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Effects of social defeat on dopamine neurons in the ventral tegmental area in male and female California mice

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Abstract

Dopamine neurons in the ventral tegmental area (VTA) have important functions related to rewards but are also activated in aversive contexts. Electrophysiology studies suggest that the degree to which VTA dopamine neurons respond to noxious stimuli is topographically organized across the dorsal–ventral extent. We used c-fos immunohistochemistry to examine the responses of VTA dopamine neurons in contexts of social defeat and social approach. Studying monogamous California mice (*Peromyscus californicus)* allowed us to observe the effects of social defeat on both males and females. Females exposed to three episodes of defeat, but not a single episode, had more tyrosine hydroxylase (TH)/c-fos-positive cells in the ventral (but not dorsal) VTA compared with controls. This observation suggests that repeated exposure to aversive contexts is necessary to trigger activation of VTA dopamine neurons. Defeat did not affect TH/c-fos colocalizations in males. We also examined the long-term effects of defeat on c-fos expression in a social interaction test. As previously reported, defeat reduced social interaction in females but not males. Surprisingly, there were no effects of defeat stress on TH/c-fos colocalizations in any subregion of the VTA. However, females had more TH/c-fos-positive cells than males across the entire VTA, and also had greater c-fos-positive cell counts in posterior subregions of the nucleus accumbens shell. Our results show that dopamine neurons in the VTA are more responsive to social contexts in females and that the ventral VTA in particular is sensitive to aversive contexts.

Introduction

Dopamine neurons in the ventral tegmental area (VTA) are highly responsive to pleasurable contexts, including natural rewards and drugs of abuse (reviewed in Wise, 2008). Furthermore, the activity of VTA dopamine neurons is strongly influenced by cues for rewards. The difference between an expected reward and the actual reward has important effects on the direction and magnitude of activity in dopamine neurons. For example, VTA dopamine neurons will respond with burst firing following an unexpected reward or become inhibited when an expected reward is absent (Hollerman & Schultz, 1998). This has been hypothesized to play an important role in Pavlovian reward learning [i.e. prediction error; reviewed in Schultz (2013)] or to trigger internal motivational states [i.e. incentive salience; reviewed in Berridge (2012)]. Although the exact mechanism of action is debated, dopaminergic signaling is clearly an important modulator of goal-directed behaviour (reviewed in Pignatelli & Bonci, 2015). The function of VTA dopamine neurons has been studied primarily in the context of appetitive rewards. However, these neurons are also highly responsive to aversive contexts.

Dopamine release in the mesolimbic system is induced by aversive stimuli such as electric shocks and social stressors (reviewed in Miczek *et al.*, 2008; Trainor, 2011; Polter & Kauer, 2014). An important question is whether the same cells activated during appetitive contexts are also activated by aversive contexts or their cues. Electrophysiological recordings of VTA neurons in Rhesus monkeys showed that most VTA neurons were excited by cues for appetitive and aversive stimuli (Matsumoto & Hikosaka, 2009). However, electrophysiological criteria were used to identify dopamine neurons, which can sometimes lead to the misclassification of cells (Margolis *et al.*, 2010). A transgenic mouse study tagged either dopaminergic or GABAergic neurons with channelrhodopsin-2 so that they could be identified during optical stimulation (Cohen *et al.*, 2012). Most dopamine neurons were sensitive to cues predicting rewards (water to water-deprived mice) and <20% of dopamine neurons responded to cues predicting an aversive stimulus (an air puff to the face). However, air puffs are not particularly robust stimuli. About 25% of putative VTA dopamine neurons (as identified using electrophysiological criteria) responded to cues predicting a 30 cm free fall

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(Wang & Tsien, 2011). An alternative line of evidence shows that aversive contexts can induce long-lasting changes in the activity of VTA dopamine neurons. Social defeat stress induces hyperactivity in VTA dopamine neurons in multiple species of rodent, and has been observed by multiple research groups (Krishnan *et al.*, 2007; Anstrom *et al.*, 2009; Cao *et al.*, 2010). Indeed, optogenetic inhibition of VTA dopamine neurons projecting to the nucleus accumbens (NAc) in mice exposed to social defeat reversed stress-induced social withdrawal, but the inhibition of VTA neurons projecting to the prefrontal cortex (PFC) facilitated social withdrawal (Chaudhury *et al.*, 2013). It is unclear whether the effect of social defeat stress on VTA dopamine neural activity is topographically organized.

