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Stress and re-stress increases conditioned taste aversion learning in rats: Possible frontal cortical and hippocampal muscarinic receptor involvement

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ABSTRACT

Symptoms of posttraumatic stress disorder are often precipitated by sensory cues in the form of visual, auditory, olfactory and gustatory "flashbacks" resulting in enhanced fear-memory consolidation and the characteristic symptoms of re-experiencing, avoidance and hyper-arousal. Single prolonged stress with and without re-stress have been used to explore the neurobiology of this disorder, particularly with respect to contextual conditioning and spatial memory impairment. However, less work has been done regarding associative sensory-related memories linked to aversive events. Although growing evidence supports a role for cholinergic pathways in stress, this has not been studied in the above animal models. We studied the effects of single prolonged stress with and without re-stress on conditioned taste aversion learning in rats, together with differential analysis of frontal cortical and hippocampal [^{3}H]-quinuclidinyl benzylate ([^{3}H]-QNB) muscarinic receptor binding. Single prolonged stress with and without re-stress both enhanced associative sensory aversion learning 7 days after stressor-taste pairing, although re-stress did not strengthen this response. Increased cortical and hippocampal muscarinic receptor density (B_{max}) was found 7 days after single prolonged stress with re-stress, although receptor affinity remained unaltered. Frontal cortical and hippocampal muscarinic receptor changes may thus underlie conditioned taste aversion learning in rats exposed to stress and re-stress. These data suggest that it may be useful to study the role of cholinergic pathways in mediating associative memory in psychiatric disorders such as posttraumatic stress disorder.

1. Introduction

Posttraumatic stress disorder may follow exposure to a severely terrifying, often life-threatening event (American Psychiatric Association, 1994), and is characterised by re-experiencing, avoidance of associative stimuli and hyper-arousal (American Psychiatric Association, 1994). These symptoms can be directly or indirectly related to a state of disorganized memory (Cahill, 1997), resulting in a disproportionate bio-behavioural response that persists beyond stressor cessation (Yehuda and Antelman, 1993; Servatius et al., 2005). Importantly, general declarative memory function as well as explicit information about the trauma is compromised while at the same time non-declarative memory relating to involuntary recollection of the trauma is bolstered (Elzinga and Bremner, 2002; Buwalda et al., 2005). The study of brain regions regulating memory is therefore central to research on posttraumatic stress disorder.

The hippocampus is particularly sensitive to emotional content allowing it to strongly influence explicit spatial and contextual memory (Elzinga and Bremner, 2002; Buwalda et al., 2005). The frontal cortex, on the other hand, not only mediates regulation of stress-related neuroendocrine function (Diorio et al., 1993; Herman and Cullinan, 1997), but also mediates interplay between emotions and memory formation (Miller and Cohen, 2001) by dampening inappropriate emotional and fear responses, particularly of the amygdala, (Morgan and Ledoux, 1995). Frontal cortical hypofunction not only help account for deficits in fear behaviour, but also for abnormal attentional processing and explicit memory function in posttraumatic stress disorder (Newport and Nemeroff, 2000). Indeed, neuroimaging studies have confirmed medial pre-frontal cortical hypofunction (Shin et al., 1999) as well as dysfunction of the hippocampus (Bremner et al., 2003; Shin et al., 2004) in the disorder.

Although the role of monamines in posttraumatic stress disorder is now well recognized (Elzinga and Bremner, 2002; Harvey et al., 2006), increasing evidence suggests an important role for cholinergic neurotransmission in anxiety, cognitive function and in stress-related psychiatric disorders (Lopez et al., 1999; Kaufer et al., 1998). The cholinergic basal forebrain complex innervates the cortex and hippocampus (Lamprea et al., 2000; Laborszky and Duque, 2000) to influence cortical arousal, consciousness, memory and learning (Sarter and Bruno, 2000; Picciotto et al., 2002). Indeed, cholinergic manipulations can regulate memory (Pepeu and Giovannini, 2004) and behavioural arousal (Picciotto et al., 2002), while muscarinic

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antagonists increase anxiety/fear responding in rats (Smythe et al., 1998; Hess and Blozovsky, 1987) and enhance hypothalamic–pituitary–adrenal-axis stress responsiveness (Jacobson and Sapolsky, 1991; Herman and Cullinan, 1997).

