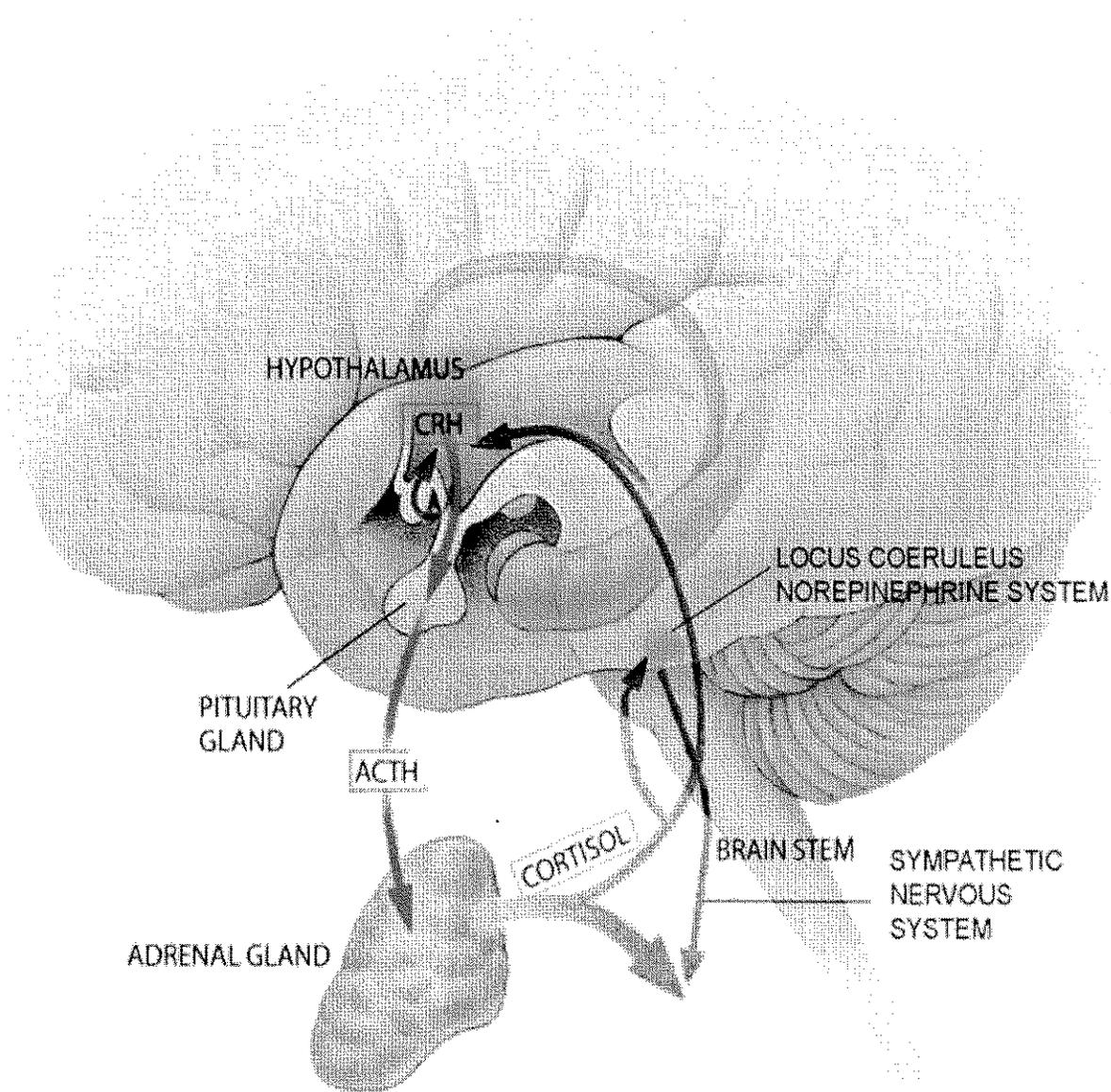


Figure 1. The hypothalamic-pituitary-adrenal axis is the body's major response system for stress. The hypothalamus secretes CRH, which binds to receptors on pituitary cells, which produce/release ACTH, which is transported to the adrenal gland where adrenal hormones such as cortisol are produced/released. The release of cortisol activates sympathetic nervous pathways and generates negative feedback to both the hypothalamus and the anterior pituitary. This negative feedback system appears to be compromised in patients with post-traumatic stress disorder. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin

inconsistent findings may result from differences in the severity and timing of psychological trauma, the patterns of signs/symptoms, comorbid conditions, personality, and genetic makeup.⁷ Studies using low-dose dexamethasone suppression testing suggest that hypocortisolism in PTSD occurs due to increased negative feedback sensitivity of the HPA axis. Sensitized negative feedback inhibition is supported by findings of increased glucocorticoid receptor binding and function in patients with PTSD.⁶ Further, sustained increases of CRH concentrations have been measured in cerebrospinal fluid (CSF) of patients with PTSD. As such, blunted ACTH responses to CRH stimulation implicate a role for the downregulation of pituitary CRH receptors in patients with PTSD.⁶ In addition, reduced volume of the hippocampus, the major brain region inhibiting the HPA axis, is a cardinal feature of PTSD.⁸ Taken as a whole, these neuroendocrine findings in PTSD reflect dysregulation of the HPA axis to stressors.⁶

In the context of the above discussion, prospective studies suggest that low cortisol levels at the time of exposure to psychological trauma may predict the development of PTSD.^{9,10} Therefore, hypocortisolism might be a risk factor for maladaptive stress responses and predispose to future PTSD. This hypothesis is supported in principle by the finding that exogenously administered hydrocortisone shortly after exposure to psychological trauma can prevent PTSD.^{11,12} In addition, it has been shown that simulation of a normal circadian cortisol rhythm using exogenously introduced hydrocortisone is effective in the treatment of PTSD.¹³ In sum, it may be that decreased availability of cortisol, as a result of or in combination with abnormal regulation of the HPA axis, may promote abnormal stress reactivity and perhaps fear processing in general. That said, it should be noted that glucocorticoids interfere with the retrieval of traumatic memories, an effect that may independently prevent or reduce symptoms of PTSD.¹⁴

The hypothalamic-pituitary-thyroid axis

The hypothalamic-pituitary-thyroid (HPT) axis is involved in regulating metabolic versus anabolic states and other homeostatic functions, which it does by controlling the blood level of thyroid hormones. A possible role for the HPT axis in stress-related syndromes has been suspected for some time because it is known that trauma can trigger thyroid abnormalities. To date, how-

ever, there has not been a significant research effort targeting the relationship between the HPT axis and PTSD. Studies have been conducted, however, on Vietnam Veterans with PTSD who were found to have elevated baseline levels of both tri-iodothyronine (T3) and thyroxine (T4). Of note, the level of T3 in these subjects was disproportionately elevated relative to T4, implicating an increase in the peripheral deiodination process.^{15,16} These findings were replicated for the most part in a study of WWII Veterans with more longstanding PTSD diagnoses. In these individuals, isolated T3 levels were elevated whereas T4 levels were normal.¹⁷ Taken together, these studies suggest that over time the impact of trauma on T4 levels may abate. The authors suggest that elevated T3 may relate to subjective anxiety in these individuals with PTSD.

Neurochemical factors

Core neurochemical features of PTSD include abnormal regulation of catecholamine, serotonin, amino acid, peptide, and opioid neurotransmitters, each of which is found in brain circuits that regulate/integrate stress and fear responses. Of note, catecholamine and serotonin (as well as acetylcholine) dysregulation is also found in patients diagnosed with TBI, presumably as a result of diffuse axonal injury.

The catecholamines

The catecholamine family of neurotransmitters, including dopamine (DA) and norepinephrine (NE), derive from the amino acid tyrosine. Increased urinary excretion of DA and its metabolite has been reported in patients with PTSD. Further, mesolimbic DA has been implicated in fear conditioning. There is evidence in humans that exposure to stressors induces mesolimbic DA release, which in turn could modulate HPA axis responses. Whether or not DA metabolism is altered in PTSD remains unclear, though genetic variations in the DA system have been implicated in moderating risk for PTSD (see below). NE, on the other hand, is one of the principal mediators of autonomic stress responses through both central and peripheral mechanisms. The majority of CNS NE is derived from neurons of the locus ceruleus (LC) that project to various brain regions involved in the stress response, including the prefrontal cortex, amygdala, hippocampus, hypothala-

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mus, periaqueductal grey, and thalamus. In addition, there is evidence for a feed-forward circuit connecting the amygdala and hypothalamus with the LC, in which CRH and NE interact to increase fear conditioning and encoding of emotional memories, enhance arousal and vigilance, and integrate endocrine and autonomic responses to stress. Like other stress pathways, this cascade is inhibited by glucocorticoids,¹⁸ which serve as a “brake” for the system. In the periphery, stress-induced sympathetic nervous system activation results in the release of NE and epinephrine from the adrenal medulla, increased release of NE from sympathetic nerve endings, and changes in blood flow to a variety of organs as needed for fight-or-flight behavior. The NE effects are mediated via postsynaptic α_1 , β_1 , and β_2 receptors, whereas another NE-activated receptor, the α_2 receptor, serves as a presynaptic autoreceptor inhibiting NE release. Because of its multiple roles in regulating arousal and autonomic stress responses, as well as promoting the encoding of emotional memories, NE has been a central focus of many studies investigating the pathophysiology of PTSD.

A cardinal feature of patients with PTSD is sustained hyperactivity of the autonomic sympathetic branch of the autonomic nervous system, as evidenced by elevations in heart rate, blood pressure, skin conductance, and other psychophysiological measures. Accordingly, increased urinary excretion of catecholamines, and their metabolites, has been documented in combat veterans, abused women, and children with PTSD. In addition, patients with PTSD exhibit increased heart rate, blood pressure, and NE responses to traumatic reminders. Decreased platelet α_2 receptor binding further suggests NE hyperactivity in PTSD.^{19,20} Administration of the α_2 receptor antagonist yohimbine, which increases NE release, induces flashbacks and increased autonomic responses in patients with PTSD.²¹ Serial sampling revealed sustained increases in CSF NE concentrations and increased CSF NE responses to psychological stressors in PTSD.^{22,23} Taken together, there is an abundance of evidence that NE accounts for certain classic aspects of PTSD symptomatology, including hyperarousal, heightened startle, and increased encoding of fear memories.²⁰

Interestingly, prospective studies have shown that increased heart rate and peripheral epinephrine excretion at the time of exposure to trauma predict subsequent development of PTSD.¹⁰ Further, administration

of the centrally acting β -adrenergic receptor antagonist propranolol shortly after exposure to psychological trauma has been reported to reduce PTSD symptom severity and reactivity to trauma cues.²⁴ Although propranolol administration in this study did not prevent the development of PTSD, it may have blocked traumatic memory consolidation,²⁵ and therefore may reduce the severity and/or chronicity of PTSD. It is important to note, however, that this finding contradicts those from an earlier study.²⁶ Various antiadrenergic agents have been tested for their therapeutic efficacy in the treatment of PTSD in open-label trials; there is a paucity of controlled trials.²⁰

Serotonin

Serotonin (5HT), is a monoamine neurotransmitter synthesized from the amino acid tryptophan. Neurons containing 5HT originate in the dorsal and median raphe nuclei in the brain stem and project to multiple forebrain regions, including the amygdala, bed nucleus of the stria terminalis, hippocampus, hypothalamus, and prefrontal cortex. 5HT has roles in regulating sleep, appetite, sexual behavior, aggression/impulsivity, motor function, analgesia, and neuroendocrine function. Not surprisingly, given its connectivity and broad homeostatic role, 5HT has been implicated in the modulation of affective and stress responses, as well as a role in PTSD. Although the mechanisms are not entirely clear, the effects of 5HT on affective and stress responses vary according to stressor intensity, brain region, and receptor type. It is believed that 5HT neurons of the dorsal raphe mediate anxiogenic effects via 5HT₂ receptors through projections to the amygdala and hippocampus. In contrast, 5HT neurons from the median raphe are thought to mediate anxiolytic effects, facilitate extinction and suppress encoding of learned associations via 5HT_{1A} receptors. Chronic exposure to stressors induces upregulation of 5HT₂ and downregulation of 5HT_{1A} receptors in animal models. Further, 5HT_{1A} knockouts exhibit increased stress responses.

The 5HT system interacts with the CRH and NE systems in coordinating affective and stress responses.^{19,27} Indirect evidence suggests a role for 5HT in PTSD-related behaviors including impulsivity, hostility, aggression, depression, and suicidality. In addition, 5HT presumably mediates the therapeutic effects of the selective serotonin reuptake inhibitors (SSRIs). A recent small

and controversial study suggests that the street drug 3, 4-Methylenedioxymethamphetamine (also known as MDMA or "ecstasy"), which alters central serotonin transmission, has therapeutic potential in the treatment of PTSD.²⁸ Other evidence for altered 5HT neurotransmission in PTSD includes decreased serum concentrations of 5HT, decreased density of platelet 5HT uptake sites, and altered responsiveness to CNS serotonergic challenge in patients diagnosed with PTSD.^{19,27} However, no differences in CNS 5HT_{1A} receptor binding were detected in patients with PTSD compared with controls using PET imaging.²⁸ Taken together, altered 5HT transmission may contribute to symptoms of PTSD including hypervigilance, increased startle, impulsivity, and intrusive memories, though the exact roles and mechanisms remain uncertain.

Amino acids

γ -Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the brain. GABA has profound anxiolytic effects and dampens behavioral and physiological responses to stressors, in part by inhibiting the CRH/NE circuits involved in mediating fear and stress responses. GABA's effects are mediated by GABA_A receptors, which are colocalized with benzodiazepine receptors that potentiate the inhibitory effects of GABA on postsynaptic elements. Uncontrollable stress leads to alterations of the GABA_A/benzodiazepine receptor complex such that patients with PTSD exhibit decreased peripheral benzodiazepine binding sites.²⁹ Further, SPECT and PET imaging studies have revealed decreased binding of radiolabeled benzodiazepine receptor ligands in the cortex, hippocampus, and thalamus of patients with PTSD, suggesting that decreased density or receptor affinity may play a role in PTSD.^{30,31} However, treatment with benzodiazepines after exposure to psychological trauma does not prevent PTSD.^{32,33} Further, a recent study suggests that traumatic exposure at times of intoxication actually facilitates the development of PTSD.³⁴ Although perhaps counterintuitive, the authors suggest that the contextual misperceptions which commonly accompany alcohol intoxication may serve to make stressful experiences more difficult to incorporate intellectually, thereby exacerbating fear. Taken together, while there are multiple studies strongly implicating the GABA/benzodiazepine receptor system in anxiety disorders, studies in

PTSD are relatively sparse and conclusive statements would be premature.¹⁹

Glutamate is the primary excitatory neurotransmitter in the brain. Exposure to stressors and the release of, or administration of, glucocorticoids activates glutamate release in the brain. Among a number of receptor subtypes, glutamate binds to N-methyl D-aspartate (NMDA) receptors that are localized throughout the brain. The NMDA receptor system has been implicated in synaptic plasticity, as well as learning and memory, thereby contributing in all likelihood to consolidation of trauma memories in PTSD. The NMDA receptor system is also believed to play a central role in the derealization phenomena and dissociation associated with illicit and medical uses of the anesthetic ketamine. In addition to its role in learning and memory, overexposure of neurons to glutamate is known to be excitotoxic, and may contribute to the loss of neurons and/or neuronal integrity in the hippocampus and prefrontal cortex of patients with PTSD. Of additional note, elevated glucocorticoids increase the expression and/or sensitivity of NMDA receptors, which may render the brain generally more vulnerable to excitotoxic insults at times of stress.

Peptides

CRH neurons in the hypothalamic PVN integrate information relevant to stress and thereby serve as a major component of the HPA axis. CRH neurons are also found in widespread circuitry throughout the brain, including the prefrontal and cingulate cortices, central nucleus of the amygdala, the bed nucleus of the stria terminalis, hippocampus, nucleus accumbens, periaqueductal gray, and locus coeruleus (LC) as well as both dorsal and median raphé. Direct injection of CRH into the brain of laboratory animals produces physiological stress responses and anxiety-like behavior, including neophobia (fear of new things or experiences), enhanced startle, and facilitated fear conditioning. Anxiety-like behaviors have been specifically linked with increased activity of amygdalar CRH-containing neurons that project to the LC. Of note, glucocorticoids inhibit CRH-induced activation of LC noradrenergic neurons, providing a potential mechanism by which low cortisol may facilitate sustained central stress and fear responses.

The effects of CRH are mediated primarily through two CRH receptor subtypes, CRH₁ and CRH₂. In animal experiments, both exogenous administration of a CRH₁

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receptor antagonist, and experimental knockout of the CRH₁ receptor, produce attenuated stress responses and reduced anxiety. A recent experiment demonstrated that CRH₁ receptor blockade impacted not only gastrointestinal measures of chronic stress, but also prevented stress-induced hair loss in rodents.³⁵ Thus, CRH₁ receptor stimulation may be involved in facilitating stress responses and anxiety. By contrast, CRH₂ knockout mice demonstrate stress sensitization and increased anxiety, suggesting a role for CRH₂ receptor activation in reducing stress reactivity.³ Given the central effects of CRH, as described in animal models, increased CNS CRH activity may promote certain of the cardinal features of PTSD, such as conditioned fear responses, increased startle reactivity, sensitization to stressor exposure, and hyperarousal. These results suggest that CRH₁ receptor antagonists and/or CRH₂ agonists might have important therapeutic potential in the treatment of PTSD.

Neuropeptide Y (NPY) may well be protective against the development of PTSD in that it has anxiolytic and stress-buffering properties. NPY has been shown to inhibit CRH/NE circuits involved in stress and fear responses and to reduce the release of NE from sympathetic neurons. As such, a lack of NPY may promote maladaptive stress responses and contribute to the development of PTSD. Indeed, patients with PTSD have been reported to exhibit decreased plasma NPY concentrations and blunted NPY responses to yohimbine challenge, compared with controls. Together, these findings suggest that decreased NPY activity may contribute to noradrenergic hyperactivity in PTSD.³⁶ Moreover, it has been suggested that NPY may be involved in promoting recovery from, or perhaps resilience to PTSD, given that combat veterans without PTSD have been shown to exhibit elevated NPY levels compared with veterans with PTSD.⁶

Endogenous opioid peptides including the endorphins and enkephalins act upon the same CNS receptors activated by exogenous opioid molecules such as morphine or heroin. Endogenous opioids exert inhibitory influences on the HPA axis. Naloxone, an opioid receptor antagonist, increases HPA axis activation as evidenced by exaggerated HPA axis response to naloxone. PTSD patients exhibit increased CSF β -endorphin levels, suggesting increased activation of the endogenous opioid system. Alterations in endogenous opioids may be involved in certain PTSD symptoms such as numbing,

stress-induced analgesia, and dissociation. Of additional interest, the nonselective opioid receptor antagonist, naloxone, appears to be effective in treating symptoms of dissociation and flashbacks in traumatized persons.^{19,37} Further, the administration of morphine has been reported to prevent PTSD.³⁸ Of note, an experiment investigating the hypothesis that PTSD may play an etiologic role in fostering opioid addiction in an opioid-dependent group of subjects rendered negative results.³⁹

Brain circuitry

Characteristic changes in brain structure and function have been identified in patients with PTSD using brain-imaging methods.⁴⁰⁻⁴² Brain regions that are altered in patients with PTSD include the hippocampus and amygdala as well as cortical regions including the anterior cingulate, insula, and orbitofrontal region. These areas interconnect to form a neural circuit that mediates, among other functions, adaptation to stress and fear conditioning. Changes in these circuits have been proposed to have a direct link to the development of PTSD.⁴⁰ Recent work raises the question as to which CNS elements are involved in circuit changes resulting from stress, and suggests a critical role for myelin.⁴³ Similar to PTSD, brain areas most impacted by TBI include inferior frontal and temporal lobes, and it is likely that myelinated circuits are subject to damage broadly as a result of shear forces.

Hippocampus

A hallmark feature of PTSD is reduced hippocampal volume. The hippocampus is implicated in the control of stress responses, declarative memory, and contextual aspects of fear conditioning. Not surprisingly, the hippocampus is one of the most plastic regions in the brain. As mentioned above, prolonged exposure to stress and high levels of glucocorticoids in laboratory animals damages the hippocampus, leading to reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis.⁴ Initial magnetic resonance imaging (MRI) studies demonstrated smaller hippocampal volumes in Vietnam Veterans with PTSD and patients with abuse-related PTSD compared with controls.⁴⁴⁻⁴⁷ Small hippocampal volumes were associated with the severity of trauma and memory impairments in these studies. These findings were generally replicated in most but not

all subsequent work. Studies using proton magnetic resonance spectroscopy further observed reduced levels of N-acetyl aspartate (NAA), a marker of neuronal integrity, in the hippocampus of adult patients with PTSD.⁴⁰ Of note, NAA reductions were correlated with cortisol levels.⁴⁸ Interestingly, reduced hippocampal volume has been observed in depressed women with a history of early life trauma⁴⁹ but not in children with PTSD.⁵⁰

Hippocampal volume reduction in PTSD may reflect the accumulated toxic effects of repeated exposure to increased glucocorticoid levels or increased glucocorticoid sensitivity, though recent evidence also suggests that decreased hippocampal volumes might be a pre-existing vulnerability factor for developing PTSD.²⁴ Indeed, hippocampal deficits may promote activation of and failure to terminate stress responses, and may also contribute to impaired extinction of conditioned fear as well as deficits in discriminating between safe and unsafe environmental contexts. Studies using functional neuroimaging have further shown that PTSD patients have deficits in hippocampal activation during a verbal declarative memory task.⁵¹ Both hippocampal atrophy and functional deficits reverse to a considerable extent after treatment with SSRIs,⁵² which have been demonstrated to increase neurotrophic factors and neurogenesis in some preclinical studies,⁵ but not others.⁵³

Amygdala

The amygdala is a limbic structure involved in emotional processing and is critical for the acquisition of fear responses. The functional role of the amygdala in mediating both stress responses and emotional learning implicate its role in the pathophysiology of PTSD. Although there is no clear evidence for structural alterations of the amygdala in PTSD, functional imaging studies have revealed hyper-responsiveness in PTSD during the presentation of stressful scripts, cues, and/or trauma reminders.⁴¹ PTSD patients further show increased amygdala responses to general emotional stimuli that are not trauma-associated, such as emotional faces.⁴¹ The amygdala also seems to be sensitized to the presentation of subliminally threatening cues in patients with PTSD,⁵⁴⁻⁵⁶ and increased activation of the amygdala has been reported in PTSD patients during fear acquisition in a conditioning experiment.⁵⁷ Given that increased amygdala reactivity has been linked to genetic traits which

moderate risk for PTSD,^{58,59} increased amygdala reactivity may represent a biological risk factor for developing PTSD.

Cortex

The medial prefrontal cortex (PFC) comprises the anterior cingulate cortex (ACC), subcallosal cortex, and the medial frontal gyrus. The medial PFC exerts inhibitory control over stress responses and emotional reactivity in part by its connections with the amygdala. It further mediates extinction of conditioned fear through active inhibition of acquired fear responses.⁴¹ Patients with PTSD exhibit decreased volumes of the frontal cortex,⁶⁰ including reduced ACC volumes.^{61,62} This reduction in ACC volume has been correlated with PTSD symptom severity in some studies. In addition, an abnormal shape of the ACC,⁶³ as well as a decrease of NAA levels in the ACC,⁶⁴ has been reported for PTSD patients. A recent twin study suggests that, unlike the hippocampus, volume loss in the ACC is secondary to the development of PTSD rather than a pre-existing risk factor.⁶⁵ Functional imaging studies have found decreased activation of the medial PFC in PTSD patients in response to stimuli, such as trauma scripts,^{66,67} combat pictures and sounds,⁶⁸ trauma-unrelated negative narratives,⁶⁹ fearful faces,⁷⁰ emotional stroop,⁷¹ and others, though there are also discordant findings.⁴¹ Reduced activation of the medial PFC was associated with PTSD symptom severity in several studies and successful SSRI treatment has been shown to restore medial prefrontal cortical activation patterns.⁴¹ Of note, in the abovementioned conditioning experiment,⁵⁷ extinction of conditioned fear was associated with decreased activation of the ACC, providing a biological correlate for imprinted traumatic memories in PTSD. Not surprisingly, given the connectivity between the amygdala and medial PFC, interactions in activation patterns between these regions have been reported in PTSD, though the direction of the relationship is inconsistent across studies.⁴¹

The origin of neurobiological abnormalities in PTSD

A number of studies have investigated the fundamental question as to whether the neurobiological changes identified in patients with PTSD represent markers of neural risk to develop PTSD upon exposure to extreme stress

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as opposed to abnormalities acquired through traumatic exposure or, most likely, a combination of both. As an example, low cortisol levels at the time of a trauma predict subsequent development of PTSD. Thus, low levels of cortisol might be a pre-existing risk factor that engenders the development of PTSD; low levels of cortisol could disinhibit CRH/NE circuits and thereby promote unopposed autonomic and neuroendocrine responses to stress, as well as augmented fear conditioning and traumatic memory consolidation. Similarly, the reduced size of the hippocampus in PTSD has remained an unresolved question for many years. There has been considerable debate as to whether this brain region shrinks as a result of trauma exposure, or whether the hippocampus of PTSD patients might be smaller prior to trauma exposure. Studies in twins discordant for trauma exposure have provided a means to address this question, though without complete resolution. Gilbertson and colleagues⁷² studied 40 pairs of identical twins, including Vietnam Veterans who were exposed to combat trauma and their twins who did not serve in Vietnam, and measured hippocampal volumes in all subjects. As expected, among Vietnam Veterans, the hippocampus was smaller in those diagnosed with PTSD as compared with those without a diagnosis. However, this brain region was abnormally smaller in non-PTSD twins as well, despite the absence of trauma exposure and diagnosis. These findings suggest that a smaller hippocampus could be a pre-existing, potentially genetic, neurodevelopmental, and almost surely multifactorial vulnerability factor that predisposes to the development of PTSD (and perhaps other stress-spectrum disorders). Recent results from the same study group indicate, as above, that gray matter loss in the ACC seems on the contrary to be an acquired feature.⁶⁵ Studies are needed to identify the timing and/or etiology of other hallmark neurobiological features of PTSD.

Risk and resilience for developing PTSD

Individuals exposed to an event that either threatens serious injury/death, or is perceived as such, respond in different ways. Most will experience minimal (seconds) to brief (hours) to short-term (days/weeks) abnormalities while a smaller number will suffer from significant psychopathology over longer-term (months) and chronic (lifetime) time frames. In short, not all individuals who face potentially catastrophic trauma go on to develop

PTSD. Why some individuals will develop PTSD following trauma, whereas others do not, is of paramount importance. Because the majority of trauma survivors do not go on to develop PTSD, it is crucial going forward to understand vulnerability and resiliency factors. In this section, the role of genetic factors, gender differences, and early developmental stress experiences in moderating risk for developing PTSD in response to psychological trauma are discussed as is the increased risk for developing PTSD in the context of co-occurring physical traumas (including TBI).

Genetic risk factors for PTSD

Studies on the genetics of PTSD have been hampered by a variety of factors, such as genetic heterogeneity (similar phenotypes develop from different genotypes) and incomplete phenotypic penetrance (a person with genetic risk for PTSD, who is not exposed to trauma, will not develop PTSD). Despite these confounds, there is accumulating evidence that risk for PTSD is heavily influenced by genetic factors. Evidence from family and twin studies has long suggested a heritable contribution to the development of PTSD. In addition, there is evidence for heritable contributions to some of the neurobiological endophenotypes of PTSD as discussed above, such as decreased hippocampal volume⁷² or exaggerated amygdala reactivity.⁵⁸ Although it is beyond the scope of this review to comprehensively discuss the genetics of PTSD, it should be noted that there is an emerging literature on genetic variations in those neurobiological systems that drive responses to trauma and, consequently, risk versus resilience to develop PTSD.⁷³ One study has linked a polymorphism in the DA transporter gene to PTSD risk. In this study, PTSD patients were found to have an excess of the SLC6A39 repeat allele. This finding suggests that genetically determined features of DA transmission may contribute to the development of PTSD among trauma survivors.⁷⁴ Several studies have suggested polymorphisms in the D₂ receptor as possible elements of PTSD risk, though results have not been consistent.⁷³ In addition, there is evidence linking a low expression variant of the serotonin transporter to stress responsiveness and risk for developing depression in relation to life stress, particularly in the presence of low social support.⁵⁹ This finding is intriguing as the same polymorphism is associated with increased amygdala reactivity⁵⁸ as well as the trait of

neuroticism,⁷⁵ which is another risk factor for PTSD. It must be noted, however, that these findings of genetic risk with regard to the serotonin transporter have recently been questioned.⁷⁶

Particularly exciting are findings that a genetic variation of the glucocorticoid receptor cochaperone protein, FKBP5, moderates risk of developing PTSD in relation to childhood abuse.⁷⁷ This study tested interactions of childhood abuse, adulthood trauma, and genetic polymorphisms in the FKBP5 gene in 900 nonpsychiatric, general internal medicine clinic patients. Childhood abuse and adulthood trauma each predicted PTSD symptoms and FKBP5 polymorphisms significantly interacted with childhood abuse to predict adult PTSD symptoms. The FKBP5 genotype was further linked to enhanced glucocorticoid receptor sensitivity, as reflected by dexamethasone hypersuppression, a hallmark feature of PTSD.⁷⁷ Most recently, Ressler and colleagues have demonstrated that a female-specific elevation of pituitary adenylate cyclase-activating peptide (PACAP) correlated not only with fear physiology and the diagnosis of PTSD⁷⁸ but also a specific single nucleotide repeat on an estrogen response element in the same subjects. These findings and this type of work may shed new light not only on the well-known differences in PTSD risk between men and women that are discussed in the next section, but on our mechanistic understanding of PTSD in general.

Gender differences and risk for PTSD

Women more frequently suffer from PTSD than men for reasons that are not entirely clear. Women and men are, in general, subjected to different types of trauma, though the differences in PTSD frequency (reportedly 2:1) are unlikely to be explained solely on the basis of exposure type and/or severity alone. In addition to those findings by Ressler described above, a number of gender-related differences in the neurobiological response to trauma have been documented.⁷⁹ Rodent studies suggest that females generally exhibit greater magnitude and duration of HPA axis responses to stress than males,⁸⁰ though findings in humans are not entirely consistent.⁸¹ Sex differences in neuroendocrine stress responses have been attributed to direct effects of circulating estrogen on CRH neurons.⁸² Sex steroids also interact with other neurotransmitter systems involved in the stress response, such as the serotonin system.⁸³ Progesterone has been

implicated in modulating these systems as well.⁸⁴ However, gender differences in HPA responses to stress have also been observed independent of acute gonadal steroid effects.⁸⁵

Factors that might determine gender differences in the stress response include genomic differences (as above) and/or developmentally programmed effects of gonadal steroids.^{81,85,86} Of note, a very recent study of female Veterans demonstrated that pregnancy raises the risk of PTSD above that for nonpregnant females.⁸⁷ In addition, sex steroids play a role in structural plasticity across the lifespan of several brain regions, including areas involved in stress responsiveness such as the hippocampus and amygdala.⁸⁶ Functional imaging studies have identified gender differences in the brain's response to fear stimuli.⁸⁸ Over time our understanding of this constellation of processes may eventually converge to allow for a better description of the basis for gender differences and, specifically, how the consequences of trauma translate into differential risk for PTSD.

Early developmental factors and PTSD

Previous experience moderates risk for developing PTSD in response to trauma, particularly when exposure to stress occurs early in life. Thus, childhood adversity is associated with increased risk to develop PTSD in response to combat exposure in Vietnam Veterans.⁵¹ There is a burgeoning literature documenting that early adverse experience, including prenatal stress and stress throughout childhood, has profound and long-lasting effects on the development of neurobiological systems, thereby "programming" subsequent stress reactivity and vulnerability to develop PTSD.^{89,91} As an example, children with a history of date violence have recently been shown at risk of developing future PTSD.⁹² Further, a study of child survivors from the Hurricane Katrina disaster indicates significantly increased risk of PTSD.⁹³ Along these lines, nonhuman primates exposed to a variable foraging demand condition, which causes unpredictable maternal care in the infant, leads to an adult phenotype with sensitization to fear cues, CRH hyperactivity and low cortisol levels, a pattern of the classic features found in PTSD.⁹⁴ Consistent with these findings, adult women with childhood trauma histories exhibit sensitization of both neuroendocrine, and autonomic stress responses.⁹⁵ Studies are needed that identify particular sensitive periods for the effects of early stress,

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determine parameters for their reversal, and scrutinize the interactions of dispositional factors (genes, gender) with developmental features in determining neurobiological vulnerability to PTSD.

The influence of physical trauma (and TBI) on the development of PTSD

It has been known for some time that physical injury concomitant with psychological trauma increases risk for the development of PTSD. In studies of Vietnam Veterans,^{96,97} and more recently in a study of Iraq and Afghanistan Veterans,⁹⁸ it was found that physical injury increased the risk of PTSD at least twofold. Similarly, a literature review of patients with documented TBI and program evaluation data from surveys of US Marines following blast exposures in Iraq⁹⁹ demonstrate that TBI presents an increased risk for the development of PTSD. Though differentiating the risk of developing PTSD in patients with TBI is complicated by the subjective and objective abnormalities common to both clinical entities, it is striking that each shares common endocrine, neurochemical, and circuit abnormalities (see above, **The biology of PTSD**). As such, it would follow that the existence of both diagnoses in an individual patient might be additive if not multiplicative from a clinical standpoint. For example, in the context of TBI (with frontal lobe damage and behavioral disinhibition) it would be reasonable to expect a very high violence risk profile for a patient suffering from the irritability and anger characteristic of comorbid PTSD. Of additional note, the helplessness that accompanies certain physical injuries (perhaps most notably TBI) is certain to compound issues of limited self-efficacy (and the overall lost sense of agency) that characterize PTSD. The psychological challenges of TBI may thereby introduce an additional chronic risk for the victimization that fosters PTSD in those patients with a tendency to become increasingly dependent over time.

A basic model of PTSD neurobiology

The biological perturbations observed in patients suffering from PTSD are numerous, and likely reflect an enduring dysregulation of multiple stress-mediating systems that occurs as a result of a psychological "shock." These pathophysiological perturbations presumably occur in patients with genetic, epigenetic, and experiential predispositions when exposed to certain extreme conditions. Presumably

these changes signify an indelible sensory imprint of a maladaptively processed experience that co-opts an imbalanced degree of emotional importance and thereafter releases (or restrains) behavioral reactions that focus on defending against future trauma via activation (or deactivation) in a losing effort to secure homeostasis.

Considering neurobiological findings in PTSD patients with this overview in mind, a relative lack of baseline cortisol at the time of a psychological trauma may facilitate overactivation of the central CRH-NE cascade, resulting in enhanced and prolonged stress responses.^{6,95} This increased stress responsiveness may be further accentuated by inadequate regulatory effects of GABA, serotonin, and NPY. Additionally, altered norepinephrine and stress hormone activity may be critically involved in processes of learning and extinction, both of which are abnormal in PTSD; for example, norepinephrine enhances the encoding of fear memories and glucocorticoids block the retrieval of emotional memories. The constellation of elevated noradrenergic activity and relative hypocortisolism may lead to the enhanced encoding of traumatic memories and the lack of inhibition of memory retrieval both of which presumably trigger re-experiencing phenomena in PTSD.¹²

Further, an abnormally functioning hippocampus may account for some of the cognitive symptoms of PTSD, such as declarative memory deficits. In addition, because the hippocampus is critical for context conditioning, an impaired hippocampus may facilitate generalization of learned fear in contexts unrelated to a previous traumatic exposure and impair the ability to discriminate between safe and unsafe stimuli. In combination with exaggerated amygdalar responses seen in patients with PTSD, a limited capacity for discerning threat due to hippocampal and amygdalar dysfunction may promote paranoia, hypervigilance, behavioral activation, exaggerated stress responses, and further acquisition of fear associations. Disrupted prefrontal cortical function may then serve to facilitate PTSD pathology further as a result of deficient suppression of stress responses, fear associations, and extinction.

Future directions

In this article, we have selected findings from a broad range of the PTSD literature to consider the impact of psychological trauma on neurobiological systems. As described, some neurobiological findings in patients with

PTSD are controversial and need to be further examined. In addition, there are a number of understudied yet important topics in the field such as factors that impact resiliency and vulnerability. For example, stress-protective neurobiological factors such as activity in oxytocin and NPY-containing circuits could, in principle, be manipulated to promote resilience. In addition, there is a general need to explore further the molecular biology of PTSD; identifying interactions between dispositional factors (genetic and epigenetic) and trauma exposure is critical to understand PTSD risk, gauge illness course, and predict treatment response. The effects of trauma on neurotrophic factors (in the hippocampus), neural plasticity (CNS-wide), circuit remodeling (myelination patterns)

and gene expression need to be assessed in detail across illness duration. Though difficult, such studies will necessitate accessing, assaying and following populations at risk for exposure to trauma before any exposure occurs (ideally, predeployment soldiers). Where possible, the distinction between PTSD and TBI must also be better understood. Though the presumed mechanism of injury from psychological trauma as opposed to brain trauma is overtly different, the etiologic abnormalities seem to involve similar neurobiological systems and produce overlapping clinical syndromes. □

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Trastorno por estrés postraumático: el impacto neurobiológico del trauma psíquico

La clásica respuesta de ataque o huida ante la percepción de una amenaza es un fenómeno nervioso reflejo que, obviamente en términos evolutivos, tiene ventajas para la supervivencia. Sin embargo, los sistemas que organizan la constelación de conductas reflejas de supervivencia que siguen a la exposición a la amenaza percibida en algunas circunstancias pueden constituirse en procesos mal regulados. La mala regulación crónica de estos sistemas puede llevar a un deterioro funcional en ciertos individuos quienes pueden convertirse en "traumatizados psicológicamente" y presentar un trastorno por estrés postraumático (TEPT). Una gran cantidad de información acumulada en varias décadas ha demostrado alteraciones neurobiológicas en los pacientes con TEPT. Algunos de estos hallazgos permiten adentrarse en la fisiopatología así como en la vulnerabilidad biológica de ciertas poblaciones que van a desarrollar un TEPT. Algunas características patológicas encontradas en pacientes con TEPT se superponen con características de pacientes con daño cerebral traumático, estableciendo un paralelo de signos y síntomas compartidos entre estos síndromes clínicos.

État de stress post-traumatique : impact neurobiologique du traumatisme psychologique

La réponse classique de lutte ou de fuite à une menace perçue est un phénomène nerveux réflexe dont les avantages pour la survie sont évidents en termes d'évolution. Cependant, les systèmes organisés en constellation de comportements réflexes de survie après exposition à une menace perçue peuvent se déréguler dans certaines circonstances. Une dysrégulation chronique de ces systèmes peut entraîner un déficit fonctionnel chez certains sujets qui deviennent « psychologiquement traumatisés » et souffrent de l'état de stress post-traumatique (ESPT). Des données recueillies pendant des dizaines d'années montrent des anomalies neurobiologiques chez les patients souffrant d'ESPT, ce qui permet de mieux comprendre la physiopathologie de l'ESPT ainsi que la vulnérabilité biologique de certaines populations à développer un ESPT. Certaines caractéristiques pathologiques de l'ESPT se superposent à celles trouvées chez des patients atteints de lésion cérébrale traumatique, en parallèle avec les signes et les symptômes partagés par ces deux syndromes.

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FOR IMMEDIATE RELEASE

EFFECTIVENESS OF MOST PTSD THERAPIES IS UNCERTAIN; RESEARCH URGENTLY NEEDED TO DETERMINE WHICH THERAPIES WORK

WASHINGTON – Many people, including significant proportions of active duty military personnel and veterans, suffer from post-traumatic stress disorder (PTSD), often in conjunction with other injuries or illnesses. While several drugs and psychotherapies are used to treat PTSD, many of the studies concerning their effectiveness have problems; as a result, they do not provide a clear picture of what works and what doesn't, says a new report from the Institute of Medicine.

Given the growing number of veterans with PTSD, the U.S. Department of Veterans Affairs (VA), Congress, and the research community urgently need to take steps to overcome the problems that often plague studies of psychiatric therapies for PTSD, and to ensure the right studies are undertaken to yield data that would help clinicians treat PTSD sufferers, said the committee that wrote the report.

The committee reviewed 53 studies of pharmaceuticals and 37 studies of psychotherapies used in PTSD treatment and concluded that because of shortcomings in many of the studies, there is not enough reliable evidence to draw conclusions about the effectiveness of most treatments. There are sufficient data to conclude that exposure therapies – such as exposing individuals to a real or surrogate threat in a safe environment to help them overcome their fears – are effective in treating people with PTSD. But the committee emphasized that its findings should not be misread to suggest that any PTSD treatment ought to be discontinued or that only exposure therapies should be used to treat PTSD.

"At this time, we can make no judgment about the effectiveness of most psychotherapies or about any medications in helping patients with PTSD," said committee chair Alfred O. Berg, professor of family medicine, School of Medicine, University of Washington, Seattle. "These therapies may or may not be effective – we just don't know in the absence of good data. Our findings underscore the urgent need for high-quality studies that can assist clinicians in providing the best possible care to veterans and others who suffer from this serious disorder."

PTSD is the most commonly diagnosed service-related mental disorder among military personnel returning from Iraq and Afghanistan. Surveys of these individuals indicate that around 12.6 percent of personnel who fought in Iraq and 6.2 percent who were in Afghanistan have experienced PTSD. Moreover, significant proportions of Vietnam veterans and veterans of earlier conflicts also report suffering from PTSD. The vast majority of people who experience the disorder also have other concurrent conditions, such as alcoholism, depression, drug use, or anxiety disorders. Sexual assault during military service is another factor that can lead to PTSD among service members.

Clinicians turn to both drugs and psychotherapeutic interventions to treat PTSD. Anticonvulsants, antidepressants – including selective serotonin reuptake inhibitors (SSRIs) – monoamine oxidase inhibitors (MAOIs), and novel antipsychotics such as olanzapine and risperidone are among the drugs used to treat these patients. Psychotherapies used in PTSD treatment include exposure to trauma-related memories or stimuli, cognitive therapy, coping skills training, and hypnosis.

The committee identified 90 studies that met its criteria for trials from which it could anticipate reliable and informative data on of PTSD therapies. However, several problems and limitations characterize much of the research on PTSD treatments, making the data less informative than expected. Many of the studies have problems in their design or how they were conducted, and high dropout rates – ranging from 20 percent to 50 percent of participants – reduced the certainty of several studies' results. Moreover, the majority of drug studies were funded by pharmaceutical firms and many of the psychotherapy studies were conducted by individuals who developed the techniques or their close collaborators. Further investigation is needed to know whether these treatments would produce the same results if tested by other researchers and in other settings.

In addition, the research has not taken into account potential differences in the effectiveness of treatments for subgroups such as those with traumatic brain injury, depression, or substance abuse; nor have studies examined the effects in ethnic minorities, women, and older individuals. Many studies excluded individuals with concurrent health problems such as depression and substance abuse, raising questions about whether the results apply to the many PTSD sufferers who have multiple conditions.

VA and other government agencies that fund clinical research should make sure that studies of PTSD therapies take necessary steps and employ methods that would handle effectively problems that affect the quality of the results. Although the nature of PTSD presents special challenges to researchers, the committee did find some high-quality studies that show it is possible to overcome the problems.

Congress should ensure that resources are available for VA and other federal agencies to fund quality research on treatment of PTSD and that all stakeholders – including veterans – are represented in the research planning, the report said.

The committee emphasized that its role was solely to review what is known about the effectiveness of various PTSD treatments, and not to offer or suggest guidelines on what health care professionals or patients should do. Efficacy is one of many factors that contribute to making decisions about treatment. Other factors include safety, clinician and patient preferences, access and availability of different treatment options, and ethical issues.

The study was sponsored by the U.S. Department of Veterans Affairs. Established in 1970 under the charter of the National Academy of Sciences, the Institute of Medicine provides independent, objective, evidence-based advice to policymakers, health professionals, the private sector, and the public. The National Academy of Sciences, National Academy of Engineering, Institute of Medicine, and National Research Council make up the National Academies. A committee roster follows.