The anterior–posterior and medial–lateral divisions of the VTA have been studied extensively in reward-related and drug-related contexts (Sanchez-Catalan *et al.*, 2014). For example, overexpression of the GluR1 subunit of the AMPA receptor in the anterior VTA increased morphine reward (as measured by place preference), whereas GluR1 subunit of the AMPA receptor overexpression in the posterior VTA produced morphine-induced aversion (Carlezon *et al.*, 2000). Similarly, the effects of cyclic AMP response element-binding protein overexpression were anatomically specific, enhancing morphine place preferences in the anterior VTA but inducing aversion in the posterior VTA (Olson *et al.*, 2005). These functional differences may be related to differences in axonal projections (see, for review Ikemoto, 2007), as more posterior VTA dopamine neurons project to the NAc than anterior neurons (Swanson, 1982). Interestingly, the cyclic AMP response element-binding protein gene transfer experiments indicated that the posterior VTA can be altered to induce aversive states. Recent evidence indicates that there is topographical organization in the dorsal–ventral extent of the VTA. Anaesthetized rats exposed to footshocks showed selective activation of dopamine neurons in the ventral but not dorsal VTA (Brischoux *et al.*, 2009). In contrast, dopamine neurons in the dorsal VTA were inhibited by footshocks. So far, no study has tested whether other types of aversive contexts, such as social defeat, selectively activate VTA dopamine neurons in a topographically specific orientation.

We used c-fos immunohistochemistry to examine the responses of VTA dopamine neurons under both positive and negative social contexts. We studied the California mouse, a monogamous species in which both males and females defend territories (Ribble & Salvioni, 1990; Ribble & Millar, 1996). This social organization is unique and allows the study of males and females exposed to an equivalent intensity of defeat stress in a consistent ethological context (Trainor *et al.*, 2013). The long-term effects of defeat stress are sex-specific and generally consistent with the reactive and proactive coping strategies described by Koolhaas *et al.* (1999). In females, the long-term effects of social defeat are more consistent with reactive coping strategies such as social withdrawal (Greenberg *et al.*, 2014), reduced aggression (Steinman *et al.*, 2015) and behavioural flexibility (Laredo *et al.*, 2015). We examined tyrosine hydroxylase (TH)/c-fos cells in mice exposed to either one or three episodes of defeat stress, and recorded behavioural observations immediately before episodes of defeat to estimate the anticipation of repeated episodes of social stress. Finally, we examined the long-term effect of social defeat on both social interaction behaviour and TH/c-fos immunostaining. The social interaction test generates strong approach responses in mice naive to defeat (Trainor *et al.*, 2011). In this study we also examined c-fos in the NAc, PFC, and bed nucleus of the stria terminalis (BNST). The NAc and PFC receive strong dopaminergic projections from the VTA, whereas the BNST is an important node connecting the VTA with circuits controlling social behaviours (O'Connell & Hofmann, 2011). We previously found that social withdrawal in stressed females during the social interaction test is mediated by neurotrophin signalling and dopamine signalling in the BNST (Greenberg *et al.*, 2014) and NAc (Campi *et al.*, 2014), respectively. Together these studies form a unique examination of the effects of different social contexts on VTA dopamine neurons in both males and females.