A hallmark characteristic of posttraumatic stress disorder is recurrent involuntary recollection of the trauma, particularly through sensory modalities that take the form of visual, auditory, olfactory and gustatory "flashbacks" (Hackmann et al., 2004). Conditioned taste aversion describes the ability to learn aversively motivated tasks, namely an unpleasant gustatory or non-gustatory experience (Nachman et al., 1977; Wenk, 1997; Everitt and Robbins, 1997), with re-exposure to a contextual cue bolstering acquisition of new associations between aversive experience and environmental stimuli (Shors and Servatius, 1997). The velocity of this association is biased more towards learning about negative consequences of food ingestion (Rozin, 1977; Rozin and Kalat, 1971) which occurs reliably, rapidly and is retained for long periods of time. Notably, forced swimming applied immediately after the intake of a taste solution results in aversion to that taste in rodents (Nakajima and Masaki, 2004). As opposed to spatial and working memory, conditioned taste aversion assesses aversion-motivated learning and as such emphasises implicit learning (Fornari et al., 2000), which has direct bearing on posttraumatic stress disorder (McGaugh, 2000). Taste memory formation involves the insular or gustatory (frontal) neocortex (Naor and Dudai, 1996; Mickley et al., 2004; Reilly and Bornovalova, 2005), as well as the hippocampus (Miranda et al., 2003; Stone et al., 2005), with cortical cholinergic projections playing a dominant role in taste memory formation (Miranda et al., 2003).

Conditioned taste aversion has not been studied in an animal model of posttraumatic stress disorder. In two related animal models, impaired spatial learning and increased contextual fear conditioning has been demonstrated following single prolonged stress (Kohda et al., 2007), while single prolonged stress with re-stress has been found to attenuate spatial memory (Harvey et al., 2003). The two procedures differ in their emphasis on re-experience, which has demonstrated differences with respect to neuroendocrine, catecholamine and behavioural responses (Harvey et al., 2006). This may also be reflected in the degree to which conditioned taste aversion is induced. This study addressed whether single prolonged stress or single prolonged stress with re-stress modulates recall of associative sensory-related memory linked to the aversive event. Moreover, considering the distinct yet separate role for the frontal cortex and hippocampus in conditioned taste aversion and posttraumatic stress disorder and the possible role for acetylcholine in stress responses, muscarinic receptor binding in these two brain regions were determined 7 days after stress/re-stress at the time of aversive memory recall.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Laboratory Animal Center, Potchefstroom campus of North-West University (NWU)) used in this study were handled in accordance with the guidelines set by the Ethical committee of the NWU (ethics approval number 05D20). Animals weighing 150–170 g were housed singularly under constant environmental conditions, viz. 21±0.5 °C; 50±5% relative humidity; full spectrum cold white light, intensity 350–400 lx, 12 hour light–dark cycle with the lights coming on at 06:00, with positively maintained air pressure and air filtration 99.7% and 99.9% effective for particle size 2 μm and 5 μm, respectively. All experimental procedures were carried out between 10:00 and 14:00 during the light period of the illumination light/dark cycle. Fluid, presented in glass bottles equipped with a metal drinking spout, was restricted to a daily drinking session of 20 min each morning.

2.2. Stress and re-stress

Stress and re-stress emphasises single prolonged stress as an initial trauma, followed by a more subtle re-stress procedure 1 week later. Single prolonged stress with re-stress demonstrates notable face-, construct- and predictive validity for posttraumatic stress disorder, including spatial memory deficits and anxiety (Harvey et al., 2003, 2006), hypocortisolemia (Liberzon et al., 1997; Harvey et al., 2003, 2006), differential serotonin receptor and monoamine involvement in the cortex and hippocampus (Harvey et al., 2003, 2006) and response to antidepressant treatment (Harvey et al., 2004). Similarly, single prolonged stress alone has demonstrated validity for posttraumatic stress disorder (Kahn and Liberzon, 2004; Kohda et al., 2007).