Pre-publication copies of *Treatment of Posttraumatic Stress Disorder: An Assessment of the Evidence* are available from the National Academies Press; tel. 202-334-3313 or 1-800-624-6242 or on the Internet at <http://www.nap.edu>. Reporters may obtain a copy from the Office of News and Public Information (contacts listed above). INSTITUTE OF MEDICINE

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Atypical Antipsychotic Drugs Directly Impair Insulin Action in Adipocytes: Effects on Glucose Transport, Lipogenesis, and Antilipolysis

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Treatment with second-generation antipsychotics (SGAs) has been associated with weight gain and the development of diabetes mellitus, although the mechanisms are unknown. We tested the hypothesis that SGAs exert direct cellular effects on insulin action and substrate metabolism in adipocytes. We utilized two cultured cell models including 3T3-L1 adipocytes and primary cultured rat adipocytes, and tested for effects of SGAs risperidone (RISP), clozapine (CLZ), olanzapine (OLZ), and quetiapine (QUE), together with conventional antipsychotic drugs butyrophenone (BUTY), and trifluoperazine (TFP), over a wide concentration range from 1 to 500 μ M. The effects of antipsychotic drugs on basal and insulin-stimulated rates of glucose transport were studied at 3 h, 15 h, and 3 days. Both CLZ and OLZ (but not RISP) at doses as low as 5 μ M were able to significantly decrease the maximal insulin-stimulated glucose transport rate by ~40% in 3T3-L1 cells, whereas CLZ and RISP reduced insulin-stimulated glucose transport rates in primary cultured rat adipocytes by ~50–70%. Conventional drugs (BUTY and TFP) did not affect glucose transport rates. Regarding intracellular glucose metabolism, both SGAs (OLZ, QUE, RISP) and conventional drugs (BUTY and TFP) increased basal and/or insulin-stimulated glucose oxidation rates, whereas rates of lipogenesis were increased by CLZ, OLZ, QUE, and BUTY. Finally, rates of lipolysis in response to isoproterenol were reduced by the SGAs (CLZ, OLZ, QUE, RISP), but not by BUTY or TFP. These experiments demonstrate that antipsychotic drugs can differentially affect insulin action and metabolism through direct cellular effects in adipocytes. However, only SGAs were able to impair the insulin-responsive glucose transport system and to impair lipolysis in adipocytes. Thus, SGAs directly induce insulin resistance and alter lipogenesis and lipolysis in favor of progressive lipid accumulation and adipocyte enlargement. These effects of SGAs on adipocytes could explain, in part, the association of SGAs with weight gain and diabetes.

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Keywords: second-generation antipsychotics; adipocyte; insulin resistance; lipolysis; glucose transport; obesity

INTRODUCTION

In age-adjusted comparisons with the general population, patients with schizophrenia have a higher prevalence of cardiovascular (Davidson, 2002) and metabolic diseases (Bridler and Umbricht, 2003), including obesity, metabolic syndrome, and type II diabetes. Second-generation antipsychotics (SGAs) are increasingly replacing the conventional neuroleptics used for the treatment of schizophrenia because of their excellent antipsychotic efficacy in the absence of extrapyramidal side effects (Lean and Pajonk,

2003). Unfortunately, patients treated with SGAs are also more likely to present with obesity, insulin resistance, dyslipidemia, abnormal glucose tolerance, and overt diabetes (Bridler and Umbricht, 2003). As early as the mid-1960s, associations between diabetes and conventional neuroleptic drugs were reported, but evidence has accumulated that the risk is significantly greater for some of the SGAs. Thus, the exacerbation of obesity, diabetes, and cardiovascular disease risk in SGA-treated patients is increasingly being recognized as a critical issue in the overall care of the schizophrenic patient (Ananth and Kolli, 2005; Dwyer *et al*, 2001; Ananth *et al*, 2002; Casey, 2005).

Although SGAs induce or exacerbate obesity and diabetes, there is a wide range of variance among individuals regarding these untoward effects (Robinson *et al*, 2006), and, although somewhat controversial, SGAs exhibit a differential ability to impact body weight and glucose tolerance (Bergman and Ader, 2005). Data indicate that clozapine is associated with the greatest weight gain,

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followed by olanzapine, and then by risperidone and quetiapine having intermediate effects, and ziprasidone and aripiprazole conferring the lowest risk for weight gain (Newcomer, 2005a,b; Harvey and Bowie, 2005; Dwyer *et al*, 2001). Obesity can increase the risk of overt diabetes in high-risk individuals, and provides at least a partial explanation for higher rates of diabetes in SGAs-treated patients. SGAs are also known to exacerbate insulin resistance (Newcomer, 2005b; Dwyer *et al*, 2001; Bergman and Ader, 2005) without commensurate increments in insulin secretion (Ader *et al*, 2005), and these events are accompanied by a predictable worsening of glucose tolerance. Although progressive obesity can reduce insulin sensitivity, it is not clear whether the ability of SGAs to induce insulin resistance is entirely explained by weight gain or whether there is a component of the insulin resistance that is independent of the obesity. The fact that hyperglycemia can improve quickly after stopping the antipsychotic medication, and that diabetes (Lean and Pajonk, 2003) can appear in some patients who do not gain weight (Cohen, 2004), suggests that SGAs can adversely affect insulin sensitivity, or other functions contributing to glucose homeostasis, independent of weight gain (Lean and Pajonk, 2003).

Although the mechanisms by which SGAs induce obesity and diabetes are unclear, the common assumption is that these effects are mediated by a central action, perhaps through the modulation of serotonergic/noradrenergic pathways in the central nervous system (Kapur and Remington, 2001). For example, clozapine was found to increase serum leptin levels days after initiation of treatment, suggestive of a central effect leading to leptin resistance at the level of the hypothalamus and a resetting upward of body's 'adipostat' (Zhang *et al*, 2004). It is unknown, however, whether SGAs could also directly impair insulin action and substrate metabolism at the level of insulin target tissues such as adipose and skeletal muscle. For this reason, we have examined whether SGAs exert direct biological effects in cultured adipocytes. As a key feature of human insulin resistance involves a defect in insulin's ability to stimulate glucose transport (Hunter and Garvey, 1998), we tested for alterations in the insulin-responsive glucose transport system. In addition, we assessed intracellular substrate metabolism, including effects on substrate oxidation, lipogenesis, and lipolysis, as these parameters could lead to alterations in cellular fat content underlying a predisposition to obesity. Finally, we investigated multiple antipsychotic drugs to determine whether there were differential effects on adipocyte biology.

MATERIALS AND METHODS

Adipocyte Isolation

Adipocytes were isolated from rat epididymal fat using collagenase digestion (Digirolamo *et al*, 1971). Briefly, minced epididymal fat pads from male Wistar rats weighing 180–200 g were placed in flasks containing 4.0 ml of EHB buffer (Earle's salts, 25 mmol/l HEPES, 4% BSA, 5 mmol/l glucose, and 1.25 mg/ml type II collagenase, pH 7.4, at 37°C) and incubated for 30 min at 37°C in an orbital shaking water bath (New Brunswick Scientific, Edison, NJ) at 120 r.p.m. The isolated adipocytes were filtered through a

fine nylon mesh (200 µm), washed three times with 25.0 ml EHB (Earle's salts, 20 mmol/l HEPES, 1% BSA, 2 mmol/l Na pyruvate, and 4.8 mmol/l NaHCO₃), pH 7.4, at 37°C, and resuspended to a final 5% (vol/vol) cell concentration. Adipocyte viability (Trypan blue exclusion) was determined as described (Digirolamo *et al*, 1971) and cell sizing was performed by microscopic measurement of cell diameter. A visual field containing 100–150 cells, depending on the cell size and density, was registered by means of a digital video camera (Nikon, Japan) mounted on the microscope. Images were captured by the digital camera, and the diameter of 100 cells registered and analyzed by IPLab v.3.6.1 for Macintosh software.

3T3-L1 adipocyte culture. 3T3-L1 fibroblasts were grown in 60 × 15 mm culture dishes to confluence in Dulbecco's minimal essential medium (DMEM) with 4 mM L-glutamine, 4.5 g/l glucose, and 10% bovine calf serum, at 37°C in a humidified atmosphere containing 5% CO₂. Two days after confluence, adipogenesis was initiated by placing cells in DMEM containing 25 mmol/l glucose, 0.5 mmol/l isobutylmethylxanthine, 1 µmol/l dexamethazone, 10 µg/ml insulin, and 10% fetal bovine serum (FBS) for 3 days, and then for 2 days in DMEM containing 25 mmol/l glucose, 10 µg/ml insulin, and 10% FBS. Thereafter, cells were maintained in and refed every 2 days with DMEM, 25 mmol/l glucose, and 10% FBS. Experiments were conducted 10–14 days after adipogenesis was initiated when between 90 and 95% of the cells exhibited a fully differentiated adipocyte phenotype.

Cell treatment with antipsychotic drugs. Isolated rat adipocytes and 3T3-L1 adipocytes were preincubated with risperidone, clozapine, trifluoperazine, butyrophenone, olanzapine, and quetiapine for the indicated time periods and at concentrations varying from 1 to 500 µM depending upon the assay being performed. Control cells were incubated in an equivalent amount of solvent vehicle.

Insulin-Stimulated 2-Deoxyglucose Transport

In 3T3-L1 adipocytes, basal and insulin-stimulated glucose transport rates were assessed as previously described (Mayor *et al*, 1992) using 2-deoxyglucose (2DOG) as a glucose analogue. Fully differentiated 3T3-L1 adipocytes were cultured in 60 × 15 mm dishes. The media were aspirated, and cells were washed twice with Krebs Ringer Phosphate Hepes Buffer (KRPH) and then incubated with or without 10 nM insulin for 1 h. At the end of the incubation, cells were pulsed with ³H-2-deoxyglucose (0.2 µCi/dish; 0.1 mM final concentration), and glucose uptake was interrupted after 3 min by aspirating the media and washing the cells with 2 ml of KRPH at 4°C containing 3 × 10⁻⁶ M phloretin. The cells were transferred into scintillation vials for counting of the radioactivity. The intracellular concentration of 2DOG was calculated by correcting for the label present in the extracellular space (³H-L-glucose) and presented as nmol/dish/3 min.

In isolated rat adipocytes, measurements of 2-deoxyglucose uptake were performed as previously described (Garvey *et al*, 1987). Briefly, isolated cells at a 5% (vol/vol) concentration were incubated with SGAs (risperidone and

clozapine) at doses of 5, 10, 20, 50, and 100 μM for 3 h. Then, cells were incubated for an additional hour in the absence and presence of insulin at the indicated concentrations. Cells were then pulsed with ^3H -2-deoxyglucose (0.2 $\mu\text{Ci}/\text{dish}$; 0.1 mM final concentration), and the reaction was interrupted after 3 min by transferring 300 μl of the assayed cells to microfuge tubes and centrifuging for 30 s at 14 000g. In all assays, the intracellular concentration of 2-DOG was calculated by subtracting the distribution space of radiolabeled L-glucose that was used to correct for non-specific carryover of radioactivity with the cells and uptake of hexose by simple diffusion from total radioactivity accumulated in the pellet. Then, values were normalized by cell surface area, which was obtained after microscopic measurement of cell diameter.

Incorporation of [^{14}C]-D-Glucose into Lipids and Oxidation to $^{14}\text{CO}_2$

Isolated cells were incubated with 100 μM of SGAs (see Cell treatment with antipsychotic drugs) for 3 h, then a 10% adipocyte suspension (final concentration about 5×10^5 cells/ml) was prepared in Krebs Ringer Phosphate Buffer (pH 7.4) containing 1% BSA and 5 mM glucose, and was saturated with a gas mixture of carbogen (CO_2 5%/O₂ 95%). Aliquots of 450 μl were then pipetted into polypropylene test tubes (17 \times 100 mm) containing 25 μl of [^{14}C]-D-glucose (0.1 $\mu\text{Ci}/\text{tube}$) and 2.0 mM of D-glucose in the presence and absence of 10 nM insulin. The mixture was incubated for 1 h at 37°C in an orbital shaking water bath. At the end of the incubation with ^{14}C -labeled glucose, a 0.5 ml eppendorf tube containing a small, loosely folded piece of filter paper (2 \times 2.5 cm) moistened with 0.2 ml of 2-phenylethylamine/methanol (1:1, v/v) was suspended in the center of the polypropylene test. The incubation medium was acidified with 0.3 ml of 8 N H₂SO₄ to rupture cells and release $^{14}\text{CO}_2$, which was then captured by the filter paper placed in the eppendorf tube, saturated with phenylethylamine/methanol during further 60 min incubation. The filter paper was then transferred into scintillation vials for measurement of radioactivity reflecting glucose oxidation. The reaction mixture left in the tube was treated with 5 ml of Dole's reagent (isopropanol : n-heptane : H₂SO₄, 4:1:0.25, v/v/v) for lipid extraction (Lima *et al*, 1994), and counts in the lipid phase represented glucose incorporation into lipid (ie lipogenesis). Oxidation and lipogenesis were calculated from the known concentration and specific activity of D-glucose.

Lipolysis/antilipolysis. A 7% adipocyte suspension was prepared (vol/vol, 2×10^5 cells/ml), and aliquots were separately incubated with each one of the indicated SGAs for 3 h at 100 μM final concentration. After 3 h incubation, 200 μl of the suspension was transferred into 1.5 ml eppendorf tubes, treated with adenosine deaminase for 5 min, and treated for 1 h with either (i) 10 μM isoproterenol, a lipolytic agent and nonselective β -adrenoreceptor agonist, (ii) 0.5 nM insulin, an antilipolytic hormone, (iii) both insulin and isoproterenol, and (iv) buffer only for the basal control. The tubes were then centrifuged at 3000g for 10 min and the infranatant was collected and used for glycerol measurement (Sigma Diagnostics—Free Glycerol

Determination Kit—enzymatic method with reading at 540 nm) as a lipolytic index.

Statistics

One-way ANOVA followed by Bonferroni post-tests for multiple comparisons were used to compare means. Student's *t*-test was used to evaluate differences between groups. Statistical significance was accepted when $P \leq 0.05$. The PRISM 3.01 software (GraphPad Inc.) was used for statistical calculations.

RESULTS

Glucose Transport

We first examined whether SGAs could differentially alter basal and insulin-stimulated glucose transport activity in cultured adipocytes. Because we were uncertain as to the time course of any effects, we first studied fully differentiated 3T3-L1 adipocytes (Figure 1) as opposed to primary cultured adipocytes (Figures 2 and 3), as the former could be maintained over multiple days in culture. In a time-course experiment (Figure 1a), 10 μM clozapine

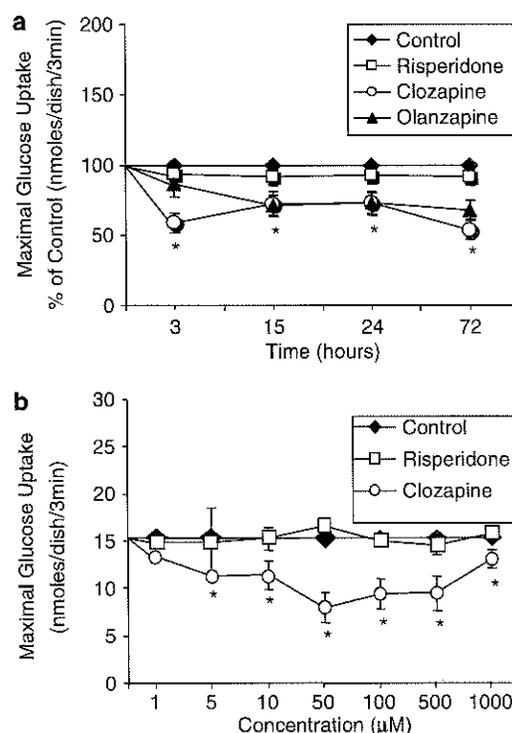


Figure 1 Effects of SGAs on maximally insulin-stimulated glucose transport rates in 3T3-L1 adipocytes. (a) Time-course experiment: In fully differentiated 3T3-L1 adipocytes, cells were treated with 10 μM clozapine, 10 μM olanzapine, 10 μM risperidone, or vehicle alone (control) for 0–72 h. At the indicated time points, cells were stimulated with a maximally effective insulin concentration (10 nM) at 37°C for 1 h, and the rate of 2-deoxyglucose transport was measured. (b) Dose-response experiment: 3T3-L1 adipocytes were incubated with the indicated concentrations of clozapine or risperidone for 3 h. Then, cells were stimulated with a maximally effective insulin concentration (10 nM) at 37°C for 1 h, and the rate of 2-deoxyglucose transport was measured. All data represent the means of results from five experiments. * $P < 0.05$ compared with control.

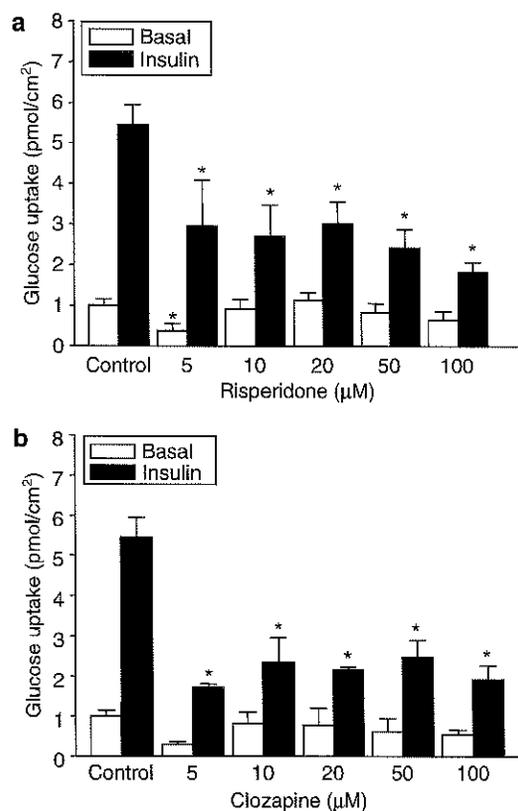


Figure 2 Effects of SGAs on glucose transport in primary cultured rat adipocytes. Isolated adipocytes were treated with risperidone (a) or clozapine (b) at the doses indicated for 3 h and then stimulated in the absence (basal) and presence (insulin) of a maximally effective insulin concentration (10 nM) at 37°C for 1 h, and the rate of 2-deoxyglucose transport was measured. All data represent the means of results from four experiments. * $P < 0.05$ compared with control.

decreased the maximally insulin-stimulated glucose transport by 40% at 3 h, and this response remained suppressed at this level for 3 days in culture. Olanzapine at 10 μM reduced maximal transport rates by 30% but this was not observed until 15 h of drug exposure. Treatment with 10 μM risperidone did not have any effects on transport compared with controls. Dose-response experiments were also conducted after 3 h of drug treatment (Figure 1b). Clozapine reduced the maximally stimulated glucose transport rate over a wide range of concentrations from 5 to 500 μM with a maximal decrement of 30–40% at doses of 50–500 μM (Figure 1b). In contrast, risperidone had no effect over this same concentration range. Conventional antipsychotic drugs (butyrophenone and trifluoperazine) did not affect basal or maximal insulin-stimulated glucose transport rates over 72 h (data not shown).

In primary cultured isolated adipocytes (Figure 2), clozapine treatment reduced maximally stimulated glucose transport rates by 50–70% at all concentrations tested from 5 to 100 μM . In contrast to the lack of effect noted in 3T3-L1 adipocytes, risperidone was observed to diminish maximal glucose transport rates, and these effects were progressive as the drug concentration was increased from 5 to 100 μM . To test for effects at submaximal insulin concentrations and

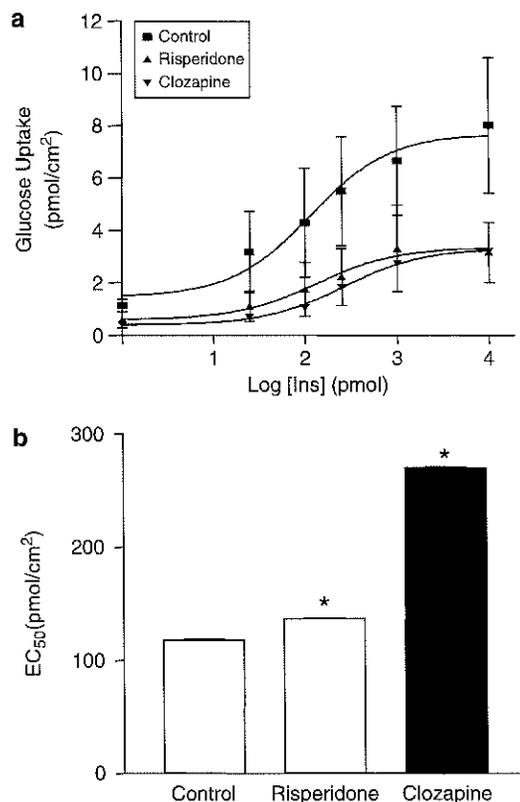


Figure 3 Effects of SGAs on insulin sensitivity in primary cultured rat adipocytes. (a) Isolated adipocytes were treated with 100 μM risperidone or 100 μM clozapine for 3 h, and then stimulated in the absence (basal) and presence of the indicated insulin concentrations at 37°C for 1 h and the rate of 2-deoxyglucose transport was measured. All data represent the means of results from four experiments. (b) Values obtained for EC₅₀ from insulin dose-response curves. * $P < 0.05$ compared with control.

on the EC₅₀ for insulin-stimulated glucose transport, insulin dose-response curves were assessed in isolated adipocytes preincubated in the absence or presence of either 100 μM clozapine or 100 μM risperidone (Figure 3). Both olanzapine and risperidone decreased insulin sensitivity as manifest by rightward shifts in the dose-response curve. However, the effects of clozapine were more profound as indicated by a greater increase in the insulin EC₅₀ (270.9 ± 1.1 pmol/ml) than the EC₅₀ with risperidone (136.5 ± 0.8 pmol/ml) compared with that in controls (EC₅₀ = 117.5 ± 1.5 pmol/ml).

Glucose Oxidation and Lipogenesis

As SGAs could affect insulin action over a relatively short time course (< 3 h), we used primary cultured adipocytes in the remaining experiments as a more physiological primary culture cell model (Lima *et al*, 1994; Garvey *et al*, 1987; Garvey *et al*, 1985) compared with the transformed cell line. In primary cultured adipocytes, we assessed the effects of SGAs on intracellular substrate metabolism distal to any influences on glucose transport. All antipsychotic drugs led to increases in basal and/or insulin-stimulated rates of glucose oxidation, except for cells treated with clozapine, where the increases did not achieve statistical significance,

as shown in Figure 4a. Specifically, drugs that produced the greatest increments in basal glucose oxidation were olanzapine and quetiapine, which increased rates by 46 and 41%, respectively, compared with control cells. All other drugs (except clozapine) led to lesser but significant increases in basal glucose oxidation including butyrophenone, trifluoperazine, risperidone, and quetiapine. With respect to maximally insulin-stimulated rates of glucose oxidation, butyrophenone led to the largest increment of 78% over that observed in controls. Additionally, maximal glucose oxidation rates were increased by olanzapine (57%) and by quetiapine (47%), whereas trifluoperazine, risperidone, and clozapine did not exert statistically significant effects.

Effects of antipsychotic drugs on lipogenesis were also assessed, as shown in Figure 4b. Both basal and insulin-stimulated rates of lipogenesis were significantly increased in cells treated with butyrophenone (78.8%; 58.6%, respectively), olanzapine (109.6%; 83.8%), clozapine (85.4%; 50.6%), and quetiapine (112.9%; 42.6%) compared with controls, whereas effects of trifluoperazine and risperidone did not achieve statistical significance.

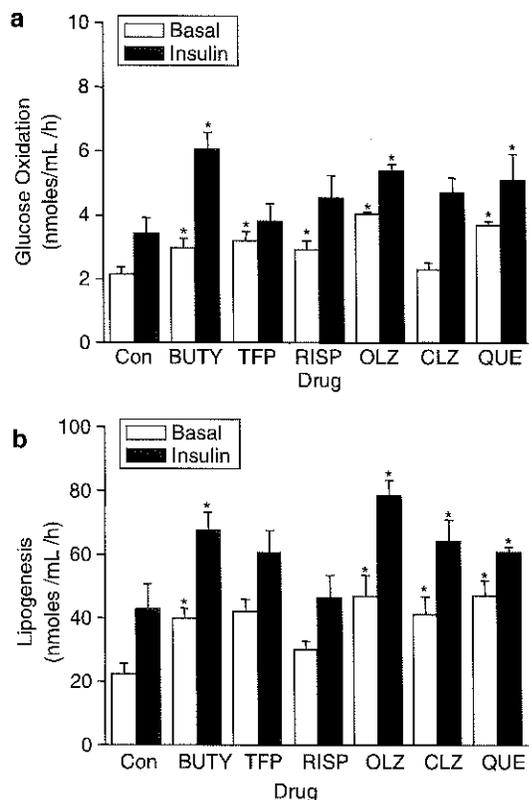


Figure 4 Effects of antipsychotic drugs on basal and insulin-stimulated rates of glucose oxidation and lipogenesis in adipocytes. Primary cultured rat adipocytes were incubated with butyrophenone (BUTY), trifluoperazine (TFP), risperidone (RISP), olanzapine (OLZ), clozapine (CLZ), and quetiapine (QUE), all at a concentration of 100 μ M, for 3 h at 37°C. Then, cells were incubated for an additional 30 min in the absence (basal) and presence of a maximal insulin concentration (10 nM). (a) Rates of glucose oxidation were assessed as 14 C-glucose label released as CO_2 . (b) Rates of lipogenesis were assessed as 14 C-glucose label incorporated into lipids. All data represent the means \pm SE of results from six experiments. * $P < 0.05$ compared with basal or insulin-stimulated rate in controls as appropriate.

Lipolysis and antilipolysis. Rates of lipolysis and the antilipolytic effect of insulin were assessed (Figure 5), as these processes are important determinants of cellular lipid content and fat cell size. Lipolysis was measured as the ability of isoproterenol, a nonselective β -adrenoreceptor agonist, to promote cellular release of glycerol (ie from triacylglycerol breakdown). When compared with control cells, the basal lipolytic response was reduced 36.6% by olanzapine, 33.6% by clozapine, and 43.7% by quetiapine, whereas butyrophenone, trifluoperazine, and risperidone did not affect lipolysis, as shown in Figures 5 and 6a. We also assessed the ability of insulin to inhibit isoproterenol-induced lipolysis, and the modulatory effects of antipsychotic drugs are shown in Figures 5 and 6b. In the presence of both isoproterenol and insulin, lipolysis was decreased by 41.2% in cells treated by quetiapine, 38.5% by clozapine, 36.8% by olanzapine, and 26.3% by risperidone, whereas lipolytic rates in butyrophenone- and trifluoperazine-pretreated cells were similar to controls. Thus, the antilipolytic effect of insulin was more pronounced

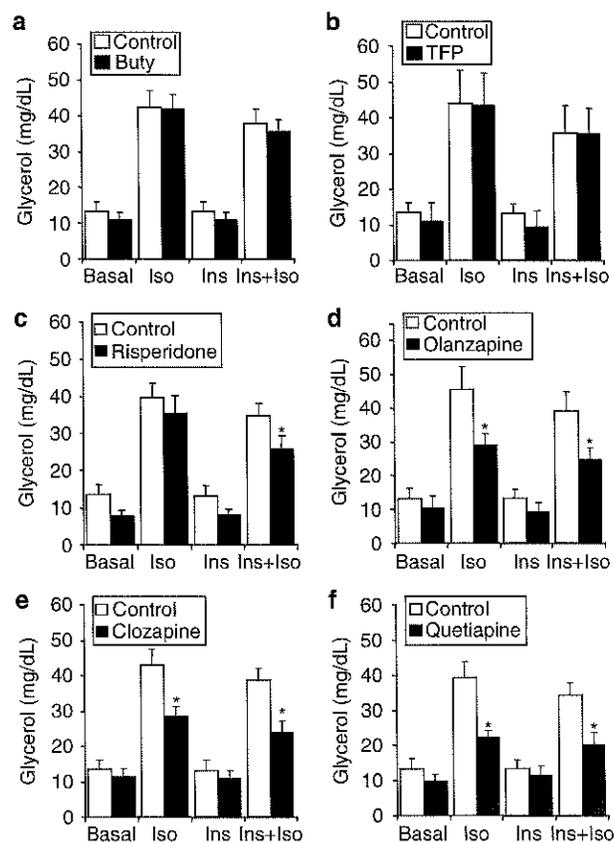


Figure 5 Effects of antipsychotic drugs on lipolysis and insulin-mediated antilipolysis in adipocytes. Primary cultured rat adipocytes were incubated with (a) butyrophenone (Buty), (b) trifluoperazine (TFP), (c) risperidone, (d) olanzapine, (e) clozapine, and (f) quetiapine, all at a concentration of 100 μ M, for 3 h at 37°C. Then, cells were incubated for an additional 1 h in the presence of 10 μ M isoproterenol (Iso) alone, 0.5 nM insulin (Ins) alone, or isoproterenol + insulin. Rates of lipolysis were assessed as the cellular release of glycerol reflecting deacylation of triglyceride, expressed as mg/dl. White bars represent control cells (no antipsychotic) and black bars represent antipsychotic-treated cells. All data represent the means \pm SE of results from eight experiments. * $P < 0.05$ compared to controls.

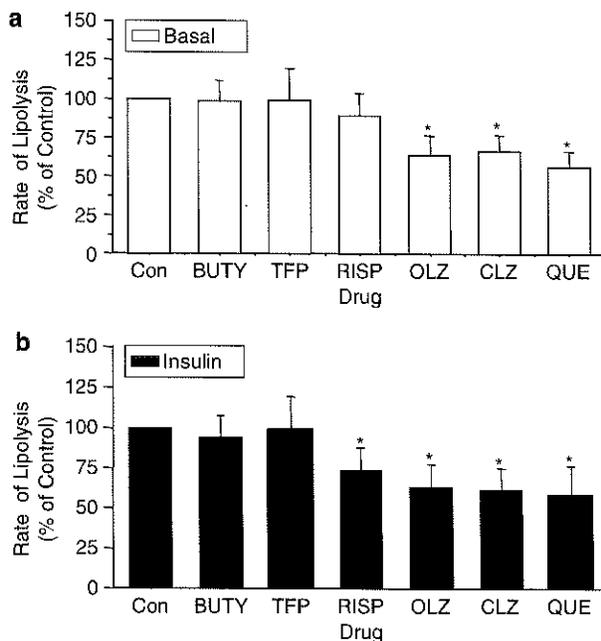


Figure 6 Relative effects of antipsychotic drugs on lipolysis and insulin antilipolysis in adipocytes. The same results presented in Figure 5 are now presented as percentage of control. (a) Lipolysis. Primary cultured rat adipocytes were incubated with butyrophenone (BUTY), trifluoperazine (TFP), risperidone (RISP), olanzapine (OLZ), clozapine (CLZ), and quetiapine (QUE), all at a concentration of 100 μ M, for 3 h at 37°C. Then, cells were incubated for an additional 1 h in the presence of 10 μ M isoproterenol. Rates of lipolysis were assessed as the cellular release of glycerol reflecting deacylation of triglyceride. (b) Antilipolysis. Primary cultured adipocytes were incubated with antipsychotic drugs as in panel a. Then, cells were incubated for an additional 1 h in the presence of both 10 μ M isoproterenol and 0.5 nM insulin. Rates of lipolysis were assessed as the cellular release of glycerol. All data represent the means \pm SE of results from eight experiments. * $P < 0.05$ compared to controls.

in cells exposed to quetiapine, clozapine, olanzapine, and risperidone.

DISCUSSION

This is the first study to show that SGAs directly modulate insulin action and metabolic processes in an insulin target tissue and the results are relevant to the high risk of obesity and diabetes conferred by these medications. We observed that antipsychotic drugs had a differential ability to reduce insulin-stimulated glucose transport activity. This is significant because glucose transport is rate-limiting for glucose metabolism in muscle and fat, and impairment in the insulin-responsive glucose transport system is the key abnormality underlying the glucose intolerance that accompanies insulin resistance (Hunter and Garvey, 1998). We also addressed effects on intracellular glucose and lipid metabolism, and found that antipsychotic drugs were variably able to increase rates of glucose oxidation and lipogenesis. Finally, certain antipsychotic drugs reduced rates of lipolysis in response to an adrenergic agent, and also enhanced insulin's ability to inhibit lipolysis under these conditions. The predicted functional consequence of

increased lipogenesis, combined with reduced lipolysis and enhanced insulin antilipolysis is an accumulation of intracellular lipid. This is directly relevant to enlarged adipocyte size and a tendency towards progressive obesity observed in patients treated with these drugs. The data suggest that untoward metabolic effects of SGAs could in part be explained by direct effects of these drugs on adipocytes, and the differential effects of SGAs on cellular metabolism could underlie relative differences in these drugs in the predisposition to obesity and diabetes observed clinically.

In studies of glucose transport in 3T3-L1 adipocytes, clozapine was able to significantly impair insulin stimulation at doses as low as 5 μ M and as early as 3 h after initial exposure. Olanzapine also reduced glucose transport activity but the initial effect was not observed until 15 h of treatment. In contrast, risperidone did not alter transport rates over a broad concentration range, neither did the conventional antipsychotic drugs butyrophenone and trifluoperazine. In a previous study, Robinson *et al* (2006) showed that lower concentrations of olanzapine (0.07–0.35 μ M) did not affect glucose transport in 3T3-L1 adipocytes, and the discrepancy with the current study may be explained by the differences in drug dosage. However, in the more physiological system of primary cultured adipocytes, both clozapine and risperidone were observed to cause even greater reductions in maximally stimulated glucose transport and a shift to the right (ie an increase in the EC_{50}) of the insulin: glucose transport dose-response curve. The relative ability of SGAs to induce insulin resistance with respect to glucose transport was not necessarily predictive of relative potency for affecting intracellular glucose and lipid metabolism. For example, clozapine did not affect glucose oxidation rates and risperidone led to an increase in basal glucose oxidation, whereas olanzapine increased glucose oxidation under both basal and insulin-stimulated conditions. Increments in glucose oxidation as a fuel source could help prime cells for lipid storage and provide glycerol for triglyceride synthesis (ie lipogenesis). In fact, clozapine, olanzapine, quetiapine, and butyrophenone all led to an augmentation in basal and insulin-stimulated rates of lipogenesis. Finally, all SGAs tested, including clozapine, olanzapine, quetiapine, and risperidone, reduced rates of lipolysis in response to isoproterenol and increased insulin antilipolysis. It is noteworthy that the conventional antipsychotic drugs (butyrophenone and trifluoperazine) did not have any effects on lipolysis/antilipolysis or on glucose transport, and this could help explain why the SGAs place patients at greater risk of weight gain, obesity, insulin resistance, and diabetes.

The idea that SGAs adversely affect metabolism through direct effects on peripheral tissues is novel, as it was commonly assumed that these effects were mediated by central nervous system actions. There are data to support central SGAs actions in this regard. Knockout of the 5-hydroxytryptamine-2C (5-HT_{2C}) receptor in mice can result in obesity and altered feeding behavior (Tecott *et al*, 1995), and clozapine and olanzapine, which have been associated with the highest risks of weight gain, also have the highest affinity for 5-HT_{2C} among SGAs (Lebovitz, 2003; Reynolds, 2004). Additionally, the -759C/T 5-HT_{2C} receptor gene polymorphism was found to be associated with

SGAs-induced weight gain (Garvin *et al*, 2002). Increased food intake observed after acute administration of clozapine in mice can be reversed by D1, D2, 5-HT_{1B}, 5HT₂, and 5HT₃ agonists (Kaur and Kulkarni, 2002), arguing for the participation of both dopaminergic and serotonergic receptors in SGAs-mediated hyperphagia, weight gain, and insulin resistance. However, these central nervous system effects may involve altered substrate metabolism in neuronal cells. A previous study showed that SGAs can alter glucose uptake in neuronal PC-12 cells, in which clozapine, quetiapine, risperidone, and various metabolites differentially affected the V_{max} but not the K_m of glucose uptake (Ardizzone *et al*, 2001). Circulating leptin levels are elevated in patients treated with SGAs (Hagg *et al*, 2001); this could be explained either by primary effects on adipocyte secretion as SGAs act rapidly in this regard without immediate change in adipocyte size (Lean and Pajonk, 2003) or could reflect central regulation as the sympathetic nervous system can inhibit leptin synthesis (Nonogaki, 2000). The current data certainly do not exclude central effects of SGAs, but do support the contention that direct effects on peripheral insulin target tissues could contribute to adverse metabolic outcomes.

It is important to consider the current results in light of drug concentrations present in serum and tissues during therapeutic administration in patients. This issue is complicated because of variability in serum levels achieved, the uncertain relationship between serum levels and therapeutic or metabolic drug effects, and the fact that several antipsychotic drugs are converted to active metabolites *in vivo*. For example, whereas olanzapine metabolites are reputed to have no antipsychotic activity, clozapine metabolites are active but are several fold lower in concentration and potency than the parent compound. In contrast, 9-hydroxy-risperidone, the major circulating metabolite for risperidone, is approximately equipotent with risperidone and is present in sera at concentrations several fold higher than the parent compound (Baldessarini *et al*, 1993). Importantly, it is unknown whether adverse metabolic effects of antipsychotic drugs relate to the parent compound or various metabolites. Another issue is that these lipophilic drugs are often concentrated in tissues such that adipocytes could be exposed to higher levels than those observed in serum. The tissue concentration of butyrophenone, for example, is 22-fold higher than plasma (Tsuneizumi *et al*, 1992), and trifluoperazine-sulfoxide accumulates to reach > 50 times higher levels in tissues than in blood (Aravagiri *et al*, 1995). However, studies have shown that certain SGAs, such as olanzapine and risperidone, do not reach high concentrations in adipose tissue (Aravagiri *et al*, 1998) and the clearance seems to occur faster than with other tissues in rats (Aravagiri *et al*, 1998, 1999). In humans, there is limited information regarding the relative tissue concentrations of SGAs and their metabolites. In any case, 5 μ M clozapine is the lowest concentration tested in the current experiments that induced insulin resistance in adipocytes, and the routine daily oral dose of 400 mg in schizophrenic patients results in plasma levels in the range of 0.2–0.7 μ g/ml (0.6–2 μ M) (Broich *et al*, 1998). In another study, intraperitoneal injection of 20 mg/kg of clozapine in rats led to tissue accumulation in the range of 16–64 μ g/g (Gardiner *et al*, 1978), which is a much higher concentration than that seen in plasma. These data highlight the fact that

SGAs are highly lipophilic and highly protein-bound with a large volume of distribution and low plasma concentration (Burns, 2001). It remains feasible that tissue concentrations *in vivo* could be sufficient to directly influence adipocyte metabolism, which raises the question as to whether individual variability regarding the untoward metabolic effects of SGAs is related to the differences in the ability of these drugs to alter adipocyte biology. This question could be resolved by careful studies in humans assessing serum and tissue levels of SGAs and their metabolites, and correlating these levels with metabolic effects in adipocytes and predisposition to obesity and glucose intolerance.

In summary, our experiments demonstrate that atypical antipsychotic drugs have a differential effect to directly alter cellular insulin action and metabolism in adipocytes. Clozapine and olanzapine, but not risperidone, were able to impair insulin-stimulated glucose transport in 3T3-L1 cells, whereas both risperidone and clozapine decreased glucose uptake in primary cultured rat adipocytes. SGAs displayed a variable ability to promote lipogenesis, impair lipolysis in response to isoproterenol, and to enhance the antilipolytic effects of insulin, whereas conventional antipsychotic drugs (butyrophenone and trifluoperazine) did not induce insulin resistance or affect lipogenesis or lipolysis/antilipolysis. Thus, SGAs were able to directly induce insulin resistance (with respect to stimulation of glucose transport) and, at the same time, promote triglyceride accumulation by stimulating lipogenesis and inhibiting lipolysis. These effects in adipocytes may partially explain increased rates of obesity, insulin resistance, and diabetes associated with SGAs relative to conventional antipsychotic drugs, as well as a differential risk of SGAs for adverse metabolic complications (Bridler and Umbricht, 2003; Lean and Pajonk, 2003; Newcomer, 2005b). However, as SGAs concentrations used to induce metabolic defects could be higher than those achieved in serum and tissues in the course of routine pharmacotherapy of schizophrenia, further research in human patients on SGAs and their metabolites is necessary to gauge the pathophysiological significance of these observations.

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A real-world data analysis of dose effect of second-generation antipsychotic therapy on hemoglobin A1C level

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ABSTRACT

Previous studies have demonstrated an association between certain second-generation antipsychotics (SGAs) and diabetes mellitus. The study assessed the impact of SGA dose on hemoglobin A1C (HbA_{1c} >6.0) levels in a real-world setting. Patients aged ≥ 18 years during 2002–2006 in Ingenix LabRx claims database were included. The database collects medical and prescription claims and a subset of laboratory results for an employed, commercially insured population distributed throughout the United States. Patients with previously diagnosed diabetes, identified by the ICD-9-CM code of 250.x or use of antidiabetic agents, were excluded. The main exposure measure was the cumulative dose over a 30 day period before the HbA_{1c} test, calculated as [sum of (number of pills per day × strength)]/100. A logistic regression was used to examine the relation with HbA_{1c} >6.0 by tertile of the cumulative dose and average daily dose, adjusted for the covariates. The study included 391 patients on olanzapine, 467 on quetiapine, and 262 on risperidone. Patients treated with aripiprazole or ziprasidone (n=212) were included as a secondary reference because of their minimal metabolic risk. Compared to lower (Tertiles 1 and 2) cumulative doses of risperidone, patients with a high cumulative dose of risperidone (Tertile 3) had a significantly higher odds ratio (OR) for HbA_{1c} >6.0 (adjusted OR=2.45; 95% confidence interval = 1.13–5.32; P=0.023). A similar increase in OR was seen in patients with high cumulative dose of olanzapine (2.41; 1.19–4.89; P=0.015). Analyses of average daily dose revealed that quetiapine ≥ 400 mg/day and risperidone ≥ 2 mg/day had an OR of 2.29 (1.04–5.06; P=0.041) and 2.28 (1.08–4.83; P=0.032), respectively, compared to aripiprazole/ziprasidone. Both olanzapine groups (≥ 10 and <10 mg/day) were associated with a significantly increased OR. All results remained similar after further adjustment for the predicated probability of having an HbA_{1c} test and additional medication covariates. In this claims data study, use of olanzapine was associated with elevated HbA_{1c} and risperidone and quetiapine appeared to have dose-related association with elevated HbA_{1c}. One of the limitations of a claims data analysis is the lack of information on potential confounders such as ethnicity and weight.

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1. Introduction

Second-generation (atypical) antipsychotics (SGAs) are of great benefit to patients with a wide variety of psychiatric disorders. Compared to first-generation (typical) antipsychotics (FGAs), SGAs cause fewer

extrapyramidal (motor) side effects. However, the potential to induce type 2 diabetes mellitus has led to FDA-mandated warning language in product labels and consensus guideline recommendations for routine laboratory surveillance for treatment-emergent metabolic abnormalities in patients treated with SGAs (American Diabetes Association et al., 2004).

Pharmacoepidemiologic studies with large claims data have demonstrated an association between SGAs, primarily clozapine and olanzapine, and diabetes mellitus (American Diabetes Association et al., 2004; Lambert et al., 2006; Leslie and Rosenheck, 2004; Ramaswamy et al., 2007; Sernyak et al., 2002; Yood et al., 2009). The pharmacoepidemiologic literature examining the diabetic risk of SGAs consists of studies of different designs (case-control or cohort),

Abbreviations: CI, confidence interval; FGAs, first-generation (typical) antipsychotics; HbA_{1c}, hemoglobin A1C; ICD-9-CM, International Classification of Diseases, Ninth Revision, Clinical Modification; OR, odds ratio; RCTs, randomized controlled trials; SGAs, second-generation (atypical) antipsychotics.

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using various claims databases, and conducted in several countries. In establishing the causal relationship (Rothman and Greenland, 1998) between a SGA and diabetes mellitus, studies have not examined if there is a dose–response relation between SGAs and diabetes mellitus. Such a relation, based on comparisons of patients with different doses of the same medication, is less prone to biases arising from unobservable confounding factors such as weight and family history, which are often not available in administrative claims data.

In a systematic review, Bushe and Leonard (2007) found no significant difference in fasting or random blood glucose levels between any of the comparative SGA treated groups in 21 randomized controlled trials (RCTs) but did find a statistically significant increase of blood glucose level in the olanzapine group ($n=22$) in one RCT (Lindenmayer et al., 2003). In a RCT of first-episode psychosis patients, no significant difference in the glucose level was found between the treatment groups (Perez-Iglesias et al., 2007) and in a recent RCT of children and adolescents, the glucose level significantly increased over a 12 week period for olanzapine group but not for other SGA groups (Correll et al., 2009). In Clinical Antipsychotic Trials of Intervention Effectiveness – Alzheimer's Disease study, no treatment effects (olanzapine, quetiapine, and risperidone) were found for change in glucose (Zheng et al., 2009).

