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Involvement of the Ventral Tegmental Area in a Rodent Model of Post-Traumatic Stress Disorder

Nadia S Corral-Frias[1], Ryan P Lahood[2], Kimberly E Edelman-Vogelsang[3], Edward D French[4] and Jean-Marc Fellous*[,5,6]

[1]Graduate Interdisciplinary Program in Neuroscience, University of Arizona, Tucson, AZ, USA; [2]Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA; [3]Undergraduate Biology Research Program, Tucson, AZ, USA; [4]Department of Pharmacology, University of Arizona, Tucson, AZ, USA; [5]Department of Psychology, Program in Applied Mathematics, University of Arizona, Tucson, AZ, USA; [6]Feinberg Foundation Visiting Faculty Program Fellow, Weizmann Institute, Rehovot, Israel

Post-traumatic stress disorder (PTSD) is an anxiety disorder of considerable prevalence in individuals who have experienced a traumatic event. Studies of the neural substrate of this disorder have focused on the role of areas such as the hippocampus, the amygdala and the medial prefrontal cortex. We show that the ventral tegmental area (VTA), which directly modulates these areas, is part of this circuitry. Using a rat model of PTSD, we show that a brief but intense foot shock followed by three brief reminders can cause long-term behavioral changes as shown by anxiety-like, nociception, and touch-sensitivity tests. We show that an intraperitoneal injection of a dopamine (DA) antagonist or a bilateral inactivation of the VTA administered immediately before the traumatic event decrease the occurrence or intensity of these behavioral changes. Furthermore, we show that there is a significant decrease of baseline VTA dopaminergic but not GABAergic cell firing rates 2 weeks after trauma. Our data suggest that VTA DA neurons undergo long-term physiological changes after trauma and that this brain area is a crucial part of the circuits involved in PTSD symptomatology.
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INTRODUCTION

About 10% of individuals exposed to trauma will eventually suffer from post-traumatic stress disorder (PTSD) and will experience vivid nightmares, emotional and social withdrawal, irritability as well as other disruptive symptoms (Kessler *et al*, 1995). The impairments resulting from PTSD symptoms usually co-occur with other disorders, such as substance abuse and mood and anxiety disorders (Kessler *et al*, 2005; Kofoed *et al*, 1993; Najavits *et al*, 1997; Yehuda and LeDoux, 2007). Many PTSD patients also suffer from the development of stress-related medical conditions, such as hypertension, respiratory conditions, and chronic pain (Asmundson and Katz, 2009; Frayne *et al*, 2011).

The neural substrate of PTSD is complex and under active investigation. Various neural models of PTSD have established the role of the amygdala and its interactions with the medial prefrontal cortex and the hippocampus (Liberzon and Sripada, 2008; Rauch *et al*, 2006). These models are based on both animal and human data and show abnormal heightened responsiveness of the amygdala to threatening stimuli and inadequate regulation of the amygdala by the medial prefrontal cortex and the hippocampus (Garcia et al, 1999; Liberzon *et al*, 1999; Pissiota *et al*, 2002; Shin *et al*, 2004). This amygdala-centered view does not exclude the possibility that other areas may be involved. The ventral tegmental area (VTA), for example, has strong reciprocal interactions with these three areas (Fields *et al*, 2007). High comorbidity of PTSD with drug addiction (Kofoed *et al*, 1993; Najavits *et al*, 1997) and evidence showing deficits in the brain reward and reinforcement circuits in PTSD patients (Elman *et al*, 2009; Hopper *et al*, 2008) also suggest the involvement of dopaminergic systems such as the VTA. Finally, a recent case study suggested that psychostimulants, which enhance dopamine (DA) release, were effective at ameliorating symptoms in three combat-related PTSD patients (Houlihan, 2011).

The mesolimbic pathway has been shown to have an important role in fear conditioning (Inoue *et al*, 2000; Pezze and Feldon, 2004) and acute (Anstrom and Woodward, 2005; Valenti *et al*, 2011) and chronic (Moore *et al*, 2001) stress. Evidence for the involvement of the dopaminergic pathways in conditioned fear has been shown using systemic administration of D1 and D2 receptor antagonist and agonists. Research has shown that reduced DA (using

*Correspondence: Dr J-M Fellous, Department of Psychology, Program in Applied Mathematics, University of Arizona, 1503 E University Building, P.O. Box 210068, Tucson 85721, AZ, USA, Tel: +1 520 621-7447, Fax: +1 520 621-9306, Email: fellous@email.arizona.edu
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systemic injections of a D1-receptor agonist) diminishes the freezing response elicited by conditioned fear (Inoue *et al*, 2000; Pezze and Feldon, 2004). Little research has been done to directly explore the role of dopaminergic systems, such as the VTA in animal models of PTSD. A recent study, however, identified the mesolimbic DA system as a key contributor to the resistance to social defeat in mice (Krishnan *et al*, 2007). This work showed that, as with human PTSD patients, vulnerable animals showed deficits in sensitivity to natural rewards (sucrose) and increases in drug-seeking behavior (cocaine).

Neurophysiologically, some studies showed that the mean firing rate of VTA DA neurons increased during an acute restraint paradigm and that the burst firing of a subset of DA neurons increased significantly compared with baseline (Anstrom and Woodward, 2005). This increase in burst firing could persist up to 24 h after a single exposure to stress. Others have shown that stressors increase the number of active dopaminergic neurons in VTA, when measured within 24 h of the last stress episode (Valenti *et al*, 2011). In contrast, other studies demonstrated that long-term (more than 17 days) chronic cold exposure yielded fewer spontaneously active VTA and medial substantia nigra neurons (Moore *et al*, 2001). However, in this study, the firing rates in the cells that remained active did not differ significantly from controls.

Taken altogether, these studies suggest that dopaminergic neurons of the VTA are affected by stress and respond differently depending on the type of stressor and the delay after which their activity is measured. Very little research has been conducted on the long-lasting effects of trauma on VTA cell activity. Here we study and validate a rodent model of PTSD, demonstrate the involvement of the dopaminergic system using systemic injections and demonstrate a specific role of the VTA by selective bilateral inactivation. We show the presence of trauma-related long-term electrophysiological changes in VTA DA cells. Preliminary results of this work were presented in abstract form (Corral-Frías *et al*, 2010).

MATERIALS AND METHODS

Animals

We used 63 male Sprague–Dawley rats weighing 300–400 g (33 behavioral, 16 injected intraperitoneally (i.p.) and 14 cannula-implanted). Animals were housed under a 12 h dark–light reversed cycle (lights off at 0900 hours). Behavioral testing was performed in the dark phase of the cycle. Animals had free access to food and water at all times. All the protocols used to complete this study were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Arizona.

In order to assess the behavioral effects of stress, each rat was subjected to a battery of tests before and after the trauma-inducing procedure, so that each rat was its own control. The anxiety tests given up to 2 weeks after trauma were given in a contextual setting different from that of the trauma procedure. The timing of the experimental procedure is shown in Figure 1a and each test is discussed in details below or in Supplementary Material.

Pre-Shock Tests

Open field test. Rats were placed in the center of a circular black arena (diameter of 152.4 cm, no obstacles) lined by a 30-cm high peripheral wall in a dim room. The animals were allowed to freely move for 5 min before being returned to their home cage. The position of the animals was recorded automatically using a video tracker (20–30 frames per second). Animals underwent open field testing before shock and on day 16 after shock. The moving time and distance were calculated. This test was used to assess initial thigmotaxis. Thigmotaxis, or the tendency to remain close to walls, has been previously used as an index of anxiety in animals, as well as in humans, and is indicative of a general bias to safety-seeking phobic behavior (Kallai *et al*, 2007; Treit and Fundytus, 1988).

Nociception and touch-sensitivity tests. There is evidence that there are increases in pain sensitivity in PTSD patients (Asmundson *et al*, 2002). To assess whether increased nociception was present in our rat model, we tested for nociception and touch sensitivity using the tail flick test and the Von Frey test, respectively. The tests were administered the day before shock procedures and again on days 8 and 17 after the shock. See Supplementary Methods for a detailed description of these procedures.

Trauma-Inducing Procedure

The trauma procedure consisted of a single exposure to an inescapable foot shock (day 1) followed by three short reexposures to the shock chamber without foot shocks (situational reminders (SRs)) on days 3, 5 and 7 (Louvart *et al*, 2005; Pynoos *et al*, 1996). The shock apparatus was made of plexiglass ($54 \times 110 \times 40\,cm^3$ high) and was subdivided into two equal-sized compartments separated by a guillotine door: a lighter first compartment and a darker second compartment (Figure 1bA and B). On the shock day, each rat was placed in the lighter compartment of the shock box. After a 3-min adaptation period, the guillotine door was opened and a bright light located in this first compartment was turned on for 20 sec. The door remained open until the animal entered the second compartment. After another 3-min adaptation period in the dark shock compartment, the rat received an inescapable continuous 2 mA foot shock for 10 sec. The sham group received the same treatment but the shock was not given. Both sham and shocked animals were reexposed to the context of the trauma (SRs) for 2 min once every 2 days for 3 days (Figure 1bB). This was achieved by placing the animal in the lighter compartment with the guillotine door opened. The reexposure to the shock box was kept short to prevent extinction. The total time spent in the white compartment and number of crosses into the shock compartment were measured.

To investigate long-term behavioral sequelae, we tested anxiety behaviors 2 weeks after the shock. Animals were tested in three different apparati: the black and white box (also referred as light and dark box (Costall *et al*, 1989)), the elevated plus maze (Pellow, 1985; Walf and Frye, 2007), and the open field (Ohl, 2003). See Supplementary Methods for a detailed description of the anxiety-testing methods used.