Briefly, animals were exposed to single prolonged stress that consists of sequential exposure to a somatosensory stressor (restraint), an inescapable or psychological stressor (forced swimming and underwater trauma), followed by a complex stress-stimuli evoked by exposure to diethyl ether. All stress procedures started at 09:00. Rats were placed in a Perspex® restrainer for 2 h with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs. Immediately thereafter, the rats were individually placed in 18 cm of ambient water (25 °C) in a cylindrical Perspex® swim tank, and allowed to swim for 10 min before being held under water with a metal net for 40 s. During forced swimming, the depth of the water is adjusted to allow the animal to keep its nose above the water by using its tail as support. Following this, each rat was then immediately exposed to 0.8 ml of 100% ether vapors in a 5 l sealed plastic container until loss of consciousness (±4 min) and then immediately removed. The animal was returned to its home cage, dried and allowed to recover over a period of 7 days. On the seventh day after exposure to single prolonged stress, the animals were re-exposed to 10 min forced swimming and 40 s underwater stress (single prolonged stress now becomes single prolonged stress with re-stress). The animals were dried and again returned to their home cages. Behavioural studies were performed 7 days after both single prolonged stress and after single prolonged stress with re-stress. Muscarinic receptor binding was determined 7 days after the combined stress and re-stress paradigm.

2.3. Conditioned taste aversion

In conditioned taste aversion, the animal learns to associate a novel taste (conditioned stimulus) with an aversive experience, the unconditioned response (US; Nachman and Ashe, 1973; Miranda et al., 2003). The paradigm is best recognized in eating behaviour where the unconditioned stimulus represents a noxious stimulus and the conditioned stimulus is related to the eating or drinking session (Garcia and Hankins, 1977; Testa and Ternes, 1977). Facilitated conditioning is reactivated days later by re-exposure to the context in which the stressful and aversive event occurred. In subjecting the animal to the same context, this re-exposure functions as a trigger or reinforcer that shapes subsequent behaviour to an anxiety provoking stimulus in a Pavlovian manner (Guitton and Dudai, 2004).

2.3.1. Conditioned taste aversion with lithium chloride as unconditioned stimulus

Standard conditioned taste aversion testing with lithium chloride utilises a 5 day period between conditioning and exposure to the contextual reminder (Nachman and Ashe, 1973; Miranda et al., 2003). However, since the single prolonged stress and single prolonged stress with re-stress study design (as outlined in Fig. 1) utilises a 7 day post-stress interval for greatest neurobiological and behavioural effect (Harvey et al., 2003, 2006; Kohda et al., 2007), it was necessary to confirm that a 7 day period between conditioning and exposure to the contextual reminder is adequate for inducing conditioned taste aversion. Various studies have confirmed an extended consolidation

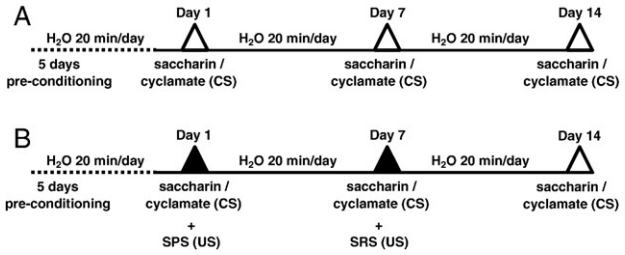

Fig. 1. Layout of conditioned taste aversion experiment with single prolonged stress (SPS) and single prolonged stress with re-stress (SRS) as unconditioned stimulus (US) and saccharin/cyclamate as conditioned stimulus (CS). (A) Control group; (B) Stress group.

of taste aversive learning for up to 10 days post-conditioning (Batsell and George, 1995). Initial conditioned taste aversion studies using lithium chloride as unconditioned stimulus were performed post-conditioned stimulus–unconditioned stimulus pairing versus initial basal saccharine/cyclamate intake (n=9) according to Wegener et al. (2001), with minor modifications. Briefly, a 0.1% solution of a 3:1 solution of sodium-cyclamate and sodium-saccharin was used as the novel taste (conditioned stimulus). Fifteen minutes after sampling the saccharin/cyclamate solution, an i.p. injection of 0.15 mEq/kg lithium chloride was administered as unconditioned stimulus, which elicits a marked gustatory response (malaise). The amount of saccharin/cyclamate consumed 7 days later upon re-challenge with the conditioned stimulus was expressed as ml/100 g of the subject's body weight. Subsequent studies evaluated the ability of single prolonged stress or single prolonged stress with re-stress to substitute lithium chloride as contextual reminder and to bolster aversive learning.