Glycosylated hemoglobin A1C (HbA_{1c}) is used primarily to identify the average plasma glucose concentration over prolonged periods of time as the HbA_{1c} level is proportional to average blood glucose concentration over the previous 4 weeks up to 4 months (Saudek et al., 2006). Therefore, measuring HbA_{1c} is recommended by the American Diabetes Association for monitoring blood glucose control in patients with diabetes mellitus and in people who may be at risk for diabetes mellitus as it provides much more insightful information on glycemic behavior than a fasting blood glucose value. There is evidence indicating that HbA_{1c} is a surrogate for metabolic abnormalities including reduced insulin action, reduced glucose effectiveness, and elevated blood pressure, and it predicts the development of diabetes (Henderson et al., 2007). Interestingly, two of the four RCTs with published HbA_{1c} data found a between-group difference in HbA_{1c} between olanzapine and ziprasidone, although not in the blood glucose level (Bushe and Leonard, 2007; Perez-Iglesias et al., 2007). A claims database analysis of 381 patients with bipolar showed a non-significant increase in HbA_{1c} in the group treated with FGAs or SGAs ($n=30$) (Castilla-Puentes 2007).

Therefore, the current study examined the potential effect of three SGAs (olanzapine, quetiapine, and risperidone) on HbA_{1c} level that is a more stable measure of glycemic control than fasting or random blood glucose levels. In order to address the issue of bias associated with comparing between treatment groups (Citrome et al., 2007), we measured the within drug group difference in HbA_{1c} level between dose groups. Specially, we test the hypothesis that high cumulative dose of a SGA, as described in Methods section, which represents the two important components of drug usage over the defined window, namely, duration (time) and strength, is associated with elevated HbA_{1c} level.

2. Methods

2.1. Study design

This retrospective study was designed to examine elevated HbA_{1c} level in patients treated with olanzapine, quetiapine or risperidone during a period of 30 days before the first HbA_{1c} test (Fig. 1). Patients treated with aripiprazole or ziprasidone were considered as a secondary reference group as described below. This study used the Ingenix LabRx claims database from 2002 to 2006. The LabRx database collects medical (in-patient and out-patient) and prescription claims and also a subset of laboratory results for an employed, commercially insured population of patients and their dependents. The research database includes over 30 million members and more than 3 million -

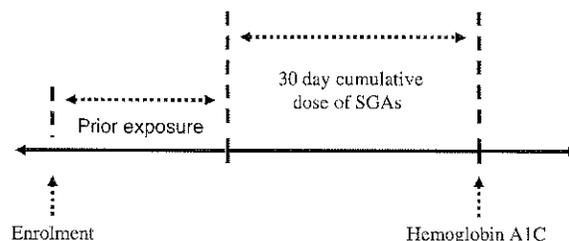


Fig. 1. Diagram of the study design.

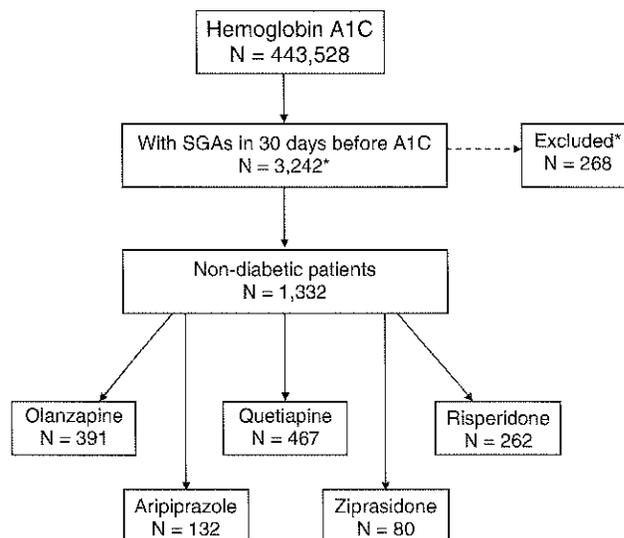
patients with at least one laboratory result. While the patient population is widely distributed throughout the United States, we did observe an over-representation of patients from the south region compared to 2000 Census data. The database is compliant with the guidelines of the Health Insurance Portability and Accountability Act. The study was conducted based on a written protocol reviewed and approved by the internal scientific committee for observational research. The HbA_{1c} data in this database has been used in scientific research (Riedel et al., 2007).

2.2. Study population

The study sample consisted of all patients aged ≥ 18 years with a paid claim for an HbA_{1c} test (the first HbA_{1c} test in the database) and a paid claim for one of the five SGAs during the 30 day period prior to the HbA_{1c} test. Patients with more than one SGA or switch to another SGA during the 30 day period and patients with previously diagnosed diabetes were excluded (Fig. 2). Patients with diabetes were identified by the ICD-9-CM code of 250.x or use of antidiabetic agents (Table 1).

2.3. Variable definition and the 30 day exposure window

The outcome variable, elevated HbA_{1c} level, was defined as $HbA_{1c} > 6.0$ (%). The cutpoint of 6.0 is commonly used in epidemiology studies (Selvin et al., 2009) and is proposed that it be used for screening diabetes



* N = 268 on more than one SGA during the 30 day window were excluded

Fig. 2. Study population of patients.

Table 1
Definition of variables.

Variables	Definition
Age	Based on year born and the testing date of hemoglobin A1C (HbA _{1c})
Gender	Women vs. men
Region (state)	Northeast: CT,ME,MA,NH,NJ,NY,PA,RI,VT Midwest: IL,IN,IA,KS,MI,MN,MO,NE,ND,OH,SD,WI South: AL,AR,DE,DC,FL,GA,KY,LA,MD,MS,NC,OK,SC,TN,VA,WV West: AK,AZ,CA,CO,HI,ID,MT,NV,NM,OR,UT,WA,WY and other areas
Medical history	Diseases were identified base on the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes from any claim before the testing date of HbA _{1c}
Schizophrenia	ICD-9-CM code: 295.X
Bipolar	ICD-9-CM code: 296.X
Diabetes	ICD-9-CM Code: 250.X
Charlson comorbidity index	References (Charlson et al., 1987; Deyo et al., 1992)
Hemolytic anemia	ICD-9-CM Code: 282.X, 283.X, 773.X
Medication	Medication use was determined by the prescription date and days of supply
Current use of antidiabetic agents	Any use within 7 days before the date of HbA _{1c}
Use of diuretics	Any use of diuretics within the 30 day window before the HbA _{1c}
Use of typical antipsychotics	Any use of typical antipsychotics within the 30 day window before the HbA _{1c} ; see Appendix A for the drug list
Use of mood stabilizers	Any use of mood stabilizers within the 30 day window before the HbA _{1c} ; see Appendix A for the drug list
Use of antidepressants	Any use of antidepressants within the 30 day window before the HbA _{1c} ; see Appendix A for the drug list
Prior use of other SGAs	Exposure of other SGAs prior to the 30 day window (after enrollment)

(Saudek et al., 2008). The assessment of SGA exposure was limited to the first HbA_{1c} test in the observation window as subsequent HbA_{1c} measurements and SGA selection may be influenced by the results of the first HbA_{1c} test. The primary exposure variable was the cumulative usage of the SGA over a period of 30 days before the HbA_{1c} test, which was calculated as [sum of (number of pills per day × strength)] / 100 and expressed as 100 mg-day. For example, 10 days with one pill of 10 mg per day will be assumed to be equal to 5 days with one pill of 20 mg per day or 10 days with two pills of 5 mg per day. Therefore, this cumulative dose represents the two important components of drug usage over the defined window, namely, duration (time) and strength. The average daily dose was calculated as the sum of (number of pills per

day × strength) divided by the total number of days. We chose a 30 day pre-testing exposure window because HbA_{1c} represents levels of blood glucose over the past 4 months and is disproportionately weighted to the glucose levels during the prior 4 weeks (Saudek et al., 2006). Recent changes in blood glucose levels are overrepresented in HbA_{1c} and about 50% of HbA_{1c} is determined by glycemia during the 1 month preceding the measurement (Saudek et al., 2006). Furthermore, we chose a 30 day window to standardize the cumulative proximal dose among patients with different durations of total exposure to a SGA. This would also limit the possibility of switching during the exposure window, and would better assess the temporal association between exposure and outcome.

Table 2
Characteristics of study samples.

	Olanzapine (n = 391)	Quetiapine (n = 467)	Risperidone (n = 262)	Aripiprazole (n = 132)	Ziprasidone (n = 80)	P ^a
Age (years)						<0.001
Mean (standard deviation)	47.2 (12.1)	45.3 (11.9)	44.5 (12.9)	40.3 (13.4)	42.7 (13.1)	
Median (interquartile range)	48.0 (17.0)	46.0 (15.0)	46.0 (16.0)	40.0 (22.0)	44.0 (22.5)	
Women (%)	223 (57.0%)	327 (70.0%)	163 (62.2%)	95 (72.0%)	62 (77.5%)	<0.001
Region (%)						0.060
Northeast	80 (20.5%)	89 (19.1%)	63 (24.0%)	32 (24.2%)	13 (16.3%)	
Midwest	95 (24.3%)	74 (15.8%)	47 (17.9%)	17 (12.9%)	17 (21.3%)	
South	200 (51.2%)	283 (60.6%)	145 (55.3%)	76 (57.6%)	47 (58.8%)	
West	16 (4.1%)	21 (4.5%)	7 (2.7%)	7 (5.3%)	3 (3.8%)	
Charlson comorbidity index						0.004
Mean (standard deviation)	0.6 (1.4)	0.7 (1.3)	0.5 (1.1)	0.5 (0.8)	0.7 (1.5)	
Median (interquartile range)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)	
Schizophrenia (%)	34 (8.7%)	18 (3.9%)	29 (11.1%)	13 (9.8%)	13 (16.3%)	<0.001
Bipolar (%)	231 (59.1%)	287 (61.5%)	141 (53.8%)	91 (68.9%)	54 (67.5%)	0.027
Hemolytic anemia (%)	4 (1.0%)	3 (0.6%)	1 (0.4%)	0 (0.0%)	0 (0.0%)	0.844
Use of diuretics (%)	30 (7.7%)	69 (14.8%)	25 (9.5%)	13 (9.8%)	4 (5.0%)	0.004
Use of first-generation (typical) antipsychotics (%)	11 (2.8%)	11 (2.4%)	10 (3.8%)	5 (3.8%)	7 (8.8%)	0.053
Use of mood stabilizers (%)	133 (34.0%)	197 (42.2%)	99 (37.8%)	66 (50.0%)	39 (48.8%)	0.004
Use of antidepressants (%)	241 (61.6%)	305 (65.3%)	192 (73.3%)	92 (69.7%)	53 (66.3%)	0.034
Prior use of other SGAs (%)	59 (15.1%)	79 (16.9%)	35 (13.4%)	57 (43.2%)	33 (41.3%)	<0.001
Hemoglobin A1C						0.053
Mean (standard deviation)	5.6 (0.9)	5.5 (0.8)	5.5 (0.7)	5.4 (0.5)	5.4 (0.6)	
Median (interquartile range)	5.5 (0.7)	5.4 (0.6)	5.4 (0.6)	5.3 (0.7)	5.3 (0.5)	
Hemoglobin A1C > 6.0 (%)	63 (16.1%)	56 (12.0%)	32 (12.2%)	8 (6.1%)	7 (8.8%)	0.028
Cumulative dose (100 mg-day)						<0.001
Mean (standard deviation)	2.1 (1.8)	50.0 (60.6)	0.4 (0.4)	3.0 (2.4)	22.7 (16.6)	
Median (interquartile range)	1.5 (2.3)	27.0 (48.5)	0.3 (0.5)	2.7 (3.0)	18.6 (25.4)	

^a P value: Wilcoxon rank-sum or Pearson's chi-square or Fisher's exact test.

Table 1 describes the pre-determined covariates and diagnosis of diabetes mellitus assessed from the database based on medical or pharmacy claims.

2.4. Statistical analysis

A logistic regression was used to examine the association with elevated HbA_{1c} level (HbA_{1c} >6.0) adjusted for all covariates listed in Table 2. The analyses focused on examining potential difference of HbA_{1c} >6.0 between the dose groups. We divided patients on a SGA evenly into three groups (tertiles) based on the cumulative dose, with risperidone tertile 1 as a pre-specified reference group. Since preliminary analyses showed that the proportion of elevated HbA_{1c} was similar between risperidone tertile 1 and 2, we combined the two groups into one reference to increase the statistical power. In a secondary analysis, we used patients treated with aripiprazole or ziprasidone, agents shown to have minimal metabolic, including diabetes mellitus, risk (American Diabetes Association et al., 2004; Kerwin et al., 2007; Lambert et al., 2006; Leslie and Rosenheck, 2004; Newcomer et al., 2008; Ramaswamy et al., 2007; Sernyak et al., 2002; Yood et al., 2009), as a reference group. Our preliminary analyses showed that the proportion of elevated HbA_{1c} was similar between these two groups. In order to increase statistical power, further analyses of average daily dose were performed in two dose groups based on at or above the FDA-recommended daily dose for each SGA (Hartung et al., 2008).

Comparison of elevated HbA_{1c} between SGAs can be biased if one drug group is more likely to receive medical monitoring of HbA_{1c}. Therefore, we imputed the HbA_{1c} testing rate for each SGA. We counted the number of HbA_{1c} tests over the total days of supply of each SGA. All HbA_{1c} tests (not only the first test) were counted if there was evidence of SGA use within the 30 days before the HbA_{1c} test based on all patients on a SGA with and without an HbA_{1c} test; SGA use was determined by the prescription date and days of supply. Each patient could have more than one HbA_{1c} test and be prescribed more than one SGA in the analysis of number of tests. The 95% confidence interval (CI) for the number of HbA_{1c} tests per 1000 person days of supply of a SGA was based on 1000 bootstrapping samples with replacement from the original population. Furthermore, we created an additional patient-level variable – the predicated probability of having an HbA_{1c} test. The probability was estimated from a Poisson regression that modeled the number of HbA_{1c} tests per person day of supply of a SGA based on all patients on a SGA with and without an HbA_{1c} test as described above. The model included age, gender, and region. A propensity score, a model-based predicted probability of having the treatment, has been used as a covariate to examine the treatment effect in an observational study (Rubin, 1997). Similar to a propensity score approach which has been extensively used, we believe that an unbiased relationship between SGAs and elevated HbA_{1c} should persist after adjustment for the probability of having an HbA_{1c} test.

Further analyses included assessing and adjusting more medication covariates, and using a 60 day window to assess SGA dose. Firstly, two additional covariates (β-blockers and corticosteroids) were included in the logistic regression models. These covariates were chosen based on their potential to increase the risk of hyperglycemia (Luna and Feinglos, 2001) and their frequency in the database. Secondly, we identified antidepressants and mood stabilizers that had a frequency of ≥5% and/or may increase the risk of hyperglycemia. We then conducted analyses adjusting for these additional medication covariates. Because of statistical concerns of over-adjustment resulting from too many covariates being included in a logistic regression model, these analyses were considered as sensitivity analyses. Lastly, we performed analyses with a 60 day window to assess SGA dose.

All statistical tests were two-tailed, with a $P < 0.05$ considered an indication of statistical significance. All analyses were performed using SAS, version 9.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Sample characteristics

The characteristics of the study samples are summarized in Table 2. There were significant differences between the SGA groups in age, gender, comorbidities and use of medications, and also in HbA_{1c} (Table 2). Monotherapy of the same SGA during the prior exposure period (Fig. 1) was 72.4% for olanzapine, 71.5% for quetiapine, 74.4% for risperidone, 43.2% for aripiprazole, and 51.3% for ziprasidone. Non-use of any SGA during the prior exposure period was 12.5% for olanzapine, 11.6% for quetiapine, 12.2% for risperidone, 13.6% for aripiprazole, and 7.5% for ziprasidone. Among those with an HbA_{1c} >6.0, 90.5% (57/63) of patients on olanzapine, 80.4% (45/56) of patients on quetiapine and 93.7% (30/32) of patients on risperidone also used the same SGA in the prior exposure period. The south region was over-represented across the five SGA groups compared to 2000 Census data (>50% vs. 35.6%, respectively).

3.2. Cumulative dose and elevated HbA_{1c}

Compared to lower (Tertiles 1 and 2) cumulative doses of risperidone, patients with a high cumulative dose of risperidone (Tertile 3) had a significantly higher odds ratio (OR) for the elevated HbA_{1c} (model 1 Table 3). Patients with high dose risperidone had similar OR for elevated HbA_{1c} as patients with high dose olanzapine. Overall, olanzapine was associated with a higher OR for elevated HbA_{1c} regardless of the reference group.

Other significant predictors for elevated HbA_{1c} were age (OR = 1.02; 95% CI = 1.01–1.04; $P = 0.003$) and use of diuretics (OR = 2.01; 95% CI = 1.27–3.18; $P = 0.003$) within 30 days before the HbA_{1c} test. Use of other SGAs prior to the 30 day period (largely compared to monotherapy of the same SGA throughout the prior exposure period and the 30 day window) was associated with a reduced likelihood of HbA_{1c} >6.0 (OR = 0.63; 95% CI = 0.38–1.03), although this was not statistically significant ($P = 0.066$).

3.3. Average daily dose and elevated HbA_{1c}

Analyses of average daily dose were performed on two dose groups based on at or above the FDA-recommended daily dose for each SGA (Hartung et al., 2008). The rate of HbA_{1c} >6.0 was generally higher among the higher dose groups of risperidone and quetiapine compared to their lower dose groups although none was statistically significant. However, interestingly, HbA_{1c} >6.0 rate was lower (not significant) among the higher dose group of aripiprazole or ziprasidone compared to their lower dose groups. Compared to patients on aripiprazole or ziprasidone, quetiapine ≥400 mg/day and risperidone ≥2 mg/day were associated with significantly increased OR for HbA_{1c} >6.0 (Table 4). Both olanzapine dose groups had significantly higher OR compared to aripiprazole/ziprasidone.

3.4. HbA_{1c} testing rates

The testing rate was significantly higher for olanzapine (0.214 per 1000 person days of supply; 95% CI = 0.201–0.227) and ziprasidone (0.216; 0.192–0.239) than for quetiapine (0.174; 0.165–0.183) or risperidone (0.168; 0.158–0.179), based on the non-overlapping 95% CIs. The rate was also higher for aripiprazole (0.195; 0.179–0.210).

The results from logistic regression analyses did not change significantly after adjustment for the HbA_{1c} testing probability. For example, the logistic model including the testing probability variable and all covariates listed in Table 2 showed an OR of 2.53 (1.16–5.51) for high dose risperidone and 2.51 (1.23–5.12) for high dose olanzapine compared to lower (Tertiles 1 and 2) cumulative doses of risperidone. Patients on quetiapine ≥400 mg/day had an OR of 2.30

Table 3Odds ratio (OR) and its 95% confidence interval (CI) for hemoglobin A1c (HbA_{1c}) >6.0 by tertile of the cumulative dose.

Cumulative dose 100 mg/day		No. of patients	HbA _{1c} >6.0 (%)	Model 1 ^a			Model 2 ^b		
Tertiles	Mean (median)			OR	95% CI	P	OR	95% CI	P
Olanzapine		391	16.1	(1.96	1.07–3.62	0.030) ^c	(2.32	1.26–4.30	0.007) ^d
Tertile 1	0.60 (0.65)	133	13.5	1.68	0.80–3.54	0.174	1.97	0.92–4.20	0.080
Tertile 2	1.63 (1.50)	128	15.6	1.82	0.87–3.80	0.111	2.12	1.01–4.43	0.046
Tertile 3	4.10 (3.15)	130	19.2	2.41	1.19–4.89	0.015	2.79	1.38–5.63	0.004
Quetiapine		467	12.0	(1.33	0.72–2.45	0.367) ^c	(1.59	0.86–2.95	0.137) ^d
Tertile 1	7.32 (7.50)	146	10.3	1.10	0.51–2.38	0.802	1.32	0.61–2.86	0.488
Tertile 2	26.87 (26.00)	165	13.3	1.50	0.74–3.05	0.264	1.74	0.85–3.55	0.129
Tertile 3	114.33 (90.00)	156	12.2	1.37	0.66–2.85	0.401	1.59	0.77–3.31	0.211
Risperidone		262	12.2	NA			(1.69	0.87–3.29	0.120) ^d
Tertile 1	0.10 (0.11)	87	8.1	1.00 (reference)			1.03	0.39–2.70	0.950
Tertile 2	0.32 (0.30)	84	9.5	1.00 (reference)			1.32	0.52–3.30	0.559
Tertile 3	0.88 (0.78)	91	18.7	2.45	1.13–5.32	0.023	2.72	1.26–5.87	0.011
Aripiprazole and ziprasidone		212	7.1	NA			1.00 (reference)		

^a Logistic regression model with risperidone tertile 1 and tertile 2 as the reference adjusted for all covariates in Table 2.^b Logistic regression model with aripiprazole and ziprasidone as the reference adjusted for all covariates in Table 2.^c A different model included olanzapine overall, quetiapine overall and risperidone by two groups as in model 1.^d A different model included olanzapine overall, quetiapine overall and risperidone overall and the reference group as in model 2.

(1.04–5.07) and patients on risperidone ≥ 2 mg/day had an OR of 2.34 (1.10–4.97) after adjustment for all covariates and the testing probability compared to patients on aripiprazole/ziprasidone.

3.5. Sensitivity analyses

Further adjustment for use of β -blockers ($n=164$, 12.3%) and corticosteroids ($n=172$, 12.9%) had little impact on the association between the three SGAs (olanzapine, risperidone, and quetiapine) and HbA_{1c} >6.0. For example, compared to patients on aripiprazole/ziprasidone, the ORs for patients with olanzapine ≥ 10 mg/day, olanzapine ≤ 10 mg/day, quetiapine ≥ 400 mg/day, and risperidone ≥ 2 mg/day were 2.28 (95% CI = 1.16–4.48; $P=0.017$), 2.31 (95% CI = 1.17–4.53; $P=0.016$), 2.30 (95% CI = 1.04–5.07; $P=0.040$), and 2.28 (95% CI = 1.08–4.84; $P=0.031$), respectively.

We identified seven antidepressants (bupropion, 11.2%; escitalopram, 8.6%; fluoxetine, 7.1%; paroxetine, 7.4%; sertraline, 11.1%; trazodone, 6.8%; and venlafaxine, 13.1%) and four mood stabilizers (lamotrigine, 11.6%; lithium, 10.4; topiramate, 5.5%; and valproate, 8.9%) with a frequency of $\geq 5\%$. We also included mirtazapine (3.6%) because of its possible effect on plasma glucose level (Luna and Feinglos, 2001). Adjusting these twelve additional covariates had no significant impact on the associations with HbA_{1c} >6.0 described above. For example, compared to risperidone tertile 1 and 2, the OR was 2.45 (95% CI = 1.11–5.40; $P=0.027$) for risperidone tertile 3 and 2.48 (95% CI = 1.21–5.08; $P=0.013$) for olanzapine tertile 3. In

addition, use of lithium was also significantly related to HbA_{1c} >6.0 (OR = 0.39; 95% CI = 0.16–0.93; $P=0.033$). Age and use of diuretics were still significant predictors for HbA_{1c} >6.0.

With the 60 day window to assess SGA dose ($n=1297$), the strength of the association was slightly reduced as expected, especially for risperidone. For example, compared to lower (Tertiles 1 and 2) cumulative doses of risperidone, patients with a high cumulative dose of risperidone (Tertile 3) had an OR of 1.86 (95% CI = 0.84–4.09) and patients with a high cumulative dose of olanzapine (Tertile 3) had an OR of 2.29 (95% CI = 1.16–4.53). However, patients with a high cumulative dose of risperidone (Tertile 3) still had a significantly higher OR (OR = 2.31; 95% CI = 1.02–5.24; $P=0.044$) compared to patients with aripiprazole/ziprasidone.

4. Discussion

Previous studies have focused on examining the risk of diabetes mellitus associated with SGAs by comparing the incidence of diabetes mellitus between drug groups. To our knowledge, this is the first study to examine if there is a dose effect of three SGAs on elevated HbA_{1c} level (>6.0) in a large claims database.

This study found a significantly higher risk of elevated HbA_{1c} associated with use of olanzapine. The relation persisted after adjustment for a number of covariates including the probability of having an HbA_{1c} test and with different reference groups. The results are consistent with previous pharmacoepidemiologic data on the

Table 4Odds ratio (OR) and its 95% confidence interval (CI) for hemoglobin A1c (HbA_{1c}) >6.0 by average daily dose.

Average daily dose (mg)	No. of patients	HbA _{1c} >6.0 (%)	Unadjusted			Adjusted ^a		
			OR	95% CI	P	OR	95% CI	P
Olanzapine								
≥ 10	182	16.5	2.59	1.35–4.99	0.004	2.27	1.15–4.46	0.017
<10	209	15.8	2.46	1.29–4.69	0.006	2.31	1.17–4.54	0.015
Quetiapine								
≥ 400	86	17.4	2.77	1.29–5.96	0.009	2.29	1.04–5.05	0.041
<400	381	10.8	1.58	0.85–2.93	0.144	1.41	0.74–2.66	0.297
Risperidone								
≥ 2	114	15.8	2.46	1.19–5.10	0.015	2.28	1.08–4.83	0.032
<2	148	9.5	1.37	0.64–2.94	0.415	1.25	0.57–2.74	0.585
Aripiprazole (ARI) and ziprasidone (ZIP)								
ARI ≥ 15 or ZIP ≥ 160	83	6.0	1.00 (reference)			1.00 (reference)		
ARI <15 or ZIP <160	129	7.8	1.00 (reference)			1.00 (reference)		

^a Adjusted for all covariates in Table 2.

relationship between olanzapine and diabetes mellitus (American Diabetes Association et al., 2004; Lambert et al., 2006; Leslie and Rosenheck, 2004; Ramaswamy et al., 2007; Sernyak et al., 2002; Yood et al., 2009).

There have been inconsistent results regarding the association of diabetes mellitus with risperidone (American Diabetes Association et al., 2004; Lambert et al., 2006; Leslie and Rosenheck, 2004; Ramaswamy et al., 2007; Sernyak et al., 2002; Yood et al., 2009). A large claim data analysis showed that the diabetic risk was greater for patients on olanzapine compared to patients on risperidone (Ramaswamy et al., 2007). In the current study, analysis of HbA_{1c} >6.0 based on tertile of cumulative dose showed that the relation with high dose risperidone was of the same magnitude as with high dose olanzapine. Analysis of average daily dose showed risperidone ≥ 2 mg/day (at or above FDA recommended daily dose) had a significantly higher risk of HbA_{1c} >6.0 compared to patients on aripiprazole/ziprasidone. Although HbA_{1c} >6.0 may not necessarily indicate that the patient is developing diabetes mellitus, we hypothesize that at higher cumulative doses, risperidone may have a significant impact on glycemic control and risk of diabetes mellitus.

Patients on therapeutic doses of quetiapine ≥ 400 mg/day had a significantly higher risk of HbA_{1c} >6.0 compared to patients on aripiprazole and ziprasidone. A quetiapine dose-related elevation of HbA_{1c} is similar to the findings from a review of quetiapine clinical trial results completed by the FDA (Clinical Review, 2009), which found a dose-related signal in quetiapine 600 and 800 mg/day groups with HbA_{1c} >6.1 of 4.9% and 7.7%, respectively, compared to 2.5% in the placebo group. It should be pointed out that the median exposure was 56, 42, and 56 days for 600 mg/day, 800 mg/day, and the placebo group, respectively. The reported lack of relation between quetiapine and diabetes may have been biased in some previous claims database studies if a large number of patients use quetiapine at subtherapeutic dose in real world. Our data showed that 81.6% patients on quetiapine had average daily dose below that approved dose for schizophrenia and bipolar disease (<400 mg/day) (Hartung et al., 2008). A recent study showed that quetiapine treatment was associated with impaired glucose homeostasis among schizophrenic patients (Chen et al., 2011).

The results of this study are of relevance primarily because they can be considered real world. That is, the data used is from the Ingenix LabRx database that includes medical and pharmacy claims in addition to a subset of laboratory data and is representative of a large national commercial health plan. Furthermore, no single RCT has provided HbA_{1c} results on a large number of patients treated with these five SGAs and none have used HbA_{1c} as a primary endpoint of interest. The uniqueness of this study is the use of a 30 day window to retrospectively assess SGA exposure in relation to HbA_{1c} level. We chose a 30 day pre-testing exposure window primarily because HbA_{1c} represents levels of blood glucose over the past 4 months and is disproportionately weighted to the glucose levels during the prior 4 weeks (Saudek et al., 2006). However, by no means, would our results suggest that a 30 day exposure be sufficient to cause elevated HbA_{1c} level (>6.0). Actually, among those with HbA_{1c} >6.0, most patients (>80%) also used the same SGA in the prior exposure period. The 30 day exposure window was pre-specified in the study protocol. The sensitivity analyses using a 60 day window to assess SGA dose revealed that the strength of the association was slightly reduced, the largest reduction being seen with risperidone tertile 3 when compared to lower (Tertiles 1 and 2) cumulative doses of risperidone.

One limitation of a claims data study is the lack of information on potential confounding variables such as ethnicity, weight, and family history of diabetes mellitus (Citrome et al., 2007), all of which are well-known risk factors for diabetes mellitus (Li et al., 2000; Manson et al., 2000; Shai et al., 2006). Furthermore, race and smoking have also been shown to affect olanzapine concentrations in plasma. For example, one study found that smokers cleared olanzapine 55% faster

than non/past smokers (Bigos et al., 2008). There is no information on reasons for HbA_{1c} testing and drug choice in any claims database. There is a possibility that unmeasured factors which are related to the outcome and/or the exposure could bias the results if these factors were unevenly distributed between the compared groups. We have tried to minimize the potential influences from these unmeasured factors by adjusting covariates including Charlson comorbidity index and comparing dose groups rather than drug groups. In addition, we created and adjusted for an additional patient-level covariate – the probability of having an HbA_{1c} test that can be driven by these unmeasured diabetic risk factors. It may be possible that there is a significant interaction between a risk factor such as obesity and a particular SGA on HbA_{1c} level (Reaven et al., 2009). Another limitation is that it is uncertain whether the first HbA_{1c} test in the observation period was actually the first HbA_{1c} test that the patient received. Patients may have been tested before 2002 or before they enrolled in the current health plan. In addition, they may have switched SGAs more than 30 days prior to the first observed HbA_{1c} test. However, such unobserved testing and switching would tend to generally reduce the chance of detecting a significant relationship between HbA_{1c} and SGA dose if it is non-differential. The quetiapine group had the highest rate of use of other SGAs prior to the 30 day study window among the three SGA groups; prior use of other SGAs was associated with a non-significantly reduced OR for elevated HbA_{1c}. This would suggest that these limitations may play a role in the lack of observed association between the cumulative dose of quetiapine and elevated HbA_{1c}. One other limitation is the generalizability of the study population. The Ingenix LabRx database over-represents the south region of the United States, and test results were available in only 30–40% of patients with lab claims. Also, a commercial health plan may not be representative of Medicaid or other public payers. A further limitation is that there are two commercial major labs used in the database, which may introduce measurement variability. Again, these limitations would generally introduce a bias toward the null hypothesis. Lastly, we cannot rule out completely the possibility that patients on olanzapine or a particular SGA who received HbA_{1c} testing were more prone to diabetes (or more likely to have diabetic risk factors) than patients on aripiprazole and ziprasidone who also received HbA_{1c} testing. However, our results clearly showed the HbA_{1c} testing rates in patients on olanzapine were similar to the rates in patients on aripiprazole and ziprasidone, indicating that the association of elevated HbA_{1c} with olanzapine cannot be explained by differentiating test rates. On the other hand, the possibility that patients at risk for diabetes are preferentially treated with low metabolic risk drugs such as aripiprazole or ziprasidone, and also monitoring for HbA_{1c} due entirely to their risk factors, argues against testing rates biasing the study results.

In summary, this large claims data analysis found dose-related effects of SGAs on elevated HbA_{1c} (>6.0). Both olanzapine and risperidone were associated with an increased risk of elevated HbA_{1c} at higher cumulative doses. Analyses of average daily dose revealed that quetiapine ≥ 400 mg/day, risperidone ≥ 2 mg/day, olanzapine ≥ 10 or <10 mg/day had an increased risk of elevated HbA_{1c} compared to aripiprazole/ziprasidone. Limitations of an administrative claims data analysis including lack of information on potential confounders and potential biases from differential testing rates have been discussed.

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Appendix A

Lists of medications

Second-generation (atypical) antipsychotics: generic (brand) name

Aripiprazole (Abilify)
 Olanzapine (Zyprexa)
 Quetiapine (Seroquel)
 Risperidone (Risperdal)
 Ziprasidone (Geodon)
 Clozapine (various generic products)

First-generation (typical) antipsychotics: generic name

Chlorpromazine
 Chlorprothixene
 Droperidol
 Flupentixol
 Fluphenazine
 Haloperidol
 Loxapine
 Mesoridazine
 Methotrimeprazine
 Molindone
 Perphenazine
 Pimozide
 Prochlorperazine
 Sulpiride
 Thioridazine
 Thiothixene
 Trifluoperazine
 Zuclopentixol

Mood stabilizers: generic (brand) name

Carbamazepine (Tegretol)
 Lamotrigine (Lamictal)
 Lithium (Eskalith)
 Oxcarbazepine (Trileptal)
 Topiramate (Topamax)
 Valproate (Depakote)

Antidepressants: generic (brand) name

Amitriptyline (Elavil)
 Amoxapine (Asendin)
 Bupropion (Wellbutrin)
 Citalopram (Celexa)
 Clomipramine (Anafranil)
 Desipramine (Norpramin)
 Doxepin (Sinequan)
 Duloxetine (Cymbalta)
 Escitalopram (Lexapro)
 Fluoxetine (Prozac)
 Fluvoxamine (Luvox)
 Imipramine (Tofranil)
 Isocarboxazid (Marplan)
 Mirtazapine (Remeron)
 Nefazodone (Serzone)
 Nortriptyline (Aventyl)
 Paroxetine (Paxil)
 Phenelzine (Nardil)
 Protriptyline (Vivactil)
 Selegiline (Emsam)
 Sertraline (Zoloft)
 Tranylcypromine (Parnate)
 Trazodone (Desyrel)
 Trimipramine (Surmontil)
 Venlafaxine (Effexor)

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Adjunctive Risperidone Treatment for Antidepressant-Resistant Symptoms of Chronic Military Service–Related PTSD

A Randomized Trial

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POSTTRAUMATIC STRESS DISORDER (PTSD) is among the most common and disabling psychiatric disorders among military personnel serving in combat theaters.¹⁻³ Antidepressants are the predominant pharmacotherapy for PTSD. Two serotonin reuptake inhibitors (SRIs), sertraline and paroxetine, have Food and Drug Administration approval for the treatment of PTSD based on multicenter trials.⁴⁻⁷ Within the Department of Veterans Affairs (VA), 89% of veterans diagnosed with PTSD and treated with pharmacotherapy are prescribed SRIs.⁸ However, SRIs appear to be less effective in men than in women⁴ and less effective in chronic PTSD than in acute PTSD.^{9,10} Thus, it may not be surprising that an SRI study in veterans produced negative results.¹¹

For editorial comment see p 549.

Context Serotonin reuptake-inhibiting (SRI) antidepressants are the only FDA-approved pharmacotherapies for the treatment of posttraumatic stress disorder (PTSD).

Objective To determine efficacy of the second-generation antipsychotic risperidone as an adjunct to ongoing pharmacologic and psychosocial treatments for veterans with chronic military-related PTSD.

Design, Setting, and Participants A 6-month, randomized, double-blind, placebo-controlled multicenter trial conducted between February 2007 and February 2010 at 23 Veterans Administration outpatient medical centers. Of the 367 patients screened, 296 were diagnosed with military-related PTSD and had ongoing symptoms despite at least 2 adequate SRI treatments, and 247 contributed to analysis of the primary outcome measure.

Intervention Risperidone (up to 4 mg once daily) or placebo.

Main Outcome Measures The Clinician-Administered PTSD Scale (CAPS) (range, 0-136). Other measures included the Montgomery-Asberg Depression Rating Scale (MADRS), Hamilton Anxiety Scale (HAMA), Clinical Global Impression scale (CGI), and Veterans RAND 36-Item Health Survey (SF-36V).

Results Change in CAPS scores from baseline to 24 weeks in the risperidone group was -16.3 (95% CI, -19.7 to -12.9) and in the placebo group, -12.5 (95% CI, -15.7 to -9.4); the mean difference was 3.74 (95% CI, -0.86 to 8.35 ; $t=1.6$; $P=.11$). Mixed model analysis of all time points also showed no significant difference in CAPS score (risperidone: mean, 64.43 ; 95% CI, 61.98 to 66.89 , vs placebo: mean, 67.16 ; 95% CI, 64.71 to 69.62 ; mean difference, 2.73 ; 95% CI, -0.74 to 6.20 ; $P=.12$). Risperidone did not reduce symptoms of depression (MADRS mean difference, 1.19 ; 95% CI, -0.29 to 2.68 ; $P=.11$) or anxiety (HAMA mean difference, 1.16 ; 95% CI, -0.18 to 2.51 ; $P=.09$; patient-rated CGI mean difference, 0.20 ; 95% CI, -0.06 to 0.45 ; $P=.14$; observer-rated CGI mean difference, 0.18 ; 95% CI, 0.01 to 0.34 ; $P=.04$), or increase quality of life (SF-36V physical component mean difference, -1.13 , 95% CI, -2.58 to 0.32 ; $P=.13$; SF-36V mental component mean difference, -0.26 ; 95% CI, -2.13 to 1.61 ; $P=.79$). Adverse events were more common with risperidone vs placebo, including self-reported weight gain (15.3% vs 2.3%), fatigue (13.7% vs 0.0%), somnolence (9.9% vs 1.5%), and hypersalivation (9.9% vs 0.8%), respectively.

Conclusion Among patients with military-related PTSD with SRI-resistant symptoms, 6-month treatment with risperidone compared with placebo did not reduce PTSD symptoms.

Trial Registration clinicaltrials.gov Identifier: NCT00099983

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Second-generation antipsychotics (SGAs) are commonly used medications for SRI-resistant PTSD symptoms, despite limited evidence support-

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ing this practice.^{12,13} In 2007, PTSD was the most common off-label diagnosis within the VA associated with an antipsychotic prescription.¹⁴ In 2009, 86 852 veterans diagnosed with PTSD (19.9%) received an antipsychotic prescription and 81 279 of these prescriptions (93.6%) were for SGAs.¹⁴ There are substantial safety concerns associated with SGAs, particularly risks for weight gain and extrapyramidal motor symptoms.¹⁵

The current study evaluated whether risperidone, an SGA, when added to an ongoing pharmacotherapy regimen would be more effective than placebo for reducing chronic military-related PTSD symptoms among veterans whose symptoms did not respond to at least 2 adequate SRI treatments. To our knowledge, this study is the first large trial of a pharmacotherapy aimed at SRI-resistant PTSD symptoms.

METHODS

Patients were eligible if they were at least 18 years old, participated in a military combat theater, met diagnostic criteria for military service-related chronic PTSD on the basis of a structured interview for making psychiatric diagnoses according to the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition) (*DSM-IV*),¹⁶ had a Clinician-Administered PTSD Scale (CAPS) score greater than 50,¹⁷ had a clinical history of intolerance of or nonresponse to 2 or more antidepressants, and had an inadequate response to 2 adequate SRI treatments (minimum of 4 weeks of pharmacotherapy each). Other eligibility criteria included having a fixed address within 50 miles of the research site or confirmed transportation for all visits, using an acceptable method of birth control (female patients), and giving written informed consent.

Patients were excluded if they met lifetime diagnostic criteria for bipolar disorder or schizophrenia; required antipsychotic medication for the treatment of psychosis; met diagnostic criteria for dependence on a substance other than nicotine in the 30 days prior

to screening; had clinical or laboratory evidence (levels of aspartate aminotransferase, alanine aminotransferase, bilirubin, blood urea nitrogen, or creatinine) of hepatic or renal compromise; had a medical disorder that might increase the risks of risperidone treatment (insulin-dependent diabetes) or complicate interpretation of study results (epilepsy, dementia); had a history of intolerance of antipsychotics; attempted suicide or assaulted someone in the prior year; or had an impending legal incarceration. Although ongoing pharmacotherapy was allowed, patients receiving SGAs, serotonergic (5HT₂) receptor antagonists (cyproheptadine, methysergide, trazodone), α_1 receptor antagonists (prazosin), and α_2 receptor agonists/antagonists (clonidine, guanfacine, mirtazapine) were excluded initially.

Race and ethnicity of the participants were determined by self-reports with concurrence by the rater.

Interventions

The human subjects subcommittees of the VA Cooperative Studies Program and each participating VA Medical Center approved this study. All patients gave written informed consent prior to study entry. An independent data safety monitoring board monitored patient safety throughout the study.

Patients were randomized to receive double-blinded 6-month treatment with risperidone or matched placebo. Study medication (risperidone 1 mg or matching placebo) was initiated at a dose of 1 tablet orally at bedtime and increased by 1 tablet per week to a dose of 3 tablets at bedtime. After participants received study medication for 4 weeks, investigators who were blinded to study medication status and were treating patients had the option of further increasing the dose by 1 tablet (1 mg), providing medications were well tolerated and a dose increase was indicated clinically.

Prior to study entry, patients and their primary mental health care clinicians developed a treatment plan that would not violate study protocol and

would be engaged if study medications were ineffective. These alternative treatments enabled some patients to remain as participants for the full 6 months of randomized treatment (eTable 1, available at <http://www.jama.com>). There were no significant differences across groups in the frequency with which these adjunctive medications from particular classes were initiated during the clinical trial.

Patients participated in a feedback program that was designed to enhance adherence to prescribed medications.^{18,19} Medication was provided in bottles with microelectronic monitor caps (MEMS; AARDEX Group, Union City, California) that recorded the date and time of each opening and showed the number of hours elapsed since the previous opening. The Medication Usage Skills for Effectiveness feedback system,¹⁸ in which data on the previous month's dosing were shown to patients at each visit, encouraged patients to take medication daily by training them to develop and use reminders that supported medication adherence.

Randomization and Treatment

Patients were recruited initially from 20 VA Medical Centers over a 2-year period. To address low recruitment rates and other issues, 8 sites were discontinued and 6 sites were added during the course of the study. A total of 26 sites were approved by the human subjects subcommittee to enroll patients into the study. In addition, the recruitment period was extended by 6 months, and patients who had initially been considered ineligible to participate in the study because they were receiving certain drugs (trazodone ≤ 100 mg, nefazodone ≤ 100 mg, quetiapine ≤ 25 mg, and mirtazapine ≤ 30 mg) were allowed if the drugs were prescribed for at least 3 months prior to screening and prescribed at the current dose for at least 1 month. A total of 83 patients (42 in the risperidone group, 41 in the placebo group) who were ultimately enrolled in this study had received at least 1 of these medications. Secondary analyses testing the effect of broaden-

ing the study entry criteria did not find any effects on the findings for the principal outcome measures.

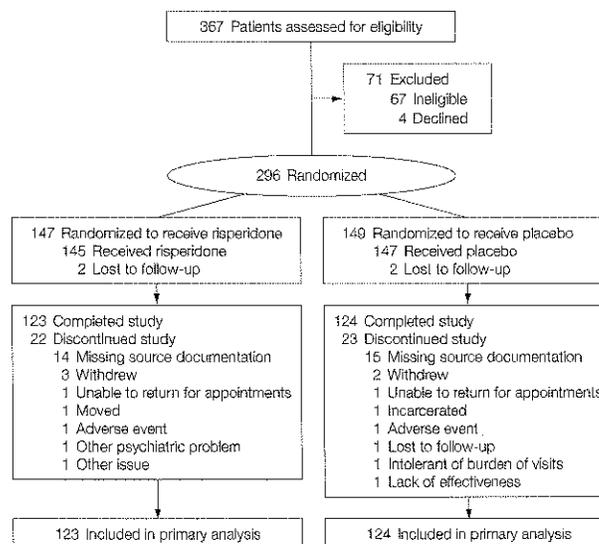
Randomized assignment of patients to treatment groups was conducted by the Cooperative Studies Program Coordinating Center (Perry Point, Maryland). Calls requesting randomization went to a central location on the day the patient was deemed eligible and ready to start medication. Separate randomization schedules were generated for each participating center, assigning equal numbers of patients to each of the groups. Block sizes of 2 and 4 were used to balance assignments across groups and to prevent decoding of the system. Assignments were stratified within centers. Patients were evaluated to ensure they met all eligibility criteria before a randomization code was provided. Treatment was initiated within a day of randomization.

Outcome Measures

The primary outcome measure for this study was the total score on the 34-item CAPS.²⁰ This scale was administered by trained raters who were blind to the randomization status of patients at baseline and weeks 6, 12, and 24. All raters underwent initial training and credentialing to administer and score the primary and secondary outcome measures. They also completed annual training and reliability checks during the study to ensure that they met at least 80% reliability of their measurement; all raters eventually met this reliability standard. Interrater reliability was assessed at 2 annual subsequent time points. All raters showed 100% diagnostic accuracy at both sessions, and median scores were within 0.5 points and 3 points at the 2 annual follow-ups, respectively.