Materials and methods

Animals

Adult male and female California mice (*Peromyscus californicus*) (3–6 months old) were bred in our laboratory colony and housed in same-sex groups of two to three per cage on Sani-Chips bedding with cotton nestlets in clear polypropylene cages. Mice were kept on a 16 h light/8 h dark cycle (lights on at 23:00 h) with Teklad 2016 food (Harlan, Hayward, CA, USA) and water provided *ad libitum*. All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Oestrous cycles were not monitored in experiments. Although the oestrous cycle can impact behaviour and neural function, a recent metanalysis of 293 studies showed that randomly cycling female mice were no more variable than males across a range of behavioural and neurobiological processes (Prendergast *et al.*, 2014). We have also demonstrated that defeat-induced social aversion is present across all stages of the oestrous cycle (Trainor *et al.*, 2011) and that ovariectomy has no effect on defeat-induced social aversion (Trainor *et al.*, 2013). The sample sizes for each experiment are listed in Table 1.

Behavioural testing and tissue collection

All behavioural testing was performed during lights out under dim red light (3 lux). For all experiments, mice were anaesthetized with isoflurane gas and killed via decapitation at 1 h following the final behavioural test. Brains were removed and fixed for 24 h in 5% acrolein (Sigma, St Louis, MO, USA) in 0.1 M phosphate-buffered saline (PBS) at 4 °C. Brains were then transferred to 20% sucrose in PBS for an additional 24 h before being frozen at −40 °C.

Behavioural testing

In experiment 1, mice were randomly assigned to a single episode of social stress or control conditions (Trainor *et al.*, 2013). Mice assigned to defeat were placed into the cage of an aggressive, same-sex resident for 7 min or until they received 10 bites from the resident, whichever occurred first. Control mice were placed in an empty cage for 7 min. At the end of this period each mouse was returned to its home cage. Mice were killed 1 h later, as described above.

TABLE 1. Sample sizes for all experiments

Study	Design	Male control	Male stress	Female control	Female stress
Experiment 1	Single defeat	4	6	4	6
Experiment 2	3 × defeat	8	9	7	15
Experiment 3	3 × defeat + social interaction	8	7	6	8

In experiment 2, mice were randomly assigned to undergo one episode of social defeat or control conditions per day for three consecutive days. After each episode of defeat or control conditions, each mouse was returned to its home cage and left undisturbed until the following day. On the third day, mice were killed at 1 h after the third episode as in experiment 1.

In experiment 3, mice were randomly assigned to social defeat or control conditions for three consecutive days. In this experiment we made additional behavioural observations immediately before each episode of defeat or control. Each cage of mice was transferred from the colony room to an adjacent behaviour-testing room. Within 1 min we started a 3 min video recording. This recording was then scored for indices of activity and anxiety-like behaviour using behavioural scoring software (Behavior Tracker 1.5, www.behaviortracker.com) by an observer without knowledge of treatment assignments. The frequencies of backflips, autogrooming, digging and rearing were recorded. The durations of autogrooming and digging were also scored. Immediately after this 3 min recording, focal mice were transferred to defeat or control conditions. After defeat or control conditions, focal mice were returned to their home cage and left undisturbed (apart from routine animal husbandry) until social interaction testing.

Two weeks following the third day of social defeat or control conditions all mice underwent social interaction testing as described previously (Trainor *et al.*, 2013; Greenberg *et al.*, 2014). Mice were first habituated to a large Plexiglas box (89 × 63 × 60 cm) after being placed in a neutral zone (the centre of the arena) from which they were free to move about. Each mouse spent 3 min in the arena during this open-field phase. A video-tracking system (Any-Maze, Stoelting, Wood Dale, IL, USA) recorded the total distance travelled and the durations that mice spent within 8 cm of the sides and within a centre zone located 14 cm from the sides. An empty wire cage was then placed against a wall of the arena for a second 3 min stage (acclimation phase). The distance travelled, time spent within 8 cm from the sides and front of the wire cage, and time spent in a corners zone opposite the cage zone were recorded. Finally, a same-sex novel 'target' mouse was placed into the wire cage, and the same variables were once again recorded. Mice were killed at 1 h after testing as in experiment 1.