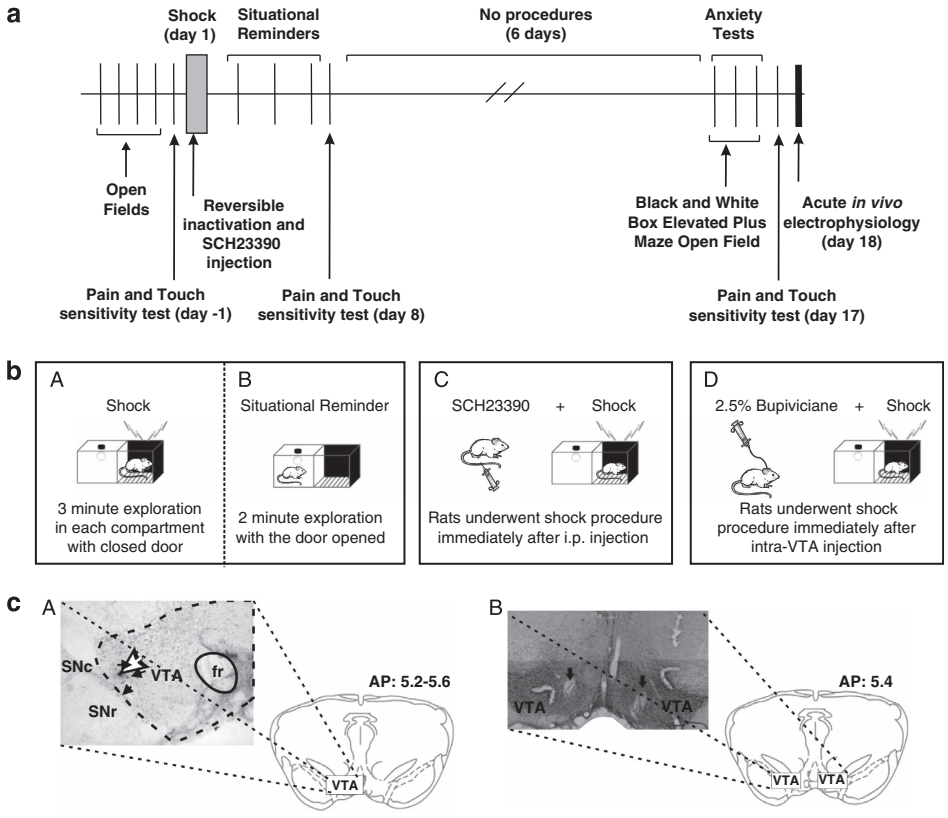

Figure 1 Behavioral protocols and histology. (a) General protocol. Animals underwent baseline measurements including 4 days of open fields and pain and touch-sensitivity tests. They were then exposed to a single inescapable foot shock (day 1), followed by reexposures to the nonshock compartment of the shock cage (SRs, days 3, 5, and 7). To assess long-term behavioral sequelae, animals were evaluated for anxiety, pain, and touch sensitivity after 6 days of rest in their home cage. The experiments ended with acute recordings in the VTA. In two groups of animals, the dopaminergic system was interfered with, immediately before foot shock. (b) Trauma-inducing procedure. The trauma-inducing procedure consisted of a single 2-mA 10-s inescapable foot shock (A) followed by three SRs on 3 different days during which the animal was exposed to the safe compartment of the shock box for 2 min (B). A group of animal received a single i.p. injection of D1-receptor antagonist (SCH23390) immediately before shock (C). Another group of animals received 2.5% bupivicaine hydrochloride or saline delivered in the VTA just before the foot shock (D). (c) Histology. Recordings were obtained from the peribrachial pigmented nucleus of the VTA and marked by ejection of Pontamine Sky Blue (white arrow, A). The location of the injection canulae was verified with tyrosine hydroxylase staining (black arrows, B).

Intraperitoneal Injections of D1 Agonists

A subset of animals was injected with the D1 antagonist SCH23390 (Sigma) prior to the shock procedure. Rats either received 0.1 mg/kg of SCH23390 (dissolved in 0.9% saline solution) or vehicle (0.9% saline solution) only once, immediately before shock (Figure 1bC). All other phases of the experiments were identical.

Intracerebral Cannulation and Injections

A group of animals underwent cannulation surgery targeting the peribrachial pigmented nucleus in the VTA (Nair-Roberts *et al*, 2008). Rats were anesthetized with isoflurane and their body temperature was maintained at 37 °C using a temperature-controlled heating pad. Two stainless steel guide cannulae (plastic one) were stereotaxically implanted bilaterally 5.4 mm posterior to bregma, 0.5 mm lateral to midline, and 7.5 mm below the skull. Five stainless steel microscrews were placed into the skull and dental cement was used to anchor the cannula guides to the screws and skull.

Rats either received bilateral injections of bupivacaine hydrochloride (2.5% dissolved in 0.9% saline solution) or vehicle (0.9% saline solution) only once, on their shock day, immediately before shock (Figure 1bD). This type of reversible local anesthetic has been previously used in the inactivation of VTA neurons (Mahmoodi *et al*, 2011; Moaddab *et al*, 2009; Seip and Morrell, 2009). All injections were done in awake rats with the internal cannulae extending 1 mm beyond the tip of the guide cannula and were performed using a microliter Hamilton syringe (Hamilton, Reno, NV) attached to flexible polyethylene tubing. See Supplementary Methods for a detailed description of spread and mechanisms of action of bupivicaine.

Acute *In Vivo* Electrophysiology

After all behavioral tests were completed, chloral hydrate (350 mg/kg, i.p., 14 rats) was used for the induction and maintenance of anesthesia (Cao *et al*, 2010). Supplemental doses of anesthetic (45 mg/kg intravenous) were given when vibrassal movements became evident. Each animal was fitted with a tracheal breathing tube and a catheter was placed in a lateral tail vein for the intravenous

injection of apomorphine. Using stereotaxic procedures, a small burr hole was drilled in the skull overlying the VTA (5.2–5.6 mm posterior to bregma and 0.5–1 mm lateral to the midline suture). Body temperature was maintained at 37–38 °C throughout the experiment by a temperature-controlled heating pad.

Action potentials were recorded by single-barrel glass-capillary electrodes that were pulled and broken back to a tip diameter of approximately 1 μm and filled with a solution of 2% Pontamine Sky Blue dye in 0.5 M Na acetate. The impedance of the electrodes ranged 12–20 MΩ. At the end of each recording session, a DC current (10 μA pulses 10 s on/10 s off for 20–30 min) was passed through the recording electrode in order to eject the dye, which allowed for the identification of the location of the recorded cells (Figure 1c).

Spike Sorting

Action potentials were isolated using Spike 2 (Cambridge Electronic Design, UK). Using custom-written Matlab code (Mathworks), the firing and action potential properties of VTA cells were determined. Putative DA and GABA neurons were defined by the mean firing rate as well as the shape of the extracellular spike waveform (Supplementary Material, Supplementary Figures S2–S4). The mean activity was calculated using 5-min time windows. A burst was defined by a minimum of three successive spikes having an initial interspike interval (ISI) $\leqslant$80 ms and ending with an ISI $>$160 ms (Grace and Bunney, 1984). Cells were classified as putative dopaminergic on the basis of their firing rate and spike waveforms (Supplementary Figures S2–S4 and (Ungless and Grace, 2012)). See Supplementary Methods for a detailed description of VTA neuron classification.

Statistical Analyses

All statistical analyses were conducted using SigmaStat software (SYSTAT, San Jose, California). Statistics were performed using the Mann–Whitney nonparametric test (sham, shock groups) or using an analysis of variance (ANOVA) (two-way repeated measures ANOVA) with time (days 3, 5, and 7 for SRs or days 1, 8, and 16 for nociception and touch-sensitivity tests) as a between-factor. A significant difference was indicated by $*p<0.05$ or $**p<0.01$.

Localization of Recording Electrodes and Cannulae and Immunohistochemistry

Each rat was perfused transcardially with 0.9% saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was extracted and cryoprotected overnight in a 30% sucrose and 0.02% sodium azide solution. Coronal sections were cut into 50-μm slices on a cryostat. The electrode tracts were localized and their position in VTA confirmed (Figure 1c). One out of three slices was stained with Cresyl Violet. Another third of the slices was processed for tyrosine hydroxylase. The primary antibody was a rabbit anti-tyrosine hydroxylase from Chemicon (1 : 10 000 in PBS-Triton-X100 with 3% normal goat serum), followed by a biotinylated secondary antibody (diluted 1 : 1000, biotinylated rabbit anti-goat, Invitrogen, Oregon). An avidin–biotin peroxidase enzyme complex was prepared and applied according to the manufacturer's instructions (Vectastain Elite ABC kit). Finally, sections were incubated for 5 min in a DAB/hydrogen peroxide substrate solution (Sigma-Aldrich). Sections were mounted with cytoseal (Stephens Scientific) and coverslipped.

RESULTS

Validation of The Rat Model of PTSD

SRs. In order to mimic the conditions in which humans experience the most traumatic events (most PTSD patients are reexposed to the context of the trauma), rats underwent three short SRs (Pynoos *et al*, 1996). On days 3, 5, and 7, animals were placed in the nonshock (safe, light) compartment of the shock cage with the guillotine door opened and the total amount of time spent in each compartment was measured. Animals in the shock group avoided the black compartment in which the trauma procedure was induced, whereas animals in the sham group spent about a third of the time in the shock compartment (Figure 2a, two-way

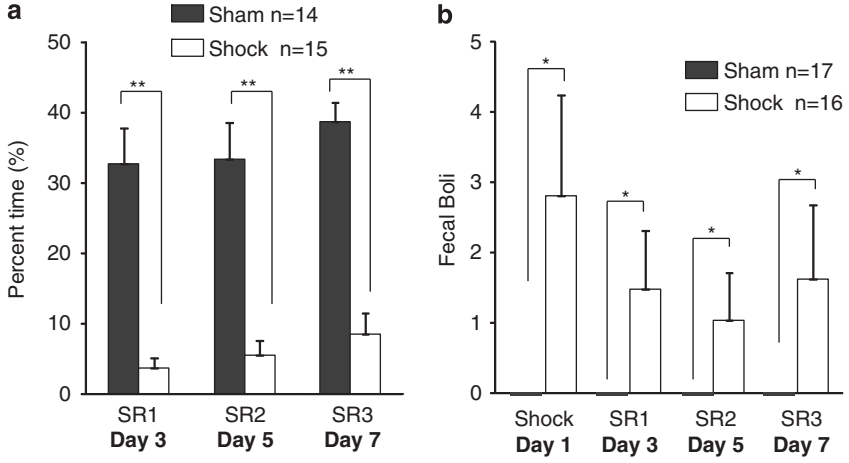

Figure 2 A single inescapable foot shock increases avoidance and excretion of fecal boli. (a) Percent time in shock compartment. Fraction of time spent in the shock compartment during SRs. (b) Fecal boli count. Number of fecal boli during shock and SRs. All error bars are SE (two-way repeated measures ANOVA and Mann–Whitney rank-sum test, $**p<0.01$, $*p<0.05$).

repeated measures ANOVA, $p<0.01$). The fraction of time spent in the shock compartment did not differ significantly throughout the three exposures in either group. A bolus count was also conducted and the animals exposed to the trauma had a significantly higher count than their control counterparts (Figure 2b None of the sham animals excreted). These results demonstrated that animals that received the foot shock showed high avoidance of the trauma chamber up to 7 days after being shocked. In the following, the trauma-inducing procedure refers to the shock day and the three SRs.