The CAPS provided an overall measure of PTSD symptom severity. Secondary outcomes were assessed each time the CAPS was administered: the observer-rated and patient-rated Clinical Global Impression scale (CGI), the Montgomery-Asberg Depression Rating Scale (MADRS),²¹ the Hamilton Anxiety Scale (HAMA),²² a scale used to rate psycho-

Figure 1. Recruitment Flowchart in Clinical Trial of Risperidone Treatment for Military Service-Related Posttraumatic Stress Disorder



Valid baseline data were collected for 267 patients; the primary outcome analysis included 247 patients for whom a valid week-24 CAPS assessment was obtained.

sis (Positive and Negative Syndrome Scale [PANSS]),²³ the Veterans RAND 36-Item Health Survey (SF-36V),²⁴ the 26-item Boston Life Satisfaction Inventory (BLSI),²⁵ and a service utilization measure. At each visit, smoking was assessed using the first 3 items of the Fagerström Scale,²⁶ and alcohol consumption was evaluated using the timeline follow-back method for the 90 days prior to study entry and the interval between each visit.²⁷ Motor adverse events associated with risperidone were assessed using the Barnes Akathisia Scale,²⁸ the Extrapyramidal Symptom Rating Scale,²⁹ and the Abnormal Involuntary Movement Scale.³⁰ On all reported outcome measures except the SF-36V, higher scores reflect higher symptom levels. On the SF-36V, higher scores reflect higher quality of life.

Data Analyses

Data were collected and analyzed by the VA Cooperative Studies Program. Baseline characteristics were compared with χ^2 and *t* tests as appropriate.

The primary outcome measure in this study was the intent-to-treat analysis of

the improvement in PTSD symptoms from baseline to week-24 follow-up as measured by the CAPS. A 2-tailed *t* test was performed on these data using an $\alpha = .05$. This study was powered initially to detect a 9-point difference between the treatment groups in the CAPS change score; assuming a 20% dropout rate and a power of 0.9, a target sample size of 205 patients per group was required. In the absence of a validated threshold for minimal important difference on the CAPS, the threshold of 9 points was derived from data suggesting the following: (1) a 9-point decrease would be predicted to produce clearly evident changes in core PTSD symptoms^{31,32}; (2) 9 points was estimated to be approximately 0.5 SD in severely symptomatic veterans with PTSD,³³ and across medical conditions score reductions of 0.5 SD are generally found to be a minimal important difference³⁴; and (3) 9- to 10-point decreases would be expected to be associated with improvements in measures of quality of life.³⁵

The recruitment rate was lower than projected, with a total of 296 randomized patients rather than the targeted 410.

However, both the dropout rate and the variance in the data were lower than projected, offsetting the effects of the actual sample size on the statistical power of the study. Two hundred forty-seven patients (123 per group, for purposes of power calculation) completed the study. Based on the original parameters for study sample size, an $\alpha = .05$, and the estimated pooled 18.4 SD, this sample size provided 96.9% power to detect a 9-point difference between the groups in the primary outcome measure—ie, the difference between baseline and week-24 CAPS scores.

In secondary and exploratory analyses, the CAPS, its subscales, and all other continuous outcome measures were analyzed using mixed models,³⁶

covarying for baseline values and using all available outcome data. The models initially had fixed effects for treatment group and time. The interactions between treatment and time effects were dropped because they were not significant in reported analyses. Site and patient were treated as random effects. Generalized least squares means of treatment effect were computed within the SAS mixed linear models procedures (MIXED and GLIMMIX) used to analyze outcome data (SAS Institute, Cary, North Carolina). These least squares means are estimators of the treatment means that would be expected for a balanced design.

In post hoc analyses, the severity of the 3 component clusters of PTSD

symptoms associated with DSM-IV-TR diagnostic criteria³⁷—reexperiencing, avoidance/numbing, and hyperarousal²⁰—were analyzed separately with Bonferroni adjustments for multiple comparisons. Also, treatment effects on PTSD severity categories based on the CAPS³² were analyzed using a 2-tailed χ^2 test. This analysis yielded an estimate of medication effects on remission rates in this study as defined by a CAPS score of less than 20.³⁸

A comparison of the treatment groups on retention in the study was based on survival analysis of time (days) receiving study medication as measured from the day of randomization to the day of last dose. Survival curves for study retention were estimated for each treatment group with Kaplan-Meier methodology (SAS procedure LIFETEST), and treatment group comparisons were based on the log-rank test.

RESULTS

Of the 26 sites that were approved to enroll patients into the study, 23 sites enrolled patients from February 2007 to August 2009, with follow-up ending in February 2010. A total of 367 patients screened yielded 296 patients diagnosed with military-related PTSD with clinically significant SRI-resistant PTSD symptoms who signed consent forms from 23 sites (FIGURE 1). Valid diagnostic and primary outcome data were collected on 267 patients randomized to receive risperidone ($n = 133$) and placebo ($n = 134$) treatment.

The study populations included severely ill patients, many of whom had disabilities related to long-standing military-related PTSD (TABLE 1, TABLE 2, and TABLE 3). The sample was predominately male ($n = 258$, 96.6%), middle-aged (mean [SD] age, 54.4 [10.7] years), non-Hispanic white ($n = 177$, 66.3%), and married ($n = 140$, 52.4%) or divorced ($n = 60$, 22.5%). Most patients served during the Vietnam war or earlier ($n = 193$, 72.3%) or the wars in Iraq and Afghanistan ($n = 63$, 23.6%). Their PTSD symptoms were attributed principally to direct participa-

Table 1. Baseline Demographic Data

	Risperidone (n = 133)	Placebo (n = 134)	Total (N = 267)	P Value
Age, mean (SD), y	54.2 (10.8)	54.5 (10.6)	54.4 (10.7)	.82 ^a
Sex, No. (%)				
Male	128 (96.2)	130 (97.0)	258 (96.6)	.75 ^b
Female	5 (3.8)	4 (3.0)	9 (3.4)	
Race/ethnicity, No. (%)				
White, not Hispanic	84 (63.2)	93 (69.4)	177 (66.3)	.57 ^b
Black, not Hispanic	25 (18.8)	25 (18.7)	50 (18.7)	
Hispanic	16 (12.0)	11 (8.2)	27 (10.1)	
Other	8 (6.0)	5 (3.7)	13 (4.9)	
Weight, mean (SD), lb ^c	205.3 (38.8)	214.0 (46.1)	209.6 (42.7)	.11 ^a
Marital status, No. (%) ^d				
Single	20 (15.0)	19 (14.2)	39 (14.6)	.29 ^b
Married	67 (50.4)	73 (54.5)	140 (52.4)	
Widowed	0	2 (1.5)	2 (0.7)	
Divorced	36 (27.1)	24 (17.9)	60 (22.5)	
Separated	5 (3.8)	10 (7.5)	15 (5.6)	
Living with partner	5 (3.8)	5 (3.7)	10 (3.7)	
Education, mean (SD), y ^e	14.2 (2.7)	14.1 (2.2)	14.1 (2.5)	.82 ^a
Employment (current), No. (%) ^d				
Full time	49 (36.8)	48 (35.8)	97 (36.3)	.94 ^b
Part time	6 (4.5)	4 (3.0)	10 (3.7)	
Irregular, part time	7 (5.3)	7 (5.2)	14 (5.2)	
Unemployed	17 (12.8)	21 (15.7)	38 (14.2)	
Other	54 (40.6)	53 (39.6)	107 (40.1)	
Military history, No. (%)				
WWI, WWII, Korea, Vietnam	95 (71.4)	98 (73.1)	193 (72.3)	.67 ^b
Gulf War, Afghanistan, Iraq	34 (25.6)	29 (21.6)	63 (23.6)	
Balkans, other war	1 (0.8)	3 (2.2)	4 (1.5)	
Peace time	3 (2.3)	4 (3.0)	7 (2.6)	

Abbreviations: WWI, World War I; WWII, World War II.

^at Test.

^bFisher exact test.

^cData were missing for 13 patients.

^dData were missing or incorrect for 1 patient in the placebo group (0.4% of total patients).

^eData were missing for 2 patients.

tion in combat (n=209, 78.3%). The majority of patients in this study also met lifetime diagnostic criteria for major depression (n=186, 69.7%) and lifetime alcohol abuse or dependence (n=167, 62.5%). Smaller numbers of patients were smokers (n=88, 33.0%) or met diagnostic criteria for other lifetime substance abuse or dependence, antisocial personality disorder, or other mood/anxiety disorders.

Most patients in this study received VA service-connected disability compensation (n=223, 83.5%), of which 181 (81.2%) and 163 (73.1%) had psychiatric and medical disability, respectively. More than one-third of patients (n=99, 37.1%) received a Social Security pension. Patients in this study received typical psychosocial treatments at the medical centers. Based on data collected with a service utilization measure, patients had received the following VA services in the month preceding study entry: 195 patients (74.1%) had received outpatient mental health treatment; 43 patients (16.4%), case management; 16 (6.1%), readjustment counseling; and 15 (5.7%), addiction services. Less than 5% of the sample received any other specified service. There were no significant differences between the groups in service utilization.

The patients in this study were highly symptomatic at study baseline despite long-standing individualized pharmacologic treatments (mean [SD] medications per patient: risperidone, 3.09 [1.69]; placebo, 2.86 [1.46]) (eTable 2 and eTable 3). There were no significant differences in the frequency with which medications other than SRIs were prescribed across the groups prior to randomization or in various combinations of medications (eTable 4). Mean (SD) CAPS total score at study entry was 78.2 (14.8), associated with high levels of reexperiencing (20.9 [6.4]), avoidance/numbing (31.5 [8.1]), and hyperarousal (25.9 [4.9]) symptoms. Patients were significantly depressed (mean [SD] MADRS score, 23.4 [8.2]) and anx-

Table 2. Disability and Service Utilization at Baseline^a

	Risperidone (n = 133)	Placebo (n = 134)	Total (N = 267)	P Value
VA disability pension, No. (%)				
Yes	112 (84.2)	111 (82.8)	223 (83.5)	.87 ^b
No	21 (15.8)	23 (17.2)	44 (16.5)	
Medical disability, No. (%)				
Yes	83 (74.1)	80 (72.1)	163 (73.1)	.76 ^b
No	29 (25.9)	31 (27.9)	60 (26.9)	
Medical disability, mean (SD), % ^d	34.5 (31.8)	31.8 (23.5)	33.2 (28.0)	.55 ^c
Psychiatric disability, No. (%)				
Yes	89 (79.5)	92 (82.9)	181 (81.2)	.61 ^b
No	23 (20.5)	19 (17.1)	42 (18.8)	
Psychiatric disability, mean (SD), % ^a	63.4 (28.1)	65.3 (25.2)	64.4 (26.6)	.62 ^c
Social Security pension, No. (%)				
Yes	50 (37.6)	49 (36.6)	99 (37.1)	.90 ^b
No	83 (62.4)	85 (63.4)	168 (62.9)	
VA service use, No. (%)				
Outpatient mental health	102 (77.9)	93 (70.5)	195 (74.1)	.40 ^b
Case management	21 (16.0)	22 (16.7)	43 (16.4)	.81 ^b
Alcohol/drug abuse clinic	8 (6.1)	7 (5.3)	15 (5.7)	.74 ^b
Rehabilitation program	6 (4.6)	1 (0.76)	7 (2.7)	.13 ^b
Readjustment counseling	9 (6.9)	7 (5.3)	16 (6.1)	.75 ^b

Abbreviation: VA, Veterans Administration.

^aVA compensation and pension boards rule on the presence or absence of a VA service-connected disability. The disability may be related to medical or psychiatric disorders. The extent of disability ranges from 0% to 100%.

^bFisher exact test.

^cT Test.

^dData were missing for 60 patients.

^eData were missing for 42 patients.

ious (mean [SD] HAMA score, 19.4 [7.8]), with low levels of psychotic symptoms (mean [SD] PANSS positive symptom score, 11.6 [3.9]).

Retention

Rates of retention while receiving randomized treatment were high and did not differ by group (log-rank test $\chi^2=0.71$, $P=.40$) (eFigure 1). However, patients treated with placebo continued receiving assigned medication on average approximately 1 week longer than patients treated with risperidone (risperidone: median, 166.5 days; mean, 133.1 days; 95% confidence interval [CI], 123.6-142.6 days; placebo: median, 167.0 days; mean, 148.9 days; 95% CI, 141.5-156.4 days; $t=2.59$; Satterthwaite $df=238.87$; $P=.01$).

Treatment Effects

There were no significant effects of risperidone treatment on the primary outcome measure, the change in CAPS total score from baseline to 24 weeks (risperidone: -16.3; 95% CI, -19.7 to

-12.9; placebo: -12.5; 95% CI, -15.7 to -9.4; mean difference, 3.74; 95% CI, -0.86 to 8.35; $t=1.6$; $P=.11$). In the mixed model of CAPS total scores, the effect of medication was also not significant ($F_{1,253}=2.30$; $P=.13$), but symptom scores decreased over time in both groups ($F_{2,488}=9.94$; $P<.001$) (FIGURE 2 and TABLE 4). Baseline CAPS score ($F_{1,253}=257.67$; $P<.001$), but not the war in which the veteran served, was associated with higher CAPS score throughout the study. Neither effect interacted significantly with medication group and controlling for their effects did not alter the findings.

To further explore whether risperidone produced clinically significant changes on the CAPS, the distribution of patients in each treatment group was determined following a published categorization of PTSD status³² (0-19, asymptomatic/few symptoms; 20-39, mild PTSD/subthreshold; 40-59, moderate PTSD/threshold; 60-79, severe PTSD symptomatology; and >80, extreme PTSD symptomatology). This

analysis did not reveal significant differences across treatment groups ($\chi^2=4.9$; $P=.30$). This analysis also provided information about the rate of remission of patients in each group because a CAPS score of less than 20 is a validated remission threshold.³⁸ The rate of remission in patients treated with placebo (4%) did not differ significantly from patients treated with risperidone (5%) (eFigure 2).

In post hoc Bonferroni-adjusted analyses ($P=.02$) of CAPS subscales using mixed regression models, risperidone was associated with significantly reduced symptoms as measured by the CAPS reexperiencing subscale ($F_{1,253}=8.16$, $P=.005$, $d=0.298$) and the CAPS hyperarousal subscale (treatment: $F_{1,253}=8.09$, $P=.005$, $d=0.318$; treatment \times week interaction: $F_{2,486}=4.11$, $P=.02$), but

not the CAPS avoidance/numbing subscale ($F_{1,253}=1.23$, $P=.27$). Assuming a 0.5-SD threshold for the minimal clinically important difference, the statistically significant findings for the CAPS subscales do not meet this threshold. This suggests that although statistically significant, the changes on the CAPS scales would not be recognized by many clinicians as meaningful.

Consistent with the CAPS findings, no medication effects on the observer-rated version ($\chi^2=3.88$, $P=.049$) or self-rated version ($\chi^2=1.88$, $P=.17$) of the CGI were significant after Bonferroni adjustments for multiple comparisons (significance threshold: $P=.008$). Also there were no significant drug effects on anxiety (HAMA score: $F_{1,249}=3.20$, $P=.08$), depression (MADRS score: $F_{1,248}=2.02$, $P=.16$), psychosis (PANSS positive symptom score: $F_{1,250}=0.43$, $P>.10$), or quality of life (SF-36V physical component score: $F_{1,248}=2.24$, $P=.14$).

Adverse Events

Adverse events that occurred in at least 5% of the overall sample are reported in eTable 5. Overall, the rate of adverse events during treatment was low but appeared related to dosing of risperidone. The study protocol targeted a risperidone dose of 3 mg/day and allowed clinicians to increase the dose to 4 mg if indicated. With these instructions, the modal medication dose was 4 mg for both groups. By the end of the study, patients randomized to receive placebo were receiving 3.35 mg of placebo on average, suggesting that clinicians were satisfied with the clinical progress of many patients treated with placebo. However, patients randomized to risperidone were receiving on average a dose of 2.74 mg. This suggests, consistent with our clinical impressions, that adverse effects limited some patients from achieving the target dose of 3 mg. This study was unable to determine whether adverse effects limited the efficacy of risperidone, but perhaps these data suggest that future studies should explore doses lower than 3 mg of risperidone.

Table 3. Mental Health Conditions and Measures at Baseline

	No. (%)			P Value
	Risperidone (n = 133)	Placebo (n = 134)	Total (N = 267)	
PTSD symptom attribution				
Direct participation in combat	108 (81.2)	101 (75.4)	209 (78.3)	.70 ^a
Other combat-related events	12 (9.0)	17 (12.7)	29 (10.9)	
Physical or sexual abuse	7 (5.3)	8 (6.0)	15 (5.6)	
Other event during military service	6 (4.5)	8 (6.0)	14 (5.2)	
Alcohol^{b,c}				
Absent	46 (34.6)	53 (39.6)	99 (37.1)	.67 ^a
Abuse	27 (20.3)	24 (17.9)	51 (19.1)	
Dependence	60 (45.1)	56 (41.8)	116 (43.4)	
Cannabis^{b,c}				
Absent	98 (73.7)	103 (76.9)	201 (75.3)	.67 ^a
Abuse	20 (15.0)	15 (11.2)	35 (13.1)	
Dependence	15 (11.3)	15 (11.2)	30 (11.2)	
Cocaine^{b,c}				
Absent	111 (83.5)	107 (79.9)	218 (81.6)	.77 ^a
Abuse	10 (7.5)	10 (7.5)	20 (7.5)	
Dependence	12 (9.0)	16 (11.9)	28 (10.5)	
No. of cigarettes per day^c				
0	86 (64.7)	92 (68.7)	178 (66.7)	.51 ^a
≥ 1	47 (35.3)	41 (30.6)	88 (33.0)	
Major depression^{b,c}				
Absent	34 (25.6)	31 (23.1)	65 (24.3)	.65 ^a
Subthreshold	9 (6.8)	6 (4.5)	15 (5.6)	
Threshold	90 (67.7)	96 (71.6)	186 (69.7)	
Dysthymia^{b,c}				
Absent	116 (87.2)	115 (85.8)	231 (86.5)	.70 ^a
Subthreshold	5 (3.8)	3 (2.2)	8 (3.0)	
Threshold	12 (9.0)	15 (11.2)	27 (10.1)	
Generalized anxiety disorder^{b,c}				
Absent	113 (85.0)	117 (87.3)	230 (86.1)	.77 ^a
Subthreshold	5 (3.8)	4 (3.0)	9 (3.4)	
Threshold	15 (11.3)	12 (9.0)	27 (10.1)	
Social phobia^{b,c}				
Absent	122 (91.7)	123 (91.8)	245 (91.8)	>.99 ^a
Subthreshold	6 (4.5)	6 (4.5)	12 (4.5)	
Threshold	5 (3.8)	4 (3.0)	9 (3.4)	
Antisocial personality disorder^b				
Absent	119 (89.5)	125 (93.3)	244 (91.4)	.20 ^a
Subthreshold	4 (3.0)	1 (0.7)	5 (1.9)	
Threshold	10 (7.5)	6 (4.5)	16 (6.0)	
Missing data	0	2 (1.5)	2 (0.7)	

(continued)

However, there were significantly more cases in the group treated with risperidone of self-reported weight gain (risperidone: $n=20$, 15.3%; placebo: $n=3$, 2.3%), fatigue (risperidone: $n=18$, 13.7%; placebo: $n=0$), somnolence (risperidone: $n=13$, 9.9%; placebo: $n=2$, 1.5%), and hypersalivation (risperidone: $n=13$, 9.9%; placebo: $n=1$, 0.8%) (eTable 5). Risperidone did not increase measured weight significantly ($F_{1,235}=2.86$, $P=.09$). Also, there were no significant effects of risperidone on the 3 measures of extrapyramidal symptoms in this study, the Barnes Akathisia Scale, the Extrapyramidal Symptom Rating Scale, and the Abnormal Involuntary Movement Scale.

COMMENT

In this study, there was no statistically significant difference between risperidone and placebo in reducing CAPS total scores when prescribed for 6 months as an adjunct to SRIs and other ongoing medication and psychosocial treatments in a group of highly symptomatic veterans with medication-resistant symptoms associated with chronic military-related PTSD. Compared with placebo, risperidone produced only a 3.74-point greater reduction from baseline in the CAPS total score. Thus, it is unlikely that clinicians could detect the magnitude of the risperidone effect over placebo that was observed in this study. In addition, risperidone was not statistically superior to placebo on any of the secondary outcomes, including the observer- and self-rated versions of the Clinical Global Impressions scale; quality of life (SF-36V or BLSI); and measures of depression (MADRS), anxiety (HAMA), or paranoia/psychosis (PANSS positive symptom subscale).

Adverse events associated with risperidone were not serious. Post hoc analyses of the CAPS, adjusted for multiple comparisons, suggested that risperidone was associated with a significant reduction in reexperiencing and hyperarousal symptoms associated with PTSD with a small effect size. Although the findings were significant sta-

Table 3. Mental Health Conditions and Measures at Baseline (continued)

	No. (%)			P Value
	Risperidone (n = 133)	Placebo (n = 134)	Total (N = 267)	
CAPS score, mean (SD)				
Total	78.2 (15.0)	78.2 (14.7)	78.2 (14.8)	>.99 ^d
Part B (reexperiencing)	20.9 (6.6)	20.8 (6.2)	20.9 (6.4)	.83 ^d
Part C (avoidance/numbing)	31.1 (8.1)	31.9 (8.1)	31.5 (8.1)	.40 ^d
Part D (hyperarousal)	26.2 (5.1)	25.5 (4.7)	25.9 (4.9)	.27 ^d
PCL score, mean (SD)				
Total ^e	64.1 (10.6)	63.6 (11.7)	63.9 (11.2)	.72 ^d
Part B (reexperiencing) ^f	18.2 (4.1)	18.4 (4.2)	18.3 (4.1)	.69 ^d
Part C (avoidance/numbing) ^g	25.8 (5.2)	25.6 (5.6)	25.7 (5.4)	.71 ^d
Part D (hyperarousal) ^h	19.9 (3.4)	19.4 (4.0)	19.7 (3.7)	.26 ^d
OGI, observer rated, mean (SD) ^f	5.1 (0.9)	5.0 (0.9)	5.0 (0.9)	.08 ^d
MADRS, mean (SD) ⁱ	24.3 (7.3)	22.5 (9.0)	23.4 (8.2)	.08 ^d
HAMA, mean (SD) ^j	19.7 (8.1)	19.2 (7.5)	19.4 (7.8)	.60 ^d
PANSS score, mean (SD) ^f				
Total	59.4 (13.8)	59.4 (14.3)	59.4 (14.1)	.97 ^d
Positive symptoms	11.5 (3.7)	11.7 (4.2)	11.6 (3.9)	.62 ^d
Negative symptoms	14.0 (4.7)	13.7 (4.9)	13.9 (4.8)	.67 ^d
General	33.9 (7.6)	34.0 (7.8)	34.0 (7.7)	.94 ^d
Pittsburgh Sleep Scale total score, mean (SD) ^k	13.8 (3.9)	13.6 (3.9)	13.7 (3.9)	.77 ^d
BLSI score, mean (SD) ^l	101.5 (25.5)	104.4 (29.6)	102.9 (27.6)	.41 ^d
SF-36V PCS score, mean (SD) ^m	30.3 (9.8)	31.3 (11.3)	30.8 (10.6)	.44 ^d
SF-36V MCS score, mean (SD) ^m	39.2 (11.8)	39.7 (10.6)	39.5 (11.2)	.69 ^d

Abbreviations: BLSI, Boston Life Satisfaction Inventory; CAPS, Clinician-Administered PTSD Scale; OGI, Clinical Global Impression; HAMA, Hamilton Anxiety Scale; MADRS, Montgomery-Asberg Depression Rating Scale; PANSS, Positive and Negative Syndrome Scale; PCL, PTSD Checklist; PTSD, posttraumatic stress disorder; SF-36V PCS and MCS, Veterans RAND 36-Item Health Survey physical component subscale and mental component subscale.

^aFisher exact test.

^bBased on lifetime DSM-IV diagnosis.

^cData were missing or incorrect for 1 patient in the placebo group (0.4% of total patients).

^d χ^2 Test.

^eData were missing for 13 patients.

^fData were missing for 1 patient.

^gData were missing for 10 patients.

^hData were missing for 7 patients.

ⁱData were missing for 2 patients.

^jData were missing for 3 patients.

^kData were missing for 23 patients.

^lData were missing for 18 patients.

^mData were missing for 4 patients.

tistically, these changes were smaller than the 0.5-SD threshold used to define the minimal important difference in estimating the sample size for this study.³⁴ Thus, it is questionable whether the observed changes on these subscales would be detected clinically.

However, this study could not rule out the possibility that risperidone treatment addressed a real clinical need for some patients. The ability of risperidone to reduce reexperiencing and hyperarousal symptoms, such as disrupted sleep and autonomic arousal, is consistent with its ability to block 5-HT_{2A} and α_1 adrenergic receptors.³⁹ This hypothesis is supported by the

widespread prescription of trazodone, a 5-HT₂ receptor antagonist, for sleep impairment associated with PTSD.⁴⁰ It is also consistent with the increasing evidence of the efficacy of prazosin, an α_1 adrenergic receptor antagonist, for treating reexperiencing and hyperarousal symptoms of PTSD.⁴¹⁻⁴³

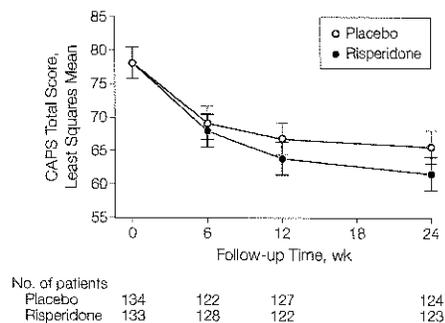
The lack of efficacy of adjunctive risperidone on CAPS total scores and global outcome measures in this study contrasts with positive findings from some smaller randomized trials^{41,44} but is consistent with a study of SRI-resistant civilian PTSD.⁴⁵ However, the lack of risperidone efficacy on avoidance/emotional numbing symptoms

Table 4. Follow-up Assessment Outcomes Based on Least Squares Mean Estimates With All Available Data Up to 24 Weeks^a

Variable	Mean (95% CI)		Mean Difference (95% CI)	P Value
	Risperidone	Placebo		
CAPS score				
Total	64.43 (61.98 to 66.89)	67.16 (64.71 to 69.62)	2.73 (-0.74 to 6.20)	.12
Part B (reexperiencing)	15.54 (14.58 to 16.49)	17.55 (16.60 to 18.50)	2.01 (0.66 to 3.37)	.004
Part C (avoidance/numbing)	27.98 (26.77 to 29.19)	26.93 (25.72 to 28.13)	-1.05 (-2.76 to 0.66)	.23
Part D (hyperarousal)	20.99 (20.16 to 21.83)	22.70 (21.87 to 23.54)	1.71 (0.53 to 2.89)	.005
HAMA	15.80 (14.86 to 16.75)	16.97 (16.02 to 17.92)	1.16 (-0.18 to 2.51)	.09
MADRS	19.24 (18.19 to 20.29)	20.43 (19.39 to 21.48)	1.19 (-0.29 to 2.68)	.11
BLSI	104.62 (102.02 to 107.22)	104.30 (101.64 to 106.95)	-0.32 (-4.04 to 3.40)	.87
SF-36V PCS	39.66 (38.63 to 40.68)	38.53 (37.50 to 39.55)	-1.13 (-2.58 to 0.32)	.13
SF-36V MCS	33.80 (32.48 to 35.13)	33.55 (32.22 to 34.87)	-0.26 (-2.13 to 1.61)	.79
PANSS score				
Total	55.77 (54.24 to 57.30)	55.56 (54.03 to 57.09)	-0.21 (-2.37 to 1.96)	.85
General symptoms	31.69 (30.81 to 32.56)	31.80 (30.93 to 32.68)	0.12 (-1.12 to 1.35)	.85
Positive symptoms	10.65 (10.26 to 11.05)	10.85 (10.46 to 11.25)	0.20 (-0.35 to 0.75)	.48
Negative symptoms	13.45 (12.96 to 13.94)	12.88 (12.39 to 13.37)	-0.57 (-1.26 to 0.13)	.11
CGI, patient rated	4.49 (4.30 to 4.67)	4.68 (4.50 to 4.86)	0.20 (-0.06 to 0.45)	.14
CGI, observer rated	4.32 (4.20 to 4.43)	4.49 (4.38 to 4.61)	0.18 (0.01 to 0.34)	.04
Weight, lb	211.86 (210.48 to 213.25)	210.18 (208.78 to 211.58)	-1.68 (-3.66 to 0.29)	.09

Abbreviations: BLSI, Boston Life Satisfaction Inventory; CAPS, Clinician-Administered PTSD Scale; CGI, Clinical Global Impression; CI, confidence interval; HAMA, Hamilton Anxiety Scale; MADRS, Montgomery-Asberg Depression Rating Scale; PANSS, Positive and Negative Syndrome Scale; PTSD, posttraumatic stress disorder; SF-36V PCS and MCS, Veterans RAND 36-Item Health Survey physical component subscale and mental component subscale.

^aFor all outcomes, the treatment comparison was a linear contrast based on a mixed-effects model with site as a random effect and with autocorrelated repeated measures over time. On all reported outcome measures except SF-36V, higher scores reflect higher symptom levels; higher scores on SF-36V reflect higher quality of life.

Figure 2. Change in CAPS Total Score During Treatment

CAPS indicates Clinician-Administered Posttraumatic Stress Disorder Scale. Error bars indicate 95% confidence intervals.

and the relatively greater efficacy for hyperarousal or reexperiencing symptoms appear to be consistent with findings of prior risperidone studies.¹² Second-generation antipsychotics have been proposed as a treatment strategy for paranoia or other psychotic symptoms associated with PTSD.^{46,47} However, positive symptoms of psychosis were at very low levels at baseline in this study. Thus, this study does not inform the question of whether risperi-

done would be a useful adjunct to treatment in paranoid or psychotic patients with PTSD.

This study has several limitations. This study did not achieve the prespecified sample size of 410 patients projected for this study. Further, source documentation for 29 patients was inadvertently lost, invalidating their data. These 29 patients (9.8% of all randomized study participants) were enrolled at 2 of the original study sites. After the

loss of data was discovered, the 29 patients were excluded from further analyses and enrollment was discontinued at both sites. At 1 of these sites, enrollment was later restarted with a new site investigator. Because our analyses controlled for clustering by study site, it is unlikely that the loss of patient data from these 2 sites would have biased the results, which were based only on patient data from the 23 other study sites. In addition, the study participants in these 2 sites were balanced with respect to treatment group (14 in the risperidone group and 15 in the placebo group), so pre-existing biases were likely to have been distributed equally across treatment groups. Even after excluding these 29 patients, our study had adequate statistical power to detect a clinically meaningful benefit of risperidone, if a true benefit had existed.

Patient retention in the study was greater than expected and variance within the data was less than expected. Based on the 247 patients who completed the study and the prespecified factors in the power analysis, this study had 96.6% power to detect a

9-point difference in the ability of risperidone and placebo to reduce CAPS total score during treatment, a change that might be considered a minimal important difference. However, even if the full projected sample had been recruited, this study most likely would not have yielded statistical significance for the small differential change in CAPS total scores produced by risperidone and placebo (3.74 points).

A second limitation is that study entry criteria were relaxed because of recruitment problems; patients were accepted who had long-standing prescriptions of low doses of commonly prescribed sleep medications, particularly trazodone and quetiapine. Although adjusting for this effect did not alter the findings with respect to the CAPS, including these patients may have reduced the expected effects of risperidone in the current study. Third, it is not clear that the findings generalize to other SGAs, such as olanzapine or quetiapine, that may have somewhat different clinical profiles in PTSD.¹⁰ Fourth, it remains to be determined whether the findings generalize to women because the study population was nearly entirely men. Analyses conducted to adjust for the effect of differing combat theaters did not alter the findings related to the primary outcome measure, but this study was not designed explicitly to explore the interaction of combat theater and treatment response. Fifth, this study evaluated the efficacy of adjunctive risperidone treatment, and the findings may not generalize to risperidone prescribed by itself for the treatment of PTSD.

In summary, risperidone, the second most widely prescribed SGA within VA for PTSD and the best data-supported adjunctive pharmacotherapy for PTSD,¹² did not reduce overall PTSD severity (CAPS total score), produce global improvement (CGI score), or increase quality of life (SF-36V) in patients with chronic SRI-resistant military-related PTSD symptoms. Overall, the data do not provide strong support for the current wide-

spread prescription of risperidone to patients with chronic SRI-resistant military-related PTSD symptoms, and these findings should stimulate careful review of the benefits of these medications in patients with chronic PTSD.

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Author Contributions: Ms Jones had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Post-traumatic Stress Disorder and Cardiovascular Disease

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Abstract: This review provides an up-to-date summary of the evidence from clinical and epidemiologic studies indicating that persons with post-traumatic stress disorder (PTSD) may have an increased risk of coronary heart disease and possibly thromboembolic stroke. Persons with PTSD, a common anxiety disorder in both veteran and nonveteran populations, have been reported to have an increased risk of hypertension, hyperlipidemia, obesity, and cardiovascular disease. Increased activity of the sympathoadrenal axis may contribute to cardiovascular disease through the effects of catecholamines on the heart, vasculature, and platelet function. Reported links between PTSD and hypertension and other cardiovascular risk factors may partly account for reported associations between PTSD and heart disease. The associations observed between PTSD and cardiovascular diseases have implications for cardiology practice and research.

Keywords: Anxiety disorders, coronary heart disease, hypertension, hyperlipidemia, post-traumatic stress disorder, stroke, veterans.

INTRODUCTION

An increasing body of evidence indicates that post-traumatic stress disorder, a common anxiety disorder in both veteran and nonveteran populations, is associated with major forms of cardiovascular disease including those attributed to atherosclerosis such as coronary heart disease and thromboembolic stroke. Persons with PTSD have also been reported to be more likely to have hypertension, hyperlipidemia, obesity, and cardiovascular disease [1]. These findings are important to the field of cardiology since coronary heart disease may develop over time as a result of hemodynamic factors (for example, elevated blood pressure with turbulence and shear stress within coronary arteries), hyperlipidemia, and events such as the rupture of atherosclerotic plaques and thrombus formation [2]. This review summarizes cardiovascular alterations linked to PTSD including results from epidemiologic and clinical studies and possible biological mechanisms.

BACKGROUND

Individuals may develop PTSD after being exposed to a traumatic event such as combat experiences, a motor vehicle crash, or sexual assault [3]. Symptoms of PTSD may include nightmares, intrusive thoughts, or other re-experiencing phenomena, the avoidance of situations that remind the person of the traumatic event, a feeling of numbness or being socially detached from family and friends, and hyper-arousal (for example, feeling angry, irritable and "on edge," or having difficulty concentrating). Hyper-arousal or hyper-vigilance includes a rapid and pronounced reaction to

stressors which may lead to a preoccupation with signs of threat and emotional distress. Persons with PTSD may have other challenges such as difficulties with employment, relationships, or other health conditions (for example, depression, alcohol abuse or drug dependency).

Effective psychological and medical treatments for PTSD include group or individual psychotherapy (for example, cognitive-behavioral therapy) and pharmacotherapy such as the use of selective serotonin reuptake inhibitors [4]. Cognitive-behavioral therapy helps patients to address their traumatic memories and distorted cognitions (for example, by providing education about the nature of PTSD and stress responses and helping the individual with the integration of the traumatic events).

CARDIOVASCULAR ALTERATIONS ASSOCIATED WITH PTSD

Cardiovascular alterations associated with autonomic arousal and cardiovascular health outcomes have long been reported to be associated with PTSD or wartime traumatic exposure [1, 5]. Persons suffering from PTSD and chronic PTSD have been shown to have increases in basal heart rate and blood pressure and increased heart rate and blood pressure in response to stimuli such as loud sounds and visual slides that remind them of the trauma [6-9]. In clinical studies involving small samples of veterans, plasma norepinephrine and 24-hour urine norepinephrine levels have been reported to be elevated among veterans with PTSD as compared to those without PTSD [10]. The increases in plasma norepinephrine are more pronounced when PTSD patients are exposed to trauma-related stimuli such as loud tones [11]. Stress and anxiety have been associated with increased plasma and urinary norepinephrine, epinephrine, and their metabolites, which are peripheral measures of the noradrenergic system, in healthy adults [8, 12].

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The effects of traumatic exposures or chronic stress on the hypothalamic pituitary adrenal axis (HPA) and the autonomic nervous system have been examined in clinical studies and in animal models. The results of these studies indicate that PTSD can result in important neurobiologic and psychophysiological changes [1]. Physiologic dysregulation of the HPA axis and altered autonomic function may contribute to increases in cardiovascular risk factors reported in persons with PTSD. Increased activity of the sympathoadrenal axis might contribute to cardiovascular disease through the effects of catecholamines on the heart, vasculature, and platelet function [8]. Platelet function is altered by elevated levels of circulating catecholamines. Catecholamines act on alpha-2a receptors on platelet membranes leading to increased platelet aggregation and other changes in platelet function [8, 13]. Catecholamine-induced alterations of platelet activity have been hypothesized to be a link between chronic stress, increased sympathoadrenal activation, and cardiovascular disease [13, 14].

Studies have shown that patients with PTSD have higher heart rates at rest and reduced heart rate variability which is consistent with increased sympathetic activity [8, 15]. The finding that baseline heart rate is higher among veterans suffering from PTSD than among those without PTSD is consistent with chronic hyperstimulation of the autonomic nervous system. Alternatively, the finding could be an artifact due to the research participants being anxious about the impending psychophysiological assessment [16]. The individuals who participated in the studies may have experienced anxiety because they were anticipating exposure to stimuli that would remind them of traumatic events [8]. McFall *et al.* [17] examined basal heart rates, systolic and diastolic blood pressures among veterans with and without PTSD over an extended period and did not find any significant differences between the two groups. However, in a separate study by Gerardi *et al.* [6] which included 32 Vietnam veterans with combat-related PTSD and 26 Vietnam era veterans with no combat exposures, those with PTSD had significantly higher heart rate, systolic and diastolic blood pressure. Buckley and Kaloupek [18] completed a meta-analysis of reported studies of basal heart rate and blood pressure among persons with and without PTSD. A total of 34 studies were included with a total sample size across studies of 2,670 subjects. Their results suggested that, on average, persons with PTSD have an elevated basal heart rate as compared with persons without PTSD or those who were not exposed to trauma [18]. The average difference in resting heart rate between persons with or without PTSD was 5 beats per minute. Their meta-analysis also suggested that PTSD is associated with blood pressure elevations [18].

STUDIES OF PTSD AND HYPERTENSION

PTSD was associated with an increased risk of hypertension in the National Comorbidity Survey and in an epidemiologic study of Vietnam veterans from Australia [19, 20]. Since elevated diastolic and systolic blood pressure are established risk factors for cardiovascular disease, the apparent link between PTSD and hypertension may partly account for reported associations between PTSD and heart disease [1]. Cohen *et al.* [15] examined associations between PTSD and hypertension and other cardiovascular risk factors using national data from veterans of Operation Enduring Freedom

and Operation Iraqi Freedom (OEF/OIF) who sought care at VA health care facilities. The majority of the PTSD patients in their cross-sectional study had comorbid mental health diagnoses including depression (53%), other anxiety disorder (29%), substance abuse disorder (10%) and other psychiatric diagnoses (33%). Veterans with mental health diagnoses had a significantly higher frequency of hypertension and other cardiovascular disease risk factors [15]. For example, among 65,603 male OEF/OIF veterans who had PTSD with or without other mental health diagnoses, the adjusted odds ratio for the association between PTSD and hypertension was 2.88 (95% confidence interval 2.79-2.97) after controlling for age, race (white, black, Hispanic, or other), component type, rank, branch of service, and multiple deployments [15]. Among 6,964 female OEF/OIF veterans who had PTSD with or without other mental health diagnoses, the adjusted odds ratio for the association between PTSD and hypertension was 2.88 (95% confidence interval 2.79-2.97) after controlling for age, race/ethnicity (white, black, Hispanic, or other), component type, rank, branch of service, and multiple deployments [15].

PTSD AND HYPERLIPIDEMIA

There is increasing evidence from clinical studies that PTSD may have effects on lipid metabolism [21, 22]. Karlovic *et al.* [23] examined total cholesterol, LDL and HDL cholesterol, and triglycerides in Croatian war veterans with PTSD and patients with major depression. Those with PTSD had higher levels of cholesterol and LDL cholesterol, and triglycerides, on average, and lower HDL cholesterol levels as compared with the patients with major depression. In the study by Cohen *et al.* [15] of associations between PTSD and cardiovascular risk factors among OEF/OIF veterans who sought care at VA health care facilities, veterans with mental health diagnoses had a significantly higher frequency of dyslipidemia [15]. For example, among 65,603 male OEF/OIF veterans who had PTSD with or without other mental health diagnoses, the adjusted odds ratio for the association between PTSD and dyslipidemia was 2.70 (95% confidence interval 2.63-2.78) after controlling for age, race/ethnicity (white, black, Hispanic, or other), component type, rank, branch of service, and multiple deployments [15]. Among 6,964 female OEF/OIF veterans who had PTSD with or without other mental health diagnoses, the adjusted odds ratio for the association between PTSD and dyslipidemia was 2.68 (95% confidence interval 2.44-2.95) after controlling for age, race/ethnicity (white, black, Hispanic, or other), component type, rank, branch of service, and multiple deployments [15]. Elevated levels of total cholesterol and triglycerides have also been observed among Brazilian police officers with PTSD [24].

STUDIES OF PTSD AND CORONARY HEART DISEASE

Positive associations between PTSD and cardiovascular disease (particularly coronary heart disease) have been observed in a growing number of studies of veterans and civilians who were exposed to combat or other traumatic experiences, as summarized in Table 1. Some of these studies were retrospective, relied upon self-reported information about cardiovascular disease, or had other design limitations [2, 25-31, 35, 36]. Nevertheless, an increasing number of

Table 1. Studies of PTSD and Cardiovascular Disease among Veterans and Civilian Populations Exposed to Traumatic Experiences

Study	Sample	Study Design	Results	Limitations	Other Information
Falger <i>et al.</i> (1992)	Male WW II Dutch Resistance veterans (n=147), aged 60-65 years, and age and sex-matched controls with recent hospitalization for MI (n=65) or surgery (n=79).	Clinical interviews of surviving veterans conducted more than 4 decades after the war had ended. PTSD was assessed using structured interviews based on DSM-III.	The Resistance veterans, especially those with PTSD, scored higher than the matched controls on angina pectoris, type A behavior, life stressors, and vital exhaustion. About 10% of the veterans reported having had an MI in the past 15 years. About 56% percent of the veterans were currently suffering from PTSD.	The use of controls with recent MI may have partly obscured associations with cardiovascular risk factors. History of MI was based on self-reported information.	Half of these Resistance veterans had been arrested and incarcerated in Nazi prisons and forced labor and death camps. All were exposed to extraordinary war-time trauma.
Boscarino (1997)	National sample of male Vietnam veterans (n=1,399) who served in the U.S. Army.	In-person interviews conducted about 20 years post combat exposure. Circulatory diseases were assessed retrospectively.	After controlling for age, race, region of birth, enlistment status, volunteer status, Army marital status, Army medical profile, smoking history, substance abuse, education, income, and other factors, lifetime PTSD status was associated with reported circulatory diseases (OR = 1.62, p = .007) and other illnesses after military service. About 63% (n=332) had a lifetime history of PTSD.	Self-reported information about disease history was used in the analysis. The response rate was 65%.	
Boscarino and Chang (1999)	National sample of male U.S. Army veterans who served in theatre during the Vietnam war (n=2,490) or during the same era (n=1,972).	Medical examinations (conducted about 17 years after combat exposures for Vietnam theatre veterans). Psychiatric evaluations included the Diagnostic Interview Schedule based on DSM-III.	After controlling for age, place of service, illicit drug use, medication use, race, body mass index, alcohol use, cigarette smoking, and education, PTSD was associated with ECG findings including atrioventricular conduction defects (OR =2.81, 95% CI 1.03-7.66, p < 0.05) and infarctions (OR=4.44, 95% CI 1.20-16.43, p < 0.05).	The overall participation rate was 60%. Soldiers who served in theatre may have had greater exposure to toxic chemicals.	The average age of first onset of PTSD was 21 years.
Boscarino (2006)	National sample of male U.S. Army veterans (n=15,288) who served during the Vietnam War era.	Cohort mortality study with 16 years of follow-up following completion of a telephone survey (or about 30 years after their military service).	After controlling for race, Army volunteer status, entry age, and discharge status, Army illicit drug abuse, age, and other factors, PTSD among Vietnam theatre veterans was associated with cardiovascular mortality (hazards ratio = 1.7, p = 0.034), all-cause mortality, cancer, and external causes of death.	Adjustment was made for pack-years of cigarette smoking only when looking at cancer mortality.	
Boscarino (2008)	National sample of male Vietnam veterans (n=4,328) who served in the U.S. Army. The men were < 65 years of age at follow-up.	Cohort mortality study	PTSD was assessed using two measures include one based on DSM-III. Having more PTSD symptoms was positively associated with early-age heart disease mortality.		
Dobie <i>et al.</i> (2004)	Female veterans (n=1,259) who received care at the VA Puget Sound Health Care System between October 1996 and January 1998	Cross-sectional postal survey	Of the eligible women who completed the survey, 21% screened positive for current PTSD (PTSD Checklist-Civilian Version score \geq 50). A statistically nonsignificant association was observed with myocardial infarction or coronary artery disease (OR = 1.8, 95% CI 0.9-3.6).	Study limitations include the cross-sectional design and the reliance on self-reported information about medical conditions.	