2.3.2. Stress and re-stress as unconditioned stimuli in the conditioned taste aversion paradigm

Sprague–Dawley rats were randomly assigned to one of the two training groups, viz. single prolonged stress with re-stress (n=20) and control (n=12). Rats were trained to receive their daily water ration within 20 min from one pipette containing 40 ml. This pre-conditioning period was implemented 5 days prior to the conditioned stimulus–unconditioned stimulus pairing (Fig. 1). The daily consumption of each rat was recorded in ml throughout the experiment and animals were weighed every 2–3 days in order to express final consumption as ml/100 g. The animals typically drank 5–8 ml (5–6 ml/100 g body weight) on the first 3 days of pre-conditioning and gradually increased their daily water consumption to 9–12 ml (6–7 ml/100 g body weight) thereafter.

On day 1, the conditioning day, the rats were offered 0.1% of a 1:3 saccharin/cyclamate solution as the only source of fluid for 20 min (basal saccharin/cyclamate intake). Five minutes thereafter, they were subjected to single prolonged stress (see schematic in Fig. 1B). The animals were then left undisturbed, but once again were presented with water for 20 min/day. On day 7, the water was replaced by the saccharin/cyclamate solution and intake determined over 20 min to quantify aversion. Five minutes thereafter, the rats were exposed to the re-stress procedure (Fig. 1B). The rats were left undisturbed for another 6 days, receiving only water for 20 min each day. On day 14 of the experiment, rats were again offered the saccharin/cyclamate solution for 20 min, and the drinking volume recorded to quantify the extent of the acquired aversion. A control group underwent exactly the same protocol over the same time period (Fig. 1A), being handled only when weighed every 2–3 days, but without any stressors. Consumption on day 7 represents aversion induced by single prolonged stress acquired on day 1, while consumption on day 14 represents aversion as a consequence of both the conditioned stimulus–single prolonged stress pairing (day 1) and the conditioned stimulus–single prolonged stress with re-stress (day 7) (see Fig. 1B). Aversion was expressed as ml/100 g body weight and compared across the two conditioning days (day 1 and day 7) and the test day (day 14).

2.4. Muscarinic M_1 receptor binding assays

With 10 animals allocated/group, neuroreceptor studies were performed in two separate groups of control and stressed animals, viz. single prolonged stress with re-stress, with the animals subjected to the same protocol as that described for the conditioned taste aversion experiments (Fig. 1). Thus, these animals were exposed to single prolonged stress with re-stress and sacrificed by decapitation on day 7 post-re-stress (i.e. day 14), with control animals handled as above and sacrificed on the same day. Hippocampi and fontal cortices were rapidly removed and placed on ice, fixed in liquid nitrogen (−196 °C) and stored at −80 °C until later analysis. The left hippocampi of three animals and the whole frontal cortices of two animals were pooled respectively for analysis.

Tissues were thawed at room temperature and homogenised in 35 ml ice-cold Tris–HCl buffer (pH 7.4), with a Brinkman Polytron PT 10 homogenizer (setting 7) for 20 s. The homogenate was centrifuged at 48,000 ×g for 10 min at 4 °C. The supernatant was discarded and the membrane pellet was re-suspended in 17.5 ml of ice-cold buffer. This procedure was repeated twice where after the final membrane suspension was made up to 100 volumes with buffer and kept on ice. Protein was analysed by the method of Bradford (1976). Muscarinic receptor binding was measured by incubating 960 μl aliquots (0.3–0.7 mg/ml protein) of the homogenate with 20 μl [^{3}H]-quinuclidinyl benzylate [^{3}H]-QNB (specific activity 36.5 Ci/mmol; 0.2–10 nM) and 20 μl buffer or atropine (3 mM) for 15 min in a shaking water bath at 25 °C (Yamamura and Snyder, 1974). The drug-receptor binding reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-soaked in Tris–HCl buffer. The filters were washed rapidly with 2×5 ml ice-cold buffer and placed in polypropylene counting vials with 3 ml Filter Count scintillation cocktail. The tubes were left for 1 h and the radioactivity counted by liquid scintillation spectrometry (Packard Tri-Carb 4660). Specific binding was defined as the total binding minus binding in the presence of 3 mM atropine. Receptor binding data was analysed by non-linear regression analysis using Prism from GraphPad Software, Inc. (www.graphpad.com) to give affinity (K_d) and receptor density (B_{max}) values.