Anxiety Tests

One of the main criteria for a valid PTSD animal model is to have long-lasting behavioral effects in response to a brief stressor (Siegmund and Wotjak, 2006; Stam, 2007; Yehuda and Antelman, 1993). To investigate long-term behavioral sequelae, we tested anxiety behaviors 2 weeks after the foot shock, using the black and white box, the elevated plus maze, and the open field tests. The black and white box is a well validated anxiety test in which more anxious animals spend less time in the white compartment, and have decreased general ambulation including decreased crosses back to the white compartment (Chaouloff *et al*, 1997; Costall *et al*, 1989; Louvart *et al*, 2005). The elevated plus maze is also used to assess the general level of anxiety of the animals in an environment entirely different from that of the trauma (Pellow *et al*, 1985). The open field test is used to assess the general level of anxiety (expressed by thigmotaxis) (Ennaceur *et al*, 2006).

Black and white box. On day 14 after the shock day, animals were tested in the black and white box and the latency to escape the white compartment, total time in the white compartment, and total number of white compartment reentries were measured. The mean latency to escape from the white compartment was 6.93 s for the sham group and the mean total time spent in the white compartment was 49.11 s ($n=17$). The animals that experienced the electrical foot shock had significantly longer latencies to escape (Figure 3a, average 15.13 s, $n=17$, Mann–Whitney rank-sum test, $p<0.05$), possibly reflecting the fact that although different, the black and white box may be reminiscent of the shock box. Shocked animals spent less

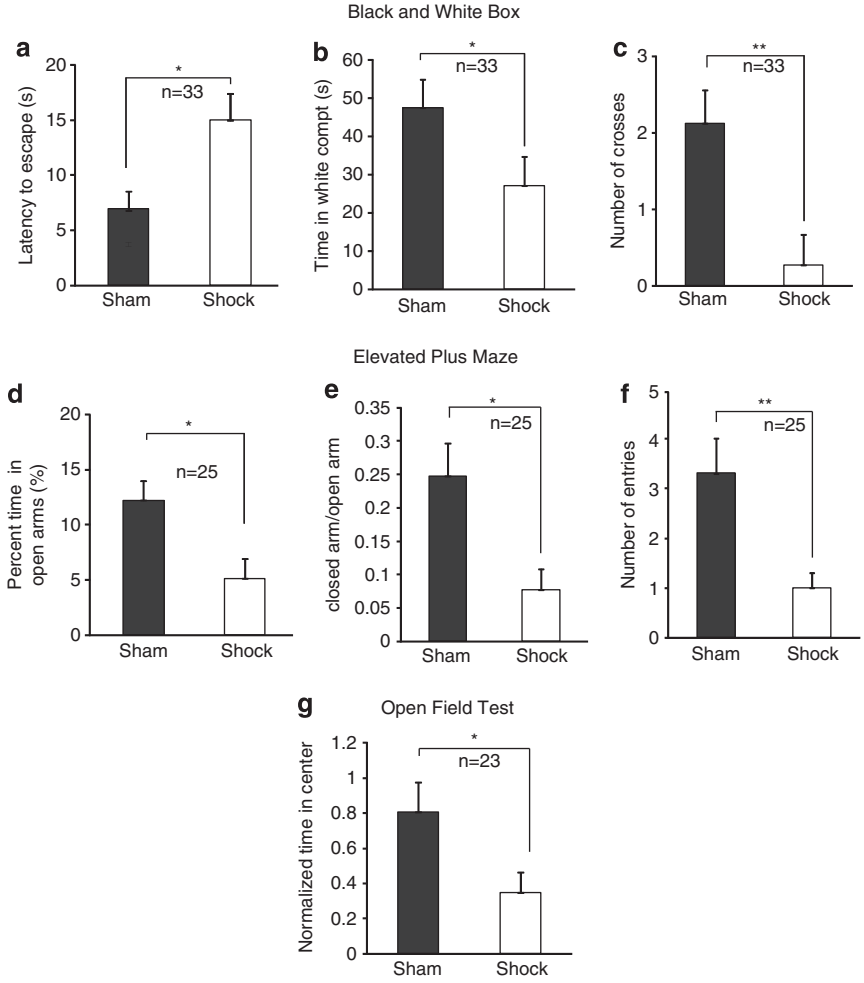

Figure 3 A single inescapable foot shock induces long-term behavioral changes in anxiety. Black and white box. (a) Latency to escape the white compartment (Day 14). (b) Total time spent in the white compartment. (c) Number of crosses back to white compartment. Elevated Plus Maze. (d) Time spent in the open arms. (e) Closed arm/open arm ratio. (f) Number of open arm entries. Open Field. (g) Normalized time in center. All error bars are SE. (two-way repeated measures ANOVA and Mann–Whitney rank-sum test $^{**}p<0.01$, $^{*}p<0.05$).

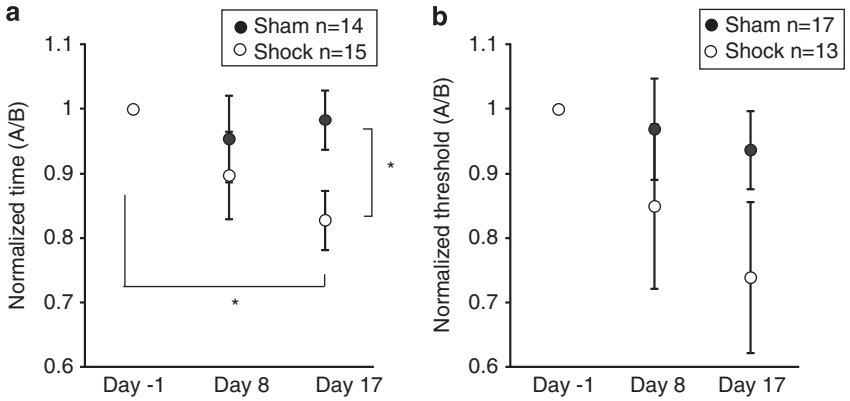

Figure 4 Trauma procedure induced changes in nociception sensitivity, but not in touch sensitivity. (a) Nociception. Normalized nociception measured by the tail flick test before foot shock (day 1) and 8 and 17 days after foot shock. (b) Touch sensitivity. Normalized touch-sensitivity test assessed by the von Frey filament tests on the same days as in a (two-way repeated measures ANOVA, $*p<0.05$).

time in the white compartment than shams (Figure 3b, average 27.26 s, $n=16$, Mann–Whitney rank-sum test, $p<0.05$). The average number of white compartment reentries was significantly different for the shock and the sham groups (Figure 3c).

Elevated plus maze. On day 15 after foot shock, rats were tested on the elevated plus maze. The total time and number of entries in the open and closed arms were recorded. The time spent in the open arms (Mann–Whitney rank-sum test, $p<0.05$, Figure 3d), the closed arm/open arm time ratio ((Mann–Whitney rank sum test, $p<0.05$, Figure 3e), and the average number of open arm entries (Mann–Whitney rank sum test, $p<0.01$, Figure 3f) were all significantly different between the shock and sham groups. These data demonstrate that the rats in the shock group expressed a higher level of anxiety than those in the sham group.

Open field test. The open field test was conducted 16 days after the foot shock. Because of the natural variability in thigmotactic behavior between rats, the time in the center was normalized on an animal-per-animal basis with respect to the times in the center obtained during the open field test performed before the trauma-inducing procedures. A significant difference in the time spent in the center of the field was observed between the two groups (Figure 3g, Mann–Whitney rank-sum test, $p<0.05$).

Nociception and Touch Sensitivity

One of the major health complaints expressed by PTSD patients is increased sensitivity to pain and other general disorders including chronic pain (Asmundson *et al*, 2002). To assess whether such an increase was present in our rat model, we tested for nociception and touch sensitivity using the tail flick test and the Von Frey test, respectively. We tested the animals on three occasions: the day before shock, and 8 and 17 days after shock. The measures were normalized on an animal-to-animal basis using data obtained before the shock procedure. On day 17, in the tail flick test, animals that underwent shock had a significantly shorter latency to remove their tail than those in the sham group as shown in Figure 4a (vertical comparison bracket, two-way repeated measures ANOVA, $p<0.05$). The latency to remove the tail did not differ significantly throughout the three different test days in the sham group (two-way repeated measures ANOVA, black points, Figure 4a). However, there was a significant difference between days 1 and 17 in the shock group (Figure 4a, white points, horizontal comparison bracket). Interestingly, this increase in nociception did not appear to result from a change in touch sensitivity because there was no significant difference in touch sensitivity between the two groups, or across days (Figure 4b).

The results shown in the black and white box, elevated plus maze, and open fields, as well as the nociception test, demonstrate that the trauma-inducing procedure used here is capable of inducing anxiety-like behaviors that persist over time and result in a change in nociception akin to that observed in humans.

Pre-shock D1-Antagonist Administration

In order to characterize the role of DA in this PTSD animal model, we examined the effect of systemically blocking D1 receptors. A D1 antagonist (SCH23390) or saline was injected i.p. immediately before the shock procedures only once. The same behavioral procedures described in the Materials and methods were used in this group of rats.

SRs. On days 3, 5 and 7, i.p. injected animals were subjected to the SRs. Rats injected with SCH23390 spent about a tenth to a quarter of the total time in the shock compartment (SR1 13.76%, SR2 7.55%, and SR3 22.80%). Rats that received vehicle injections had higher avoidance for the shock compartment than the SCH23390 animals (SR1 5.94%, SR2 6.26%, and SR3 7.18%). A significant difference in the total percent time spent in the shock compartment was found on the last SR (Figure 5a, two-way repeated measures ANOVA, $p<0.05$). Fecal boli counts were also conducted but did not reach statistical significance (Figure 5b).