Table 1. contd....

Study	Sample	Study Design	Results	Limitations	Other Information
Kang <i>et al.</i> (2006)	Former WW II prisoners of war (n=19,442) and non POW controls (n=9,728)	Review of healthcare utilization data for 10 years (1991-2000) from VA and non-VA healthcare providers.	After adjustment for age and race, former POWs with PTSD had statistically significant increased risks of CVD, including ischemic heart disease and hypertension, as compared with both non-POWs and POWs without PTSD. The magnitude of the increased risk of ischemic heart disease was modest.	POWs might be more likely than the study controls to be in VA medical treatment files.	
Schnurr <i>et al.</i> (2000)	Male combat veterans of WW II and the Koren conflict (n=605). The average age at study entry was 43.9 years. The majority of the men (98%) were white.	Follow-up study. Medical examinations were performed periodically beginning in 1960. PTSD symptoms were assessed in 1990.	PTSD was assessed using the Mississippi Scale for Combat-Related PTSD. PTSD symptoms were positively associated with the onset of arterial disorders (hazard ratio =1.3, 95% CI 1.2-1.5) after controlling for age, smoking, alcohol consumption, and body mass index. The hazard ratios for hypertensive and ischemic cardiovascular disease were not significantly different than one.	PTSD was not measured at the beginning of the study but rather in 1990 after many of the outcomes had already occurred.	
Kubzansky <i>et al.</i> (2007)	Community dwelling men (n=1,002) from the greater Boston, Massachusetts area who were aged 21 to 80 years in 1961. Over 90% of the men are veterans and most were white. Men with preexisting coronary heart disease or diabetes	Prospective cohort study.	PTSD was assessed using the Mississippi Scale for Combat-Related PTSD. For each standard deviation increase in PTSD symptom level, the age-adjusted relative risk for nonfatal and fatal myocardial infarction combined was 1.3 (95% CI 1.05-1.5).		The data were from the VA Normative Aging Study.
Kubzansky <i>et al.</i> (2009)	Community dwelling women who participated in the Baltimore cohort of the Epidemiologic Catchment Area Study (n=1,059)	Prospective cohort study that assessed incident coronary heart disease over a 14-year period	Past year trauma and associated PTSD symptoms were assessed using the NIMH Diagnostic Interview Schedule. Women with 5 or more symptoms of PTSD were over three times more likely to develop coronary heart disease than those with no symptoms (age-adjusted OR = 3.2, 95% CI 1.3-8.0). The association persisted after further adjustment was made for coronary risk factors and depression or trait anxiety.		
Dirkzwager <i>et al.</i> (2007)	Sample of adult survivors (n=896) of a fire disaster in Enschede, Netherlands that killed 23 persons and destroyed or damaged almost 1,500 houses.	Longitudinal design. Electronic medical records from family practitioners (1 year and 4 years post disaster) were used. Survey data were also collected at 3 weeks and 18 months post disaster to assess PTSD and physical health.	The Self-Rating Scale for PTSD was used to assess the condition. After controlling for demographic factors, smoking, and predisaster physical health, PTSD was positively associated with risk of new vascular problems (OR = 1.9, 95% CI 1.04-3.6).		

Table 1. contd....

Study	Sample	Study Design	Results	Limitations	Other Information
Spitzer <i>et al.</i> (2009)	Community dwelling adults in Germany (n=3,171)	Cross-sectional survey	PTSD was assessed using the Structured Clinical Interview for DSM-IV. After controlling for demographic factors, smoking, body mass index, blood pressure, depression, and alcohol use disorders, PTSD was positively associated with angina (OR = 2.4, 95% CI 1.3-4.5), heart failure (OR = 3.4, 95% CI 1.9-6.0), and peripheral arterial disease.	Study limitations include the cross-sectional design and the reliance on self-reported information about medical conditions.	
Johnson <i>et al.</i> (2010)	Male residents of four U.S. communities (n=5,347)	Population-based study of the prevalence of subclinical atherosclerosis (carotid intima thickness and carotid plaque) measured noninvasively at two study visits (1987-1989 and 1990-1992).	Compared to non-combat veterans, non-veterans and combat veterans had higher age-adjusted mean carotid intima thickness. Differences remained for combat veterans after adjustment for race, father's education, and age at service entry but not years of service. No differences in carotid plaque were noted.	PTSD was not assessed in this study.	The data were from the Atherosclerosis Risk in Communities (ARIC) Study.

studies have prospectively examined PTSD as a predictor of physician-diagnosed cardiovascular disease [32-34]. Taken overall, these results from observational research provide considerable evidence that persons with PTSD have an increased risk of coronary heart disease morbidity and mortality.

PTSD AND CEREBRAL VASCULAR DISEASE

There is some evidence from epidemiologic studies of an association between PTSD and cerebrovascular disease. Brass and Page [37] found that former World War II prisoners of war (POWs) had a statistically nonsignificant increased risk of stroke. Among the 475 former POWs, 12.7% (20 of 158) of those with PTSD had strokes, compared with 7.6% (24 of 317) without PTSD (relative risk = 1.7, 95% confidence interval 0.95 to 2.9). In a cross-sectional survey of female veteran's who received care at the VA Puget Sound Health Care System, Dobie *et al.* [29] found an association between PTSD and self-reported history of stroke. About 5% (13 of 256) of the female veterans with PTSD reported a history of stroke as compared with 3% (28 of 905) of those without PTSD (age-adjusted odds ratio = 2.9, 95% confidence interval 1.4-6.0) [29]. A study of trauma and PTSD among 3,171 male and female adults living in the general population of a German community found that persons with a history of trauma had a higher odds of stroke (odds ratio = 1.2, 95% confidence interval 1.0-1.5), angina pectoris, and heart failure after adjustment for demographic factors, blood pressure, smoking, body mass index, depression, and alcohol-related disorders [35].

DISCUSSION

This review of the published literature highlights evidence from epidemiologic and clinical studies that persons

with PTSD are at increased risk of cardiovascular disease including coronary heart disease and possibly stroke. These findings have several implications for cardiologists and cardiovascular researchers. Anxiety disorders such as PTSD are common in the general population [4]. In addition, combat veterans have an increased risk of PTSD [3, 4]. Clinicians who see patients in primary care practice or in cardiology clinics should be aware that longstanding PTSD may have deleterious effects on the cardiovascular system including increased risk of coronary heart disease and hypertension. Cardiologists routinely talk with patients about alcohol drinking and cigarette smoking, both of which may be more frequent among persons suffering from PTSD. Effective evidence-based treatments are available for persons with PTSD including those who suffer from dual diagnoses such as PTSD and alcohol dependence [4].

The biological mechanisms that account for the observed associations between PTSD and cardiovascular disease may relate to the effects of traumatic exposures and chronic stress on the HPA axis and the autonomic nervous system [8]. Dysregulation of the HPA axis and chronic over-stimulation of the autonomic nervous system may contribute to the increases in blood pressure and lipid levels that have been observed in PTSD patients. Catecholamine-induced alterations of platelet activity may also contribute to the apparent link between PTSD and cardiovascular disease. Changes in immune function seen in some PTSD patients may also have a role including circulating levels of interleukin-6 (IL-6), IL-1, tumor necrosis factor, and C-reactive protein [38]. Inflammatory mediators such as IL-6, tumor necrosis factor, and C-reactive protein have been reported to stimulate atherosclerosis [38]. Interactions among the immune and neuroendocrine systems may partly account for associations between PTSD and chronic disease outcomes.

Previous authors have proposed general models of possible mechanisms underlying the relationship between PTSD and physical health including cardiovascular disease [31]. The models take into account biological function (e.g., HPA axis, heightened noradrenergic function, immune function), psychological comorbidities such as depression, health risk behaviors, symptom reports and functional status, and disease morbidity and mortality. A similar model of PTSD outcomes proposed by Boscarino accounts for different pathways leading to changes in health status; the influences of heredity, shared environment, history of trauma, behavior and perceptions, biological changes, and stressful life event exposures are taken into account in the model [5].

In conclusion, persons with PTSD have been reported to have an increased risk of hypertension, hyperlipidemia, obesity, and cardiovascular disease. Such persons have been observed to have an increased risk of coronary heart disease and possibly thromboembolic stroke. The reported link between PTSD and hypertension and other cardiovascular risk factors may partly account for the observed associations between PTSD and heart disease.

ABBREVIATIONS

DSM	=	Diagnostic and Statistical Manual of Mental Disorders
LDL	=	Low Density Lipoprotein
HPA	=	Hypothalamic Pituitary Adrenal Axis
IL-6	=	Interleukin-6
LDL	=	Low Density Lipoprotein
OR	=	Odds Ratio
OEF/OIF	=	Operation Enduring Freedom/Operation Iraqi Freedom
POWs	=	Prisoners of War
PTSD	=	Post-traumatic Stress Disorder
VA	=	Department of Veterans Affairs

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Hypercoagulation in Chronic Post-Traumatic Stress Disorder

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ABSTRACT: **Background:** Whereas procoagulation abnormalities in acute stress are well established, little is known about the mechanism of hypercoagulation in chronic stress, such as post-traumatic stress disorder (PTSD). This is crucial, given the fact that chronic coagulation disturbances have been associated with increased morbidity and premature mortality due to thromboembolism and cardiovascular disorders, complications recently described in PTSD patients.

Objectives: To explore the mechanisms of hypercoagulation in chronic PTSD.

Methods: Thirty patients diagnosed with chronic PTSD were enrolled and compared with a control group matched for age, gender and ethnicity. Hypercoagulation state was evaluated by levels of fibrinogen, D-dimer, prothrombin fragment F 1+2, von Willebrand factor (vWF) antigen, factor VIII activity, activated protein C resistance, ProC Global assay, and tissue factor antigen. Psychiatric evaluation was performed using the Mini-International Neuropsychiatric Interview and Clinician Administered PTSD Scale (CAPS).

Results: vWF antigen levels were significantly higher in patients with chronic PTSD compared with the controls (121.3 ± 42 vs. 99.7 ± 23 , respectively, $P = 0.034$). Higher levels of vWF antigen and factor VIII activity were found in patients with severe chronic PTSD (CAPS > 80), compared to controls and patients with chronic PTSD and less severe symptoms (CAPS ≤ 80). However, no differences were observed in any other studied coagulation parameters between patients and controls.

Conclusions: Increased levels of vWF antigen and factor VIII activity were documented in severe chronic PTSD. These findings suggest that the higher risk of arterial and venous thromboembolic events in PTSD patients could be related to endothelial damage or endothelial activation.

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KEY WORDS: chronic post-traumatic stress disorder (PTSD), hypercoagulation, von Willebrand factor, factor VIII, thromboembolic events

Acute stress has been extensively studied and is known to correlate with procoagulant changes such as increased levels of fibrinogen, clotting factors VII, VIII, XII and von Willebrand factor [1], especially in the elderly. However, the data on chronic psychological stress and coagulation are still contradictory. This information is particularly important, since chronic stress diseases, such as post-traumatic stress disorder, may affect 8% of the population [2] and persist for a lifetime in the vast majority of cases. PTSD was recently described as a concurrent psychiatric and somatic disorder [3], given the common cardiovascular complication, which could be partially explained by possible coagulation disturbances. PTSD may develop as a result of life-threatening traumatic events and is diagnosed by the presence of three clusters of symptoms: re-experiencing, avoidance, and hyperarousal for at least 1 month [2]. In acute PTSD the duration of symptoms is limited to 3 months; after this period it is considered to be chronic [2]. In a recent study, PTSD-like symptoms were found in approximately 10% of Israelis exposed to a long wave of terrorist attacks in 2002 [4]. Chronic PTSD has been associated with poor physical health [5] and premature mortality due to venous and arterial thromboembolism, even when depression is controlled [3,6]. In PTSD, biological factors such as lower cortisol levels, increased sympathetic activity [7] and resting mean blood pressure [8] have been shown to be related to a hypercoagulable state, reflected by an increased amount of procoagulant molecules, providing a plausible biopsychological link to coronary artery disease [9]. Patients with PTSD develop a low grade systemic inflammatory state [10], suggesting a mechanism that could contribute to coronary heart disease. The mediators of this mechanism could be stress hormones (norepinephrine) producing a cascade of inflammatory reactions (interleukin 6, IL-1, C-reactive protein, tumor necrosis factor-alpha, leptin, resistin and angiotensin II) [11], which may culminate with the metabolic syndrome, elevated blood pressure, obesity, dyslipidemia, diabetes, heavy smoking and low physical activity level that are associated with PTSD and are major risk factors for coronary artery disease [12].

PTSD = post-traumatic stress disorder
IL = interleukin

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Inflammation can induce local thrombosis which, in turn, can amplify inflammation and this cross-talk contributes to atherosclerosis progression [13]. A positive and partially independent correlation was revealed between the severity of acute PTSD and plasma levels of both factor VIII and fibrinogen [14]. In addition, markers of endothelial dysfunction, i.e., soluble tissue factor, and vWF were found to be associated with acute PTSD and partly affected by psychobiological distress [15]; however, the data concerning a possible link between chronic PTSD, its symptom severity and hypercoagulation are relatively limited.

The aim of the current study was to assess the levels of hypercoagulation parameters in patients with chronic PTSD compared to matched controls who were exposed to the same trauma.

PATIENTS AND METHODS

The study group included 30 civilians diagnosed as suffering from chronic PTSD after the Second Lebanon War in summer 2006. Study participants were recruited at the Center for Anxiety and Trauma Disorders of the Rambam Health Care Campus between October and December 2007 and were diagnosed by senior psychiatrists as suffering from chronic PTSD for more than a year. The study was approved by the Rambam Institutional Review Board and all patients signed an informed consent form.

Inclusion criteria were age 18–70, diagnosis of chronic PTSD, and the ability to give informed consent. Participants were excluded from the study if they had a history of psychiatric disorder, major cardiac or thromboembolic events, evidence of acute infectious disease, a diagnosis of cancer, or if they were pregnant, drug abusers, or receiving anticoagulant therapy. The control group included 30 healthy civilians matched for age, gender and ethnicity, exposed to the same war trauma, who had not developed PTSD or other psychiatric disorders.

PSYCHIATRIC ASSESSMENT

All study participants were invited for one clinic visit during which they underwent psychiatric assessment to confirm the diagnosis of chronic PTSD and its severity. Assessment instruments included the MINI and the CAPS. The MINI (Mini-International Neuropsychiatric Interview) is an abbreviated psychiatric structured interview evaluating major adult Axis I disorders using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and International Classification of Diseases-10 (ICD-10). MINI elicits all the symptoms listed in the symptom criteria for DSM-IV and for ICD-10 for 15 major Axis I diagnostic categories, one Axis II disorder and for suicidality. Its diagnostic algorithms are con-

sistent with DSM-IV and ICD-10 diagnostic algorithms. The CAPS (Clinician Administered PTSD Scale) is a structured interview assessing PTSD diagnostic status, symptom severity, core and associated symptoms. It evaluates the frequency and intensity of each symptom using standard prompted questions and explicit, behaviorally anchored rating scales [16]. In the current study, a CAPS score > 80 corresponded to severe PTSD, and a CAPS score ≤ 80 corresponded to mild PTSD.

COAGULATION STUDIES

During the same visit, venous blood samples of patients and controls were collected by venipuncture into tubes with 3.2% sodium citrate for coagulation studies. Blood samples were centrifuged at 2000 g for 15 minutes. Prothrombin time, activated partial thromboplastin time, fibrinogen, ProC Global assay and activated protein C resistance tests were performed on fresh plasma samples. All other coagulation assays were performed on thawed frozen plasma samples. Plasma samples were frozen after a second centrifugation at 2000 g for 15 minutes in aliquots at -70 ± 5°C. Prior to testing, plasma aliquots were thawed in a 37 ± 0.5°C water bath for 15 min. PT, PTT, fibrinogen, ProC Global assay and APC-R were performed on the STA-R evolution analyzer (Diagnostica Stago, Gennevilliers, France). Recombinant human thromboplastin Dade Innovin® (Dade Behring Marburg GmbH, Germany) was used for PT assay. STA-PTT®, STA-FIBRINOGEN and STA-LLATEST® D-DI kits were employed for PTT, fibrinogen and D-dimer assays, respectively (Diagnostica Stago). The ProC Global assay kit (Dade Behring) and Coatest APC resistance kit (Chromogenix – Instrumentation Laboratory SpA Milan, Italy) were used for the relevant tests.

Levels of coagulation factor VIII activity were determined by one-stage assay using factor VIII deficiency plasma (Diagnostica Stago). Levels of vWF antigen were evaluated using the STA-LLATEST® vWF:ag kit (Diagnostica Stago). Prothrombin fragment F1+2 concentration, as a marker of prothrombin activation, was measured by an enzyme-linked immunosorbent assay using Enzygnost® F1+2 (monoclonal) (Dade Behring). Tissue factor antigen levels were determined with the IMUBIND® Tissue Factor ELISA kit (American Diagnostica Inc., Stamford, CT).

STATISTICAL ANALYSIS

Data were analyzed using the SPSS statistical software package. Differences between the two groups in coagulation parameter levels and other continuous variables (age, years of education) were estimated with the *t*-test. Differences

CAPS = Clinician Administered PTSD Scale
 PT = prothrombin time
 PTT = partial thromboplastin time
 APCR = activated protein C resistance
 vWF = von Willebrand factor
 F1+2 = fragment F1+2

MINI = Mini-International Neuropsychiatric Interview

between the two groups in categorical demographic variables (gender, marital status, employment status and CAPS) were checked by Pearson chi-square or Fisher's exact test. Linear correlation between CAPS scores and the level of each of the coagulation factors as well as between CAPS scores and education duration was analyzed using Pearson correlation. $P < 0.05$ was considered significant.

RESULTS

Table 1 shows key demographic and ethnic characteristics of the two groups. The patients with chronic PTSD and the controls were matched in all criteria apart from education duration and employment status. Patients had higher PTSD symptom scores compared with controls; the mean CAPS score was 89 ± 25 for patients and 0.9 ± 1.4 for controls. In addition, PTSD patients had higher symptom levels of anxiety, suicidal thoughts and depression; almost 73% of PTSD patients had comorbid major depression and 26% had suicidal thoughts.

vWF antigen levels were found to be significantly higher in patients with chronic PTSD as compared with controls. No differences were documented in other studied coagulation factor levels between the two groups [Table 2]. Significantly higher levels of vWF antigen and factor VIII activity were observed in patients with severe chronic PTSD (CAPS > 80) compared with controls and 10 patients with mild chronic PTSD (CAPS ≤ 80) [Table 3].

Factor VIII levels correlated with those of vWF antigen in both patient and control groups ($r = 0.7$, $P < 0.0001$). However, factor VIII levels correlated with F1+2 only in the patient group ($r = 0.4$, $P = 0.04$), and with fibrinogen levels only in the control group ($r = 0.4$, $P = 0.02$). No correlation was found between the CAPS scores and the level of any of the coagulation factors, or education duration.

Among patients with chronic PTSD, no differences were found in any of the studied coagulation parameters between subjects with comorbid major depression or suicidal thoughts and those with PTSD only, or between employed and unemployed individuals. In the patient group, no correlation was

Table 2. Plasma coagulation parameters in PTSD patients compared with controls

Plasma parameters (mean ± SD)	Patients (n=30)	Controls (n=30)	P value
PT (sec)	9.8 ± 1	9.9 ± 1	0.651
PTT (sec)	33.7 ± 3	34.8 ± 3	0.178
D-dimer (mg/L)	0.4 ± 0.4	0.35 ± 0.3	0.842
Fibrinogen (mg/dl)	322.8 ± 60	343.1 ± 66	0.324
Protein C global assay (PCAT-NR)	0.77 ± 0.13	0.78 ± 0.11	0.796
APC sensitivity ratio**	2.43 ± 0.23	2.42 ± 0.25	0.8
vWF antigen (u/ml)	121.3 ± 42	99.7 ± 23	0.034
Factor VIII activity (u/ml)	123.8 ± 31	111.3 ± 28	0.208
Tissue factor (pg/L)	36.4 ± 21	39.8 ± 20	0.279
Prothrombin F1+2 (pmol/L)	208.7 ± 109	191.3 ± 71	0.734

PCAT-NR = protein C activation time-normalized ratio, APC = activated protein C sensitivity ratio, vWF = von Willebrand factor
 $P < 0.05$ considered significant

Table 3. Levels of FVIII activity and vWA antigen in severe PTSD compared with mild PTSD and controls

	CAPS > 80	CAPS ≤ 80	P value
Patients (n)	20	10	-
Controls (n)	0	30	-
vWF antigen (u/ml)	130.4 ± 32	111.1 ± 27	0.043
FVIII activity (u/ml)	128.5 ± 48	101.5 ± 21	0.022

CAPS = clinician administered PTSD scale
 CAPS > 80 = severe PTSD, CAPS ≤ 80 = mild PTSD

observed between education duration and the CAPS score ($r = -0.005$, $P = 0.979$). In addition, no significant correlation was demonstrated between vWF and education duration or between factor VIII activity levels and education duration either in patients ($r = -0.12$, $P = 0.52$ or $r = -0.16$, $P = 0.38$, respectively) or in controls ($r = -0.04$, $P = 0.81$ or $r = -0.01$, $P = 0.95$, respectively).

Table 1. Demographic characterization of PTSD patients and controls

	Patients (n=30)	Controls (n=30)	P value
Gender (male/female)	11/19	11/19	NS
Age (yrs, mean ± SD)	39.63 ± 11	39.83 ± 10.9	NS
Marital status (married/not married)	15/15	14/16	NS
Ethnicity (Jewish/Arabs)	14/16	12/18	NS
Employment status (employed/unemployed)	18/12	30/0	< 0.001
Education (yrs, mean ± SD)	12 ± 1.6	16.43 ± 2.2	< 0.001

NS = not significant

DISCUSSION

The lifetime prevalence of PTSD is estimated to be about 8% in the general population, although an additional 5–15% may experience subclinical forms of the disorder [2]. The symptoms are chronic and often life-lasting, disabling people and causing a financial burden on society. PTSD patients are at increased risk of mortality, especially from cardiovascular disease and thromboembolic events that could be associated with hypercoagulation [2,5,17].

The aim of the current study was to assess the levels of hypercoagulation parameters in patients with chronic PTSD compared to matched controls who were exposed to the

same trauma. Among all the hypercoagulation parameters evaluated, including D-dimer, F1+2, tissue factor levels and protein C pathway activity, only levels of vWF antigen were found to be significantly higher in the patient group compared to the controls. These results suggest that the higher risk of arterial thrombosis in PTSD patients may be related to endothelial damage or endothelial activation and is not associated with coagulation activation or decreased activity of the protein C pathway. In patients with severe PTSD (CAPS > 80), elevated levels of vWF were accompanied by high FVIII activity levels.

vWF plays an important role in hemostasis and thrombosis, both as a cofactor in platelet adhesion and aggregation and as a circulating carrier protein for factor VIII [18]. Meta-analyses of prospective studies have suggested that increased circulating vWF levels are associated with a high risk of CAD [19,20], and elevated plasma levels of factor VIII are related to an increased risk of venous thrombosis [21,22].

Von Kanel et al. [14] found a positive and partially independent association between dimensional aspects of acute PTSD and plasma levels of both factor VIII and fibrinogen, suggesting a correlation between the severity of acute PTSD symptomatology and concentration of these procoagulant factors. Their data also suggest that traumatic stress could increase levels of factor VIII [14]. Factor VIII and fibrinogen are known to be acute-phase reactants [23-25]; however, in our study no differences were found in fibrinogen levels between patients and controls, but the high levels of factor VIII were associated with elevated levels of vWF. These findings may imply that fibrinogen levels return to normal in the chronic stress state and that higher levels of factor VIII and vWF are associated with chronic endothelial cell activation, but this should be further investigated.

In a later study, von Kanel and co-authors [15] revealed elevated levels of soluble tissue factor, which is another endothelial marker, in 14 patients with acute PTSD developed after an accident compared to 14 controls who did not have PTSD. In this study, no differences were documented in the levels of vWF antigen. In our study, the levels of soluble tissue factor, measured by the method used by von Kanel et al., did not differ between the study and control groups. Differences in the results of endothelial marker evaluation might stem from chronic versus acute state, variations in study populations and the type of trauma that patients were exposed to.

The aim of this study was to explore hypercoagulation parameters in a chronic stress disorder (PTSD). Our findings of elevated levels of vWF and FVIII activity support the hypothesis that hypercoagulation persists after an acute period in PTSD, which could contribute to the morbidity and mortality in these patients and should be addressed in the treatment program.

Compared to previous studies [14,15], the current trial included larger cohorts of patients with chronic PTSD (dura-

tion ≥ 1 year) and controls who were exposed to the same trauma. However, the limitation of our study is related to the small size of the group with severe chronic PTSD (CAPS > 80). Another limitation is associated with the higher education and employment levels found in the control group compared to the patient group.

Levels of vWF and factor VIII were not found to be affected by any concomitant psychiatric illness (e.g., depression, suicidal thoughts), education duration or employment status; however, they were associated with the incidence and severity of chronic PTSD.

CONCLUSIONS

Severe chronic PTSD is associated with high levels of vWF and factor VIII, which may explain the increased risk of developing arterial and venous thrombosis among patients with this disorder. These findings could contribute to the improvement of PTSD diagnosis and treatment. The results obtained in the current study may be considered preliminary. To assess their significance, prospective clinical trials larger cohorts of patients with severe chronic PTSD are warranted. It is also crucial that follow-up of arterial and venous thrombosis be incorporated in protocols of these studies.

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Cannabinoid Receptor Activation in the Basolateral Amygdala Blocks the Effects of Stress on the Conditioning and Extinction of Inhibitory Avoidance

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Despite the efficacy of behavior therapy for human anxiety disorders, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress. The endocannabinoid system has recently emerged as important in the regulation of extinction learning and in the regulation of the hypothalamic–pituitary–adrenal axis. Here, we aimed to examine the involvement of the cannabinoid CB₁ receptor in the basolateral amygdala (BLA) in inhibitory avoidance (IA) conditioning and extinction and to test whether cannabinoid activation would reverse the effects of stress on these memory processes. The synthetic full agonist of the CB₁/CB₂ receptor WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate] (5 μg/0.5 μl) microinjected into the BLA had no effect on IA conditioning or extinction by itself. However, microinjecting WIN55,212-2 into the BLA before exposing the rats to a stressor reversed the enhancing effects of the stressor on IA conditioning and its impairing effects on IA extinction. Importantly, WIN55,212-2 microinjected into the BLA reduced stress-induced elevations in corticosterone levels. Control experiments demonstrated the following: (1) the effects of WIN55,212-2 could not be attributed to sensorimotor deficits, because these parameters seemed unchanged by WIN55,212-2 microinjected into the BLA; and (2) the CB₁ receptor in the BLA is crucially involved in the extinction of IA, because the CB₁ receptor antagonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] (6 ng/0.5 μl) microinjected into the BLA significantly blocked extinction. Together, our findings may support a wide therapeutic application for cannabinoids in the treatment of conditions associated with the inappropriate retention of aversive memories and stress-related disorders.

Introduction

Fear inhibition is most often studied through a procedure in which a previously fear-conditioned organism is exposed to a fear-eliciting cue in the absence of any aversive event. This procedure results in a decline in conditioned fear responses that is attributed to a process called extinction (Myers and Davis, 2007).

Despite the efficacy of behavior therapy for human anxiety disorders, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress. We recently showed (Akirav and Maroun, 2007) that 30 min of exposure to the elevated platform stressor disrupts the extinction of both auditory and contextual fear conditioning. Others have reported that stress reduces cued fear extinction (Shumake et al., 2005; Izquierdo et al., 2006; Maren and Chang, 2006) or impairs its recall (Maren and Chang, 2006; Miracle et al., 2006; Garcia et al., 2008). In parallel, exposure to stress facilitates the initial fear learning, thus further enhancing the fear response (Shors et al., 1992; Cordero et al., 2003).

Manipulation of the endogenous cannabinoid system has become a major focus of current search for novel therapeutics to treat many common mental illnesses, including anxiety disorders, depression, and drug addiction (Porter and Felder, 2001; Kathuria et al., 2003). It is generally appreciated that the recreational use of cannabinoids is related to their positive modulatory effects on brain-rewarding processes along with their ability to positively influence emotional states and remove stress responses to environmental stimuli (Gardner and Vorel, 1998). Indeed, the potential therapeutic value of cannabinoid modulation is underscored by the dense expression of the cannabinoid CB₁ receptor in regions known to be significant for anxiety and emotional learning, particularly the basolateral amygdala (BLA) (Katona et al., 2001; Haller et al., 2002).

The endocannabinoid system has recently emerged as important in the regulation of extinction learning (Marsicano et al., 2002; Varvel and Lichtman, 2002; Suzuki et al., 2004; de Oliveira Alvares et al., 2005) and of the hypothalamic–pituitary–adrenal (HPA) axis and its end product corticosterone (CORT) (Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008). Studies so far suggest that environmental stress and CB₁ receptor activity interact in the regulation of the HPA axis and that the augmentation of endocannabinoid signaling can suppress stress-responsive systems (Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008).

Our main goal was to test whether cannabinoid activation in the BLA would inhibit stress-induced alterations in inhibitory

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avoidance (IA) conditioning and extinction and to examine the possible association with the HPA axis. To that end, we examined the following: (1) the effects of administering cannabinoid receptor agonist into the BLA on the conditioning and extinction of IA, (2) whether cannabinoid activation in the BLA would reverse the effects of stress on IA conditioning and extinction, and (3) whether cannabinoid activation in the BLA would affect plasma CORT levels.

Materials and Methods

Subjects. A total of 434 male Sprague Dawley rats (~60 d old, 250–300 g) were used for the experiments. Animals were caged individually at 22 ± 2°C under 12 h light/dark cycles. Rats had access to water and laboratory rodent chow *ad libitum*. The experiments were approved by the University of Haifa Ethics and Animal Care Committee, and adequate measures were taken to minimize pain or discomfort in accordance with the guidelines laid down by the National Institutes of Health in the United States regarding the care and use of animals for experimental procedures.

Drug treatments. Three drugs were investigated: the synthetic CB₁/CB₂ receptor agonist WIN55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate] (WIN); an inhibitor of endocannabinoid reuptake and breakdown, AM404 [*N*-(4-hydroxyphenyl)-arachidonamide]; and the CB₁ receptor antagonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] (Tocris Bioscience). Each drug was initially dissolved in dimethylsulfoxide (DMSO) and further diluted with saline (0.9% NaCl).

The final DMSO concentration was <7%. This was also used as the vehicle. The final concentration of DMSO did not affect performance in the inhibitory avoidance task. Drug concentrations are based on reports in the literature (Martin et al., 1999; Chhatwal et al., 2005; de Oliveira Alvares et al., 2005; Moreira et al., 2007; Pamplona et al., 2008) and our preliminary results. For microinjection, WIN55,212-2 was used at 2.5 μg/0.5 μl, 5 μg/0.5 μl, or 10 μg/0.5 μl. AM404 was used at 200 ng/0.5 μl or 800 ng/0.5 μl, and AM251 was used at 6 ng/0.5 μl. For intraperitoneal administration, WIN 55,212-2 was used at 0.25 mg/kg.

Cannulation and drug microinjection. Rats were anesthetized with 4.8 ml/kg Equithesin (2.12% w/v MgSO₄ 10% ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotaxic apparatus (Stoelting), and implanted bilaterally with a stainless steel guide cannula (23 gauge, thin walled) aimed at the BLA (anteroposterior, −3 mm; lateral, ±5 mm; ventral, −6.7 mm). The cannulae were set in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations.

For microinjection, the stylus was removed from the guide cannula, and a 28 gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The injection cannula was connected via polyethylene PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (CMA/100; Carnegie Medicine). Microinjection was performed bilaterally in a 0.5 μl volume per side delivered over 1 min. The injection cannula was left in position for an additional 30 s before withdrawal to minimize dragging of the injected liquid along the injection tract.

Light–dark inhibitory avoidance. Animals were placed in an inhibitory avoidance apparatus with a metal grid floor. The apparatus was divided into a light side and a dark side, and the rats were placed in the light side, facing the left rear corner of the box.

For conditioning (Cond), when the rats crossed over to the dark side of the box (with four paws on the grid), they received a 2 s, 0.7 mA scrambled footshock. After administration of the footshock, the opening between the two sides of the box was blocked, and the rats remained in the dark side for an additional 60 s, after which they were removed back to the home cage.

For extinction, rats were submitted to a non-reinforced test trial every 24 h for three days (Ext1–Ext3), beginning 24 h after conditioning. Each

rat was placed in the light side of the box, and the time elapsed until it crossed over to the dark side (i.e., latency) was measured. If, after 180 s, the rat did not cross over on its own, the experimenter gently guided it to the dark side. The opening between the two sides of the shuttle was then blocked, no footshock was administered, and the rat was allowed to explore the dark side freely for 180 s, after which it was removed back to the home cage.

A drug (the CB₁ receptor antagonist AM251 or one of the agonists WIN55,212-2 or AM404) was microinjected into the BLA at different time points to address various phases of memory processing. Drugs were administered 20 min before conditioning (Pre-Cond), 20 min before the first extinction trial (pre-Ext1), or immediately (i.e., 2 min) after the first extinction trial (post-Ext1). The vehicle was administered at the same time points.

Elevated platform stress. An elevated platform (EP) (12 × 12 cm) stressor was used to examine the effects of exposure to a stressful experience on IA conditioning and extinction. Individual animals were placed on an elevated platform for 30 min in a brightly lit room, which elicits stress responses in the form of behavioral “freezing,” that is, immobility for up to 10 min, defecation, and urination (Maroun and Akirav, 2008).

Exposure to the EP occurred immediately before conditioning (Pre-Cond), immediately before Ext1 (Pre-Ext1), or immediately after Ext1 (Post-Ext1). The EP groups (i.e., EP Pre-Cond, EP Pre-Ext1, and EP Post-Ext1) experienced the EP stressor in the absence of any microinjection, whereas the WIN+EP groups were microinjected with WIN55,212-2, 2 min before experiencing the EP stressor. The vehicle groups were microinjected with vehicle when the WIN+EP groups received WIN but did not experience the EP stressor.

Open field. The open field consisted of a closed wooden box. The walls were painted black, and the floor was white and divided by 1-cm-wide black lines into 25 squares measuring 10 × 10 cm each. A video image of the entire open field was displayed on a television monitor, and the movements of the rat, which was initially placed in a corner of the field, were manually recorded and analyzed to measure motor activity over a period of 5 min. Recordings were made of the time the rat spent in the central and the peripheral squares, the number of instances of rearing, and the total distance covered. The open-field arena was thoroughly cleaned between each trial.

Rats were microinjected with the different drugs into the BLA and, after 20 min, tested in the open-field arena. For rats that were placed on the EP for 30 min with or without previous microinjection of WIN55,212-2 into the BLA, the open-field test was performed immediately after the EP stressor.

Pain sensitivity. Pain sensitivity was assessed by determining the footshock intensity (in milliamperes) that elicited a discomfort response (i.e., flinch or vocalization) (Kim et al., 1991). Rats were individually placed in a Plexiglas box (25 × 25 × 34 cm) with a floor consisting of 13 stainless steel rods of 5 mm diameter, spaced every 1 cm. Each rat received a continuously ascending mild electric footshock (beginning at 0.0 mA and ending as soon as the animal flinched or vocalized) via the metal grid floor to determine current thresholds at which each animal would exhibit a flinch or a vocalization response. Two observers scored flinch and vocalization thresholds. Rats were taken for the pain sensitivity test 5 min after the open-field test.

Corticosterone measurement. Trunk blood was collected after decapitation between 9:00 and 11:00 A.M. for 4 consecutive days (from one-quarter of the rats per group per day). Samples were centrifuged at 3000 rpm for 20 min at 4°C. Serum was stored at −80°C and analyzed for CORT using ELISA kits (DSL Inc.).

Histology. On completion of the inhibitory avoidance experiments, the animals were deeply anesthetized with 4.8 ml/kg Equithesin (see above) and microinjected into the BLA with 0.5 μl of ink, to verify the location of the cannulae. Figure 1 shows a representative schematic drawing of the placements of the cannulae in the BLA (coronal view at position 3.14 and 3.30 mm posterior to bregma) (Paxinos and Watson, 1998).

Statistical analysis. The results are expressed as means ± SEM. For statistical analysis, repeated-measures ANOVA, one-way ANOVA, and *t* tests were used as indicated. All *post hoc* comparisons were made using the least-significant difference multiple-comparison test.

Results

Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA has no effect on inhibitory avoidance conditioning or extinction

First, we asked whether stimulation of cannabinoid receptor signaling in the BLA might accelerate the IA extinction rate or affect IA conditioning. Thus, vehicle or the CB₁/CB₂ receptor agonist WIN55,212-2 were microinjected into the BLA before conditioning, before Ext1, or immediately after Ext1.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of the rats to enter the dark side of the box ($F_{(2,9)} < 1$; NS). Consequently, all vehicle groups for the light–dark IA experiments involving WIN55,212-2 (5 $\mu\text{g}/0.5 \mu\text{l}$) were pooled for all analyses (vehicle, $n = 12$). For WIN55,212-2 (5 $\mu\text{g}/0.5 \mu\text{l}$) microinjected before conditioning (Pre-Cond WIN_5, $n = 8$), before Ext1 (Pre-Ext1 WIN_5, $n = 9$), or immediately after Ext1 (Post-Ext1 WIN_5, $n = 9$), repeated-measures ANOVA [treatment \times days (4×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,34)} < 1$; NS) (Fig. 2*a*). Also, there were no within-subject differences in the latency between the days ($F_{(1,34)} < 1$; NS), nor was there an interaction effect ($F_{(3,34)} < 1$; NS). Because of the apparent reduction in latency in the Pre-Ext1 WIN_5 group on the first extinction day, we analyzed the latency on Ext1 using one-way ANOVA, which did not reveal a significant effect ($F_{(3,34)} = 1.43$; NS).

Because dose–response issues may have been responsible for the failure of a microinjection of WIN55,212-2 into the BLA to affect latency, we examined the effects of other doses. Thus, the effect on latency was examined after microinjection of a lower [2.5 $\mu\text{g}/0.5 \mu\text{l}$ (WIN_2.5), $n = 7$] or a higher [10 $\mu\text{g}/0.5 \mu\text{l}$ (WIN_10), $n = 7$] dose of WIN55,212-2 into the BLA after Ext1. Repeated-measures ANOVA [treatment \times days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,21)} < 1$; NS) (Fig. 2*b*). Also, there were no within-subject differences in the latency between the days ($F_{(1,21)} = 1.81$; NS), nor was there an interaction effect ($F_{(2,21)} < 1$; NS). Thus, together with the results from Figure 2*a*, WIN55,212-2 microinjected into the BLA appears to have no effect on IA conditioning or extinction by itself.

A previous report (Chhatwal et al., 2005) showed that the CB₁/CB₂ receptor agonist WIN55,212-2, and an inhibitor of endocannabinoid reuptake and breakdown, AM404, have different effects on the extinction of contextual fear. Hence, we examined the effects of AM404 on the conditioning and extinction of IA.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of rats to enter the dark side of the box ($F_{(2,10)} < 1$; NS). Consequently, all vehicle groups in the light–dark IA experiments involving AM404 were pooled for all analyses (vehicle; $n = 13$).

For AM404 microinjected before conditioning (Pre-Cond 404, $n = 12$), before Ext1 (Pre-Ext1 404, $n = 7$), or immediately after Ext1 (Post-Ext1 404, $n = 10$), repeated-measures ANOVA

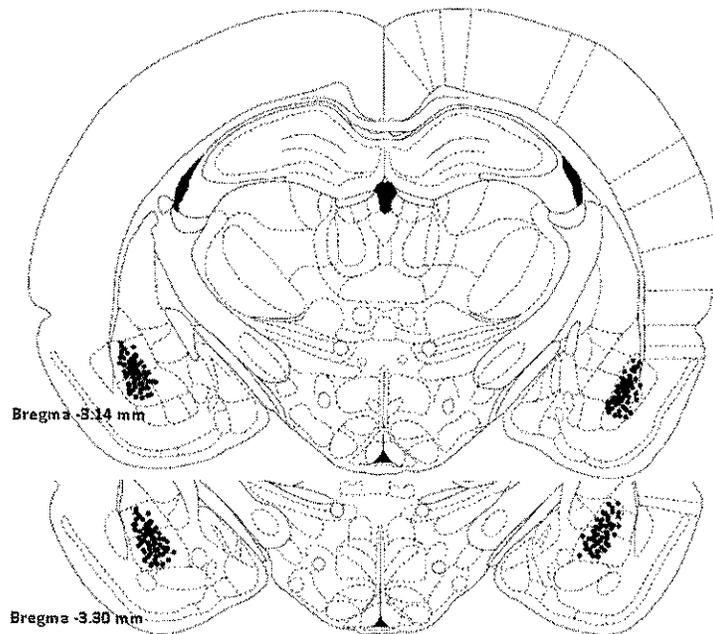


Figure 1. Representative schematic drawing of cannulae tip positions in the BLA. A coronal view at position 3.14 and 3.30 mm posterior to bregma.

[treatment \times days (4×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,38)} < 1$; NS) (Fig. 2*c*). Also, there were no within-subject differences in the latency between the days ($F_{(1,38)} < 1$; NS), nor was there an interaction effect ($F_{(3,38)} = 1.157$; NS). Because of the apparent reduction in latency in the Pre-Ext1 404 group on the first extinction day, we analyzed the latency on Ext1 using one-way ANOVA, which revealed a significant group effect ($F_{(3,38)} = 4.04$; $p = 0.014$). *Post hoc* comparison showed a significant difference between the vehicle and the Pre-Ext1 404 group ($p = 0.002$) on Ext1, indicating a reduction in the latency to enter the dark side after microinjection of AM404 that recovered the following day. Using a higher dose of AM404 (800 ng/0.5 μl) before the first extinction trial resulted in a similar effect, i.e., reduced latency to enter the dark side on Ext1 (vehicle, 118.03 ± 4.1 s, $n = 7$; Pre-Ext1 404_800, 31.74 ± 3.72 s, $n = 7$; $t_{(12)} = 5.17$; $p < 0.0001$), with no effect on Cond, Ext2, or Ext3 (data not shown). Thus, except for the transient effect on latency on Ext1, AM404 had no effect on IA conditioning or extinction.

Because the cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA had no effect on IA conditioning or extinction, we next examined whether the CB₁ receptor in the BLA is essential for IA conditioning or extinction. Hence, rats were microinjected with vehicle or the CB₁ receptor antagonist AM251 before conditioning, before Ext1, or immediately after Ext1.

Microinjecting vehicle into the BLA before conditioning, before Ext1, or immediately after Ext1 had no effect on the latency of rats to enter the dark side of the box ($F_{(2,11)} < 1$; NS). Consequently, all vehicle groups for light–dark IA experiments involving AM251 were pooled for all analyses (vehicle; $n = 14$).