2.5. Statistical analyses

All conditioned taste aversion data were first analysed using a one-way analysis of variance (ANOVA) (Statsoft, Inc.2001: Statistica Data Analysis Software System, version 7), followed by post-hoc multiple comparisons using the Tukey test. Conditioned taste aversion validation studies using LiCl as unconditioned response were analysed with the Student's t test. All receptor binding data were analysed with a Student's t test. In all cases, statistical significance was defined at 5% (P<0.05), and all data are expressed as the mean±S.E.M.

3. Results

3.1. Conditioned taste aversion studies

3.1.1. Conditioned taste aversion with lithium chloride as unconditioned stimulus 7 days post-conditioning

Lithium chloride as unconditioned stimulus was found to be successful in evoking taste aversion on day 7 post-conditioned stimulus–unconditioned stimulus pairing versus initial basal saccharine/cyclamate intake (4.4±0.31 ml/100 g initial intake versus 0.83±0.13 ml/100 g on day 7; P<0.0001; Student's t test). Conditioned taste aversion with lithium chloride therefore develops and is

sustained over 7 days. Subsequent studies would therefore replace lithium chloride with either single prolonged stress or single prolonged stress with re-stress as unconditioned stimulus.

3.1.2. Induction of conditioned taste aversion with stress and stress plus re-stress as the unconditioned stimulus

In the absence of a stressor co-presented with the conditioned stimulus (control), one-way ANOVA failed to indicate significant group effects between conditioning on day 1 and subsequent aversion assessments on day 7 or day 14 [$F(2.33) = 1.54$, $P = 0.23$] (Fig. 2A, Tukey test). However, in animals receiving single prolonged stress and single prolonged stress with re-stress, one-way ANOVA indicated significant group differences across days 1, 7 and 14 [$F(2, 57) = 11.18$, $P < 0.05$].

Post-hoc Tukey multiple comparisons revealed that animals consumed significantly less saccharin/cyclamate on day 7 after single prolonged stress ($P < 0.001$; Fig. 2B, Tukey test) compared to the first day of introduction to the novel taste (day 1). Similarly, animals receiving single prolonged stress with re-stress showed a significant suppression of saccharin/cyclamate ingestion on day 14 (day 7 after re-stress; $P < 0.001$, Fig. 2B, Tukey test) compared to the first day of introduction to the novel taste (day 1). However, no differences in aversion were apparent between the single prolonged stress and single prolonged stress plus re-stress groups.

3.2. Muscarinic receptor binding studies

Muscarinic receptor density (B_{max}) and affinity (K_d) were determined 7 days post-conditioning in rats exposed to single prolonged stress plus re-stress (i.e. day 14). Significantly elevated hippocampal muscarinic receptor densities were discernable in animals subjected to single prolonged stress with re-stress groups compared to control animals 7 days after re-stress ($P < 0.05$; Student's *t* test; Fig. 3A). No significant change was discernable with respect to hippocampal muscarinic receptor K_d-values between the 2 groups analysed (Student's *t* test, Table 1). A significant increase in muscarinic receptor density was also observed in the frontal cortex 7 days after re-stress ($P < 0.001$; Fig. 3B; Student's *t* test), with no change evident in affinity values between the two groups (Student's *t* test, Table 1).