Anxiety Tests

Black and white box. On day 14 after the shock day, SCH23390 and vehicle-injected animals were tested in the

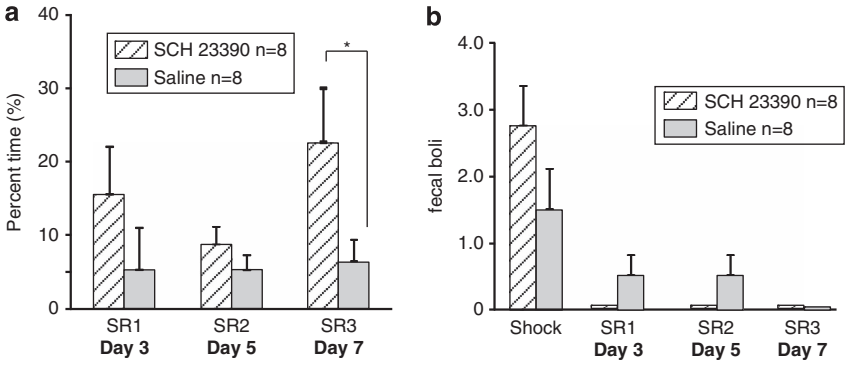

Figure 5 Behavior during SRs of D1-receptor antagonist injected animals. (a) Percent time in shock compartment. Fraction of time spent in the shock compartment during SRs. (b) Fecal boli count. Number of fecal boli excreted during shock and SRs. All graphs are means plus SE (two-way repeated measures ANOVA, $^{**}p<0.01$,$^{*}p<0.05$).

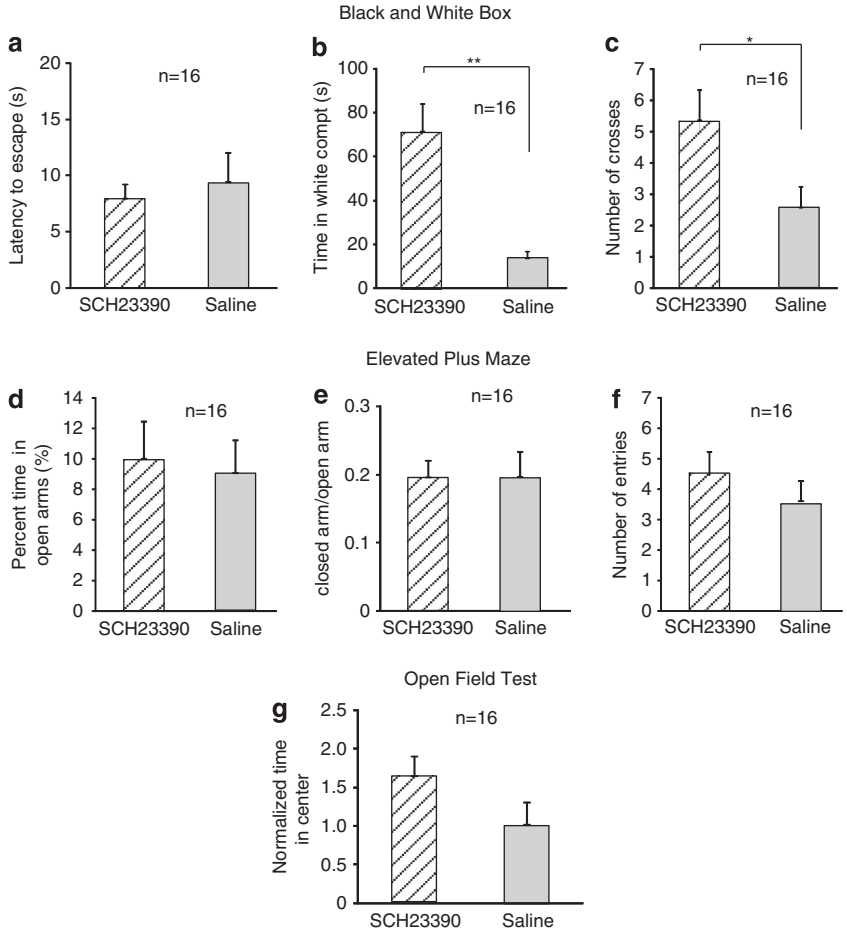

Figure 6 Behavioral changes in response to trauma are prevented by pre-shock D1-receptor antagonist injections. Black and white box. (a) Latency to escape the white compartment (Day 14). (b) Total time spent in the white compartment. (c) Number of crosses back to white compartment. Elevated plus maze. (d) Time spent in the open arms. (e) Closed arm/open arm ratio. (f) Number of open arm entries. Open Field. (g) Normalized time in center. All error bars are SE. (two-way repeated measures ANOVA and Mann–Whitney rank-sum test, $^{**}p<0.01$,$^{*}p<0.05$).

black and white box. The mean latency to escape from the white compartment was on average 9.81 s for the vehicle group and 7.51 s for the SCH23390-injected animals (Figure 6a). The mean total times spent in the white compartment were 12.96 and 69.54 s for the vehicle and D1-receptor antagonist animals, respectively (Figure 6b, Mann–Whitney rank-sum test, $p<0.05$). The mean total number of crosses back to white compartment was 2.87 and 5.37 for the vehicle and SCH23390 animals, respectively (Figure 6c, Mann–Whitney rank-sum test, $p<0.05$).

Elevated plus maze. The total mean percent time spent in the open arms was not significantly different between the SCH23390-injected animals and the vehicle animals

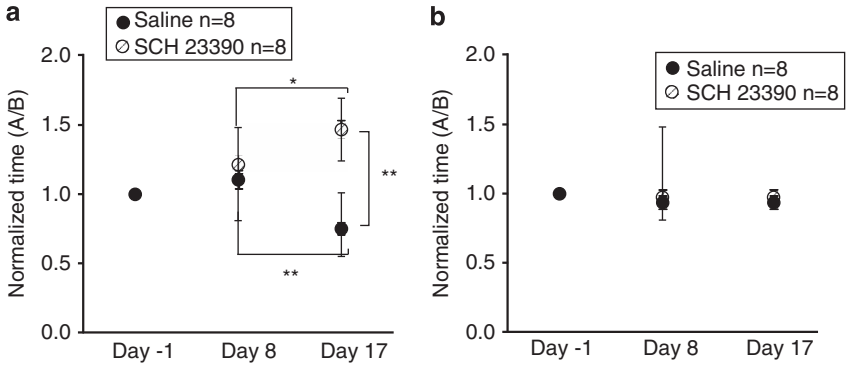

Figure 7 D1-receptor antagonist decreased nociception but not touch sensitivity. (a) Nociception. Normalized nociception measured by the tail flick test before foot shock (day 1) and 8 and 17 days after foot shock. (b) Touch sensitivity. Normalized touch-sensitivity test assessed by the von Frey filament tests on the same days as in A (two-way repeated measures ANOVA, $*p<0.05$).

(Figure 6d). The close arm/open arm time ratio (Figure 6e), as well as the number of open-arm entries (Figure 6f), was also not significantly different between the two groups. A significant difference was found, however, in the total mean percent time spent in the closed arms, with saline-injected animals spending significantly more time in the closed arms than SCH23390 animals (64.1% and 57.1%, respectively; Mann–Whitney rank-sum test, $p<0.05$, not shown).

Open field. The time in the center was normalized with respect to the time spent in the center before the shock was given. There was a trend indicating that the SCH23390 spent more time in the center of the field, but no significant difference was found (Figure 6g).

Nociception and Touch Sensitivity

As with the behavioral animals (Figure 4), the same animals were tested for nociception and touch sensitivity. There was a significant decrease in nociception between day 8 and 17 in the SCH23390 group (Figure 7a, black circles) and a significant increase in the vehicle group (Figure 7a, white-stripped circles). There were no significant differences in touch sensitivity in either group (Figure 7b).

Pre-shock Reversible VTA Inactivation

To test the hypothesis that the VTA is specifically involved in the long-term anxiety exhibited in our animal model, a separate group of animals was implanted bilaterally with cannulae targeted to the this area. Animals were subjected to the behavioral procedures described previously. All animals received microinjections of either bupivacaine hydrochloride or vehicle (saline) only once, immediately before the electrical foot shock on day 1 (Figure 1).

SRs. During the SRs, animals in the saline group avoided the shock compartment of the shock cage significantly more than the animals in the bupivacaine group (Figure 8a). The fraction of time spent in the shock compartment was larger for the bupivacaine group than for the saline group in all three SRs (two-way repeated measures ANOVA test, $p<0.05$). Fecal boli counts were also conducted but no significant differences were found, although saline-treated rats tended to have a higher fecal boli count than the bupivacaine group (Figure 8b).

Anxiety Tests

Black and white box. Cannula-implanted animals were also tested in the black and white box. The latency to escape from the white compartment was not found to be significantly different between bupivacaine and saline groups, although a trend is observed (Figure 9a). The total time spent in the white compartment was, however, significantly different (Figure 9b, 22.85 *vs* 88.33 s for saline and bupivacaine animals, respectively). The number of crosses back into the white compartment was also significantly different between the two groups (Figure 9c, Mann–Whitney rank-sum test, $p<0.05$).

Elevated plus maze. The total time spent in open arms was significantly different between the groups (Figure 9d, Mann–Whitney rank-sum test, $p<0.01$). The close arm/open arm ratio was also found to be significantly different (Figure 9e, Mann–Whitney rank-sum test, $p<0.05$). The average number of open-arm entries in the saline and bupivacaine groups were significantly different as well (Figure 9f).

Open field test. The last anxiety test conducted in the cannula-implanted animals, 16 days after shock procedures, was the open field test. There was a trend for bupivacaine animals to spend more time in the center (ie, showing less thigmotaxis) than saline animals (Figure 9g).

Altogether, these results demonstrate that VTA inactivation had protective effects in this PTSD paradigm.

Nociception and Touch Sensitivity

In the tail flick test, animals that received saline intracranial injections had significantly shorter latencies to remove their tail on day 17 when compared with days 1 and 8 (increased nociception, gray points, Figure 10a). VTA-inactivated animals did not show significant changes in nociception (Figure 10a, black points). There was no significant change in touch sensitivity in either group of animals, although a small decreasing trend was apparent in the saline group (Figure 10b).

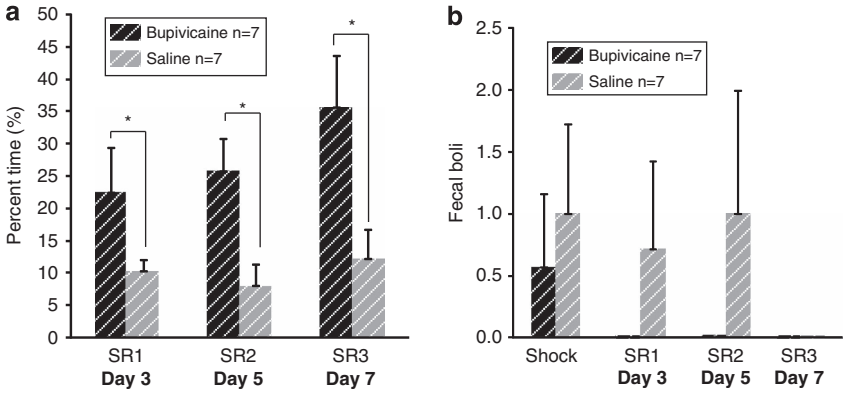

Figure 8 VTA inactivation decreased avoidance. (a) Percent time in shock compartment. Fraction of time spent in the shock compartment during SRs. (b) Fecal boli count. Number of fecal boli during shock and SRs. All error bars are SE (two-way repeated measures ANOVA and Mann–Whitney rank sum test, $*p<0.05$).