For AM251 microinjected rats, repeated-measures ANOVA [treatment \times days (4×4)] revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(3,38)} = 9.63$; $p < 0.001$) (Fig. 2*d*). *Post hoc* comparison unveiled a significant difference between the vehicle group and the groups microinjected with AM251 before conditioning (Pre-

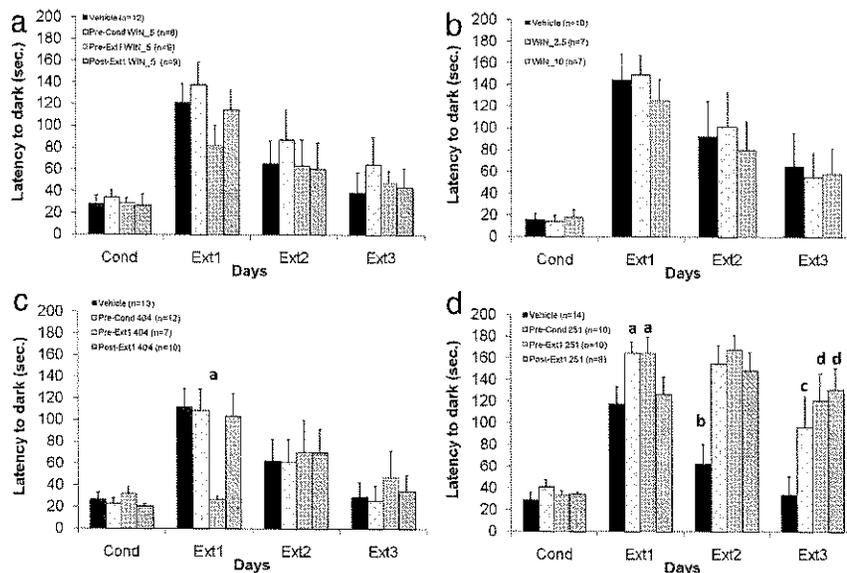


Figure 2. Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA has no effect on inhibitory avoidance conditioning or extinction. *a*, Rats were microinjected into the BLA with vehicle ($n = 12$), with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) before conditioning (Pre-Cond WIN_5, $n = 8$), before the first extinction trial (Pre-Ext1 WIN_5, $n = 9$), or immediately after that trial (Post-Ext1 WIN_5, $n = 9$). There were no significant differences between the latencies of the groups. *b*, Rats were microinjected into the BLA with vehicle ($n = 10$) or with a lower ($2.5 \mu\text{g}/0.5 \mu\text{l}$; WIN_2.5, $n = 7$) or a higher ($10 \mu\text{g}/0.5 \mu\text{l}$; WIN_10, $n = 7$) dose of WIN55,212-2 immediately after Ext1. There were no significant differences between the latencies of the groups. *c*, Rats were microinjected into the BLA with vehicle ($n = 13$) or with AM404 ($200 \text{ ng}/0.5 \mu\text{l}$) before conditioning (Pre-Cond 404, $n = 12$), before the first extinction trial (Pre-Ext1 404, $n = 7$), or immediately after that trial (Post-Ext1 404, $n = 10$). The latency of the Pre-Ext1 404 group was significantly shorter than that of the vehicle group on the first extinction day (Ext1, $^a p < 0.01$) (for details, see Results). *d*, Rats were microinjected into the BLA with vehicle ($n = 14$) or AM251 ($6 \text{ ng}/0.5 \mu\text{l}$) before conditioning (Pre-Cond 251, $n = 10$), before the first extinction trial (Pre-Ext1 251, $n = 10$), or immediately after that trial (Post-Ext1 251, $n = 8$). The latencies of all the AM251-injected groups were significantly longer than that of the vehicle group, indicating enhancement of inhibitory avoidance acquisition and/or consolidation and impaired extinction. (Ext1, $^a p < 0.05$, vehicle different from Pre-Cond 251 and Pre-Ext1 groups; Ext2, $^b p < 0.001$, vehicle different from all the groups; Ext3, $^c p < 0.05$, vehicle different from Pre-Cond 251; $^d p < 0.01$, vehicle different from Pre-Ext1 251 and Post-Ext1 251 groups).

Cond 251, $n = 10$; $p < 0.001$), before Ext1 (Pre-Ext1 251, $n = 10$; $p < 0.001$), or after Ext1 (Post-Ext1 251, $n = 8$; $p = 0.001$).

One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the AM251-treated groups and the vehicle group throughout the extinction days (Ext1, $F_{(3,38)} = 3.12$, $p = 0.037$; Ext2, $F_{(3,38)} = 9.44$, $p < 0.001$; Ext3, $F_{(3,38)} = 4.5$, $p = 0.008$) but not on the conditioning day. *Post hoc* comparison revealed a significant difference between the vehicle group and the Pre-Cond 251 and Pre-Ext1 251 groups ($p = 0.02$) on Ext1, and between the vehicle group and all the treatment groups on Ext2 ($p < 0.001$) and Ext3 (Pre-Cond 251, $p = 0.039$; Pre-Ext1 251, $p = 0.005$; Post-Ext1 251, $p = 0.004$).

Thus, AM251 microinjected before conditioning enhanced IA acquisition and/or consolidation, as indicated by a higher latency to enter the dark side of the box on Ext1, and impaired extinction, as indicated by a higher latency to enter the dark side on Ext2 and Ext3. When AM251 was microinjected before the first extinction trial, it enhanced IA retrieval and impaired extinction. Finally, AM251 microinjected after Ext1 impaired the consolidation of IA extinction, as shown by the increased latency on Ext2 and Ext3 (but not before microinjection on Ext1). Repeated-measures ANOVA also revealed significant within-subject differences in the latency between the days ($F_{(1,38)} = 22.09$; $p < 0.001$) and a significant interaction effect ($F_{(3,38)} = 4.92$; $p = 0.005$). Hence, the cannabinoid receptor in the BLA is crucially involved in the conditioning and extinction of IA.

Cannabinoid receptor agonist WIN55,212-2 microinjected into the BLA blocks the effects of stress on inhibitory avoidance conditioning and extinction

To examine the effects of exposure to a stressful experience on the conditioning and extinction of IA, rats were exposed to the EP stress before conditioning, before Ext1, or immediately after Ext1. To examine whether cannabinoid receptor agonist would reverse the effects of stress on IA conditioning and extinction, WIN55,212-2 was microinjected into the BLA immediately before placing the rats on the EP (WIN+EP groups).

Before conditioning, rats were microinjected with vehicle ($n = 12$), placed on the EP for 30 min (EP Pre-Cond, $n = 9$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 7$). Repeated-measures ANOVA [treatment \times days (3×4)] revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,25)} = 4.57$; $p = 0.02$) (Fig. 3*a*). *Post hoc* comparison unveiled a significant difference between the vehicle and the EP Pre-Cond group ($p = 0.006$).

One-way ANOVA applied on the different days revealed that the significant main effect stemmed from a difference in latency between the groups on Ext1 ($F_{(2,25)} = 4.184$; $p = 0.027$) but not afterward. *Post hoc* comparison showed significantly increased latency in the EP group compared with the vehicle group ($p = 0.008$).

There were no within-subject differences in the latency between the days ($F_{(1,25)} < 1$; NS), nor was there an interaction effect ($F_{(2,25)} = 1.48$; NS). Thus, exposure to the EP stressor before conditioning enhanced IA acquisition and/or consolidation on Ext1, and microinjecting WIN55,212-2 into the BLA before exposure to the EP reversed the effects of the stressor on IA conditioning, because no significant differences were observed between the vehicle and WIN_5+EP group throughout the days of the experiment.

The experiment was then repeated on another set of rats with stress exposure and drug administration placed before the first extinction day. Before Ext1, rats were microinjected with vehicle ($n = 12$), placed on the EP for 30 min (EP Pre-Ext1, $n = 9$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 10$). Repeated-measures ANOVA [treatment \times days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,28)} = 1.04$; NS) (Fig. 3*b*). Also, there were no within-subject differences in the latency between the days ($F_{(1,28)} = 1$; NS), nor was there an interaction effect ($F_{(2,28)} = 1.04$; NS). However, rats that were placed on the EP avoided entering the dark side on Ext1 altogether (all rats reached the maximum latency of 180 s). Thus, using one-way ANOVA on the different days, we found a significant effect on latency on Ext1 ($F_{(2,28)} = 4.81$; $p = 0.017$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared

with the vehicle ($p = 0.022$) and WIN_5+EP ($p = 0.007$) groups on the first extinction day. Thus, exposure to the EP stressor before the first extinction trial enhanced IA retrieval and microinjecting WIN55,212-2 into the BLA before exposure to the EP blocked the effects of the stressor on retrieval, because no significant differences were observed between the vehicle and WIN_5+EP groups throughout the days of the experiment.

The experiment was then repeated again on a third set of rats with stress exposure and drug administration placed after the first extinction day. After Ext1, rats were microinjected with vehicle ($n = 14$), placed on the EP for 30 min (EP Pre-Ext1, $n = 8$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP for 30 min (WIN_5+EP, $n = 8$). Repeated-measures ANOVA [treatment \times days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,27)} = 1.86$; NS) (Fig. 3c). Also, there were no within-subject differences in latency between the days ($F_{(1,27)} < 1$; NS), nor was there an interaction effect ($F_{(2,27)} = 1.37$; NS). However, rats that were placed on the EP showed increased latency to enter the dark side of the box on Ext2, and, using one-way ANOVA on the different days, we found a significant effect on the latency on Ext2 ($F_{(2,27)} = 3.4$; $p = 0.048$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared with the vehicle ($p = 0.019$) and WIN_5+EP ($p = 0.05$) groups.

Thus, exposure to the EP stressor after the first extinction trial disrupted the consolidation of extinction, and microinjecting WIN55,212-2 before exposure to the EP reversed the impairing effects of the stressor, because no significant differences were observed between the vehicle and WIN_5+EP groups on the second and third extinction days.

Next we examined whether a lower dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) microinjected into the BLA after Ext1 would also block the impairing effects of the stressor on the consolidation of IA extinction. After Ext1, rats were microinjected with vehicle ($n = 8$), placed on the EP for 30 min (EP Post-Ext1, $n = 8$), or microinjected with a lower dose of WIN55,212-2 and immediately afterward placed on the EP for 30 min (WIN_2.5+EP, $n = 8$). Repeated-measures ANOVA [treatment \times days (3×4)] did not reveal a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,21)} = 1.03$; NS) (Fig. 3d). Also, there were no within-subject differences in latency between the days ($F_{(1,21)} = 2.7$; NS), nor was there an

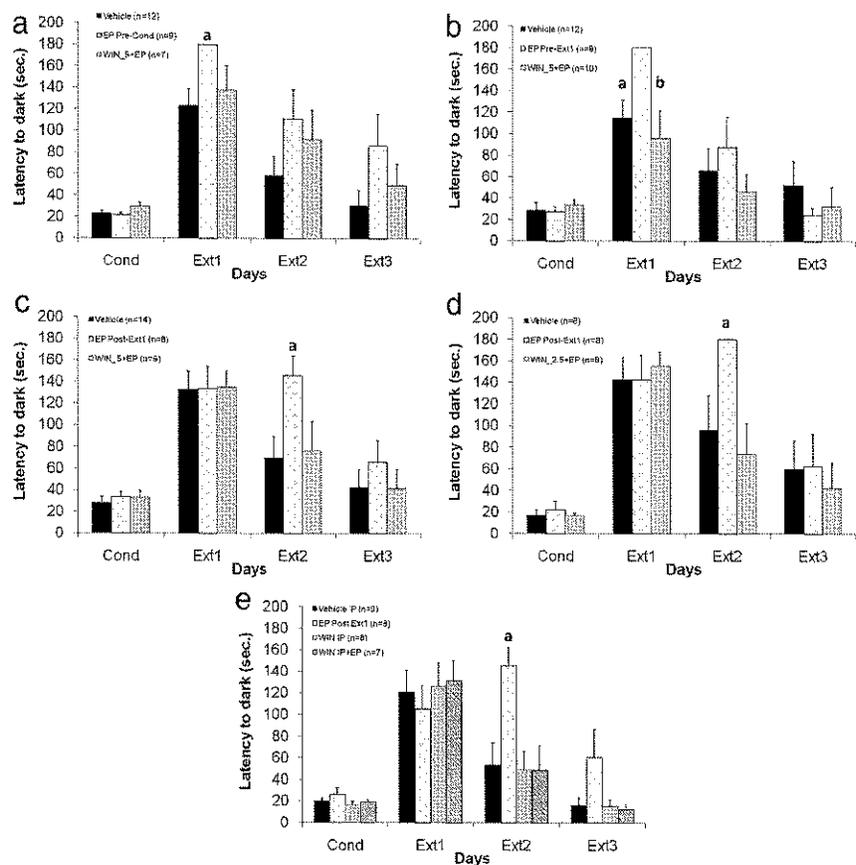


Figure 3. Cannabinoid receptor agonist WIN55,212-2 blocks the effects of EP stress on IA conditioning and extinction. **a**, Before conditioning, rats were microinjected with vehicle ($n = 12$), placed on the EP (EP Pre-Cond, $n = 9$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP (WIN_5+EP, $n = 7$). The EP Pre-Cond group showed a significantly increased latency to enter the dark side on the first extinction day compared with the vehicle group (Ext1, $^*p < 0.01$). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the enhancing effect of the stressor on IA acquisition and/or consolidation. **b**, Before the first extinction trial, rats were microinjected with vehicle ($n = 12$), placed on the EP (EP Pre-Ext1, $n = 9$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP (WIN_5+EP, $n = 10$). The EP Pre-Ext1 group showed a significantly increased latency to enter the dark side on the first extinction day (Ext1, $^*p < 0.05$, EP differs from vehicle; $^*p < 0.01$, EP differs from WIN_5+EP). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the enhancing effect of the stressor on IA retrieval. **c**, After the first extinction trial, rats were microinjected with vehicle ($n = 14$), placed on the EP (EP Post-Ext1, $n = 8$), or microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP (WIN_5+EP, $n = 8$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day compared with the other groups (Ext2, $^*p < 0.05$). Thus, WIN55,212-2 administered into the BLA before stressor exposure reversed the disrupting effect of the stressor on IA extinction. **d**, After the first extinction trial, rats were microinjected with vehicle ($n = 8$), placed on the EP (EP Post-Ext1, $n = 8$), or microinjected with a low dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) and immediately afterward placed on the EP (WIN_2.5+EP, $n = 8$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day (Ext2, $^*p < 0.01$, EP Post-Ext1 differs from WIN_2.5+EP). Thus, a lower dose of WIN55,212-2 administered into the BLA before stressor exposure also reversed the disrupting effect of the stressor on IA extinction. **e**, After the first extinction trial, rats were intraperitoneally injected with vehicle ($n = 9$), placed on the EP (EP Post-Ext1, $n = 8$), intraperitoneally injected with WIN (0.25 mg/kg ; WIN IP, $n = 8$), or intraperitoneally injected with WIN and immediately afterward placed on the EP (WIN IP+EP, $n = 7$). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day compared with all the other groups (Ext2, $^*p < 0.01$). Thus, intraperitoneal administration of WIN55,212-2 before stressor exposure also reversed the disrupting effect of the stressor on IA extinction.

interaction effect ($F_{(2,21)} < 1$; NS). However, rats that were placed on the EP showed increased latency to enter the dark side of the box on Ext2 (i.e., all EP Post-Ext1 rats reached the maximum latency of 180 s). Thus, using one-way ANOVA on the different days, we found a significant effect on the latency on Ext2 ($F_{(2,21)} = 4.42$; $p = 0.027$). *Post hoc* comparisons revealed significantly increased latency in the EP group compared with the WIN_2.5+EP group ($p = 0.009$) and a marginally significant difference compared with the vehicle group ($p = 0.061$). Thus,

Table 1. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on locomotion and anxiety in the open-field test

	Vehicle (<i>n</i> = 6)	AM404 (<i>n</i> = 6)	WIN55,212-2 (<i>n</i> = 6)	AM251 (<i>n</i> = 6)
Time in center (s)	7.83 ± 1.25	6.33 ± 1.31	4.66 ± 1.08	4.5 ± 1.28
Time in periphery (s)	292.16 ± 1.25	293.66 ± 1.31	295.33 ± 1.08	295.5 ± 1.28
Number of rearing events	20.33 ± 1.74	21.66 ± 2.03	22 ± 1.69	19.16 ± 2.10
Distance covered (s)	1758.33 ± 114.32	1916.66 ± 158.46	1675 ± 107.04	1729.16 ± 231.16

Rats microinjected into the BLA with the CB₁ receptor antagonist (AM251, *n* = 6), one of the agonists (WIN55,212-2 or AM404, *n* = 6 each), or vehicle (*n* = 6) showed no differences in any of the parameters measured in the open-field test.

microinjecting a lower dose of WIN55,212-2 into the BLA before exposure to the EP also reversed the impairing effects of the stressor on the consolidation of extinction.

Finally, we were interested in investigating whether the same effects would be seen after systemic treatment with WIN55,212-2 (0.25 mg/kg, i.p.). Hence, immediately after Ext1, rats were intraperitoneally injected with vehicle (Vehicle IP, *n* = 9), placed on the EP for 30 min (EP Post-Ext1, *n* = 8), intraperitoneally injected with WIN55,212-2 (WIN IP, *n* = 8), or intraperitoneally injected with WIN55,212-2 and immediately afterward placed on the EP for 30 min (WIN IP+EP, *n* = 7). Repeated-measures ANOVA [treatment × days (3 × 4)] revealed a strong trend in terms of the latency to enter the dark side of the box ($F_{(3,28)} = 2.61$; $p = 0.07$) (Fig. 3e). One-way ANOVA applied on the different days revealed a significant difference in latency between the groups on Ext2 ($F_{(3,28)} = 5.94$; $p = 0.003$). *Post hoc* comparison showed significantly increased latency in the EP group compared with the other groups ($p = 0.002$). Thus, systemic administration of WIN55,212-2 before exposure to the EP also reversed the impairing effects of the stressor on the consolidation of extinction. Repeated-measures ANOVA also revealed a significant interaction effect ($F_{(3,28)} = 5.68$; $p = 0.004$) but no within-subject differences in latency between the days ($F_{(1,28)} = 1.4$; NS).

The effects of the different manipulations on anxiety and sensorimotor parameters

Next, we performed two types of control experiments (the open-field and pain sensitivity tests) to exclude the possibility that the effects of the drugs on IA acquisition, consolidation, or extinction were caused by sensorimotor deficits or by increased anxiety under the experimental conditions used. Hence, rats were microinjected into the BLA with the CB₁ receptor antagonist (AM251, *n* = 6; 6 ng/0.5 μl), agonists [WIN₅, *n* = 6 (5 μg/0.5 μl) and AM404, *n* = 6 (200 ng/0.5 μl)], or vehicle (*n* = 6) and then tested in the open-field arena and in the pain sensitivity test. One-way ANOVA did not reveal a significant difference in any of the parameters measured in the open-field test (Table 1), namely, time spent in the center ($F_{(3,20)} = 1.65$; NS), time spent in the periphery ($F_{(3,20)} = 2.8$; NS), number of rearing events ($F_{(3,20)} < 1$; NS), or the distance covered ($F_{(3,20)} = 2.44$; NS). Also, ANOVA did not reveal significant differences in pain sensitivity ($F_{(3,20)} < 1$; NS) (Table 2).

Although WIN55,212-2 microinjected into the BLA had no effect on locomotion, anxiety, or pain sensitivity by itself, the combination of WIN55,212-2 and the EP could conceivably have a different effect on those parameters than either component alone. Hence, experiments were undertaken in which the rats were microinjected into the BLA with vehicle (*n* = 6), placed on the EP (*n* = 5), or microinjected with WIN55,212-2 and placed on the EP (WIN₅+EP, *n* = 6) and then tested in the open-field arena and in the pain sensitivity test. In the open field, one-way ANOVA did not reveal a significant difference between the

Table 2. The effects of cannabinoid receptor agonists and antagonist microinjected into the BLA on pain sensitivity

	Vehicle (<i>n</i> = 6)	AM404 (<i>n</i> = 6)	WIN55,212-2 (<i>n</i> = 6)	AM251 (<i>n</i> = 6)
Pain threshold for foot shock (mA)	0.36 ± 0.04	0.31 ± 0.03	0.30 ± 0.01	0.34 ± 0.03

Rats microinjected into the BLA with the CB₁ receptor antagonist (AM251, *n* = 6), one of the agonists (WIN55,212-2 or AM404, *n* = 6 each), or vehicle (*n* = 6) showed similar pain sensitivity responses to electric footshock.

Table 3. The effects of WIN 55,212-2 and the EP on locomotion and anxiety in the open-field test

	Vehicle (<i>n</i> = 6)	EP (<i>n</i> = 5)	WIN55,212-2 + EP (<i>n</i> = 6)
Time in center (s)	9.5 ± 0.76	7.8 ± 4.18	5.5 ± 1.91
Time in periphery (s)	290.5 ± 0.76	292.2 ± 4.18	294.5 ± 1.91
Number of rearing events	19.16 ± 1.25	10.4 ± 2.28*	12.83 ± 1.1**
Distance covered (s)	1525 ± 163.17	1080 ± 180.62	1258.33 ± 84.07

Rats placed on the EP (*n* = 5) showed increased rearing in the open-field test compared with groups that received a microinjection of vehicle (*n* = 6) or WIN55,212-2 before being placed on the platform (WIN₅+EP; *n* = 6) (* $p < 0.05$, vehicle group differs from WIN₅+EP group; ** $p < 0.01$, vehicle group differs from EP group).

Table 4. The effects of WIN55,212-2 and the EP on pain sensitivity

	Vehicle (<i>n</i> = 6)	EP (<i>n</i> = 5)	EP + WIN55,212-2 (<i>n</i> = 6)
Pain threshold for foot shock (mA)	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01

Rats microinjected into the BLA with vehicle (*n* = 6), placed on the EP (*n* = 5), or microinjected with WIN55,212-2 and placed on the EP (WIN₅+EP, *n* = 6) showed similar pain sensitivity responses to electric footshock.

groups in terms of time spent in the center ($F_{(2,14)} < 1$; NS), time spent in the periphery ($F_{(2,14)} < 1$; NS), or the distance covered ($F_{(2,14)} = 2.17$; NS) (Table 3). However, a significant difference was found between the groups in terms of the number of rearing events ($F_{(2,14)} = 7.74$; $p = 0.005$). *Post hoc* comparisons revealed that the vehicle group reared significantly more times than the EP ($p = 0.002$) and the WIN₅+EP ($p = 0.013$) groups. Rearing behavior characterizes individual differences in reactivity to novelty, and, thus, more frequent rearing may indicate greater novelty seeking behavior (i.e., less anxiety) (Thiel et al., 1999). The EP group showed a reduced number of rearing events and a trend toward a reduced distance covered in the open-field test compared with the control group, thus suggesting an increased stress level that may have contributed to the enhanced IA acquisition or consolidation and disrupted extinction shown in the previous figures.

Finally, one-way ANOVA did not reveal significant differences in pain sensitivity ($F_{(2,14)} < 1$; NS) (Table 4).

WIN55,212-2 microinjected into the BLA or administered intraperitoneally reduces stress-induced increases in corticosterone levels

Because it has been suggested that the augmentation of endocannabinoid signaling can suppress stress-responsive systems

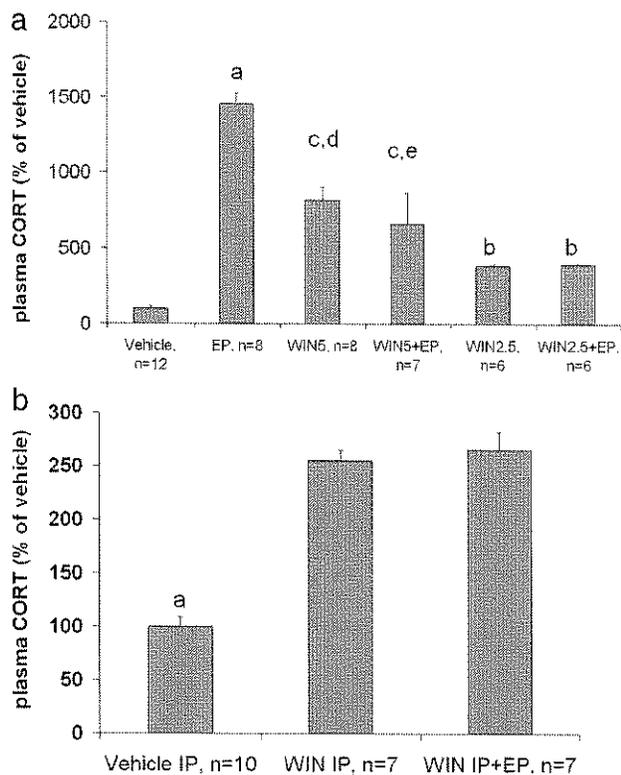


Figure 4. The effects of the cannabinoid receptor agonist WIN55,212-2 and EP stress on CORT levels. *a*, CORT levels were measured in rats microinjected with vehicle into the BLA (vehicle, $n = 12$), placed on the EP ($n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA and placed on the EP (WIN_5+EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_2.5, $n = 6$), or microinjected with the lower dose of WIN55,212-2 and placed on the EP (WIN_2.5+EP, $n = 6$). Data represent the means \pm SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, $95.52 \pm 16.7 \text{ ng/ml}$) ($^a p < 0.001$, EP group differs from all other groups; $^b p < 0.05$ and $^c p < 0.001$, vehicle group differs from all other groups; $^d p < 0.01$, WIN_5 group differs from WIN_2.5 and WIN_2.5+EP groups; $^e p < 0.05$, WIN_5+EP group differs from WIN_2.5 and WIN_2.5+EP groups). *b*, CORT levels were measured in rats injected intraperitoneally with vehicle (Vehicle IP, $n = 10$), WIN55,212-2 (WIN IP, $n = 7$), or injected with WIN55,212-2 intraperitoneally and placed on the EP (WIN IP+EP, $n = 7$). Data represent the means \pm SEM expressed as a percentage of the CORT values of the vehicle animals (CORT levels in the vehicle group, $381.01 \pm 64.39 \text{ ng/ml}$) ($^a p < 0.001$, vehicle group differs from all other groups).

(Patel et al., 2004; Cota, 2008; Steiner and Wotjak, 2008), we sought to examine whether WIN55,212-2 given in conjunction with EP had a different effect on CORT levels than did exposure to the stressor alone.

In the first CORT experiment, rats were microinjected with vehicle to the BLA (vehicle, $n = 12$), placed on the EP ($n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_5, $n = 8$), microinjected with WIN55,212-2 ($5 \mu\text{g}/0.5 \mu\text{l}$) and placed on the EP (WIN_5+EP, $n = 7$), microinjected with a lower dose of WIN55,212-2 ($2.5 \mu\text{g}/0.5 \mu\text{l}$) into the BLA (WIN_2.5, $n = 6$), or microinjected with the lower dose of WIN55,212-2 and placed on the EP (WIN_2.5+EP, $n = 6$).

Thirty minutes after microinjection (vehicle and WIN groups) or immediately after the EP (EP and WIN+EP groups), trunk blood was collected for CORT measurement. One-way ANOVA on CORT levels unveiled a significant difference between the groups ($F_{(5,41)} = 32.7$; $p < 0.001$) (Fig. 4*a*). *Post hoc* comparisons revealed that rats that were exposed to the EP in the

absence of previous WIN microinjection, i.e., the EP group, showed the highest CORT levels when compared with all the groups ($p < 0.001$). The vehicle group showed the lowest CORT levels and was significantly different from all the groups (WIN_5 and WIN_5+EP, $p < 0.001$; WIN_2.5 and WIN_2.5+EP, $p < 0.05$). Also, the WIN_2.5 and WIN_2.5+EP groups showed significantly lower CORT levels than the WIN_5 ($p < 0.01$) and WIN_5+EP groups ($p < 0.05$). Hence, WIN55,212-2 microinjection into the BLA ($2.5 \mu\text{g}/0.5 \mu\text{l}$ or $5 \mu\text{g}/0.5 \mu\text{l}$) in itself increased CORT levels compared with those of the vehicle group, but it reduced CORT levels in rats that were exposed to the EP stress when compared with rats exposed to the EP without WIN microinjection. Furthermore, although both WIN doses reversed the stress-induced increase in CORT levels, the effect was dose dependent, because a lower dose of WIN resulted in less CORT activation than did the higher dose of WIN.

In the second CORT experiment, rats were injected intraperitoneally with vehicle (Vehicle IP, $n = 10$) or WIN55,212-2 (WIN IP, $n = 7$), or injected with WIN55,212-2 and placed on the EP (WIN IP+EP, $n = 7$).

Thirty minutes after injection (vehicle and WIN groups) or immediately after the EP (WIN+EP group), trunk blood was collected for CORT measurement. It seems that the injection of the vehicle intraperitoneally is stressful by itself because the intraperitoneal vehicle group showed relatively enhanced CORT levels (CORT levels in the vehicle group, $381.01 \pm 64.39 \text{ ng/ml}$). Nevertheless, one-way ANOVA on CORT levels unveiled a significant difference between the groups ($F_{(2,21)} = 39.11$; $p < 0.001$) (Fig. 4*b*). *Post hoc* comparisons revealed that the vehicle rats showed significantly lower CORT levels than the WIN IP and WIN IP+EP groups ($p < 0.001$). Hence, WIN55,212-2 injected intraperitoneally in itself increased CORT levels compared with those of the vehicle group, but it reduced CORT levels in rats that were exposed to the EP stress when compared with rats exposed to the EP without WIN injection (EP) (shown in Fig. 4*a*).

Finally, we examined whether the effects of AM251 microinjected into the BLA on IA conditioning and extinction are associated with alterations in CORT levels. *t* test unveiled a significant increase in CORT levels in rats microinjected with AM251 into the BLA [AM251 BLA, $n = 7$; plasma CORT levels (% of vehicle), $199.8 \pm 40.8 \text{ ng/ml}$] compared with the vehicle group (Vehicle BLA, $n = 10$; CORT levels, $100 \pm 25.72 \text{ ng/ml}$) ($t_{(15)} = 2.16$; $p = 0.047$).

Discussion

The main finding of the present study is that cannabinoid receptor activation in the BLA reverses the enhancing effects of environmental stress on IA conditioning and its impairing effects on extinction. We also find that WIN55,212-2 microinjected into the BLA inhibits stress-induced corticosterone elevation, thus suggesting that the reversal of the effects of stress on memory caused by cannabinoid activation in the BLA may be associated with influences on the HPA axis. Furthermore, the results show the crucial involvement of the CB₁ receptor in the BLA in the extinction of avoidance behavior because the CB₁ receptor antagonist impairs IA extinction. The control experiments demonstrate that the effects of WIN55,212-2 cannot be attributed to sensorimotor deficits, because these parameters seemed unchanged by WIN55,212-2 microinjected into the BLA. Together, these findings suggest that the BLA could be an important neural substrate relevant to the effects of cannabi-

noids on emotional responses and that cannabinoids may have a potential therapeutic value in the treatment of fear- and stress-related disorders.

The effects of CB₁ receptor antagonist AM251 on inhibitory avoidance learning

Administration of the CB₁ receptor antagonist into the BLA before conditioning or before/after the first extinction trial potentiates the aversive response or blocks extinction of IA. Indeed, the importance of CB₁ receptors in the extinction of aversive memories has been substantiated by several groups in different behavioral paradigms using systemic administration. CB₁ receptor antagonists were found to impair extinction in fear-related (Marsicano et al., 2002; Suzuki et al., 2004; Chhatwal et al., 2005; Reich et al., 2008) and non-fear-related paradigms (Varvel and Lichtman, 2002), with no effect on appetitively motivated learning tasks (Hölter et al., 2005; Niyuhire et al., 2007; Harloe et al., 2008). Reich et al. (2008) found that administering AM251 enhances acquisition of freezing behavior and impairs extinction in trace and delay pavlovian fear conditioning. However, several studies did not find the CB₁ receptor antagonist to have any effect on memory acquisition or consolidation (Marsicano et al., 2002; Suzuki et al., 2004; De Oliveira Alvares et al., 2008). Recently, it has been suggested that the endocannabinoid system prevents the expression of inappropriate generalized and learned responses during aversive learning and retention (Reich et al., 2008), thus, possibly explaining the enhancing effects of the CB₁ receptor antagonist on IA learning and its impairing effects on extinction.

Memory retrieval is thought to activate a second memory consolidation cascade (i.e., reconsolidation) or it may initiate the opposite process of extinction (Nader et al., 2000; Sara, 2000; Dudai, 2002; Alberini, 2005). Reconsolidation acts to stabilize, whereas extinction tends to weaken, the expression of the original memory. It has been suggested that, after retrieval, there is a brief time window for reconsolidation, whereas extinction only occurs after prolonged reexposure, and that the process that prevails is determined (at least partly) by the duration of the reexposure (Suzuki et al., 2004). Here, the latencies of the control rats to enter the dark side decreased over repeated tests, thus supporting the extinction process. Accordingly, we suggest that AM251 microinjected into the BLA impairs IA extinction rather than facilitates reconsolidation.

The effects of cannabinoid receptor agonists WIN55,212-2 and AM404 on inhibitory avoidance learning

WIN55,212-2, in doses ranging from 2.5 to 10 $\mu\text{g}/0.5 \mu\text{l}$, administered into the BLA has no effect on IA conditioning or on extinction kinetics. AM404 microinjected before the first extinction trial reduces the latency to enter the dark side on Ext1, with latency recovering the following day. Thus, the drug may elicit a general decrease in the inhibitory response that temporarily affects the rats' latency. Chhatwal et al. (2005) have shown that AM404 facilitates the retention of extinction of conditioned fear, whereas WIN55,212-2 has no effect. However, Pamplona et al. (2006) found that WIN55,212-2 facilitates the extinction of both contextual fear memory and a reversal task in the water maze. Using intracerebral injection, Kobilov et al. (2007) found that WIN55,212-2 has no effect on the extinction of conditioned taste aversion. Thus, the alleviating effects of cannabinoid receptor activation on extinction have not been observed consistently.

Many studies have shown that the administration of CB₁ receptor agonists impairs memory (Lichtman et al., 1995; Hampson and Deadwyler, 1999; Davies et al., 2002). However, several other studies have indicated differently, in particular with regards to aversive or fear-based paradigms. For example, CB₁ receptor agonist enhances the acquisition of contextual fear conditioning (Mikics et al., 2006) but has no effect on the acquisition of other aversive tasks (De Oliveira Alvares et al., 2008; Yim et al., 2008). Thus, cannabinoids may have various effects that may result from differences in experimental protocols (e.g., aversive vs nonaversive protocols, mass vs spaced extinction trials, time of drug injection or time between extinction learning and testing, central or systemic drug administration, the use of different drugs, etc).

Cannabinoid receptor agonist in the BLA reverses the effects of stress on inhibitory avoidance learning

Exposing rats to acute stress before conditioning or before/after the first extinction trial enhances inhibitory acquisition/consolidation and disrupts extinction. This corroborates several studies that examined the effects of stress on different memory processes (Cordero et al., 2003; Izquierdo et al., 2006; Akirav and Maroun, 2007). Although administering the cannabinoid receptor agonist into the BLA has no effect on IA conditioning and extinction by itself, environmental stress and cannabinoid receptor activity interact in their regulation of memory in the BLA. Thus, cannabinoid activation in the BLA acts to modulate the effects of stress on conditioning and extinction. In support, Patel et al. (2005) found a synergistic interaction between environmental stress and CB₁ receptor activation in the amygdala, because the combination of restraint stress and CB₁ agonist administration produces robust Fos induction within the BLA and the central amygdala.

The effects of cannabinoids and stress on corticosterone levels

Intra-BLA WIN55,212-2 by itself dose dependently enhances CORT levels when compared with the control group, because the higher dose (5 $\mu\text{g}/0.5 \mu\text{l}$) resulted in more CORT secretion than the lower dose (2.5 $\mu\text{g}/0.5 \mu\text{l}$). This is consistent with findings that cannabinoid activation in both human and animal models stimulates glucocorticoid secretion (Murphy et al., 1998). Most importantly, the CORT levels of rats microinjected with WIN55,212-2 into the BLA without exposure to the EP stressor do not differ significantly from those of rats microinjected with WIN55,212-2 and then exposed to the stressor. Similarly we found that an intraperitoneal administration of WIN55,212-2 (0.25 mg/kg) reversed the stress-induced increase in CORT levels. Hence, acute stress elevates corticosterone levels, and CB₁ receptor activation in the BLA significantly reduces this stress-induced elevation. These findings may suggest that cannabinoid activation in the BLA modulates the effects of stress on learning, at least partially, via inhibition of the HPA axis. Similarly, Patel et al. (2004) have demonstrated that mice treated systemically with CB₁ receptor agonists show significantly decreased or eliminated restraint-induced CORT release. In our study, the abolishment of the effects of stress on CORT levels by WIN55,212-2 was localized to the BLA. Interestingly, microinjecting the CB₁ receptor antagonist AM251 (6 ng/0.5 μl) also resulted in the enhancement of CORT levels.

A model that explains the possible interaction between the endocannabinoid system, stress and the HPA axis has been

suggested previously (Patel et al., 2005; Cota, 2008). On exposure to an acute stressor, a reduction in endocannabinoid signaling would result in increased synaptic activity at glutamatergic afferents to the paraventricular nucleus (PVN), thus allowing stressful stimuli to activate the HPA axis (Di et al., 2003; Patel et al., 2004). The BLA has received considerable attention as a stress-regulatory structure, but there is limited evidence of direct innervations of the PVN by the BLA or other intra-amygdalar projections of the BLA, such as the medial and central nuclei (Herman et al., 2003). Hence, the mechanism by which WIN55,212-2 administered into the BLA inhibits the HPA axis during stress needs additional investigation. In any case, it is important to note that pharmacological administration of exogenous cannabinoids may lead to a different action than that induced by the endogenous agents of the endocannabinoid system. Thus, exogenous CB₁ receptor activation, as in our study, may not resemble endocannabinoid signaling and its role in HPA axis regulation (Steiner and Wotjak, 2008).

It has been shown recently (Campolongo et al., 2009) that the endocannabinoid system is involved in modulating the consolidation of memory for IA training and that CB₁ activity within the BLA is essential for mediating glucocorticoid effects on long-term IA memory. Specifically it has been shown that AM251 administered into the BLA prevented CORT effects on memory consolidation. Steiner et al. (2008) have shown that mice lacking CB₁ in cortical glutamatergic neurons showed decreased immobility in the forced swim test with normal corticosterone release compared with controls. In our study, AM251 into the BLA was found to facilitate and impair IA conditioning and extinction, respectively, and to increase CORT levels. Exposure to the EP stress had similar effects on both IA learning and CORT levels. Together, it seems that additional investigation regarding the possible interaction between the CB₁ receptor antagonist and the HPA axis is required.

The modulation of emotional processes by cannabinoids

Cannabis is widely used, primarily because of its euphorant, anti-anxiety, and stress-reducing properties (Green et al., 2003). The effects of cannabinoid agonists on anxiety are biphasic, with low doses being anxiolytic and high doses anxiogenic (Viveros et al., 2005). Although the precise mechanisms by which CB₁ receptors modulate neuronal activity within the BLA are not fully understood, various studies have reported that cannabinoids serve to attenuate the neuronal and behavioral responses to aversive environmental stimuli (Patel et al., 2005). Indeed, pharmacological augmentation of cannabinoids reduces anxiety-related behavioral responses (Berrendero and Maldonado, 2002; Kathuria et al., 2003) and suppresses restraint stress-induced corticosterone release (Patel et al., 2004). In addition, cannabinoid exposure was shown to decrease corticotropin-releasing hormone levels in the amygdala, which may account for reduced stress responses (Rodríguez de Fonseca et al., 1997).

Within the BLA, high concentrations of CB₁ receptors are found localized on a subpopulation of inhibitory interneurons (McDonald and Mascagni, 2001), suggesting an important regulatory role for CB₁ receptor transmission within the BLA through endocannabinoid signaling. Several studies have reported strong inhibition of BLA interneurons after application of CB₁ receptor agonists (Azad et al., 2004; Pistis et al., 2004), which is expected to decrease local inhibitory feedback on pyramidal amygdalar outputs neurons. Katona et al. (2001) suggested that, by reducing the tonic GABAergic inhibitory

control over pyramidal cells in the BLA, cannabinoids indirectly inhibit neuronal activity in the central nucleus, which mediates stress and fear responses to aversive stimuli. Nevertheless, cannabinoids were found to control synaptic transmission in the lateral amygdala by also modulating glutamatergic synapses (Azad et al., 2003). Thus, this suggests that the effects could also result from CB₁-mediated suppression of excitatory neurotransmission.

It has been suggested that the endocannabinoid system has a specific involvement in the habituation component of fear extinction (Kamprath et al., 2006) and that this involvement resembles its role in adaptation of stress responses (Viveros et al., 2005). Patel et al. (2005) showed that the endocannabinoid system mediates habituation to repeated restraint stress and suggested that pharmacological augmentation of endocannabinoid signaling is a good target for the treatment of affective disorders (Patel and Hillard, 2008). Altogether, these studies indicate that extinction of aversive memories via a habituation-like process and the adaptation to stress responses via the alleviation of the stress axis are, in part, controlled by endocannabinoids (for review, see Lutz, 2007).

Conclusions

Our findings give preclinical support to the suggestion that cannabinoids could represent a therapeutic target for the treatment of diseases associated with the inappropriate retention of aversive memories, such as posttraumatic stress disorder (Marsicano et al., 2002). Importantly, because of the effects of the drug on the stress response, it is likely that potential patients treated with cannabinoids or related compounds might benefit also from the stress-reversing effects of the drug. Nevertheless, studies show that cannabinoids elicit dose-dependent, biphasic effects on emotionality (Onaivi et al., 1990; Haller et al., 2004; Viveros et al., 2007; Moreira et al., 2009). Thus, the dose together with the context in which cannabinoids are administered should be taken into consideration.

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Enhancing Cannabinoid Neurotransmission Augments the Extinction of Conditioned Fear

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The endogenous cannabinoid (eCB) system represents a major therapeutic target for the treatment of a variety of anxiety-related disorders. A recent study has demonstrated that pharmacologic or genetic disruption of CB1-receptor-mediated neurotransmission decreases the extinction of conditioned fear in mice. Here, we examined whether CB1 blockade would similarly disrupt extinction in rats, using fear-potentiated startle as a measure of conditioned fear. We also examined whether pharmacologic enhancement of CB1 activation would lead to enhancements in extinction. Our results indicate that systemic administration of the CB1 antagonist rimonabant (SR141716A) prior to extinction training led to significant, dose-dependent decreases in extinction. While the administration of the CB1 agonist WIN 55,212-2 did not appear to affect extinction, administration of AM404, an inhibitor of eCB breakdown and reuptake, led to dose-dependent enhancements in extinction. In addition to showing decreased fear 1 and 24 h after extinction training, AM404-treated animals showed decreased shock-induced reinstatement of fear. Control experiments demonstrated that the effects of AM404 could not be attributed to alterations in the expression of conditioned fear, locomotion, shock reactivity, or baseline startle, as these parameters seemed unchanged by AM404. Furthermore, coadministration of rimonabant with AM404 blocked this enhancement of extinction, suggesting that AM404 was acting to increase CB1 receptor activation during extinction training. These results demonstrate that the eCB system can be modulated to enhance emotional learning, and suggest that eCB modulators may be therapeutically useful as adjuncts for exposure-based psychotherapies such as those used to treat Post-Traumatic Stress Disorder and other anxiety disorders. *Neuropsychopharmacology* (2005) **30**, 516–524, advance online publication, 22 December 2004; doi:10.1038/sj.npp.1300655

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INTRODUCTION

Manipulation of the endogenous cannabinoid (eCB) system has become a major focus of current research, especially in the search for novel therapeutics to treat many common mental illnesses, including anxiety disorders, depression, and drug addiction (Porter and Felder, 2001; Kathuria *et al*, 2003). Indeed, the potential therapeutic value of cannabinoid modulation is underscored by the dense expression of the CB1 receptor in regions known to be important for anxiety and emotional learning, including the amygdala, hippocampus, and throughout the mesolimbic dopamine reward system (Katona *et al*, 1999, 2000, 2001; Freund *et al*, 2003; van der Stelt and Di Marzo, 2003).

Recent studies of CB1 knockout mice have demonstrated that the genetic deletion of the CB1 receptor leads to increased anxiety in several well-studied measures (Haller *et al*, 2002, 2004a,b; Martin *et al*, 2002). Furthermore, the elegant studies of Marsicano *et al* (2002) have demonstrated that CB1 knockout mice also show profound deficits in the learned inhibition of fear (heretofore referred to as extinction), while the acquisition of the initial fear response was normal. In the same study, the authors demonstrated that pharmacologic blockade of the CB1 receptor led to a similar deficit in extinction in mice, demonstrating the importance of CB1 receptor activation for extinction in mice.

The critical involvement of cannabinoid-mediated transmission in extinction potentially has important clinical implications, as numerous similarities link the expression of fear and anxiety in humans suffering from phobias, Post-Traumatic Stress Disorder (PTSD), and other anxiety disorders to the expression of classically conditioned fear in animals. Perhaps the most important of these similarities is the persistence of fear memories in both humans and animal models. In this context, studying extinction in animals may further the development of experimental

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therapeutics for the treatment of these disorders. Indeed, recent results from our laboratory have shown that the administration of a partial NMDA agonist both enhances the extinction of conditioned fear in rodents (Walker *et al*, 2002), and can increase the efficacy of behavioral exposure therapy in human phobics (Ressler *et al*, 2004).

Furthermore, several recent studies have suggested that prolonging the action of released cannabinoids through the inhibition of the enzyme fatty-acid amide hydrolase (FAAH), which is critically involved in cannabinoid catabolism and reuptake, leads to anxiolysis in rodents (Kathuria *et al*, 2003). The results of these and other studies have highlighted the sizable potential of the CB1 receptor and the eCB-degradative enzyme FAAH as targets of experimental therapeutics (eg Porter and Felder, 2001; Kathuria *et al*, 2003).