4. Discussion

This study has established that single prolonged stress alone and single prolonged stress plus re-stress, a putative animal model of posttraumatic stress disorder, can enhance the development of an associative aversive-conditioned response in the conditioned taste aversion paradigm, with neither stressor being more robust than the other. Importantly, increased muscarinic receptor density, corresponding to the increased aversion observed on day 14 post-re-stress, was noted in both the hippocampus and frontal cortex of rats exposed to single prolonged stress with re-stress. Receptor affinity (K_d), however, remained unchanged in both brain regions.

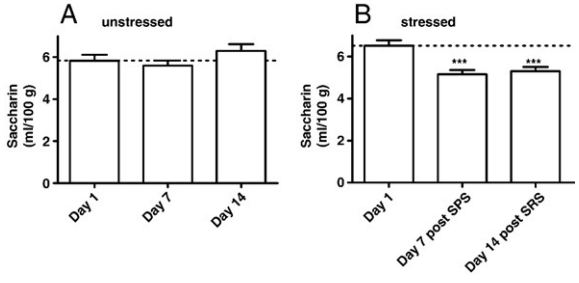

Fig. 2. Conditioned taste aversion in animals subjected to pairing of saccharin/cyclamate consumption 7 days after saccharin/cyclamate with single prolonged stress (SPS) pairing (day 7) and 7 days after saccharin/cyclamate with single prolonged stress with re-stress (SRS) pairing. (A) Unstressed controls received only handling (mean ± S.E.M., $n = 12$), (B) Stressed groups (mean ± S.E.M., $n = 20$). ***$P < 0.001$ versus day 1.

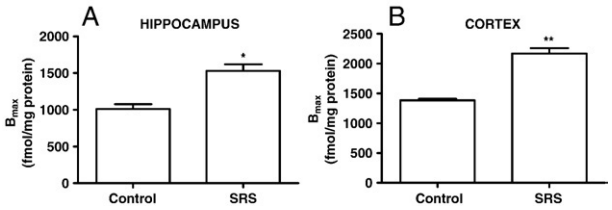

Fig. 3. Effects of stress and re-stress (SRS), as evoked by single prolonged stress with re-stress, on muscarinic receptor binding density in the hippocampus (A; $n = 3$) and frontal cortex (B; $n = 4$) as determined on day 14 (mean ± S.E.M.). *$P < 0.05$ SRS versus control. **$P < 0.01$ SRS versus control (Student's *t* test).

Stress procedures like swim stress have been successfully implemented as aversive stimuli in the conditioned taste aversion model (Nakajima and Masaki, 2004). However, despite conditioned taste aversion being a robust tool for assessing aversion-motivated memory, it has not been used to probe conditioned taste aversion in an analogous animal model of posttraumatic stress disorder. Posttraumatic stress disorder is characterised by an increase in fear-memory (Van Oyen Witvliet, 1997) and a loss of explicit memory function (Elzinga and Bremner, 2002). Indeed, single prolonged stress with re-stress (Harvey et al., 2003) and more recently single prolonged stress alone (Kohda et al., 2007), have been found to induce deficits in spatial memory performance. Moreover, Kohda et al. (2007) have demonstrated an increase in contextual fear conditioning 7 days after single prolonged stress. However, that single prolonged stress with or without re-stress should associate with taste to evoke consolidation of aversive memory has not been studied. Single prolonged stress with or without re-stress differs with respect to the emphasis on re-experience. Since these two paradigms have demonstrated different neuroendocrine, catecholamine and behavioural responses (Harvey et al., 2006), re-experience may evoke a different pattern of conditioned taste aversion.

Although the conditioned taste aversion procedure (using lithium chloride as unconditioned stimulus) is widely used as a test for aversive memory over a period of 5 days post-conditioned stimulus/unconditioned stimulus pairing (Wegener et al., 1997, 2001; Miranda et al., 2003), consolidation of aversive memory has been found to be preserved for up to 10 days post-conditioning (conditioned stimulus–unconditioned stimulus) (Batsell and George, 1995). Similarly, we found lithium chloride-pairing with saccharin/cyclamate to demonstrate successful associational recollection of digestive malaise upon re-exposure to saccharin/cyclamate 7 days later.