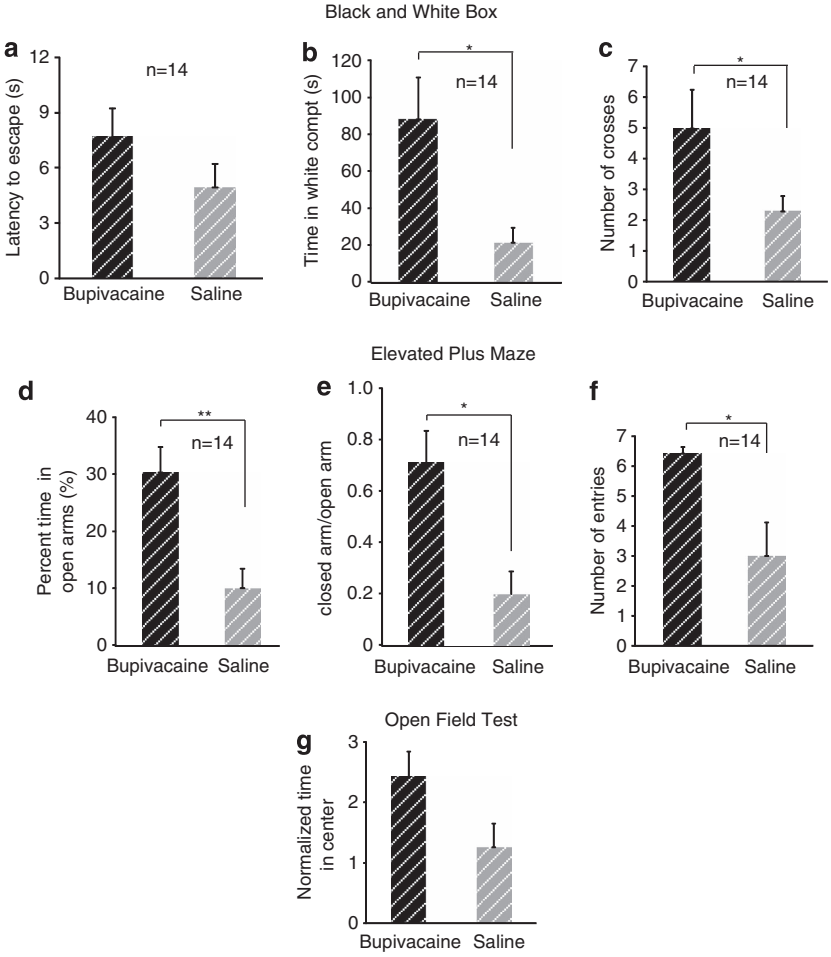

Figure 9 Behavioral changes in response to trauma are prevented by pre-shock VTA inactivation. Black and white box. (a) Latency to escape the white compartment (day 14). (b) Total time spent in the white compartment. (c) Number of crosses back to white compartment. Elevated plus maze. (d) Time spent in the open arms. (e) Closed arm/open arm ratio. (f) Number of open arm entries. Open Field. (g) Normalized time in center. All error bars are SE. (two-way repeated measures ANOVA and Mann–Whitney rank-sum test, $**p<0.01$, $*p<0.05$).

To control for possible analgesic effects of bupivicaine during the shock procedure, we assessed nociception in a separate group of rats. Using the Hargreaves test showed no difference in nociception before and after bupivacaine injections, excluding the possibility that bupivacaine animals were rendered insensitive to the foot shock by the intracranial injection (see Supplementary Figure S1). These results demonstrate that animals that underwent reversible inactivation of the VTA have less long-term increases in nociception than control.

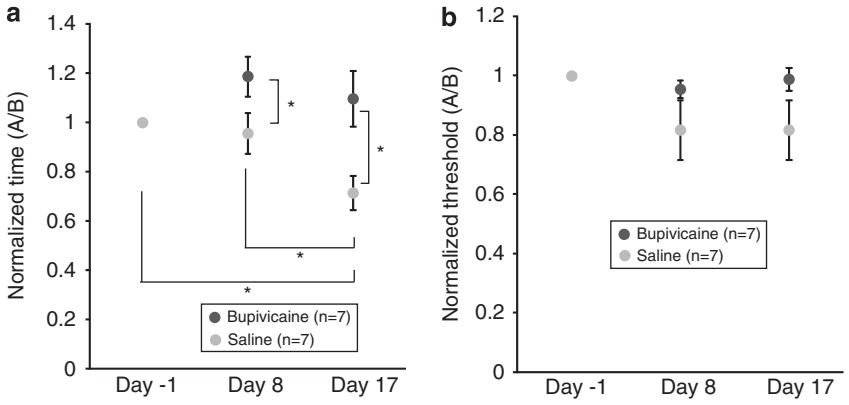

Figure 10 Increases in nociception were not present in animals with VTA inactivation. (a) Normalized nociception measured by the tail flick test before foot shock (day 1) and 8 and 17 days after foot shock. (b) Touch sensitivity. Normalized touch-sensitivity test assessed by the von Frey filament tests on the same days as in A (two-way repeated measures ANOVA, $*p<0.05$).

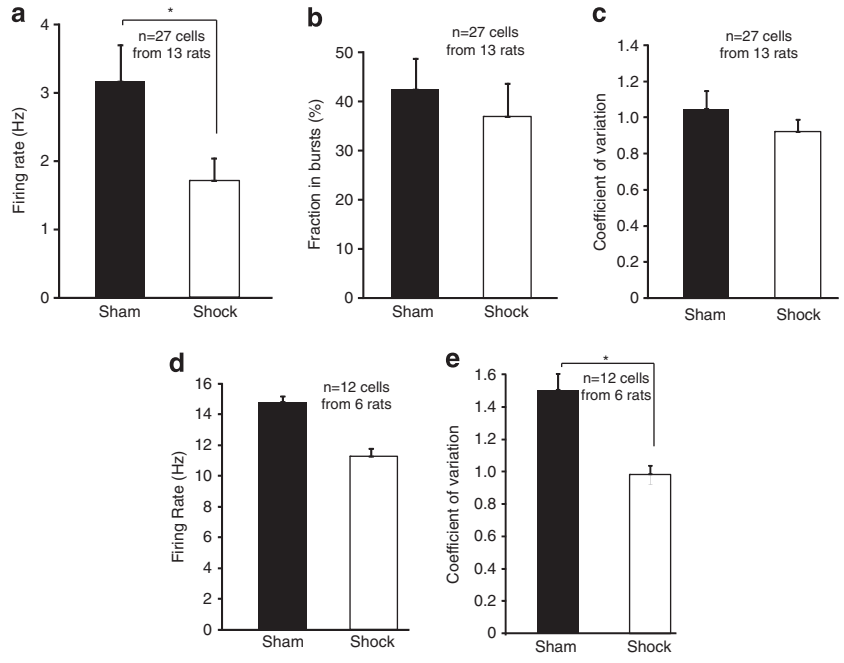

Figure 11 Long-term (day 18) changes in VTA putative dopaminergic and GABAergic cell electrophysiology after trauma. (a) Mean firing rate for putative DA cells. (b) Fraction of action potentials in bursts in putative DA cells. (c) Coefficient of variation of putative DA cells. (d) Mean firing rate of putative GABA cells. (e) Coefficient of variation of putative GABA cells. All error bars are SE (Mann–Whitney rank-sum test, $*p<0.05$).

Electrophysiology Experiments

Following the last nociception and touch-sensitivity test, on day 18, VTA cells from the shock and sham animals were recorded. The mean baseline firing rate of putative VTA DA cells of shocked animals was significantly lower than that of control animals (Figure 11a).

The bursting mode in VTA cells is thought to be important to boost the gain of neural signaling of salient stimuli by enhancing transmitter release and dendritic depolarization (Cooper, 2002). There was no significant difference between the fraction of action potentials fired in bursts between the shock and sham groups (Figure 11b). The coefficient of variation measures the regularity of cell firing (CV close to zero is regular; CV close to 1 is near-Poisson). There was no significant difference between the shock and sham groups (Figure 11c).

The firing rates of putative GABAergic cells were also calculated. There was no significant difference between shock and sham group baseline firing rates (Figure 11d). However, there was a significant difference in the coefficient of variation between shock and sham group. GABAergic cells in the sham animals had a CV >1, reflecting irregular bursting patterns whereas the CV was near 1 in the shocked animals (Figure 11e).

DISCUSSION

The number of PTSD diagnoses is rapidly increasing. There is to this day no specific pharmacological treatment for this disorder, and most patients are given antidepressants or antipsychotics, mainly to treat their symptoms. Animal models of PTSD have the potential to provide insights into

the etiology, course and possible novel treatments of this disease. A widely accepted family of animal models relies on the inescapable foot-shock paradigm. This type of animal model has demonstrated many symptomatic and physiological similarities with human PTSD (Stam, 2007). Animal models based on inescapable foot stocks have shown increases in anxiety (Louvart *et al*, 2005; Stam *et al*, 2002; Van Dijken *et al*, 1992), hypervigilance (object burying) (Langevin *et al*, 2010; Mikics *et al*, 2008), decreases in social interaction (Louvart *et al*, 2005), as well as increases in nociception (Geerse *et al*, 2006). The inescapable foot-shock paradigm is also a suitable model because the trauma that gives rise to PTSD, such as rape or combat-related injuries, often occurs without the control of the individual. An imperative criterion to be met for a valid PTSD model is that the behavioral sequelae persist over time. It has been shown that a brief intense inescapable foot shock together with subsequent SRs cause increased anxiety-like behavior and blunted corticosterone levels and that these effects remain up to 6 weeks after the traumatic event (Louvart *et al*, 2005; Pynoos *et al*, 1996). Consistent with previous animal models, we demonstrated here that a brief intense inescapable foot shock followed by SRs can cause long-term behavioral sequelae such as avoidance for the traumatic spatial context and increased anxiety (Figures 2 and 3). Furthermore, these long-term anxiety-like behaviors are accompanied by increases in nociception (Figure 4), a feature often noted in PTSD patients.