Immunohistochemistry

A cryostat was used to collect 40-μm-thick coronal sections through the hypothalamus. Previous studies of the VTA have used both coronal and horizontal sections. Coronal sections have been used to examine rostral vs. caudal topography in anatomical (Carlezon *et al.*, 2000) and functional (Mahler & Aston-Jones, 2012) analyses. Horizontal sections provide an alternate approach for examining the VTA in a dorsal–ventral axis. Cell bodies in the VTA that express TH have a horizontal orientation, and horizontal sections allow for better visualization of these cells (Phillipson, 1979). Horizontal sectioning also facilitates discrimination of the VTA from the substantia nigra (SN) by having both the interpeduncular nucleus (IPN) and medial terminal nucleus of the accessory optic tract in the same section (Margolis *et al.*, 2006b; Margolis *et al.*, 2012). We used horizontal sections because we predicted that dorsal and ventral subregions of the VTA would have different responses to defeat stress.

The two primary antibodies used in our studies have been previously evaluated for specificity by pre-absorption with an immunizing peptide (Schiltz & Sawchenko, 2007) or immunoblotting (Haycock & Waymire, 1982). In addition, these antibodies produced immunostaining congruent with previously published reports for TH (Margolis *et al.*, 2010) and c-fos (Justice *et al.*, 2008). In a control experiment, omission of TH or c-fos primary antibody abolished positive staining for TH and c-fos, respectively. In a second pilot experiment, we ran both primary antibodies with respective secondary antibodies in striatal brain slices in which TH is selectively expressed in fibres. We only detected signal for TH (green staining) in fibres and signal for c-fos (red staining) in nuclei. This experiment indicates that the antibodies used for our experiments do not cross-react.

For all experiments, every third section (every 120 μm) containing the VTA and SN (40 μm thick) was processed for immunostaining, resulting in one 'ventral', one 'middle', and one 'dorsal' section (Fig. 1) (Margolis *et al.*, 2006a,b). Immunofluorescent procedures were used to facilitate the determination of colocalizations. Chromagenic immunostaining procedures have a greater ability to amplify signals, but are less optimal for determining colocalizations because separate images of proteins cannot be obtained. Sections were first washed three times for 5 min in PBS. Sections were then incubated for 10 min in 0.1 M sodium borohydride in PBS and then blocked in 10% normal donkey serum in PBS for 2 h. Following a 5 min wash in PBS, they were then incubated overnight at 4 °C in rabbit anti-c-fos (1 : 2500, catalogue no. PC38T, EMD Biosciences) in PBS with 0.5% Triton X (Tx) and 2% normal donkey serum. The next day, sections underwent three washes (5 min each) in PBS followed by incubation in Alexa Fluor 555-conjugated donkey anti-rabbit lgG (1 : 250, catalogue no. A31572, Life Technologies) for 2 h at 25 °C. Following three 5 min washes in PBS, sections were blocked in 10% normal rabbit serum in PBS for 2 h before being incubated for 48 h at 4 °C in sheep anti-TH (1 : 600, catalogue no. AB1542 Millipore) diluted in PBS with 0.5% Tx and 2% normal rabbit serum. After this incubation, sections were washed three times for 5 min each in PBS. Sections were then incubated for 2 h at room temperature with biotin-conjugated rabbit anti-sheep (1 : 500, catalogue no. BA-6000 Vector Laboratories) in PBS with 0.5% Tx and 2% normal rabbit serum for 2 h. After three 5 min washes in PBS, sections were incubated for 30 min in Alexa Fluor 488-conjugated streptavidin (1 : 250, S-11223, Life Technologies) diluted in PBS with 0.5% Tx. Sections underwent three last washes: first in PBS alone for 5 min, a second wash for 10 min in PBS, followed by a 30 min wash in PBS with 0.1% Tx and a final wash in PBS alone. Stained sections were then dipped in Nanopure water before being mounted onto Superfrost plus slides (Fisher, Pittsburgh, PA, USA) and coverslipped using Vectashield Mounting Medium (Vector Laboratories).