Given the mounting clinical interest in modulators of the eCB system, we examined whether the CB1 antagonist rimonabant (SR141716A) would block extinction of fear in rats as measured with fear-potentiated startle. We then examined if administration of an agonist (WIN 55,212-2) or of an inhibitor of eCB reuptake and breakdown (AM404) would enhance the extinction of conditioned fear. In so doing, we addressed whether manipulation of the eCB system could lead to enhancements as well as decrements in extinction, a clinically relevant form of fear modulation important in the understanding of emotional learning and in the treatment of anxiety-related behaviors.

MATERIALS AND METHODS

Animals

The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. A total of 216 adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing between 350 and 450 g were used. Animals were housed in pairs in a temperature-controlled (24°C) animal colony, with *ad libitum* access to food and water. They were maintained on a 12 h light/dark cycle with lights on at 0800, with all behavioral procedures performed during the rats' light cycle.

In Situ Hybridization

In situ hybridization was performed as previously described (Ressler *et al*, 2002). A cDNA clone containing the coding sequence of the rat cannabinoid receptor type 1 (CB1) (I.M.A.G.E. expressed sequence tag clone, GI Accession #11375084) was linearized after sequence verification. An antisense riboprobe was generated with T3 RNA polymerase. Slide-mounted sections of snap-frozen rodent brain tissue were postfixated, proteinase K digested, blocked, and hybridized overnight at 52°C with ³⁵S-UTP-labeled riboprobes. After a stringent wash protocol, slides were apposed to autoradiography film and hybridization density was qualitatively assessed.

Fear Conditioning

Animals were trained and tested in 8 × 15 × 15 cm Plexiglas and wire-mesh cages, with floors consisting of four 6.0-mm-diameter stainless-steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed 90 × 70 × 70 cm ventilated sound-attenuating chamber. Background noise (60-dB wide-band) was provided by a General Radio Type 1390-B noise generator (Concord, MA) and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (sound pressure level) were made with a Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (Type 4176) located 7 cm from the center of the speaker (approximating the distance of the rat's ear from the speaker). Startle responses were evoked by 50-ms, 95-dB white-noise bursts generated by a Macintosh G3 computer soundfile (0–22 kHz), amplified by a Radio Shack amplifier (100 W; model MPA-200; Tandy), and delivered through the same speakers used to provide background noise. An accelerometer (model U321AO2; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage produced a voltage output proportional to the velocity of cage movement. This output was amplified (model 483B21; PCB Piezotronics) and digitized on a scale of 0–2500 U by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after onset of the startle-eliciting stimulus. The CS was a 3.7-s light (82 lux) produced by an 8 W fluorescent bulb (100 μs rise time) located 10 cm behind each cage. Luminosity was measured using a VWR light meter (Atlanta, GA). The US was a 0.5-s shock, delivered to the floorbars and produced by a shock generator (SGS-004; Lehigh Valley, Beltsville, MD). Shock intensities (measured as in Cassella and Davis, 1986) were 0.4 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc., Newton, CT). Animals were pre-exposed to the chambers for 10 min on each of 2 days prior to training to habituate them to handling and the test chambers and to minimize the effects of contextual conditioning. On 2 consecutive days following habituation, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 s) coterminating with a 0.4-mA, 0.5-s shock (3.6-min intertrial interval).

Matching

At 24 h following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-ms, 95-dB white-noise bursts) in the presence or absence of the light-conditioned stimulus (15 light-startle compounds and 15 startle alone). Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented in compound with the startle stimulus *vs* when it was omitted (fear-

potentiated startle or FPS). Using these measurements, animals were divided into groups displaying approximately equal levels of FPS prior to drug treatment and extinction training.

Extinction Training

At 5 days following the last fear-conditioning trial, animals were injected intraperitoneally with a test compound or its vehicle in 1 ml/kg volumes and then immediately returned to the same chambers and presented with 30 or 90 presentations of the light-CS in the absence of footshock (3.7-s light, 30-s intertrial interval). At 1 h following this extinction training session, animals were given a short test consisting of startle stimuli in the presence or absence of the light-CS (2–5 light-startle compounds and 2–5 startle alone, values shown are averages of all trials). At 24 h postextinction training, all animals were tested for the presence of fear-potentiated startle (15 light-startle compounds and 15 startle alone). As animals showed a large amount of extinction within the 24-h testing session (within-session extinction), the FPS values shown for all drug studies are the average FPS during the first five light-startle compounds.

Reinstatement

Previously fear-conditioned and extinction-trained animals were returned to the testing chamber 48 h following extinction training and presented with three footshocks in the absence of the light-CS (0.4 mA, 0.5 s shock, 2 min intertrial interval). Immediately following the unpaired shocks, animals were tested for the presence of fear-potentiated startle (15 light-startle compounds, and 15 startle alone).

Shock Reactivity, Startle, and Activity Measures

A separate group of fear-conditioned animals was injected with AM404, placed in the training/testing chambers, and presented with three unpaired shocks and 42 startle stimuli (0.4-mA, 0.5-s shocks, 95-dB noise-burst startle). The same group of animals was returned to the same chambers 3 days later, injected with vehicle, and presented with an identical behavioral test. The values shown are the mean integrated voltages of the accelerometers measured over 200-ms periods beginning at the onset of either the shocks or the startle stimuli. Additionally, a measure of spontaneous motor activity was derived from the mean displacement of the accelerometers in the 2 min prior to delivery of the first shock, while animals were exploring the chambers.

Drugs

Rimonabant (SR141716A, NIMH Drug Supply Program, Bethesda, MD) and WIN 55,212-2 (Biomol, Plymouth Meeting, PA) were dissolved in 100% DMSO. AM404 (Biomol, Plymouth Meeting, PA) was dissolved in 70% DMSO, 30% PBS. In experiments in which both rimonabant and AM404 were used, all drugs were dissolved in 100% DMSO.

Statistics

Comparisons were made across drug-treatment groups at each test (eg 24-h groups were compared across treatment groups) using ANOVA or Student's *t*-test with drug or dose as the independent measure, and using Fischer's LSD test for *post hoc* analysis.

RESULTS

CB1 is Enriched in the Rat Basolateral Amygdala (BLA)

The BLA has been repeatedly implicated in the extinction of conditioned fear in both direct pharmacological inactivation and augmentation studies (Falls et al, 1992; Walker et al, 2002; Davis et al, 2003). *In situ* hybridization was used to determine if CB1 mRNA was expressed within the rat amygdala and whether it was differentially expressed in the basolateral, medial, and central amygdaloid nuclei. Representative sections from these *in situ* hybridization studies (Figure 1), suggest that CB1 mRNA is highly enriched in the BLA, with very little CB1 mRNA expression seen in the central (CeA) or medial nuclei (MeA) of the amygdala. Additionally, the presence of the mRNA for the CB1 protein within the BLA itself suggests that the CB1-mediated signaling taking place in the BLA is part of the intrinsic neurocircuitry of the BLA. These hybridization results are in close agreement with previous studies using immunohistochemical and hybridization techniques (Katona et al, 1999; Marsicano and Lutz, 1999; McDonald and Mascagni, 2001).

CB1 Antagonist Blocks Extinction

The next experiment examined whether pharmacologic antagonism of the CB1 receptor would disrupt extinction in rats and the dose-response relationship for this interaction. Parametric studies were performed to identify a set of behavioral manipulations that could reliably induce extinction in rats. In these studies (outlined in Figure 2a), animals

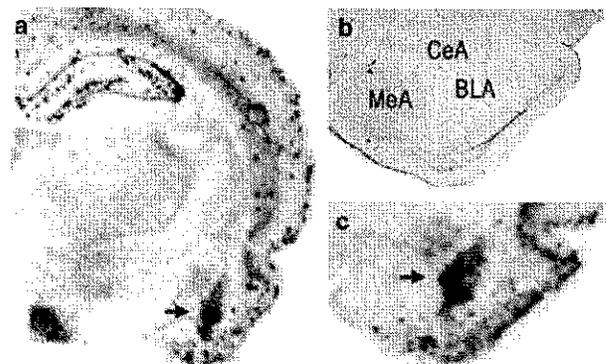


Figure 1 CB1 is densely expressed within rat BLA. Expression patterns of CB1-receptor mRNA are shown following *in situ* hybridization with a ³⁵S-labeled antisense riboprobe. (a) Dense CB1 mRNA expression is seen within amygdala (arrow) and hypothalamus, with more sparse cellular expression throughout hippocampus and cortex. (b) Cresyl violet-stained sections of the temporal lobe. (c) CB1 is most densely expressed within the basolateral amygdala (BLA, arrow). CeA = central amygdaloid nucleus, MeA = medial amygdaloid nucleus.

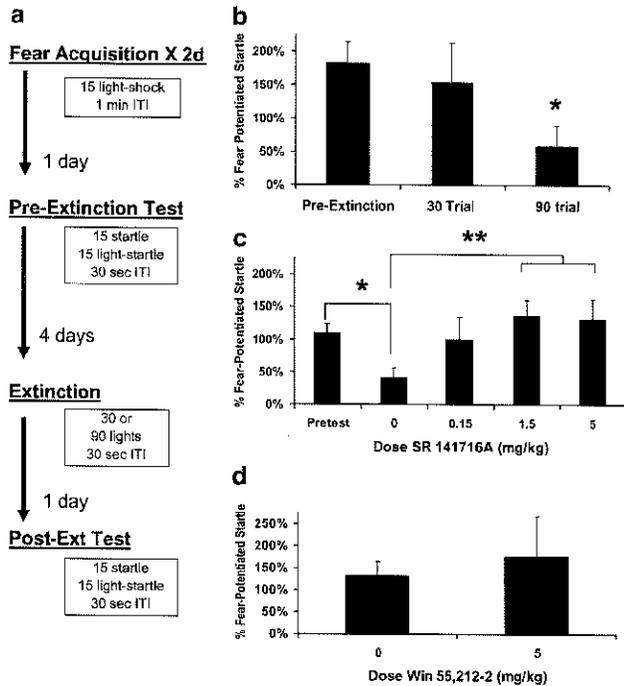


Figure 2 Effect of CB1 antagonist on extinction of fear. (a) Timeline for behavioral experiments. (b) Percent fear-potentiated startle (% FPS) 24 h following extinction training (lights without shocks). Animals receiving 90 light exposures showed significant extinction compared to pretest. (c) % FPS is shown for the pre-extinction test and 24 h postextinction tests of four groups of animals that received SR 141716A (0, 0.15, 1.5, 5 mg/kg, ip.) prior to extinction training ($n = 16$ for 0, 1.5, and 5 mg/kg groups; $n = 8$ for 0.15 mg/kg group). Only the vehicle group (0 mg/kg) demonstrated significant extinction to the light, and animals receiving the two higher doses of rimonabant displayed significantly greater % FPS than vehicle-treated controls (*denotes $p < 0.05$, **denotes $p < 0.01$). (d) % FPS following extinction in the presence of the CB1 agonist, WIN 55,212-2 ($n = 5$ per group).

showed robust fear conditioning prior to extinction training and varying the number of nonreinforced light-CS presentations decreased the amount of fear animals showed in subsequent testing trials (Figure 2b). We found that 90 trials of nonreinforced lights led to significant extinction retention, whereas only 30 trials led to a more modest, nonsignificant reduction in fear (compared to pre-extinction: 90 trials, $F_{(1,27)} = 4.05$, $p < 0.05$; 30 trials, $p > 0.05$). In all subsequent studies, the 90-trial extinction protocol was used when trying to block extinction, while the weaker 30-trial extinction protocol was used when trying to enhance extinction.

This 90-trial extinction protocol was used to test the effect of systemic administration of the CB1 antagonist rimonabant on extinction in rats. The acute administration of rimonabant to rats immediately prior to extinction training led to a profound disruption of extinction retention, as evidenced by the fact that rimonabant-treated animals showed significantly higher levels of fear in the presence of the light-CS 24 h following extinction training (Figure 2c, ANOVA dose \times postextinction FPS, $F_{(3,55)} = 3.40$, $p < 0.05$). This disruption in extinction appeared to be dose-dependent, as animals receiving 1.5 or 5 mg/kg of rimonabant showed significantly higher levels of conditioned fear

than vehicle-treated controls, and appeared to show virtually no reduction in conditioned fear following extinction training (*post hoc*, $p < 0.01$ for 1.5 and 5 mg/kg compared to vehicle). The ability of rimonabant to disrupt extinction at the relatively low doses used here suggested that the neural process underlying extinction may be extremely sensitive to the level of CB1 receptor activation during extinction training.

A Direct CB1 Agonist has No Effect on Extinction

The next experiment examined whether the application of the CB1 direct agonist WIN 55-212,2 (WIN) prior to extinction training might enhance extinction retention. A relatively high dose of WIN (5 mg/kg) was administered prior to a 30 trial-extinction training protocol, to determine if increasing CB1 activation would augment the modest extinction normally induced by this weak training protocol. The administration of 5 mg/kg WIN prior to extinction training did not enhance extinction; in contrast, WIN-treated animals actually showed a nonsignificant, but slightly higher, level of conditioned fear 24 h following extinction training (Figure 2d). Notably, the well-documented emergence of prominent locomotor and analgesic effects following administration of higher doses of WIN (eg Tsou *et al*, 1996; Herzberg *et al*, 1997) limited our ability to test the effects of doses of WIN greater than 5 mg/kg.

One explanation for this lack of agonist effect on extinction is that the CB1 receptor could be rapidly downregulated following direct agonist administration (Coutts *et al*, 2001; Hsieh *et al*, 1999). The next experiments examined whether augmentation of endogenously released eCBs, instead of direct agonist administration, would have a different effect.

AM404, an Inhibitor of Cannabinoid Reuptake and Breakdown, Enhances Extinction

In contrast to the potential compensatory decrease in efficacy of CB1-mediated transmission following direct agonist administration, an inhibitor of eCB reuptake or breakdown may enhance extinction by prolonging the action of released eCBs. This, in turn, would lead to increases in activity-dependent CB1-receptor activation.

Consistent with this hypothesis, administration of AM404 prior to 30-trial extinction training led to an enhancement of extinction retention, as AM404 animals showed significantly less fear in the presence of the CS 24 h following extinction training (Figure 3a, main effect of drug treatment $F_{(1,70)} = 4.06$, $p < 0.05$). This enhancement of extinction appeared to be dose-dependent, as animals treated with 10 mg/kg AM404 showed less fear than those treated with 2 mg AM404 and significantly less than vehicle-treated animals (10 mg vs control, *post hoc* $p < 0.05$).

A subset of AM404-treated animals was tested 1 h following extinction to assess whether the effects of AM404 were likely taking place during the acquisition phase of extinction. The AM404-induced enhancement of extinction was evident 1 h postextinction, as animals that received the 10-mg/kg dose of AM404 showed significantly less fear than vehicle-treated controls (Figure 3b, ANOVA

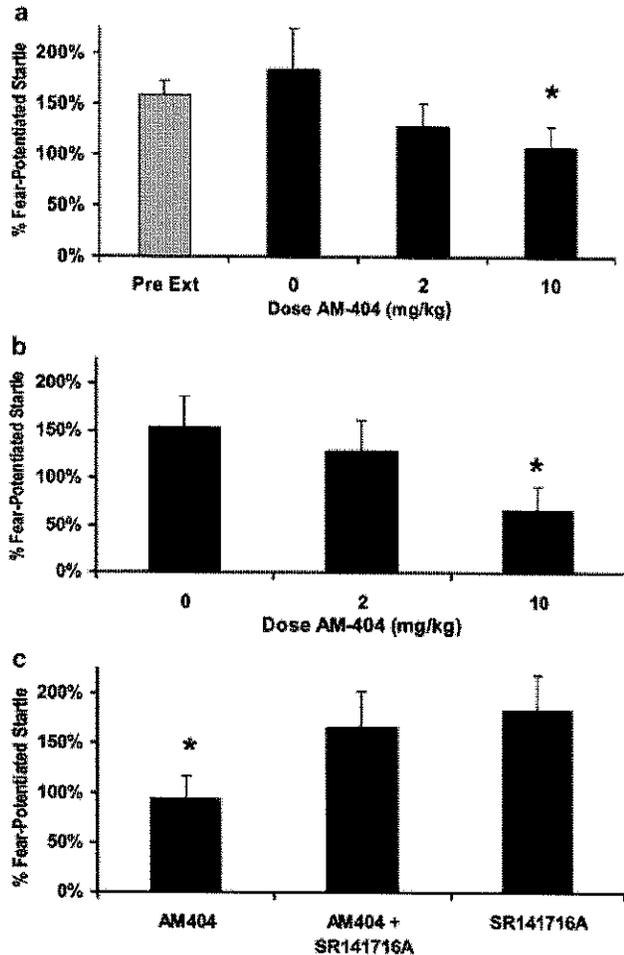


Figure 3 CB1 reuptake inhibitor enhances extinction. AM404 was given prior to extinction training in rats previously fear conditioned as in Figure 2a. (a) % FPS during 24 h postextinction testing in animals that received 0, 2, or 10 mg/kg AM404, i.p., prior to extinction training ($n = 21$ for 0 and 2 mg/kg; $n = 29$ for 10 mg/kg). These data demonstrate increasing extinction (decrement in % FPS) with increasing doses of AM404. (b) % FPS during 1 h postextinction testing in animals that received 0, 2, or 10 mg/kg AM404, i.p., prior to extinction training ($n = 13$ for 0 and 10 mg/kg, $n = 12$ for 2 mg/kg). (c) % FPS during 24 h postextinction testing in animals that received 10 mg/kg AM404 i.p., 5 mg/kg rimonabant i.p., or the combination prior to extinction training ($n = 8$ per group), demonstrating that coadministration of a CB1 antagonist prevented AM404 from enhancing extinction. (*denotes $p < 0.05$)

linear contrast $F_{(1,37)} = 4.89$, $p < 0.05$; *post hoc* comparison, 10 mg/kg vs vehicle, $p < 0.05$).

AM404-Dependent Enhancement of Extinction Appears to be Mediated by Activation of CB1 Receptor

AM404 has been implicated in the inhibition of eCB reuptake as well as in inhibiting FAAH (Jarrhian *et al*, 2000; Beltramo *et al*, 2000; Giuffrida *et al*, 2001), but does not itself activate CB1 receptors (eg Beltramo *et al*, 1997). As the enzyme FAAH participates in the breakdown of a number of neuroactive arachidonic-acid derivatives (Giuffrida *et al*, 2001) and some have suggested that AM404 may also act at the vanilloid receptor (VR1, Smart and Jerman,

2000), a series of experiments was performed to determine if the AM404-induced enhancement of extinction requires CB1 activation.

Animals were fear conditioned and extinction trained (30 trial extinction) as in the previous study, and prior to extinction training administered 10 mg/kg AM404, 10 mg/kg AM404 + 5 mg/kg rimonabant, or 5 mg/kg rimonabant alone. During testing, animals administered AM404 + rimonabant and rimonabant alone showed virtually no decrease in FPS 24 h following extinction. In contrast, animals treated with 10 mg/kg AM404 alone showed significant extinction ($t_{(26)} = 2.36$, $p < 0.05$ AM404 alone as compared to pre-extinction), and significantly less fear than animals receiving AM404 + rimonabant or rimonabant alone (Figure 3c, $F_{(1,23)} = 5.40$, $p < 0.05$, rimonabant and rimonabant + AM404 groups pooled for comparison). Taken together, these results suggest that the enhancement of extinction seen in AM404-treated animals is mediated via CB1-receptor activation.

AM404-Induced Enhancement of Extinction Requires Cue-Exposure and is not Due to Drug-Induced Changes in the Expression of Conditioned Fear

A series of control experiments was performed to rule out the possibility that AM404 administration itself could lead to decreases in the expression of conditioned fear, even in the absence of cue re-exposure during extinction training. To this end, a parallel set of rats was fear conditioned and matched for equivalent levels of FPS as in the above studies. On the day on which extinction training was to be performed, animals were administered 10 mg/kg AM404, 5 mg/kg rimonabant, or vehicle, but cue re-exposure was omitted. At 1 h following drug administration, animals were tested for FPS using a procedure similar to the above studies. The results from these studies indicate that the highest doses of AM404 and rimonabant used here had no effect on FPS if cue-exposure was omitted, as all drug groups showed similar levels of conditioned fear 1 h following drug administration (Figure 4a).

AM404 Treatment Does not Lead to Obvious Analgesic or Locomotor Effects

To better understand the behavioral effects engendered by AM404 treatment, a series of control experiments was performed. These included testing the effects of 10 mg/kg AM404 on (1) shock reactivity as a measure of pain sensitivity, (2) baseline startle as one measure of anxiety, and (3) general motor activity within the training chambers. Animals were fear conditioned and then returned to the training chamber several days later and administered 10 mg/kg AM404. Following drug administration, animals were presented with three shocks and 42 startle stimuli identical to those used in the above studies. Subsequently, the same animals were returned to the testing chamber 3 days later, injected with vehicle, and similarly tested. The results from these studies (Figures 4b–d) showed that the administration of AM404 had little effect on shock reactivity or overall locomotor activity levels in the testing chamber ($p > 0.5$ for both comparisons). The apparent decrease in baseline startle observed following AM404 administration

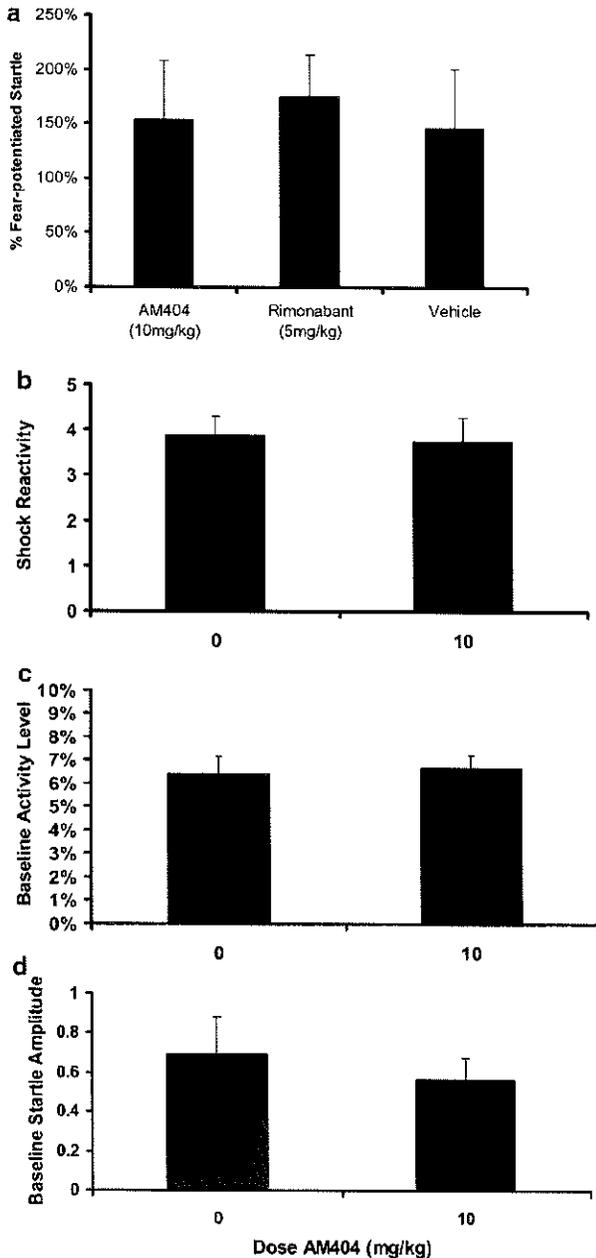


Figure 4 AM404 effect on extinction is independent of effects on the expression of conditioned fear, pain, locomotion, and baseline anxiety. (a) Animals fear-conditioned as in the above studies were administered AM404 (10 mg/kg), rimonabant (SR141716A, 5 mg/kg), or vehicle and 1 h later tested for FPS (cue re-exposure was omitted, $n=8$ per group). Neither AM404 nor rimonabant treatment led to significant alterations in the expression of conditioned fear when cue re-exposure was omitted. (b) Average shock reactivity is shown in arbitrary units and represents the average response to three footshocks. (c) Average baseline activity level is shown in arbitrary units as determined by mean displacement of accelerometers during the 2 min prior to the delivery of any shocks in the test chambers. (d) Average baseline startle amplitude shown in arbitrary units during the presentations of startle stimuli ($n=8$ per group for b–d).

was not significant ($p=0.45$). Taken together, these results suggest that the administration of AM404 at the doses used in this study are insufficient to generate obvious motor or

analgesic effects, and do not likely affect anxiety levels as measured by baseline startle amplitude.

AM404 Treatment Prior to Extinction Training Decreases Shock-Induced Reinstatement of Fear

Shock-induced reinstatement was then examined 2 days following treatment with AM404 or vehicle during extinction. Previous studies have shown that the level of fear following reinstatement is dependent both on the level of the stressor and the amount of previous extinction, as long as the stressor is delivered in the same context as the original training context (Rescorla and Heth, 1975; Bouton and King, 1983). Thus, diminished reinstatement following extinction serves as an additional measure of the strength of the extinction process. As animals were matched for equivalent FPS prior to extinction training, the susceptibility of animals to reinstatement can be taken as a secondary measure of the strength of extinction training, and perhaps as a preliminary measure of the resiliency of these inhibitory extinction memories to stressors.

In these studies, animals that had previously been fear conditioned, extinction trained, and tested for extinction retention were returned to the training chambers and presented with three footshocks (in the absence of light-CS presentation) followed by a test for the presence of FPS to the light-CS. During these reinstatement tests, AM404-treated animals showed less reinstatement-induced conditioned fear, whereas control animals showed a transient but robust re-emergence of conditioned fear following the unpaired footshocks. This effect was especially prominent during the first two testing trials, where vehicle-treated animals showed significantly more fear to the light CS than their AM404-treated counterparts (Figure 5a $t_{(63)}=4.5$, $p<0.05$, 2 and 10 mg/kg AM404-treated groups pooled for comparison to vehicle). Additionally, examination of within-session extinction demonstrated a significant decrease in FPS among vehicle-treated groups, but little change among AM404-treated groups (Figure 5b, repeated measures ANOVA, Trial \times Drug interaction, $F_{(1,62)}=5.67$, $p<0.02$). Note that within this period of extinction testing, neither group reached terminal levels of extinction.

DISCUSSION

These experiments demonstrate that: (1) CB1 mRNA is expressed densely and relatively specifically within the rat BLA, a region implicated in the extinction of conditioned fear, and there is little expression seen in the medial and central nuclei; (2) systemic application of a specific CB1 antagonist (SR 141716A) to rats dose-dependently blocks the extinction of fear as it does in mice; (3) this dose-dependent blockade of extinction is robust and easily measured using fear-potentiated startle as a measure of fear; (4) systemic application of AM404, an inhibitor of eCB breakdown and membrane transport, dose-dependently enhances extinction of fear as measured at different times following cue re-exposure; (5) this enhancement of extinction is not likely due to changes in baseline anxiety, locomotion, or nociception; (6) the enhancement of extinction with AM404 is likely CB1-dependent, as this

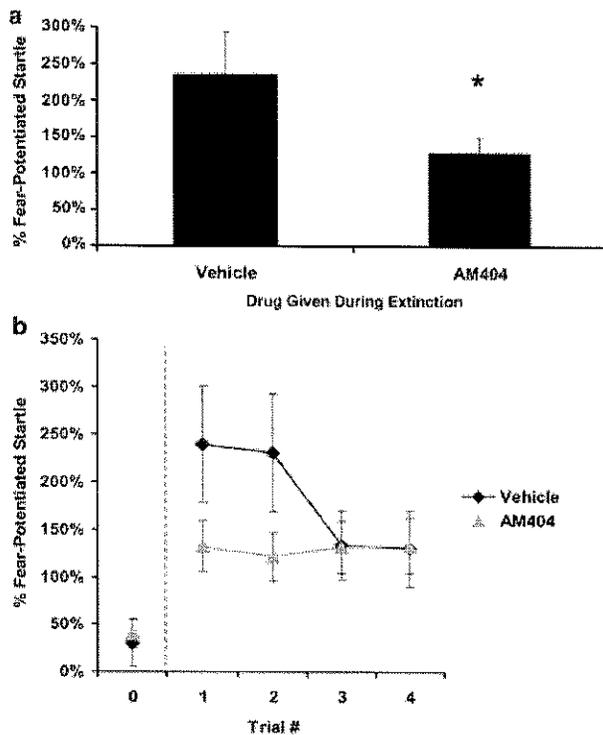


Figure 5 AM404-enhanced extinction decreases shock-induced reinstatement. (a) % FPS is shown during testing following reinstatement with three footshocks. Animals had received vehicle ($n = 21$) or AM404 (2-mg and 10-mg groups combined, $n = 42$) prior to extinction training (ie, 48 h prior to reinstatement). Animals that received AM404 prior to extinction training demonstrated significantly less % FPS following reinstatement than did control animals. (*denotes $p < 0.05$) (b) Within-session extinction is shown for the first four trials during the testing of FPS following the reinstatement experiment described in (a). The terminal level of FPS in the last testing trial prior to reinstatement (shown as trial 0) indicates that vehicle and AM404-treated animals showed similar FPS levels prior to reinstatement.

effect is blocked almost completely by coadministration of rimobant; and (7) this enhancement of extinction appears to diminish reinstatement of fear following footshock.

As has been shown previously, eCB function is normally required for extinction of fear in mice using both pharmacological (Marsicano *et al*, 2002; Suzuki *et al*, 2004) and genetic approaches in CB1 knockout animals (Marsicano *et al*, 2002). In this study, we demonstrate that blockade of the CB1 receptor with rimobant also prevents extinction of fear in rats as measured with the fear-potentiated startle paradigm.

That a similar effect did not occur with systemic application of the direct CB1 agonist, WIN 55,212-2, could be due to a rapid downregulation or desensitization of the CB1 receptor following prolonged activation (Coutts *et al*, 2001; Hsieh *et al*, 1999). Direct CB1 agonists have been shown to lead to downregulation of CB1 receptors (Hsieh *et al*, 1999) and to uncoupling of the CB1 receptor from its effector G-protein, G_i (Mato *et al*, 2004). The presence of a physiologic mechanism to rapidly decrease the efficacy of CB1-mediated transmission would have important implications for future and ongoing clinical studies examining the use of direct CB1 agonists for the treatment of anxiety

disorders and drug addiction. Future studies examining a broader range of doses of WIN 55,212-2 would be necessary to definitely determine if a lower dose of this drug might enhance extinction without leading to potential CB1 downregulation. These concerns make the use of inhibitors of cannabinoid reuptake and of FAAH more clinically attractive, since the cannabinoid system has very low basal levels of activation (Giang and Cravatt, 1997; Cravatt *et al*, 2001).

Reinstatement of fear is one of the principle behavioral processes upon which the idea is based that extinction does not lead to an 'erasure' of memory, but rather to a parallel inhibitory process that masks the previous fear memory (for a review, see Myers and Davis, 2002). We found that animals that had received AM404 during the extinction exposure showed less initial fear-potentiated startle when tested following reinstatement in the absence of any drug (Figure 5a and b). This finding is consistent with previous findings in which pharmacological enhancement of extinction with D-cycloserine (DCS) leads to less reinstatement (Ledgerwood *et al*, 2004), and provides further support for the hypothesis that the extinction seen following AM404 treatment is more robust and less susceptible to subsequent stress than the extinction seen in vehicle-treated controls. Future studies using more clinically relevant stressors and contexts are needed to clarify whether AM404 reduces susceptibility to reinstatement in a therapeutically useful way.

Drugs that can be given only at the time of extinction may provide for a new and powerful way to treat anxiety disorders. We and others have previously shown that extinction, which is known to be NMDA-dependent (Falls *et al*, 1992; Santini *et al*, 2001; Suzuki *et al*, 2004), can be enhanced with systemic or local administration into the amygdala of DCS, a partial NMDA agonist (Walker *et al*, 2002; Ledgerwood *et al*, 2003, 2004). Follow-up clinical trials have now demonstrated that this approach may be successful in humans as well (Ressler *et al*, 2004; Rothbaum and Davis, 2003).

AM404 and other inhibitors of the anandamide transporter, such as the newly identified AM1172 (Fegley *et al*, 2004), may enhance the process of extinction through an alternate mechanism. Since DCS can potentially activate all NMDA receptors, it is possible that it could enhance fear learning as well as extinction, although this has not been observed experimentally (Ledgerwood *et al*, 2003). In contrast, the CB1-receptor knockout mice have no decrement in fear learning, and pharmacological blockade of CB1 does not affect fear conditioning (Marsicano *et al*, 2002). Therefore, it appears that the activation of the cannabinoid system may be relatively specific to effects on inhibitory learning within the BLA, and it may not be required for or have a substantial impact on excitatory learning such as fear conditioning. This idea fits well with recent physiologic studies suggesting that CB1 activation may lead to enhanced LTD by presynaptically decreasing GABA release within the BLA (Azad *et al*, 2003; Chevaleyre and Castillo, 2003), perhaps via coincident activation of both presynaptic NMDA and cannabinoid receptors, as has been elegantly shown in the neocortex (Sjostrom *et al*, 2003). Lastly, it has been shown that extinction learning critically requires the activation of MAP kinase and calcineurin (Lu *et al*, 2001;

Lin *et al*, 2003a,b), and that CB1-receptor activation regulates the activity of a variety of kinases and phosphatases within the BLA (including MAP kinase and calcineurin, Cannich *et al*, 2004), providing a putative mechanistic link between eCB modulation and the plasticity underlying extinction.

Taken together, the findings in the present study suggest that augmenting eCB-mediated neurotransmission by inhibition of eCB transport or breakdown may provide a novel mechanism for enhancing the extinction of fear. As such, eCB reuptake inhibitors may serve as useful adjuncts in the treatment of anxiety disorders (such as PTSD, panic disorder, and OCD) as well as drug addiction and other disorders that respond to behavioral treatments utilizing extinction processes.

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Altered amygdala resting-state functional connectivity in post-traumatic stress disorder

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Post-traumatic stress disorder (PTSD) is often characterized by aberrant amygdala activation and functional abnormalities in corticolimbic circuitry, as elucidated by functional neuroimaging. These "activation" studies have primarily relied on tasks designed to induce region-specific, and task-dependent brain responses in limbic (e.g., amygdala) and paralimbic brain areas through the use of aversive evocative probes. It remains unknown if these corticolimbic circuit abnormalities exist at baseline or "at rest," in the absence of fear/anxiety-related provocation and outside the context of task demands. Therefore the primary aim of the present experiment was to investigate aberrant amygdala functional connectivity patterns in combat-related PTSD patients during resting-state. Seventeen Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) veterans with combat-related PTSD (PTSD group) and 17 combat-exposed OEF/OIF veterans without PTSD [combat-exposed control (CEC) group] underwent an 8-min resting-state functional magnetic resonance imaging scan. Using an anatomically derived amygdala "seed" region we observed stronger functional coupling between the amygdala and insula in the PTSD group compared to the CEC group, but did not find group differences in amygdala–prefrontal connectivity. These findings suggest that the aberrant amygdala and insula activation to fear-evocative probes previously characterized in PTSD may be driven by an underlying enhanced connectivity between the amygdala, a region known for perceiving threat and generating fear responses, and the insula, a region known for processing the meaning and prediction of aversive bodily states. This enhanced amygdala–insula connectivity may reflect an exaggerated, pervasive state of arousal that exists outside the presence of an overt actual threat/danger. Studying amygdala functional connectivity "at rest" extends our understanding of the pathophysiology of PTSD.

Keywords: amygdala, resting-state, functional magnetic resonance imaging, post-traumatic stress disorder

INTRODUCTION

Post-traumatic stress disorder (PTSD) is characterized by various altered emotional responses as a result of trauma exposure (e.g., combat, assault, and disasters). Patients with PTSD not only experience intense negative emotional reactions when reminded of their trauma but also report exaggerated arousal (poor sleep, restlessness, hypervigilance), anhedonia, social withdrawal, and decreased emotional expressivity, referred to as "emotional numbing." Characterizing the neural basis of these diverse, distorted emotional responses poses a major challenge to contemporary psychiatric research. Functional neuroimaging techniques have focused primarily on the study of brain function related to fear perception and response, and have consistently implicated aberrant amygdala reactivity to fear-relevant probes and other abnormalities in a broad aberrant amygdala-linked circuitry involving the medial prefrontal cortex (mPFC), insula, anterior cingulate cortex (ACC), and hippocampus (Rauch and Shin, 1997; Pitman et al., 2001; Nemeroff et al., 2006; Rauch et al., 2006; Rabinak and Wager,

2007; Liberzon and Sripada, 2008; Shin, 2009; Shin and Liberzon, 2010). Together these interconnected regions form a disrupted functional network thought to be responsible for impaired regulation of fear responses, enhanced attention to threat-related stimuli, and biased memory for adverse events (Shin, 2009). Anxiety disorders, such as PTSD, are believed to manifest from dysfunction in a complex integrated functional network, largely, between cortical and limbic regions (Gilboa et al., 2004; Lanius et al., 2005; Simmons et al., 2008; Bluhm et al., 2009; Shaw et al., 2009; Daniels et al., 2010). Some studies have begun to examine these brain circuits and region-to-region interactions, by measuring the extent to which activity in one region is correlated with activity in another during a particular task. Although these dysfunctional networks have been implicated in mediating several characteristics of PTSD, such as, hyperarousal, abnormal reactivity to emotional stimuli, and avoidance of emotionally distressing memories (Nemeroff et al., 2006; Shin and Liberzon, 2010), little is known about how these regions may interact dynamically within individual subjects.

Some clues exist from anatomical and functional studies that these brain regions may indeed form a network responsible for emotion processing. Tracer studies in non-human primates (Amaral and Price, 1984; Saunders et al., 1988; Barbas and De Olmos, 1990; Stefanacci et al., 1996; Ghashghaei and Barbas, 2002; Ghashghaei et al., 2007; Freese and Amaral, 2009) and, more recently, diffusion tensor imaging studies in humans (Crosson et al., 2005; Johansen-Berg et al., 2008; Bracht et al., 2009) have identified robust bidirectional projections between the amygdala and the mPFC, rostral ACC (rACC), insula, and hippocampus. Consistent with known anatomical connections, several studies that have examined functional connectivity of the amygdala have found significant co-activation and/or functionally correlated activation of the amygdala and the mPFC, insula, hippocampus, and rACC (Phan et al., 2002; Wager et al., 2003, 2008; Stein et al., 2007a; Kober et al., 2008; Etkin et al., 2009; Roy et al., 2009). It is well established that negatively valenced emotional stimuli activate the amygdala, which mediates subjective and attentional-vigilance aspects of threat processing (Liberzon et al., 1999; Phan et al., 2002, 2004; Liberzon and Phan, 2003; Taylor et al., 2003; Wager et al., 2003; Etkin and Wager, 2007; Kober et al., 2008; Liberzon and Sri-pada, 2008; Etkin, 2009; Shin and Liberzon, 2010). Similarly, insula activity also increases in response to emotionally aversive stimuli that evoke visceral or somatic sensations (Simmons et al., 2004). Increased amygdala and insula activation during fear conditioning have been shown to be reliably associated with one another (Etkin and Wager, 2007). Amygdala activity is decreased in response to suppression of negative affect via reappraisal and during inhibition of conditioned fear responses as a result of increased activation in the mPFC and rACC, which exert top-down inhibitory influences on amygdala reactivity to fear and threat (Ochsner et al., 2002; Taylor et al., 2003; Phelps et al., 2004; Etkin et al., 2006, 2011; Urry et al., 2006; Delgado et al., 2008; Quirk and Mueller, 2008). The magnitude of task-dependent functional coupling between the amygdala and mPFC/rACC has been shown to be negatively correlated with intensity of subjective reports of negative affect (Banks et al., 2007). Increased functional connectivity between the amygdala and the hippocampus has been attributed to the persistence of memories for emotionally arousing events (Hamann et al., 1999; Kalpatrick and Cahill, 2003; Phelps, 2004; Ritchey et al., 2008; Murty et al., 2011). Specifically, the hippocampus forms episodic representations of the emotional significance and interpretation of events, and influences amygdala activity when emotional stimuli are encountered (Phelps, 2004). These lines of convergent evidence suggests that how the amygdala interacts with other regions may mediate the control, or lack thereof, of fear perception and emotional arousal in humans.

Dysfunctions within discrete areas that form an amygdala-paralimbic/frontal network have been implicated in mediating several characteristics of PTSD, such as, hyperarousal, abnormal reactivity to emotional stimuli, and avoidance of emotionally distressing memories (Nemeroff et al., 2006; Shin and Liberzon, 2010). In particular, many studies have shown amygdala hyperactivity in PTSD in response to trauma-related imagery (Shin et al., 1997, 2004a), combat-related sounds or smells (Liberzon et al., 1999; Pissioti et al., 2002; Vermetten et al., 2007), trauma-related photographs or words (Hendler et al., 2003; Driessen et al.,

2004; Protopopescu et al., 2005; Morey et al., 2009), and fearful facial expressions (Rauch et al., 2006; Shin et al., 2005; Williams et al., 2006; Bryant et al., 2008). Exaggerated amygdala reactivity observed in PTSD has been posited to be a result of insufficient top-down regulation from the mPFC and ACC, consequently leading to hyperarousal and deficits in extinction as well as the inability to suppress enhanced fear perception or exaggerated fear responses to trauma-related stimuli (Rauch and Shin, 1997; Rauch et al., 1998; Pitman et al., 2001; Liberzon and Phan, 2003); for example, Shin et al. (2004a, 2005) have observed that exaggerated amygdala reactivity is negatively correlated with responses in the dorsal and ventral mPFC across individuals with PTSD. However, Gilboa et al. (2004) found little evidence for failure of inhibition of ACC over the amygdala in individuals with PTSD related to civilian trauma during symptom provocation and in fact found that amygdala activity significantly influenced ACC activity. Insula hyperactivity has been observed in PTSD patients and given its role in the experience (e.g., somatic sensation) of negative emotions and structural connectivity to amygdala (Augustine, 1996; Aggleton and Saunders, 2000; Freese and Amaral, 2009), the insula may be working in concert with aberrant amygdala responses (Bremner et al., 2003, 2005; Lanius et al., 2007; Vermetten et al., 2007; Lindauer et al., 2008; Simmons et al., 2008; Werner et al., 2009; Whalley et al., 2009). Although less commonly implicated, abnormal hippocampal function, and diminished hippocampal volumes in PTSD patients have been associated with deficits in contextual processing, as well as memory impairments, and neuroendocrine dysregulation (Bremner et al., 1999, 2003; Bonne et al., 2001; Shin et al., 2004a,b, 2006; Werner et al., 2009).

Recently these functional connectivity techniques have been applied to the study of corticolimbic circuitry abnormalities at baseline or "at rest" (resting-state functional connectivity). Studies of functional interconnectivity of brain regions derived from "resting-state" scans provides insight into the relationship of spontaneous brain activity between brain regions without being confounded by task influences on activation and has even been shown to reflect structural connectivity between brain regions (Greicius et al., 2009; van den Heuvel et al., 2009). In healthy humans resting-state functional connectivity of the amygdala has revealed patterns of connectivity consistent with task-based connectivity patterns (Stein et al., 2007a; Roy et al., 2009). Moreover, resting-state functional connectivity has been a useful tool for identifying abnormalities in the functional organization of brain systems in several anxiety and mood disorders (Greicius, 2008). However, little is known about what abnormalities, if any, in amygdala connectivity exist at rest in PTSD. Therefore the primary aim of the present experiment was to investigate aberrant amygdala functional connectivity patterns in returning Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) veterans with combat-related PTSD (PTSD group) and combat-exposed OEF/OIF veterans without PTSD [combat-exposed control (CEC) group] during resting-state. We hypothesized that amygdala connectivity to the ACC, mPFC, insula, and hippocampus would differentiate the PTSD group from the CEC group. If observed, such findings would extend our understanding of the pathophysiology of PTSD by identifying a disturbed network that exists

outside of the presence of an overt threat/danger or in the absence of stimulus or task-induced negative emotional processing.

MATERIALS AND METHODS

PARTICIPANTS

Thirty-four, right-handed, male veterans returning from OEF/OIF with documented exposure to combat-related trauma participated in this study. Based on the DSM-IV (APA, 1994), 17 participants met criteria for current PTSD (PTSD group; age: 30.12 ± 7.70 years; Caucasian = 16; Hispanic or Latino = 1) and the other 17 participants were combat-exposed matched controls without PTSD (CEC group; age: 33.71 ± 9.12 ; Caucasian = 16; Asian = 1). Psychiatric diagnoses were established via the Structured Clinical Interview for DSM-IV (First et al., 1996). Additional standardized clinical instruments including the Clinician Administered PTSD Scale (CAPS; Blake et al., 1995), the PTSD Checklist: Military (PCL-M; Blanchard et al., 1996), the Combat Exposure Scale (CES; Keane et al., 1989), the Hamilton Depression Inventory (HAM-D; Williams, 1988), and the Beck Depression Inventory (BDI-II; Beck et al., 1996) were administered to quantitatively characterize PTSD symptoms, severity of trauma exposure, and depression.