The study of the neural substrate of PTSD is best tackled using a multidisciplinary approach including medical, neuroscientific, and pharmacological studies. Most conceptual models investigating this neurocircuitry include the amygdala, the hippocampus, and the prefrontal cortex (Liberzon *et al*, 1999; Rauch *et al*, 2006). We present evidence for the involvement of the dopaminergic system by showing that injecting systemically a D1-receptor antagonist just before trauma decreases the appearance of avoidance and long-term anxiety behaviors in some of the tests conducted (Figures 5 and 6). We further show evidence for the involvement of the VTA by demonstrating that the long-term behavioral consequences induced by a traumatic event can be prevented if VTA cells are inactivated immediately before the event (Figures 8 and 9). This result assumes that bupivacaine inactivation in VTA does not have long-term consequences, as suggested by several studies in other brain areas (see Supplementary Material). Overall, the reduction in anxiety due to SCH23390 administration is less pronounced than that of VTA inactivation (compare Figures 6, 7, 9 and 10, respectively). This difference may be attributed to a number of factors including overall system-wide short-term compensation for SCH23390 D1 antagonism, the effect of SCH23390 on areas other than VTA, the dosage of the drug, the role of the intact D2 system, and the fact that bupivacaine inactivates all neurons in VTA, not just dopaminergic ones.

These findings are compatible with other studies that have indirectly implicated the VTA in PTSD. Others have, for example, suggested the involvement of the VTA in PTSD by observing high comorbidity with drug addiction (Kofoed *et al.*, 1993; Najavits *et al.*, 1997) and reduced reward sensitivity among PTSD patients (Elman *et al*, 2009; Hopper *et al*, 2008). Moreover, DA released from VTA cells has the potential to modulate the activity in areas important for the consolidation and retrieval of emotional memories, such as the hippocampus and the amygdala, as well as modulate areas essential for the extinction of memories such as medial prefrontal cortex (Fields *et al*, 2007). The VTA has also been shown to be an important factor in the nociception pathways (Becerra *et al*, 2001; Borsook *et al*, 2007; Saade *et al*, 1997). Altogether, this evidence strongly suggests that the VTA may have an important role in PTSD symptomatology.

In this study, we show that VTA DA cells in trauma-exposed rats have lower baseline firing rates 18 days after the shock procedure when compared with sham rats (Figure 11a). This difference in firing rates may be due to an increase in intrinsic VTA GABAergic activity from a neural population not recorded here, an increase of external-VTA inhibitory inputs, and/or changes in intrinsic membrane excitability. Further experiments using optogenetic techniques (Tan *et al*, 2012) or simultaneous recordings in areas such as the basolateral nucleus of the amygdala, or the medial prefrontal cortex *in vivo* and work *in vitro* would be required to tease these possibilities apart. These changes in VTA DA cell electrophysiology can give rise to changes in baseline tonic DA levels in areas mediating emotional and cognitive processes and can lead to the inadequate responses to stress often seen in PTSD patients.

Given the heterogeneity of PTSD symptoms (hyperarousal, avoidance, and reexperiencing symptoms) and the medical (chronic pain, respiratory, and cardiac disorders) and psychiatric (other anxiety and mood disorders and substance abuse) consequences, it is likely that the neural substrate involved also varies heterogeneously from patient to patient. A possible explanation for the wide differences in relative severity of symptoms may reside in the deregulation of a brain region that has widespread connections to various areas involved in perception, learning, memory, and other cognitive and emotional functions. Compatible with other models (Liberzon and Sripada, 2008; Sigurdsson *et al*, 2007), we propose that the hippocampus processes contextual information related to the trauma (Payne *et al*, 2007; Payne *et al*, 2003) and that the medial prefrontal cortex regulates the degree to which the amygdala expresses emotional responses (Figure 12a). In our conceptual model, the VTA modulates the activity of these three areas and indirectly regulates emotional responses to the traumatic event both in the short term and in the long term.

We propose that the hyperresponsiveness of the amygdala to stress and the inadequate regulation of the medial prefrontal cortex and the hippocampus after trauma are in part due to a deregulation of the mesolimbic dopaminergic modulatory system (Figure 12b). We propose that traumatic stress increases VTA DA activity transiently to high levels, as has been observed in acute stress paradigms (Anstrom *et al*, 2009; Anstrom and Woodward, 2005; Brischoux *et al*, 2009; Morrow *et al*, 2000; Valenti *et al*, 2011). This increase of DA activity during a traumatic event has the short-term consequence of enhancing the consolidation of the traumatic memory. This increase in consolidation may rely on well-established synaptic plasticity mechanisms, such as long-term potentiation and depression (LTP/LTD).

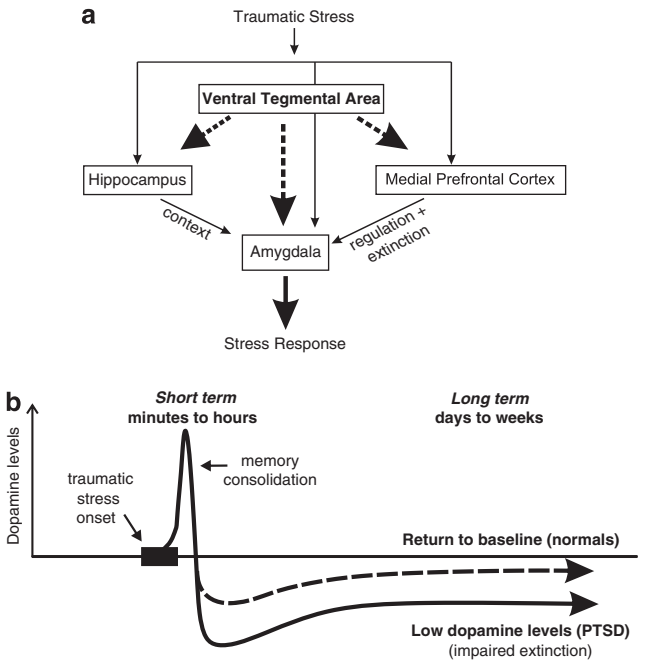

Figure 12 Conceptual model. (a) Neural circuitry involved in PTSD. DA released from VTA cells modulate the activity and synaptic plasticity in areas important for the consolidation and retrieval of emotional memories, such as the hippocampus and the amygdala. The VTA also projects to areas essential for the extinction of memories such as medial prefrontal cortex. (b) PTSD as mesolimbic deregulation. During and immediately after a traumatic event DA levels increase above baseline levels. This increase strengthens memory consolidation. The circuitry compensates for this increase by a large decrease below baseline. In most individuals, DA levels return to baseline. We propose that in a PTSD patient, the return to baseline does not occur, possibly preventing the extinction of traumatic memories.

Experiments in hippocampal slices show indeed that LTP in CA1 depends on the activation of DA receptors (Frey *et al*, 1990; Lisman *et al*, 2011; Lisman and Grace, 2005). Furthermore, evidence from behavioral experiments show that DA enhances learning (Morris *et al*, 2003). Finally, the increase of DA levels by administration of L-DOPA-enhanced memory and COMT (enzyme that metabolizes DA) inhibitors also showed memory improvements in various tasks in human subjects (Apud *et al*, 2007). We then propose that this short-term increase of DA activity may be followed by a compensatory mechanism that aims at returning DA to normal levels. In most subjects, after perhaps a transient period of hypodopaminergic activity, the DA firing rates return to normal (dashed curve, Figure 12b). In a small fraction of subjects, however, the down stroke of the compensatory mechanism may cross a phenomenological nonrecovery threshold such that the return to baseline DA levels is prevented or significantly slowed down (continuous curve, Figure 12b). The firing rate of DA cells remains lowered (hypoactive), as we showed here in rats and compatible with other studies in which lower dopaminergic activity was measured over 2 weeks after trauma onset (Moore *et al*, 2001). In this group of subjects, traumatic stress decreases DA levels in the long term and this decrease would cause a failure to reconsolidate and failures to extinguish traumatic memories, an active process that requires LTP/LTD mechanisms. The subjects would develop PTSD. This theory is consistent with pharmacological experiments showing that brain infusions of D4 antagonists into prefrontal cortex impair fear-conditioning extinction (Pfeiffer and Fendt, 2006) and by evidence showing decreased DA release in PFC (Goto *et al*, 2007; Gresch *et al*, 1994). Moreover, it has been shown that prolonged stress impairs LTP induction (Goto and Grace, 2006). If DA activity is prevented from increasing at the time of trauma, as in our inactivation experiments, no upstroke and compensatory down stroke occur, and DA levels are unaffected. Further evidence for the long-term hyporesponsiveness of the mesolimbic pathway is suggested by successful treatment of PTSD patients with psychostimulants (Houlihan, 2011), which enhance DA release in areas such as the prefrontal cortex and may alleviate symptoms by increasing DA to normal levels. This hypodopaminergic view on PTSD suggests that the co-occurrence of drug addiction and alcohol abuse often observed in PTSD patients may in fact be a coping (self-medication-like) response to compensate for abnormally low levels of DA. Altogether, our results suggest that VTA dopaminergic neurons may be key in understanding the onset and symptomatology of PTSD, and that drugs specifically targeted to the dopaminergic system may be of interest for the prevention and treatment of PTSD.

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DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Involvement of the ventral tegmental area in a rodent model of post-traumatic stress disorder

Abbreviated Title: Involvement of the VTA in PTSD

Nadia S. Corral-Frias PhD[1], Ryan P. Lahood[2], Kimberly E. Edelman-Vogelsang[3], Edward D. French PhD[4] and Jean-Marc Fellous PhD[5]

Supplemental Figures

S1. **Intra VTA bupivicaine infusion does not cause anelgesia**. A group of rats was implated with bilateral cannulae targeted to the VTA. Pain sensitivity was assessed with the plantar Hargreaves tests (see methods) All error bars are standard error.

S2. **Characterization of putative dopamine neurons.** A. Action potential width for putative DA and GABA cells. Inset shows overlaid action potentials from 1 putative dopamine cell (black) and 1 putative GABA cell. B. Example traces of putative DA cell action potentials, and comparison with Grace and Bunney (1983). C. Mean firing rate for putative DA and GABA cells. D. Putative DA and GABA cells mean firing rates plotted against action potential width. Inset shows similar plot form Anstrom and Woodward (2005).

S3. **Characterization of putative dopamine neurons**. A. Dopamine and non-dopamine cells half width against action potential width. B. Dopamine and non-dopamine cells total action potential width against peak to trough ratio. C. Dopamine and non-dopamine cells half with against peak to trough ratio. D. Dopamine and non-dopamine cells firing rate against peak to trough ratio.