For experiment 3, coronal sections containing the PFC, NAc and BNST (40 μm thick) were collected on the cryostat prior to collection of horizontal sections for the VTA and SN. Every third section (every 120 μm) was processed for immunostaining. These sections were stained for c-fos alone using the methods previously described for experiments 1 and 2. For all immunostaining, both sides of the section were quantified to increase the accuracy of the counts.

Immunohistochemistry micrographs and quantification

Images were captured with a Zeiss Axioimager microscope (Carl Zeiss Meditec, Inc., Dublin, CA, USA) with a monochromatic Zeiss Axiocam (MRm). Axioimage software was used to take images with an exposure time of 4–10 ms. Quantification was performed using ImageJ software (NIH, Bethesda, MD, USA). Although it is possible that some cells may have been over-counted or under-counted, we took steps to ensure that inaccuracies in counts were not confounded

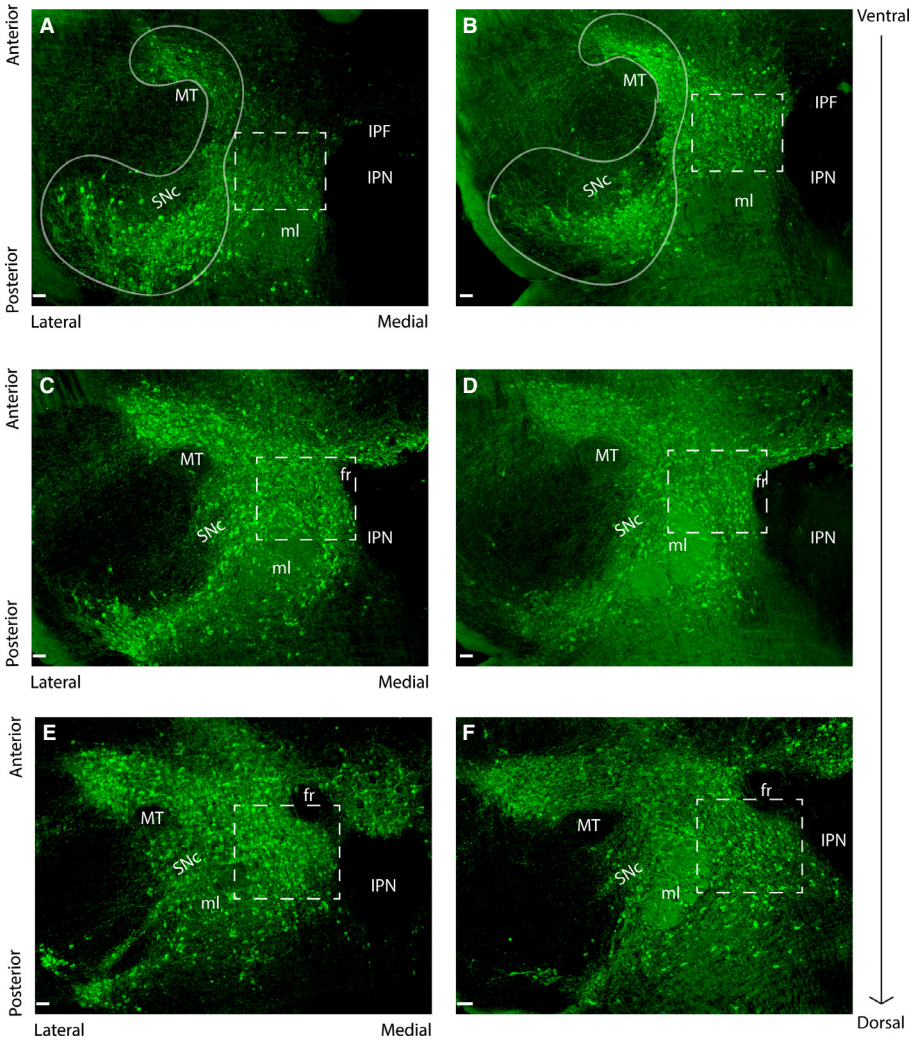

FIG. 1. Representative photomicrographs depicting regions of the VTA (dashed lines) and SNc (solid lines). Fluorescent staining is for TH in the ventral (A), middle (C) and dorsal (E) subdivisions of a representative control female. Stressed females had more TH-ir neurons in the ventral (B) and dorsal (F) VTA compared with control females. Stressed females did not differ from control females for the number of TH-positive cells in the middle VTA (D). Scale bars = 100 μm. fr, fasciculus retroflexus; IPF, interpeduncular fossa; ml, medial lemniscus; MT, medial terminal nucleus of the accessory optical tract.

with group differences. First, mice were randomly assigned to control or defeat conditions. Second, quantifications were performed by observers who had no knowledge of experimental conditions. Our data should be considered estimates of relative differences in TH and c-fos immunoreactivity between groups rather than estimates of actual cell numbers.