In the studies exploiting stress as unconditioned stimulus, control animals showed no associational aversion learning to the novel taste (Fig. 2A). However, stressed animals (Fig. 2B) linked a pronounced negative association to saccharin/cyclamate 7 days post-acute stress and 7 days post-re-stress, proving the ability of both single prolonged stress alone and with re-stress to be equally effective as an unconditioned stimulus in the conditioned taste aversion paradigm. Swim stress enhances aversive memory in a dose-dependent way (Nakajima and Masaki, 2004). Indeed, swimming forms a pivotal role

Table 1
Effects of combined single prolonged stress plus re-stress (SRS; 7 days after re-stress) on muscarinic receptor binding affinity (K_d) in the hippocampus and frontal cortex, versus control

Hippocampus mean ± S.E.M.		Frontal cortex mean ± S.E.M.	
Control ($n = 2$)	SRS ($n = 3$)	Control ($n = 3$)	SRS ($n = 2$)
1.67 ± 0.44	0.75 ± 0.11	2.20 ± 0.34	4.12 ± 2.48

Statistical analysis: $P > 0.05$, Student's *t* test.

in both the initial acute stressor (single prolonged stress) and re-stress (single prolonged stress with re-stress) procedures. Conditioned taste aversion can also be induced by an anxiety-like emotional state (Guitton and Dudai, 2004). This is of importance in the current context since single prolonged stress with re-stress increases anxious behaviour in rats (Harvey et al., 2006).

Interestingly, re-stress on day 7 post-single prolonged stress (i.e. day 14) had no significant impact on the strengthening of aversive memory over that induced 7 days after single prolonged stress, suggesting that re-experience more likely plays a role in maintaining aversive memory rather than strengthening the aversion. However, with conditioned taste aversion not being assessed on day 14 post-single prolonged stress in the absence of a re-stress procedure in between, this premise could not be confirmed. Animals therefore reacted to the conditioned stimulus with a level of aversion not unlike the way posttraumatic stress disorder patients show aversion when encountering a situational reminder of a previous fearful experience (Rau et al., 2005).

Traditionally, the neuroanatomical site of action in conditioned taste aversion has been in close proximity to the vomiting center or the lateral hypothalamus. Lesions of the area postrema attenuate lithium induced conditioned taste aversion (Ritter et al., 1980) while lateral hypothalamus lesions produce failure to learn specific food aversions (Roth et al., 1973). Recent studies also suggest involvement of the insular or gustatory (frontal) cortex (Yamamoto et al., 1994; Bermudez and McGaugh, 1991; Reilly and Bornovalova, 2005). Evidence for a role for the hippocampus in conditioned taste aversion is at present ambivalent (Purves et al., 1995; Yamamoto et al., 1995; Stone et al., 2005). In posttraumatic stress disorder, however, the hippocampus and frontal cortex play important yet distinct roles, with the former implicated in spatial and contextual memory related to an event, while the cortex is more critical in top-down control over sub-cortical processes governing emotional and fear responses. Lack of appropriate cross-talk between these two areas undoubtedly underlies the deficits in fear behaviour, attentional processing and explicit memory function in posttraumatic stress disorder (Newport and Nemeroff, 2000).

Infusion of muscarinic receptor antagonists into the hippocampus (Lydon and Nakajima, 1992) or the pre-frontal cortex (Broersen et al., 1995; Broersen, 2000) impair cognitive performance. Since cortical cholinergic projections play an important role in taste memory formation (Miranda et al., 2003; Ramirez-Lugo et al., 2003), these studies highlight not only the important contribution of both the cortex and hippocampus in explicit and also associative memory, but also emphasise the pivotal role of the cholinergic system in these processes.

An abundant expression of muscarinic receptors in the cerebral cortex and hippocampus (Adem et al., 1997) underscores extensive cholinergic involvement in the function of these brain regions. Stress promotes acetylcholine release in the hippocampus and frontal cortex (Mark et al., 1996). Moreover, acetylcholine activates the cardiovascular, sympathetic, adrenal-medullary, behavioural-affective, analgesic and neuroendocrine systems involved in the stress response (Janowsky and Overstreet, 2000) and thus highlights its functional role during stress. However, while the general involvement of the cholinergic system in stress has attracted attention (Kaufer et al., 1998; Smythe et al., 1998; Gilad, 1987), its role in posttraumatic stress disorder has not been studied to any great degree.