S4. **Response of 2 single putative dopamine neurons to apomorphine injections.** Example of two putative dopaminergic cell firing rate in response to cumulative doses of Apomorphine injected i.v..All firing rates were computed in 5 min windows after each injection.

Supplemental Methods

Nociception and Touch Sensitivity

Tail Flick (nociception)

The withdrawal latency of the tail to a temperature stimulus was measured by immersing the distal third of the tail into a 52 ± 0.5 ˚C water bath. Cut-off time was set at 10 s to avoid skin damage. Rats underwent this test three times with a 5 minute interval between each trial. The three measurements were averaged each day to obtain the withdrawal latency in seconds. This test was conducted 3 times throughout the protocol, one day before shock and days 8 and 17 after shock.

Nociception control experiment with the Hargreaves test

To control for possible short-term anesthetic effects of 2.5% bupivacaine hydrochloride infusions in the VTA, a separate group of rats was tested. Nociceptive changes in response to acute intracerebral injections of bupivacaine were assessed using the Hargreaves test which assessed paw-withdrawal latency to a thermal nociceptive stimulus. Rats were allowed to acclimate within a Plexiglas enclosure on a clear glass plate in a quiet testing room. A radiant heat source was focused onto the plantar surface of the hindpaw, and paw-withdrawal latency was determined by a motion detector that halts both the lamp and timer when the paw was withdrawn. A maximal cut-off time of 32 s was used to prevent tissue damage (Hargreaves *et al*, 1988). The tail flick sensitivity test was not used here because it would have required 6 warm-water tail dips in less than 30 minutes (effective time for bupivacaine action, see above), and would have yielded habituation.

Von Frey (touch sensitivity)

Using a set of von Frey filaments, the foot withdrawal threshold for mechanical stimulation to the hind paw was determined (Chaplan *et al*, 1994). Each von Frey filament was applied perpendicularly to the plantar surface with sufficient force to bend it slightly and held for 2–3 s. An abrupt withdrawal of the foot during stimulation or immediately after the removal of stimulus was considered a positive response. When there was a positive or negative response, the next weaker or stronger filament was applied, respectively. The test continued until four stimuli after the first change in response was obtained.

Anxiety testing

Black and White Box Test

Fourteen days after shock or sham procedures, rats were tested in the black and white box (Costall *et al*, 1989). The apparatus consisted of a plexiglass chamber subdivided into two compartments: a black compartment (30 x 32 x 40 cm high) and a white one (45 x 32 x 40 cm high). The compartments were connected by a small divider (10 x 15 cm high). Each rat was placed in the white compartment facing the wall opposite to the opening. The latency to enter the dark compartment, time spent in each compartment and the total number of crosses to the white compartment were assessed for 5 minutes. A four-paw criterion was used for compartment entries.

Elevated Plus Maze Test

On day 15 after the shock or sham procedures, rats were tested on the elevated plus maze (Pellow *et al*, 1985). The wooden apparatus consisted of two open arms (50 x 10 cm) alternating at right angles with two arms enclosed by 40 cm high walls. The four arms delimited a central area of 10 cm^2. The apparatus was placed 60 cm above the floor. The test began with the placement of the rat at the center of the maze with its head facing a closed arm. The time spent in the open and closed arms were recorded and were expressed as a percentage of the total time spent in the apparatus. A four-paw criterion was used for arm entries.

Intracerebral injections

The volume injected was 1.0µL bilaterally and the rate of injection was approximately 1 µL per minute. The injection cannulae remained in place for an additional 1 minute to allow adequate absorption of the substance. Autoradiographic analyses estimate the spread of bupivacaine to be approximately 1.4 mm from the injection site (Martin, 1991). However, based on electrophysiological evidence (Tehovnik and Sommer, 1997) it has been suggested that the functional spread of intracerebrally infused anesthetics is considerably less than the autoradiographic spread and closely conforms to the spherical volume equation ($V=4/3\pi(r)^3$). According to this analysis, the functional spread of intra-cerebral bupivacaine infusion for the present parameters would be approximately 0.63 mm (Hsu et al 2002). Given the area that VTA covers (0.8 mm medial to lateral and 1 mm rostral to caudal in a 3 month old rat) the infusions would be expected to be largely confined within the VTA. Moreover, the lack of spillover was also confirmed by the lack of behavioral responses (rotating behavior due to invasion of the bupivacaine injection into the Substantia Nigra, nearby). The shock was delivered 2 to 3

minutes after the micro-injections. The same behavioral testing detailed above was used for all cannula implanted animals.

Bupivacaine hydrochloride is an amide-linked local anesthetic of the same type as lidocaine that binds to the intracellular portion of sodium channels and blocks sodium influx into nerve cells preventing depolarization (Scholz *et al*, 1998). Bupivacaine injections have a rapid onset (within tens of seconds) and a relative short duration (30 to 40 minutes) (Lomber, 1999). This drug has been used effectively in neurobehavioral studies employing intracerebral infusions in various brain regions in the rat, including the nucleus accumbens, amygdala, prefrontal cortex and the ventral tegmental area (Floresco *et al*, 2008; Haralambous and Westbrook, 1999; Hsu *et al*, 2002; Mahmoodi *et al*, 2011; Moaddab *et al*, 2009; Seip and Morrell, 2009). Given the heterogeneity of VTA circuitry, inactivation of the entire VTA is the first step in determining whether the VTA is a critical component for the circuitry involved in PTSD symptomatology.

Recent work using lidocaine-induced reversible inactivation of VTA showed the role of this area in the acquisition of inhibitory avoidance memory in rats (Mahmoodi *et al*, 2011). The experimental procedure (e.g. 2-compartment black-and-white box, foot shocks) was very similar to that used in our study. In one of their experiments, the authors compared a non-shocked lidocaine to non-shocked saline and demonstrated that these two groups behaved similarly, showing that reversible inactivation of VTA alone did not in and of-itself interfere with the behavior of the animal in the shock box (a test that corresponds to our situational reminders). This result and the results from other studies using bupivacaine cited above suggest that a one-time injection of bupivacaine in the VTA is unlikely to produce long lasting effects 2 weeks later. For this reason, a control group of non-shocked VTA inactivated animals was not included in this study.

Neuron Classification

There has been controversy over the electrophysiological identification of dopamine neurons in the rat *in vivo* (Ungless and Grace, 2012). The debate has centered on both the electrophysiolgical and pharmacological characterization of VTA dopamine neurons. *In vitro* studies have shown that although some tyrosine hydroxylase positive neurons do fit electrophysiological characterizations, there are neurons that do not and that there are tyrosine hydroxylase negative neurons that have characteristics that were thought to be exclusive to dopamine neurons (Cameron *et al*, 1997; Johnson and North, 1992; Margolis *et al*, 2006). Many have argued that because these studies have been performed *in vitro* they may not fully serve to characterize these cells. Also, some of these studies have been done in immature rats in

which the activity patterns of dopamine neurons and the expression of dopaminergic autoreceptors may be significantly different from those of adult rats (Grace *et al*, 2007). Moreover, recent work has shown that whole-cell patch clamp recording in combination with immunohistochemical detection of tyrosine hydroxylase expression can guarantee positive but not negative dopamine identification in the VTA (Zhang *et al*, 2010).

An additional difficulty to the previously used classification system is the discovery of a third population of neurons in VTA that have glutamate as their neurotransmitter ((Margolis *et al*, 2006; Nair-Roberts *et al*, 2008). This third population shares many electrophysiological and pharmacological characteristics with tyrosine hydroxylase containing neurons making it difficult to distinguish between them. A fruitful approach that has been used to separate different populations of neurons consists in the delineation of clusters using different characteristics of the neurons firing rate and action potentials shape (Anstrom and Woodward, 2005; Roesch *et al*, 2007; Wang and Tsien, 2011). We choose this method here.

Putative dopamine neurons were characterized on the basis of electrophysiological and pharmacological measurements used in previous studies (Anstrom *et al*, 2005; Grace and Bunney, 1983). Dopamine cell characteristics include long-duration waveforms, firing rates between 1 and 7 Hz, and inhibitory responses to apomorphine, a D2 receptor agonist (see supplemental Figures 2-4). Two populations of neurons were identified on the basis of these criteria.

Spike waveforms were measured from the first inflection point to the time point when the voltage returned to within 10% of the baseline. The average duration of total extracellular spike waveforms for putative dopamine neurons was 3.92 ms and 1.69 ms for putative GABA neurons. Some of the extracellular spike waveforms were triphasic as seen in previous studies (supplemental S2B). In the conditions of our experiments the average firing rate recorded for putative dopamine neurons was 2.78 Hz and putative GABAergic neurons had firing rates that averaged 14.34 Hz. When average action potential duration was plotted against average firing rate, 2 distinct clusters were found, compatible with other studies (Anstrom and Woodward, 2005) and supplemental Figure S2D.

To further confirm that the cells that we were recording from were dopamine cells we injected increasing doses of apomorphine i.v. (1, 2, 4, 8 µg/kg). Dose response curves were then plotted and a near-linear decrease in activity with higher concentrations was observed (Figure S4, 4 µg/kg was sufficient to turn the cells off in most cases). Note that these experiments were performed only once per animal, after all data were collected, so at most one cell per animal was characterized in this fashion.

Together with the tyrosine hydroxylase immunohistochemistry and Nissl staining procedures (Figure 1), these data demonstrate that putative dopaminergic and GABAergic cells in the VTA could be effectively discriminated.