The subdivisions of the VTA have been increasingly refined for their functional specificity, cytoarchitecture, and heterogeneity of axonal projections (see, for review Ikemoto, 2007; Lammel *et al.*, 2014). In horizontal sections, the number of TH-immunoreactive (ir) neurons, c-fos-ir cells and TH/c-fos colocalizations were counted for the SN pars compacta (SNc) and VTA. The growing consensus is that the use of TH immunohistochemistry on horizontal sections is the best approach for identifying dopaminergic cells within the VTA (Ikemoto, 2007; Margolis *et al.*, 2010). We delineated boundaries between the SNc and VTA by combining methods using horizontal sectioning in rats (Margolis *et al.*, 2006a,b) and by use of tracer injections in the dorsal striatum that we have previously described (Campi *et al.*, 2013). Due to the presence of dopamine neurons in midline nuclei, such as the IPN and interfascicular nucleus, earlier studies incorporated these regions as parts of the VTA, but they have been more recently excluded from analyses due to anatomical differences (reviewed in Ikemoto, 2007). We did not include the midline nuclei in our VTA analyses, and we used the IPN as a medial boundary for the VTA, as previously described (Campi *et al.*, 2013). Cell counts were made from three sections spanning the ventral–dorsal extent of the VTA, and these three sections were termed 'ventral', 'middle', and 'dorsal' (Fig. 1; Margolis *et al.*, 2006a,b). This quantification area consisted of box sizes of 0.52 × 0.47 mm for the ventral VTA, 0.63 × 0.50 mm for the middle VTA, and 0.67 × 0.51 mm for the dorsal VTA. Boxes were placed between the lateral edge of the IPN and the dorsal edge of the medial lemniscus for the ventral and middle sections. Boxes were placed between the lateral edge of the IPN and the medial edge of the medial lemniscus for the dorsal sections. Boxes were aligned with the anterior edge of the VTA, which has been shown by previous electrophysiological studies to be more responsive to aversive contexts (Brischoux *et al.*, 2009). The box sizes and their placement across all three sections allowed for quantification of VTA neurons and avoided counts of 'wrapping' TH-positive neurons around the medial terminal nucleus of the accessory optic tract, as described in Campi

et al. (2013) and illustrated in Fig. 1. These neurons were previously determined to be part of the SNc by their configuration and from cell labelling in this area by retrograde tracers infused into the dorsal striatum.