In both frontal cortex and hippocampus, muscarinic receptor density was significantly increased 7 days after single prolonged stress with re-stress (day 14) compared to unstressed animals (Fig. 3A and B). Importantly, behavioural changes were evident on day 7 post-initial stress (Fig. 2B) and sustained on day 14 (7 days after re-stress). No changes in muscarinic receptor affinity were noted in either brain region. Thus, single prolonged stress with re-stress was associated with marked perturbations in cholinergic receptor binding in both the frontal cortex and hippocampus, and were associated with increased recall of aversive memory (Fig. 2B).

Although there is no doubt that changes in cholinergic function are extremely important in cognitive function and stress response, the precise ways in which it is altered are dependent on the intensity, duration and context of the stressor as well as conditioning circumstances which may influence the occurrence, direction and magnitude of the response. Cortical and hippocampal cholinergic neurons are activated by conditions that produce arousal, such as novelty and fear (Acquas et al., 1996; Pepeu and Giovannini, 2004). Chronic immobilization stress increases muscarinic receptor density in the hippocampus, suggesting activation of the hippocampal cholinergic system by chronic stress (Gonzalez and Pazoz, 1992). Moreover, cholinergic receptors are involved in neuroplastic events related to memory encoding, particularly in learning and memory performance in humans (Everitt and Robbins, 1997) and animals (Van der Zee and Luiten, 1999; Miranda et al., 2003). Thus, the increase in cortical and hippocampal muscarinic receptor density following single prolonged stress with re-stress indicate a distinct change in cortical cholinergic activity that may underlie the increased expression of aversive memory recall. Although determination of acetylcholine release/levels is needed to corroborate this, it is clear that single prolonged stress with re-stress evokes conditioned taste aversion through the recruitment of cortical as well as hippocampal cholinergic pathways.

The hippocampus is involved in explicit recall of sensory cues relating to the trauma (Elzinga and Bremner, 2002) by relating contextual experiences, such as its novelty or familiarity, risk and association with each other (Adamec, 1991). Cholinergic mechanisms appear to occupy a central role in the response of the hippocampus to conditioned taste aversion (Naor and Dudai, 1996; Stone et al., 2005). Increased acetylcholine release is positively correlated with enhanced spatial and reference memory (Kopf et al., 2001; Stancampiano et al., 1999), while evidence described herein of the muscarinic receptor changes in the hippocampus also attests to noteworthy cholinergic involvement in this region during conditioned taste aversion. These data are thus supportive of earlier studies describing a role for the hippocampus in conditioned taste aversion memory (Stone et al., 2005).

Cholinergic lesioning in the frontal cortex also causes marked decrease in conditioned taste aversion learning (Lopez-Garcia et al., 1993). Considering the dual role of the hippocampus and cortex in the response to stress, and in the regulation of the stress response particular in PTSD (Newport and Nemeroff, 2000), the observed muscarinic receptor changes in both brain regions, and their co-presentation with changes in aversive memory recall, are noteworthy. The current work now for the first time extends these associations using an animal model of PTSD. It is of note that while hippocampal spatial memory function may be compromised following single prolonged stress or single prolonged stress with re-stress (e.g. Harvey et al., 2003; Kohda et al., 2007), aversive memory formation is bolstered, with the latter linked to cortical and hippocampal muscarinic receptor changes. This is not unlike the paradoxical state of memory function in posttraumatic stress disorder, with enhanced involuntary recollection of the trauma, particularly through visual, auditory, olfactory and gustatory "flashbacks" (Hackmann et al., 2004), occurring at the cost of explicit memory functions (Elzinga and Bremner, 2002).

In conclusion, single prolonged stress with and without re-stress increases sensory aversion-associated learning, although re-stress does not strengthen this response. Stress plus re-stress associated aversive learning may involve increased muscarinic receptor density (B_{max}) in the frontal cortex and hippocampus, without associated changes in receptor affinity. Cholinergic dysfunction thus underlies aversive learning in rodents exposed to a posttraumatic stress disorder-like paradigm, and as such re-kindles evidence for cholinergic involvement in posttraumatic stress disorder.

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