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Supplemental Material

Figure S1

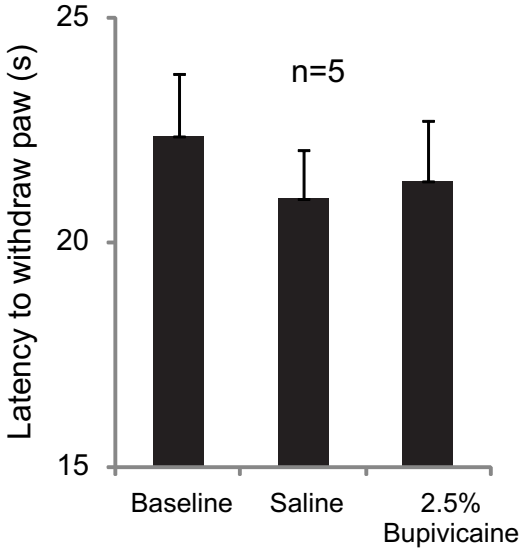
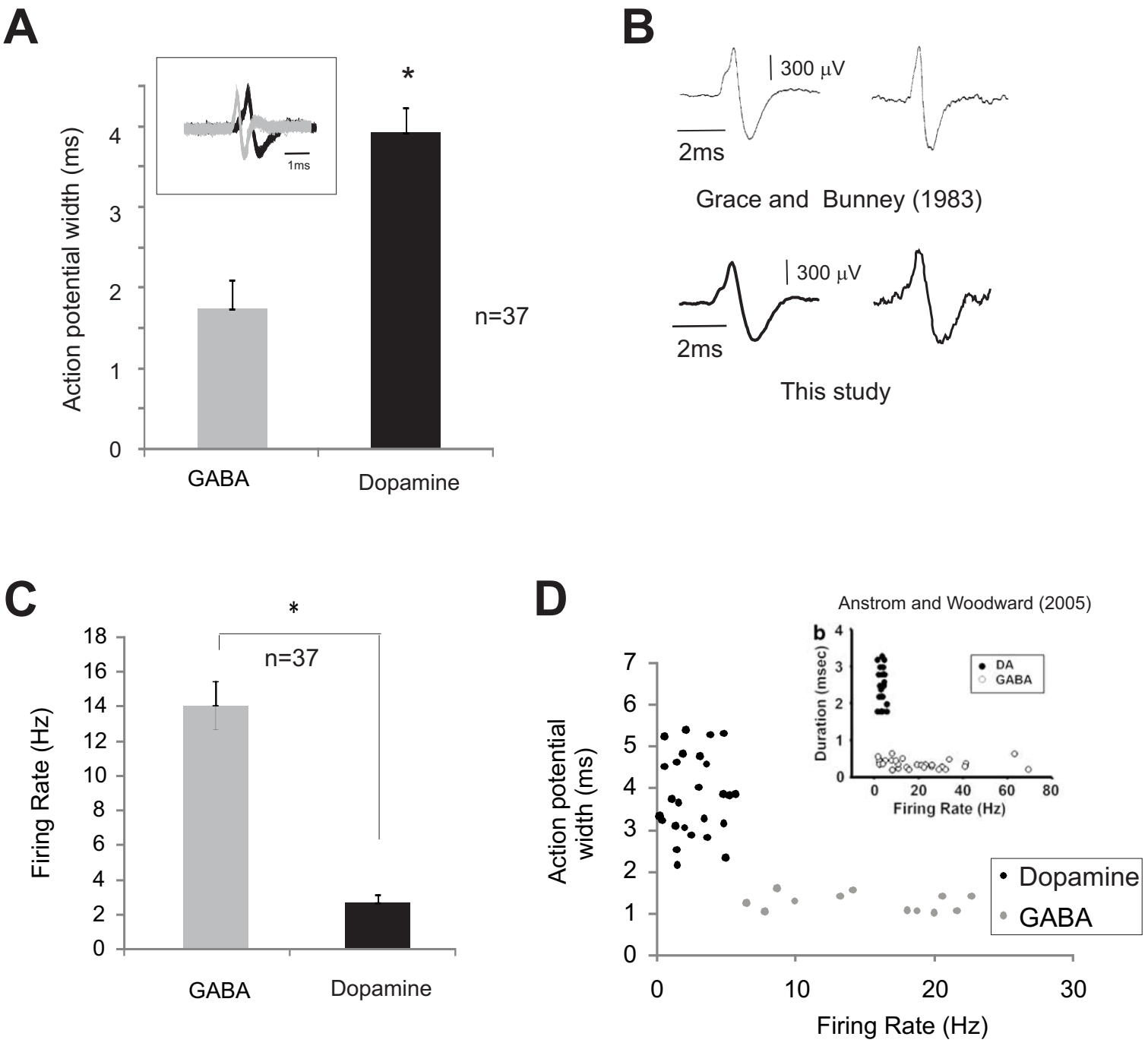


Figure S2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2
3
4
1ms
*
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GABA
Dopamine
B
300 μV
2ms
Grace and Bunney (1983)
300 μV
2ms
This study
C
Firing Rate (Hz)
0
2
4
6
8
10
12
14
16
18
*
n=37
GABA
Dopamine
D
Anstrom and Woodward (2005)
Action potential width (ms)
0
1
2
3
4
5
6
7
0
10
20
30
Firing Rate (Hz)
Dopamine
GABA
b
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3
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Firing Rate (Hz)
DA
GABA

Figure S3

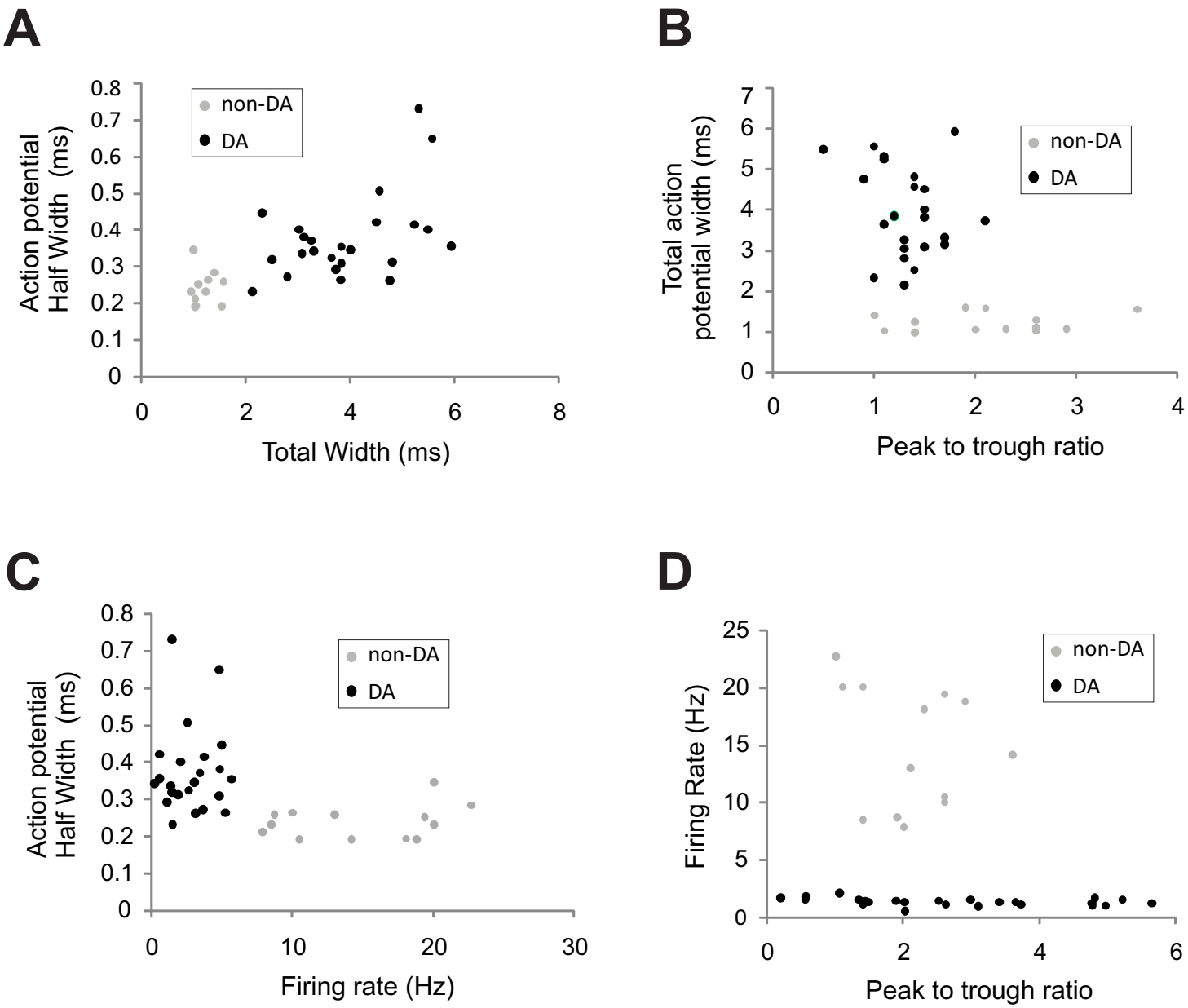
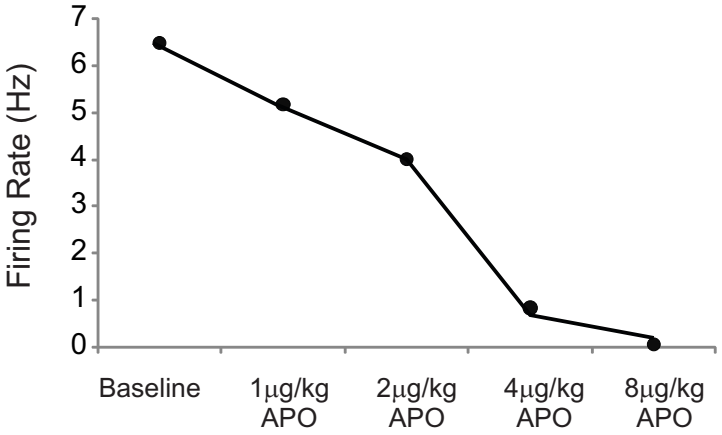

A
Action potential Half Width (ms)
non-DA
DA
Total Width (ms)
B
Total action potential width (ms)
non-DA
DA
Peak to trough ratio
C
Action potential Half Width (ms)
non-DA
DA
Firing rate (Hz)
D
Firing Rate (Hz)
non-DA
DA
Peak to trough ratio

Figure S4


Firing Rate (Hz)
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5
4
3
2
1
0
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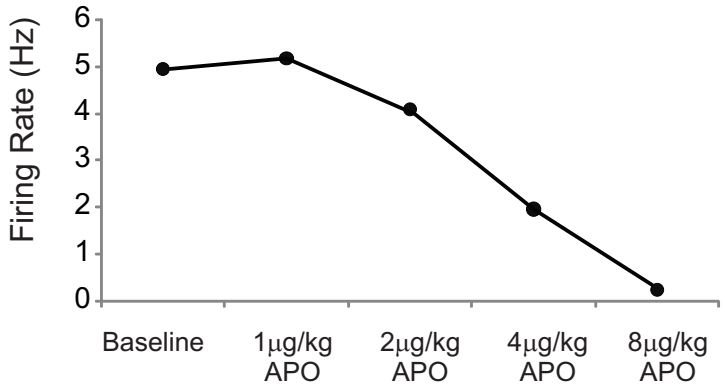
2μg/kg APO
4μg/kg APO
8μg/kg APO


Firing Rate (Hz)
6
5
4
3
2
1
0
Baseline
1μg/kg APO
2μg/kg APO
4μg/kg APO
8μg/kg APO