In experiment 3, every third coronal section was used to quantify c-fos cell counts in the PFC, NAc and BNST. In the PFC a box (0.5 × 0.67 mm) was aligned just lateral to the corpus callosum beginning at approximately Bregma 1.70 mm for the prelimbic cortex. Images of the infralimbic cortex were taken on the same sections just dorsal to the prelimbic cortex with a box of the same size. Subregions of the NAc and BNST were quantified using previous descriptions of California mouse NAc and BNST (Trainor *et al.*, 2011; Campi *et al.*, 2013; Greenberg *et al.*, 2014). For the NAc core, a box (0.5 × 0.67 mm) was placed medial to the anterior commissure (AC) and ventral to the lateral ventricle. A box of identical size was placed directly medial and ventral to this NAc core box to quantify the NAc shell. Anterior (Bregma 1.23 mm) and posterior (Bregma 0.51 mm) subregions of the NAc were quantified separately based on previous reports that glutamate signalling in these subregions had opposing effects on motivation valence (Richard & Berridge, 2011). For the anterolateral BNST, a box (0.5 × 0.67 mm) was placed adjacent to the dorsal edge of the AC and ventral to the lateral ventricle beginning at approximately Bregma 0.45 mm. For the anteromedial BNST, a box was placed dorsal to the AC and directly medial to the box for the anterolateral BNST. For the ventrolateral BNST and ventromedial BNST, quantification areas were placed on the ventral edge of the AC and aligned medial–laterally on the same section with the anterolateral BNST and anteromedial BNST, respectively. Identical quantification areas from middle BNST sections were positioned similarly but posterior to anterior BNST regions. For the posterior dorsal division of the BNST, a box (0.45 × 0.37 mm) was placed just lateral to the fornix and dorsal to the AC at approximately Bregma 0.10 mm. For the posterior lateral division of the BNST, a box (0.59 × 0.29 mm) was placed just lateral to the AC at approximately Bregma 0.10 mm.

Statistical analysis

Normality for each variable was assessed using Q–Q plots and histograms. Levene's test was used to confirm homogeneity of variance. Two-way ANOVA was used to test for effects of sex and social defeat stress on behaviour and cell counts. All significant sex*treatment interactions were followed up with planned comparisons to test for effects of treatment within sex. When criteria for normality and homogeneity were not met, data were corrected with log transformations. The autogrooming data and c-fos cell count data in experiment 3 were positively skewed, and log transformations were used to normalize the distributions of these variables for ANOVA. Colocalization data (TH/c-fos) from all three experiments had substantial positive skew that could not be normalized by transformations, so the raw data were analysed with non-parametric Mann–Whitney *U*-tests. For experiment 3, repeated-measures three-way ANOVA, using day of testing as a within-subjects factor, was used to test for effects of sex and stress on behaviour immediately prior to episodes of social defeat or control conditions. Follow-up paired *t*-tests were used to compare individual time points. In order to test connectivity between regions, non-parametric Spearman correlations were used correlate colocalizations in the VTA with c-fos counts in other forebrain areas. False discovery rate adjustments were applied to *P*-values obtained from forebrain ANOVA analyses and correlational analyses. Untransformed means and SE are presented in all figures and tables.

Results

Experiments 1 and 2: effects of social defeat on dopamine circuitry immediately following acute and chronic social defeat stress

There were no differences in VTA TH cell number or TH/c-fos colocalizations in experiment 1 in which mice were exposed to one episode of defeat or control conditions (Table 2). In contrast, in experiment 2, in which three episodes of social defeat or control conditions were used, there was a sex*stress interaction on the total number of TH-ir cells ($F_{1,35}$ = 4.06, P = 0.05) in ventral VTA sections. Stressed females (Fig. 1B) had more TH-ir cells than control females (Figs 1A and 3A) ($F_{1,35}$ = 6.37, P = 0.02) and there was no difference in males. In ventral VTA sections, stressed females had significantly more TH/c-fos colocalizations than control females (Figs 2 and 3B) (Mann–Whitney U = 21.00, P = 0.03). There were no differences in total c-fos-ir cells in the ventral VTA (Table 2). In middle sections of the VTA, there were no significant effects of sex or stress on total TH neurons or TH/c-fos colocalizations (Figs 1C and D, and 3C and D). There was a sex*stress interaction for total c-fos-ir cells ($F_{1,34}$ = 5.86, P = 0.02). In the middle VTA, planned

TABLE 2. Mean ± SE cell counts from mice undergoing a single episode of defeat or control conditions