Table 1 shows the participant's demographic and clinical characteristics. Relative to the CEC group, the PTSD group had significantly higher scores on the CAPS, PCL-M, and HAM-D and BDI-II. Of note, the groups did not differ in severity of trauma exposure. Some of the PTSD patients had current psychiatric co-morbidity ($n = 2$ with current major depressive disorder; $n = 2$ with current alcohol abuse) or had a past co-morbidity more than 6 months ago ($n = 1$ had major depressive disorder; $n = 4$ had alcohol abuse, one of whom also had past opioid abuse; $n = 1$ had alcohol dependence in full sustained remission) at the time of scanning. In addition, some PTSD patients had a history of psychotropic medication usage ($n = 8$ had taken a selective serotonin reuptake inhibitor, one of whom had also taken a norepinephrine-dopamine reuptake inhibitor; $n = 1$ had taken a tri-cyclic antidepressant; $n = 2$ had taken a serotonin antagonist-reuptake inhibitor), but none of the PTSD patients were currently taking any psychotropic medications at the time of scanning. All participants were free

of any clinically significant medical or neurologic condition that would affect brain blood flow/metabolism or function and/or task performance. None of the subjects had a positive urine toxicology screen at the time of scanning. All participants gave written informed consent after explanation of the experimental protocol, as approved by the VA Ann Arbor Healthcare System and University of Michigan Institutional Review Boards.

FUNCTIONAL IMAGING ACQUISITION

All participants underwent an 8-min resting-state fMRI scan in which they were instructed to fixate on a white crosshair that was centrally projected against a black background and let their mind wander without falling asleep. fMRI scanning was performed on a 3T GE Signa System (General Electric; Milwaukee, WI, USA) using a standard radiofrequency coil at the University of Michigan Functional MRI Laboratory. Whole-brain functional images (i.e., blood oxygenated level-dependent, BOLD) were collected from 43 axial, 3-mm-thick slices using a T_2^* -sensitive gradient echo reverse spiral acquisition sequence (repetition time, 2000 ms; echo time, 30 ms; 64×64 matrix; 220 mm field of view; flip angle, 90°), optimized to minimize susceptibility artifacts (signal loss) at the medial temporal lobe (including the amygdala; Stenger et al., 2000). Cardiac and respiratory cycles were recorded with MRI vendor supplied pulse-oximeter and respiratory belt for physiological corrections on resting-state data. A T_1 -weighted anatomical image was collected in the same planes as the functional data, but with higher in-plane resolution (1 mm^2 , T_1 -overlay) to aid in later co-registration. A high resolution, T_1 -weighted volumetric anatomical scan (T_1 -SPGR; three-dimensional spoiled gradient echo) was also acquired for precise anatomical localization and normalization.

FUNCTIONAL IMAGING ANALYSIS

Data from 32 participants (CEC = 17; PTSD = 15) met criteria for high quality and scan stability with minimum motion correction and were subsequently included in fMRI analyses ($<3 \text{ mm}$ displacement in any one direction; two PTSD patients were excluded for poor data quality due to excessive head movement). The first four volumes were discarded to allow for T_1 equilibration effects. Functional data were processed and analyzed using Statistical Parametric Mapping software (SPM8; Wellcome Trust Centre for Neuroimaging, London¹) using similar methods previously published from our lab (Jelsone-Swain et al., 2010). Images were corrected for physiological signal fluctuations using a custom code written in MATLAB (MathWorks, Natick, MA, USA; Noll et al., 1991). Slice timing and movement correction was done to the time-series data using SPM8. Each participant's T_1 -overlay was co-registered to the time-series data and the T_1 -SPGR was then co-registered to the co-registered T_1 -overlay image. The co-registered T_1 -SPGR was then segmented into gray matter, white matter, and cerebrospinal fluid (CSF) and normalized to Montreal Neurological Institute (MNI) space using VBM8 toolbox of SPM8 and the resulting normalization matrix was applied to the time-series data. These normalized time-series data were subsequently re-sampled

Table 1 | Group demographic and clinical characteristics.

	Group mean (\pm SD)		t Value	p Value
	PTSD	CEC		
Age	30.12 (7.70)	33.71 (9.12)	-1.24	0.22
CAPS	67.35 (12.41)	5.24 (5.75)	18.72	<0.001
PCLM	54.59 (9.78)	25.06 (7.34)	9.96	<0.001
BDI-II	22.76 (7.46)	5.53 (6.25)	7.30	<0.001
HAM-D	10.18 (3.75)	2.18 (2.38)	7.44	<0.001
CES	23.88 (5.98)	21.47 (5.50)	1.22	0.23

PTSD, post-traumatic stress disorder; CEC, combat-exposed controls; CAPS, Clinician Administered PTSD Scale; PCL-M, PTSD Checklist: Military; BDI-II, Beck Depression Inventory; HAM-D, Hamilton Depression Inventory; CES, Combat Exposure Scale.

¹www.fil.ion.ucl.ac.uk/spm

to 2 mm³ voxels and smoothed with an 8-mm Gaussian kernel to minimize noise and effects due to residual differences in functional and gyral anatomy during inter-subject averaging. Then the resulting white matter and CSF segments were further defined using a custom algorithm previously described (Welsh et al., 2007). Each voxel's time-series was detrended to correct for linear drift over time. Nine nuisance covariates (time-series predictors for global signal, white matter, CSF, and the six movement parameters, including the first derivative, obtained during realignment to account for motion-related effects in BOLD) were sequentially regressed from the time-series. The resulting time-series were then band-pass filtered between the frequencies of 0.01 and 0.10-Hz to limit the analysis to resting-state frequencies of interest.

To determine amygdala connectivity during resting-state, seed regions in the left and right amygdala were defined by an anatomically based amygdala mask in each hemisphere (from MAsk of region of interest analysis software, MARINA; Tzourio-Mazoyer et al., 2002; Walter et al., 2003). We then extracted the averaged time course from these seed regions in each participant's data and calculated correlation coefficients between these average time courses and all other voxels of the brain resulting in an *r*-image for amygdala connectivity. The resulting correlation coefficients were then transformed into *Z*-scores using a Fisher *r*-to-*Z* transformation and the resulting *Z* images were analyzed at the second level in a random-effects statistical model. Two-tailed independent samples *t* tests were used to identify areas of the brain that exhibited activity that covaried with the amygdala differentially during resting-state between the two groups (PTSD > CEC; CEC > PTSD). Significant activations were identified with a whole-brain voxel-wise threshold of $p < 0.005$ with a minimum cluster extent of >387 contiguous voxels (3096 mm³), to correct for multiple comparisons at a corrected $p < 0.05$ calculated using Monte-Carlo simulations (AFNI 3dClustSim²). Previous studies interested in differences in brain connectivity between patients with PTSD and trauma-exposed controls without PTSD have used similar significance

thresholding approaches to balance Type I and II error rates (Yin et al., 2011a,b). To clarify the signal direction, variance, and specificity of differences in strength of connectivity between the CEC and PTSD groups during resting-state, we extracted individual subject's *Z*-score values from activated voxels that fell within an anatomically based mask for each *a priori* region from the between-group contrast (PTSD > CEC; Tzourio-Mazoyer et al., 2002; Walter et al., 2003). Of note, we did not conduct statistical tests on these measures, as they were defined from significant activations resulting from whole-brain maps of group differences in connectivity.

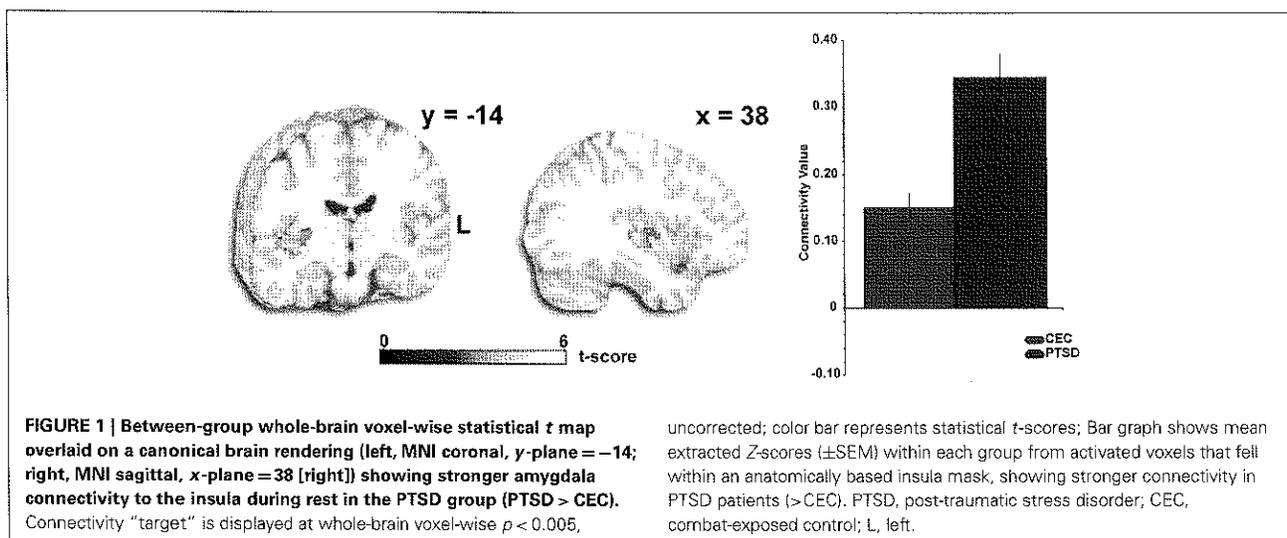
RESULTS

Across the entire brain, we observed a discretely localized difference in amygdala connectivity pattern between groups. From the right amygdala anatomical "seed" region, we observed that PTSD patients exhibited stronger connectivity with the insula than CEC subjects (MNI peak: [38, -18, -2], *Z*-score = 4.29, volume = 440 voxels; Figure 1); this pattern was not detected from the left amygdala seed. Follow-up ROI analyses on the extracted *Z*-scores of the strength of connectivity from the insula revealed that both groups exhibited positive amygdala-insula coupling, however, the extent of connectivity between the amygdala and insula was greater in the PTSD group than the CEC group (Figure 1). To explore the clinical relevance of the observed amygdala-insula connectivity abnormalities, we performed correlational analyses between the extracted values of the strength of connectivity and PTSD symptom severity measures (CAPS, BDI-II, PCL-M, and HAM-D) but did not observe any significant correlations (all $ps > 0.05$, corrected for multiple comparisons). Of note, we did not observe group differences in any other *a priori* areas that we predicted, such as the ACC, mPFC, and hippocampus in relation to amygdala connectivity at rest.

DISCUSSION

This is the first study to our knowledge that examines intrinsic amygdala functional connectivity patterns during rest in returning OEF/OIF veterans with combat-related PTSD compared to a

²http://afni.nimh.nih.gov/pub/dist/doc/program_help/3dClustSim.html



group of OEF/OIF veterans with combat exposure, but without PTSD. We found stronger amygdala–insula resting-state connectivity in the PTSD group compared to the CEC group. Of note, this connectivity pattern was from the right amygdala “seed” and was not shown from the left amygdala. Although we did not make an *a priori* prediction about lateralization of amygdala resting-state connectivity several findings of amygdala hyperactivity in PTSD and correlations between PTSD symptom severity and amygdala activity have been right-sided (Rauch et al., 1996, 2000; Pissiota et al., 2002; Fredrikson and Furmark, 2003; Driessen et al., 2004; Shin et al., 2004a). Contrary to our original hypothesis we did not observe any significant differences in amygdala connectivity to any other *a priori* regions (mPFC, ACC, hippocampus) in the PTSD group compared to the CEC group at rest. This is a notable negative finding and requires replication; however, we acknowledge that the absence of differences in amygdala–frontal or amygdala–hippocampal connectivity between groups could have resulted from: (1) Our stringent, whole-brain correction for multiple comparisons to detect significance coupled with a small sample size may have led to false negatives and/or more subtle connectivity abnormalities; and/or (2) The resting-state task may be insensitive to detecting amygdala–prefrontal and amygdala–hippocampal connectivity abnormalities, which may require engagement by an overt task.

Both the amygdala and insula have been separately implicated in the pathophysiology of anxiety and PTSD (Rauch et al., 2000; Osuch et al., 2001; Pissiota et al., 2002; Shin et al., 2004a; Protopopescu et al., 2005; Nemeroff et al., 2006; Hopper et al., 2007; Carrion et al., 2008; Shin and Liberzon, 2010). The amygdala plays an important role in the subjective and attentional-vigilance aspects of threat processing, and thus abnormalities in amygdala activity may be associated with hyperarousal and hypervigilance to threat in PTSD (Etkin, 2009). Moreover, several studies have shown that the amygdala is hyperresponsive to both trauma-related (Rauch et al., 1996; Shin et al., 1997, 2004a; Liberzon et al., 1999; Pissiota et al., 2002; Hendler et al., 2003; Driessen et al., 2004; Protopopescu et al., 2005; Vermetten et al., 2007) and unrelated stimuli in PTSD (Rauch et al., 2000; Armony et al., 2005; Shin et al., 2005; Williams et al., 2006), amygdala activation is positively correlated with PTSD symptom severity (Rauch et al., 1996; Shin et al., 2004a; Armony et al., 2005; Protopopescu et al., 2005) and self-reported anxiety (Pissiota et al., 2002; Fredrikson and Furmark, 2003), and symptom reduction after treatment is associated with decreased amygdala activation (Felmington et al., 2007). Likewise, PTSD patients display exaggerated insula activation during script-driven imagery (Lanius et al., 2007; Liödauer et al., 2008), fear conditioning and extinction (Bremner et al., 2005), the anticipation of negative images (Simmons et al., 2006), the retrieval of emotional or neutral stimuli (Bremner et al., 2003; Werner et al., 2009; Whalley et al., 2009), and aversive smells and painful stimuli (Vermetten et al., 2007) and is also positively correlated with PTSD symptom severity (Osuch et al., 2001; Hopper et al., 2007; Carrion et al., 2008). The insula controls evaluative, experiential, and expressive aspects of internal emotional states via visceral and somatic changes (e.g., autonomic “flight-or-fight” responses) evoked during presentations of aversive stimuli (Phan et al., 2002; Anderson et al., 2003; Dupont et al., 2003; Critchley

et al., 2004; Simmons et al., 2004; Paulus and Stein, 2006) and it has been posited that the insula relays interoceptive information to the amygdala to help guide behavioral responses (Augustine, 1996; Craig, 2002; Simmons et al., 2004; Paulus and Stein, 2006). In fact, the insula provides some of the strongest cortical connections to the major output division of the amygdala responsible for generating fear responses to symptom-provoking stimuli (Augustine, 1996; Aggleton and Saunders, 2000; Froese and Amaral, 2009) and abnormalities in these structures has been suggested to underlie exaggerated fear responses and the persistence of traumatic memories (Shin and Liberzon, 2010), as well as anxiety proneness (Paulus and Stein, 2006; Simmons et al., 2006; Stein et al., 2007b). Furthermore, evidence from a recent study suggests that a functional network between the amygdala and insula mediates anxious anticipation of negative events and anxious individuals display exaggerated activity within this network during anticipation of aversive stimuli (Carlson et al., 2010). Individuals with PTSD display excess anticipation of negative events and because of this are preoccupied with studying their environment for possible threats (i.e., hypervigilance) and increased amygdala–insula functional coupling may be a mechanism supporting hypervigilance in patients with PTSD.

Besides the present study, others have investigated baseline connectivity patterns in patients with PTSD and observed abnormalities in functional connectivity within the default-mode network when compared to healthy controls (although sometimes inconsistent; Bluhm et al., 2009; Daniels et al., 2010; Lanius et al., 2010), not directly related to amygdala connectivity. Our study extends these findings to resting-state amygdala coupling within a corticolimbic network known to be dysfunctional during trauma-related anxiety provocation, emotionally based tasks, and evocative stimuli in PTSD patients. However, our study has some important limitations. First, our study only included males and therefore cannot be generalized to females. Second, the resting-state analysis of changes in amygdala–insula connectivity do not allow for inferences about directionality or causality, which await task-based path or dynamic causal analyses. In addition, we have interpreted our resting-state findings based on previous functional and structural imaging studies, however, research with converging methods (i.e., task-dependent and -independent fMRI, diffusion tensor imaging) are much needed to link connectivity at rest with brain structure and function. Lastly, the cross-sectional nature of our measurement does not allow us to ascertain whether enhanced amygdala–insula resting-state connectivity was present before the traumatic experience and if so, makes it a potential vulnerability maker for PTSD.

Despite these limitations, our findings demonstrate that alterations in these connectivity patterns in a network involved in emotional processing and regulation may be relevant to a brain model of PTSD that involves baseline abnormalities in amygdala–insula functional connectivity that exist even without task induction. These findings suggest that the aberrant amygdala and insula activation to fear-evocative probes previously characterized in PTSD may be driven by an underlying enhanced connectivity between amygdala, a region known for perceiving threat and generating fear responses, and the insula, a region known for processing the meaning and prediction of aversive bodily states. This enhanced

amygdala–insula connectivity may reflect an exaggerated, pervasive state of arousal that exists outside the presence of an overt, actual threat/danger. Studying amygdala functional connectivity “at rest” extends our understanding of the pathophysiology of PTSD, and the current findings prompt further investigation in this emerging area of neuroimaging research.

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Posttraumatic stress symptoms and health-related quality of life: a two year follow up study of injury treated at the Emergency Department

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Abstract

Background

Among injury victims relatively high prevalence rates of posttraumatic stress disorder (PTSD) have been found. PTSD is associated with functional impairments and decreased health-related quality of life (HRQoL). Previous studies that addressed the latter were restricted to injuries at the higher end of the severity spectrum. This study examined the association between PTSD symptoms and health-related quality of life (HRQoL) in a comprehensive population of injury patients of all severity levels and external causes.

Methods

We conducted a self-assessment survey which included items regarding demographics of the patient, accident type, sustained injuries, EuroQol health classification system (EQ-5D) and Health Utilities Index (HUI) to measure functional outcome and HRQoL, and the Impact of Event Scale (IES) to measure PTSD symptoms. An IES-score of 35 or higher was used as indication for the presence of PTSD. The survey was completed by 1,781 injury patients two years after they were treated at the Emergency Department (ED), followed by either hospital admission or direct discharge to the home environment.

Results

Symptoms indicative of PTSD were associated with more problems on all EQ-5D and HUI3 domains of functional outcome and a considerable utility loss in both hospitalized (0.23-0.24) and non-hospitalized (0.32-0.33) patients. Differences in reported problems between patients with IES scores higher or lower than 35 were largest for EQ-5D health domains pain/discomfort (82% versus 28%) and anxiety/depression (53% versus 11%) and HUI

domains emotion (92% versus 33%) and pain (84% versus 38%). After adjusting for potential confounders, PTSD remained strongly associated with adverse HRQoL.

Conclusions

Among patients treated at an ED posttraumatic stress symptoms indicative of PTSD were associated with a considerable decrease in HRQoL in both hospitalized and non-hospitalized patients. PTSD symptoms may therefore raise a major barrier for full recovery of injury patients of even minor levels of severity.

Key words: posttraumatic stress disorder, injury, functional outcome, quality of life

Background

Posttraumatic stress disorder (PTSD) may result from any event that involves an injury, or threatened or actual death. Regarding injury victims PTSD prevalence rates up to 37% have been found three months after the injury [1]. At long-term follow-up (>1 year) PTSD prevalence rates from 5% [2] to 32% [3] have been reported.

A substantial share of studies that investigated prevalence rates and predictors of PTSD following injury addressed certain injury subgroups, such as victims of motor vehicle accidents [4-7], burn victims [8-10] or patients who required admission to hospital or the Intensive Care Unit [3, 11-15]. Those previous studies were mainly conducted in clinical patient populations and were therefore restricted to accidents and injuries at the higher end of the severity spectrum.

PTSD generally originates from cumulative exposure to traumatic stressors, which also influence the probability of spontaneous remission from PTSD [16, 17]. The level of traumatic stressors in the population of study may therefore affect to a large extent the prevalence rates found in studies on injury victims and which focus on a single stressor.

PTSD is associated with functional impairments and decreased health-related quality of life (HRQoL) [18, 19]. In one of the scarce studies addressing the latter, Holbrook et al. [20] showed that in a subgroup of injury patients admitted to a trauma centre PTSD has a substantial impact on health-related quality of life. Similar results were found among adolescents and children [21, 22]. However, these studies were again restricted to victims at the higher end of the severity spectrum and the association between PTSD and health-related quality of life among a comprehensive population of injury patients has yet to be studied.

The objective of this study was to assess the association between posttraumatic stress symptoms indicative of PTSD and HRQoL among this comprehensive injury population.

Methods

Study design

A patient-follow-up study, which was previously published [23], was conducted among a population-based sample of injury patients of all severity levels. This study followed injury patients aged 15 years and older who attended the ED of the Dutch Injury Surveillance System (a representative continuous registry of intentional and unintentional injuries of 17 hospitals in the Netherlands). Surveys were conducted at 2 months, 5 months, 9 months and two years after initial treatment. This study was conducted with the approval of the Ethics Committee Erasmus MC University Hospital.

Subjects

Between 8 October 2001 and 31 December 2002 a sample was selected of 8,564 patients aged 15 years and older who attended the ED of the Dutch Injury Surveillance System [23]. The patients were treated at the ED, followed by either hospital admission or direct discharge to the home environment. The sample of patients consisted of victims of traffic, home and leisure, occupational and sport accidents. The sustained injuries varied from minor to severe injury, single and multiple injury and hospitalized and non-hospitalized patients. The sample of patients was stratified, over sampling patients who were hospitalized. Each injury patient of the selected sample received a postal questionnaire 2½ months after the injury and 3,167 (37%) responded. The first questionnaire was made anonymous for privacy reasons. At 5, 9 and 24 months a follow-up questionnaire was sent to patients that responded to the preceding questionnaire. For these questionnaires the patients needed to give permissions by an informed consent form. The 5, 9 and 24 months follow-up questionnaire were completed by respectively 2,384, 2,295 and 1,781 patients. The present study used a sample of 1,781

respondents (i.e 21% of the original sample) on the two year post-trauma survey, which assessed both posttraumatic stress symptoms and HRQoL [23]. To adjust the data for non-response, a non-response analysis was conducted [23]. Multivariate logistic regression analysis was used to examine if variables age, sex, type of injury, external cause of the injury, hospitalization and length of stay, health status and ambulance transport were possible determinants of non-response. The significant variables were used to adjust for response bias by inverse probability weighting [24]. Additionally, the data were adjusted for stratification of the sample of ED patients [23]

Questionnaire

The follow-up questionnaire included items regarding demographics of the patient, accident category, type of injury, health care use and the Impact of Event Scale (IES), which was used to assess symptoms of posttraumatic stress indicative of PTSD [25]. The IES consists of 15 items, which measure intrusive re-experiences of the trauma and avoidance of trauma-related stimuli. By combining the 15 items the total IES-score, ranging from 0 through 75, can be calculated. Wohlfarth et al. showed that a cut-off score of 35 on the total IES-score produced a sensitivity of .89, and a specificity of .94 against the DSM-IV diagnostic criteria for PTSD as the gold standard [26]. Therefore, we assumed that an IES-score higher than 35 ($IES \geq 35$) represents symptoms of posttraumatic stress indicative of PTSD. The Dutch translation of the IES has been found to be valid and reliable [27].

Additionally, the questionnaire included items to measure functional outcome and HRQoL. HRQoL is an index of perceived functional outcome of an illness and disability that is anchored between 0 (worst imaginable health state or death) and 1 (full health), thus allowing comparison between the health status of patients with distinct diseases. To measure HRQoL, multi-attribute utility instruments (MAUIs) such as the Health Utility Index (HUI) or the EQ-

5D may be used [28, 29]. These instruments require the patient to report his or her health state with a standardised generic health state classification system, which is then converted into a health utility score using utility weights derived from the general population. Despite the similarities in obtaining the health utility score, there are important variations between the instruments regarding the health domains included in the health classification system and the methods applied to derive the utility weights [30]. As a result of these variations, the distinct instruments yield different utilities for similar health states. To overcome omissions in measuring HRQoL it is important to use several instruments that have complementary health domains [31].

Therefore, to measure functional outcome and HRQoL, the questionnaire included the EQ-5D and the HUI mark 3 (HUI3). With the EQ-5D classification system, respondents describe their health in three levels of severity on the health domains mobility, self-care, usual activities, pain/discomfort and anxiety/depression [32]. Subsequently, the weight of that health state is computed by a formula that firstly yields a partial weight score for each domain depending on the reported level and secondly adds the utility weights (also referred to as the 'tariff'), which are based on preference data of the general population of the UK [33].

For instance, a patient reports some problems with walking and performing usual activities, as well as moderate pain or discomfort (EQ-5D profile 21221). Full health has a utility value of 1. Because the health state of the patient deviates from the best possible health state (EQ-5D profile 11111), a fixed reduction of -.081 is applied. For the problems with walking, performing usual activities and moderate pain or discomfort reductions are applied of -0.069, -0.036 and -0.123 respectively. This results in a utility of 0.691. The complete algorithm to calculate EQ-5D utilities is published by Dolan et al. [34]

The questionnaire included 19 items regarding the presence of one or more chronic disease(s) prior to the injury to assess comorbidity [35]. Comorbidity is defined as the presence of any

coexisting medical conditions or disease processes additional to the injury that the injury patients sustained [36].

Primary data-analysis

For analysis of the data the Statistical Package for the Social Sciences version 14.0 was used (SPSS Inc, Chigaco, Ill). The IES-score can be calculated if all IES items are completed. In 8% of the cases data of one of the 15 IES items was missing. For these cases, the missing IES item was estimated by calculating the median value of 5 nearby points. The missing data was then imputed by the estimated values [37]. If more than one of the 15 IES items was missing, data were not imputed. Chi-square statistics (dichotomous variables) and Student t tests (continuous variables) were used to test for differences between injury patients with IES scores higher or lower than 35.

Univariate logistic regression and multivariate logistic regression analyses (enter method) were used to determine the predictive value of patient demographics, accident category and severity level of the sustained injuries with regard to posttraumatic stress symptoms indicative of PTSD ($IES \geq 35$) at two-year post-trauma. To dichotomize severity level, the injury diagnoses were categorized into two severity classes (mild versus moderate to severe) as previously tested by an international expert group [38]. The injury severity class moderate to severe comprises injuries such a skull-brain injury, fracture/dislocation of the vertebral column, fracture of pelvis and hip fracture. The injury severity class mild comprises injuries such as superficial injury, concussion and wrist fracture.

For the analysis of the association between $IES \geq 35$ and HRQoL, we selected participants that filled in both EQ-5D and HUI3. To test differences between participants with and without PTSD regarding their responses on each of the EQ-5D and HUI3 health domains, the non-parametric Wilcoxon-Mann-Whitney test was conducted. Differences regarding the mean EQ-

5D and HUI3 summary scores were tested with a one-way ANOVA. P-values < 0.05 were considered to indicate statistical significance.

Stepwise multiple regression analyses (enter method) was applied to investigate the association between demographics (block 1), hospitalization and comorbidity (block 2) posttraumatic stress symptoms indicative of PTSD (IES \geq 35) (block 3) and HRQoL measured with the EQ-5D and HUI3.

Results

Study population

Regarding the respondents on the 24-month follow-up questionnaire, the average age was 44.5 years old and 46% were female. Over one half (54%) was injured due to home and leisure accidents. The sustained injuries of all the respondents consisted mostly of superficial injury/open wounds (51%) and upper extremity fractures (13%). After treatment at the ED, 9% of the respondents were admitted to hospital. Approximately one third (31%) had one or more pre-existing comorbid conditions. Table 1 shows the characteristics of the injury patients, accident category and hospitalization status.

Association of posttraumatic stress symptoms indicative of PTSD (IES \geq 35) with HRQoL

With reference to the 1,781 respondents that completed the 24-month follow-up questionnaire, 1,585 (89%) filled in the EQ-5D and the HUI3 and 1,380 (77.5%) filled in the IES.

EQ-5D – Table 2 shows the responses on the EQ-5D of injury patients with IES scores higher or lower than 35. The calculated mean EQ-5D summary score for injury patients with IES scores \geq 35 was 0.56, whereas for injury patients with lower IES scores the mean EQ-5D

summary score was 0.87 ($t=112.0$; $p<0.001$). Respondents with posttraumatic stress symptoms indicative of PTSD reported significantly more problems on all five EQ-5D health domains ($p<0.001$). Differences in reported problems between patients with IES scores higher or lower than 35 were largest for EQ-5D health domains pain/discomfort (82% versus 28%) and anxiety/depression (53% versus 11%).

When the responses of hospitalized and non-hospitalized injury patients with $IES\geq 35$ and $IES<35$ are presented separately, again patients with symptoms indicative of PTSD ($IES\geq 35$) report significantly more problems on each of the EQ-5D health domains, resulting in a mean EQ-5D utility loss of 0.32 for non-hospitalized patients ($t=112.2$; $p<0.001$) and 0.23 for hospitalized patients ($t=22.1$; $p<0.001$). Compared to the injury patients without PTSD indications, injury patients with symptoms indicative of PTSD ($IES\geq 35$) at 24-months post-trauma also had significantly lower mean EQ-5D utility scores at the 2½-month ($t=105.0$, $p<0.001$), 5-month ($t=100.1$, $p<0.001$) and 9-month ($t=38.1$, $p<0.001$) follow-up.

Figure 1 shows the mean EQ-5D utility score of non-hospitalized and hospitalized patients with and without symptoms indicative of PTSD at 2½, 5, 12 and 24 month follow-up

HUI3 – Table 2 also shows the responses on the HUI3 domains reported by injury patients with IES-scores higher or lower than 35. For patients with $IES\geq 35$ the calculated mean HUI3 summary score was 0.51 and for patients with lower IES-scores 0.83 ($t=81.1$; $p<0.001$).

Respondents with posttraumatic stress symptoms indicative of PTSD ($IES\geq 35$) reported significantly more problems on all HUI3 health domains, except hearing where a reverse association was found ($p<0.001$). Differences in reported problems between patients with $IES\geq 35$ and $IES<35$ were largest for the HUI3 health domains emotion (92% versus 33%) and pain (84% versus 38%). Analysing the responses of non-hospitalized and hospitalized patients with and without PTSD indications ($IES\geq 35$) separately shows that hospitalized patients with

symptoms indicative of PTSD ($IES \geq 35$) reported most problems. Non-hospitalized patients with lower IES-scores reported least problems on the HUI3 health domains. Symptoms indicative of PTSD ($IES \geq 35$) were associated with a mean utility loss of 0.33 in non-hospitalized patients ($t=80.8$; $t<0.001$) and 0.24 in hospitalized patients ($t=15.9$; $t=0.001$). The models tested to predict HRQoL measured with EQ-5D and HUI3 were both statistically significant (EQ-5D: $F = 80.27$, $p < 0.001$; HUI3: $F = 118.55$, $p<0.001$). Table 3 shows that posttraumatic stress symptoms indicative of PTSD ($IES \geq 35$) are associated with decreased HRQoL, even after controlling for possible confounders.

Discussion

Posttraumatic stress symptoms indicative of PTSD were associated with more problems on almost all domains of functional outcome and a considerable decrease of HRQoL in both non-hospitalized and hospitalized injury patients two years post-injury.

Previous studies on PTSD and HRQoL were conducted in clinical patient populations and were therefore restricted to accidents and injuries at the higher end of the severity spectrum [20-22]. This study was not restricted to particular injury subgroups, such as adolescent victims or victims with severe injuries [4, 8, 11, 12, 20]. The high variety in injuries included in this study and the relatively large sample size allowed examination of the association of a number of injury characteristics and posttraumatic stress symptoms indicative of PTSD.

We found that injury patients with posttraumatic stress symptoms indicative of PTSD reported significantly more problems on all EQ-5D and almost all HUI3 health domains. A study that investigated HRQoL with EQ-5D among patients with PTSD following cardiac arrest reported similar findings [39]. Among adolescent victims PTSD was associated with impairments in Role/Social Behavioral, Role/Social Physical, Bodily Pain, General Behavior, Mental Health, and General Health Perceptions subscales of the 87-item Child Health

Questionnaire [22]. The resulting EQ-5D and HUI3 utility scores of injury patients with PTSD found in the current study are approximately in the range of the utility scores that Holbrook et al. derived with the multi-attribute utility instrument Quality of Well-being scale (QWB) (0.58 – 0.62) [20]. Although the HUI3 instrument yielded significantly lower health utility scores compared to the EQ-5D, which accords with results of other studies [40-42], both HUI3 and EQ-5D showed that PTSD was associated with a mean utility loss of 0.17 – 0.25. This concurs with the utility loss of anxiety disorders social phobia, generalized anxiety disorder and agoraphobia [43].

It should be noted that Holbrook et al. focused on injury patients admitted to a trauma centre with a length of stay of more than 24 hours and patients injured due to unintentional and intentional injury, whereas the current study included all admitted injury patients to general and university hospitals who were injured due to unintentional injury. Moreover, Holbrook et al. used an IES-score greater than 24 to identify patients with PTSD, whereas in the current study a cut off of 35 was used. Evidence from studies on this matter suggests that to avoid overestimation of the number of cases with PTSD, an IES-score of greater than 35 is more appropriate [26, 44]. Using the DSM-IV as the diagnostic criteria for PTSD, a cut-off score of 35 produced sensitivity of .89, specificity of .94 [26]. With a cut-off point of 24, the sensitivity is 0.91 and the specificity 0.46 [45]. To avoid over diagnosing of PTSD in a comprehensive population with a relative low PTSD prevalence, it is important to use a high IES cut-off score that incurs a high specificity.

Nonetheless, an important shortcoming of this study was that existence of PTSD symptoms was measured with the IES rather than Clinician-Administered PTSD Scale for DSM-IV (CAPS). The IES is a self-report questionnaire that measures only two of the three main PTSD symptoms, namely intrusion and avoidance. It is not a diagnostic tool, i.e., it is not designed to diagnose mental disorders according to the DSM-IV (the fourth edition of the

diagnostic and statistical manual for psychiatric disorders). Consequently, cases that in the current study were identified as having PTSD symptoms might not meet the DSM-IV criteria of clinical PTSD, and inversely. Due to differences in assessment of PTSD symptoms it is difficult to compare the results found in this study to previous studies on PTSD and health-related quality of life (HRQoL).

Both hospitalized and non-hospitalized injury patients with symptoms indicative of PTSD at 24 months post-injury reported a decrease in health status after 9 months, which may indicate that the sample is starting a deterioration process. On the other hand, patients might have overestimated their 9-month health status (and possibly also their 5-month health status), because their frame of reference has changed as a result of a temporary decrease in health status after the injury (response shift) [46, 47]. However, without information on PTSD status at previous measure points, the reasons for the reductions in HRQoL at 24-months post-injury can only be speculated about.

Functional consequences of injury, both temporary and permanent, show large variations dependent on the injury location and injury type. In the current study we used the European injury classification EUROCCOST [48]. This classification is compatible with the International Statistical Classification of Diseases, Injuries and Causes of Death – Ninth revision (ICD-9) classification system and consists of 39 injury groups that are homogeneous in terms of healthcare use, disability, as well as treatment and prognosis. In terms of anatomical classification the EUROCCOST classification is simple compared to the ICD, which provides very detailed information on injury diagnoses by location and type of injury.

A second limitation of this study was the low response rate of the follow-up questionnaires [23]. The 24-month follow-up questionnaire, which included the IES, was sent only to those patients who responded to the preceding three follow-up questionnaires sent at 2½, 5 and 9 months. This meant that only 21% of the patients of the initial sample selected for the follow-

up study filled in the 24-month follow-up questionnaire. However, the data were adjusted for non-response and possible response bias, because the PTSD prevalence rates were calculated using data that were weighted with respect to the original sample size and composition by inverse probability weighting. For some aspects, such as the severity of sustained injuries, the adjustments of non-response could be improved, since injury severity scores were not available.

Evidence suggested that patients with very severe health problems are less likely to respond to a survey [49]. Differential underreporting by level of severity cannot be excluded, since we found a larger proportion of hospitalized patients among those with PTSD at 2 years post-injury. This could partly be caused by missing a larger share of the more severely injured hospitalized patients among those without PTSD (e.g. comatose patients). This may have led to a slight overestimation of the utility losses due to PTSD. However, severely injured patients are only a minor part of the total sample and PTSD remained significantly associated to adverse HRQoL, even after adjustment for confounders including hospitalization status. In the current study PTSD is measured at 24 months follow-up only. A longitudinal study on PTSD and HRQoL among injury patients might elucidate any causal relationship between PTSD and subsequent reduced HRQoL. Furthermore, the influence of earlier HRQoL on PTSD remains to be investigated.

Conclusions

We conclude that among patients admitted to an ED due to injuries of all causes and severity levels posttraumatic stress symptoms indicative of PTSD are associated with decreased HRQoL even after correction for possible confounders such as comorbidity. PTSD seems a major barrier for full recovery of injury patients of even minor levels of severity, and the

development and evaluation of ED based policies for its early diagnosis and treatment should therefore be stimulated.

Abbreviations

ED, emergency department; HRQoL, health-related quality of life; HUI, health utilities index; IES, impact of event scale; MAUI, multi-attribute utility instrument; PTSD, posttraumatic stress disorder.

Conflict of Interest

The authors declare that they have no competing interests.

Author's contribution information

JAH executed the statistical analysis and drafted the manuscript. SP participated in the design of study, assisted with the statistical analysis and drafting of the manuscript. HT participated in the design of the study and data collection. MO participated in the design of study and drafting of the manuscript. GJB participated in the design of study and drafting of the manuscript. EFvB supervised, participated in the design of study and drafting of the manuscript. All authors read and approved the final manuscript.

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Figure title

Figure 1. Mean EQ-5D utility score of non-hospitalized and hospitalized patients with and without symptoms indicative of post-traumatic stress disorder (PTSS) at 2½, 5, 12 and 24 month follow-up

Tables

Table 1. Characteristics of the injury patients, accident category and hospitalization status

Characteristics	(n=1781) ^a
Patient demographics	
Age	44.9 (sd ^b 23.1)
Female sex	46%
Comorbid disease	31%
Accident category	
Home and leisure	54%
Traffic	16%
Occupational	13%
Sport	16%0
Hospitalization	8%

^a Weighted for stratification of the sample of injury patients and non-response.

^b sd = standard deviation

Table 2. Mean utility scores and percentage of reported problems on the EQ-5D and HUI3 health domains of the respondents without and with posttraumatic stress symptoms (PTSS) indicative of posttraumatic stress disorder

	No posttraumatic stress symptoms (IES-score <35; n=1708)	Posttraumatic stress symptoms (IES-score ≥35 n=73)	P
EQ-5D			
Mean EQ-5D utility score	0.87 (sd ^a 0.15)	0.56 (sd ^a 0.26)	<0.001
Problems with mobility	15.4%	47.0%	<0.001
Problems with self-care	5.1%	18.9%	<0.001
Problems with usual activities	16.9%	53.2%	<0.001
Pain/discomfort	28.1%	82.3%	<0.001
Anxiety/depression	11.4%	53.9%	<0.001
HUI3			
Mean HUI3 utility score	0.83 (sd ^a 0.24)	0.51 (sd ^a 0.26)	<0.001
Problems with vision	54.1%	65.2%	<0.001
Problems with hearing	8.6%	1.6%	<0.001
Problems with speech	4.6%	28.7%	<0.001
Problems with ambulation	12.8%	20.4%	<0.001
Problems with dexterity	10.6%	23.0%	<0.001
Problems with emotion	32.5%	91.6%	<0.001
Problems with cognition	14.3%	52.6%	<0.001
Pain	38.4%	84.4%	<0.001

^a sd = standard deviation

Table 3. Predictors of health-related quality of life at 2-year follow-up[§]

	Predictors	R2	Standardized B	p
EQ-5D	Age		-0.023	0.335
	Sex		-0.134	<0.001
	Hospitalization		-0.246	<0.001
	Comorbidity		-0.238	<0.001
	PTSS		-0.234	<0.001
		0.217		
HUI3	Age		-0.159	<0.001
	Sex		-0.058	0.008
	Hospitalization		-0.098	<0.001
	Comorbidity		-0.371	<0.001
	PTSS		-0.211	<0.001
		0.278		

[§] Analysis based on stepwise multivariate regression analysis with demographics (age, sex) as block 1; comorbidity, hospitalization and severity level of the injury as step 2, and posttraumatic stress symptoms (PTSS) indicative of posttraumatic stress disorder as step 3.

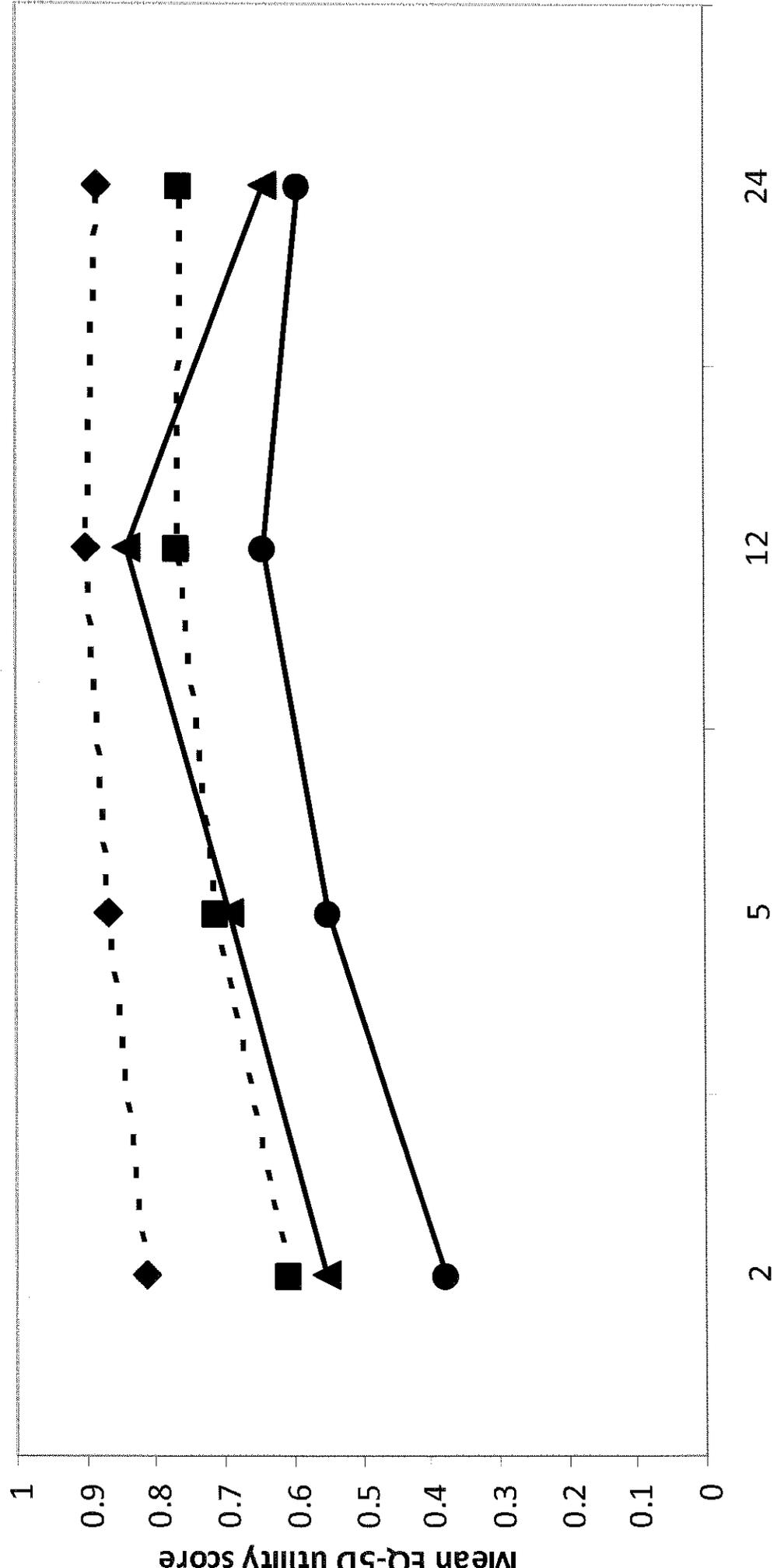
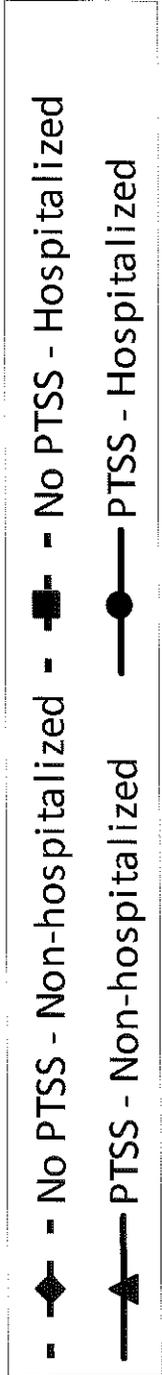


Figure 1