Region and count	Male control	Male stress	Female control	Female stress
Ventral SN TH	112.0 ± 10.0	139.7 ± 17.2	173.0 ± 2.0	134.5 ± 12.2
Middle SN TH	123.0 ± 51.0	174.0 ± 31.5	230.5 ± 7.5	155.3 ± 11.4
Dorsal SN TH	157.5 ± 26.5	172.0 ± 10.1	170.5 ± 14.5	154.8 ± 7.2
Ventral SN c-fos	12.5 ± 2.5	13.3 ± 2.2	18.0 ± 6.0	17.3 ± 2.3
Middle SN c-fos	13.0 ± 7.0	8.0 ± 2.5	13.5 ± 4.5	10.3 ± 1.8
Dorsal SN c-fos	13.5 ± 6.5	7.0 ± 3.1	11.5 ± 2.5	10.2 ± 2.0
Ventral SN TH/c-fos	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	1.3 ± 0.6
Middle SN TH/c-fos	0.5 ± 0.5	1.0 ± 0.6	0.0 ± 0.0	0.3 ± 0.2
Dorsal SN TH/c-fos	1.0 ± 1.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
Ventral VTA TH	74.0 ± 13.1	79.2 ± 5.3	69.3 ± 6.9	54.5 ± 7.5
Middle VTA TH	101.0 ± 20.9	116.4 ± 9.7	125.0 ± 19.5	97.2 ± 15.8
Dorsal VTA TH	77.0 ± 13.0	70.2 ± 6.0	73.2 ± 9.2	48.0 ± 8.2
Ventral VTA c-fos	17.0 ± 6.2	29.2 ± 7.5	24.3 ± 15.8	17.3 ± 6.0
Middle VTA c-fos	23.3 ± 4.4	18.0 ± 5.4	17.3 ± 10.7	31.6 ± 12.2
Dorsal VTA c-fos	22.3 ± 9.4	22.8 ± 6.0	23.5 ± 15.0	21.0 ± 10.5
Ventral VTA TH/c-fos	8.0 ± 3.8	6.5 ± 2.3	2.8 ± 1.0	5.2 ± 1.9
Middle VTA TH/c-fos	7.5 ± 3.4	8.2 ± 2.2	4.0 ± 2.5	6.8 ± 2.5
Dorsal VTA TH/c-fos	4.0 ± 3.4	3.6 ± 2.2	9.7 ± 3.3	9.6 ± 2.9

Data are shown through the ventral–dorsal extent of the VTA and SNc.

comparisons showed that stressed females had fewer c-fos-ir cells than control females ($F_{1,34}$ = 11.87, P = 0.002) and there was no effect of stress in males (Table 2). In the dorsal VTA, there was a sex*stress interaction (Fig. 3E) ($F_{1,34}$ = 3.98, P = 0.05) on TH-ir neurons. Planned comparisons indicated a trend for stressed females (Fig. 1F) to have significantly more TH-ir cells than control females (Fig. 1E) ($F_{1,34}$ = 3.96, P = 0.06) and there was no difference between control and stressed males. There were no significant effects of sex or stress on TH/c-fos colocalizations (Fig. 3F) or total c-fos counts in dorsal VTA sections.

In the SNc, there were no effects of sex or stress on total dopamine neurons, TH/c-fos colocalizations or total c-fos cells within ventral, middle or dorsal sections following three episodes of social defeat stress or control conditions (Table 3). Following a single episode of social defeat stress, there were no effects of sex or stress on SN TH neurons, TH/c-fos colocalizations, or total c-fos cell counts in the ventral, middle, or dorsal sections (Table 3).

Experiment 3: long-term effects of social defeat on mesolimbic circuits

Immediately prior to social defeat episodes or control conditions, the duration of autogrooming increased over 3 days in stressed mice but not controls (repeated-measures ANOVA, $F_{2,50}$ = 6.66, P < 0.01), and there were no sex differences (P-values > 0.5). The duration of autogrooming increased on day 3 compared with day 1 in stressed mice (Fig. 4A) (paired t_{14} = 4.812, P < 0.001) but not controls. The effect of stress on rearing was different in males and females across

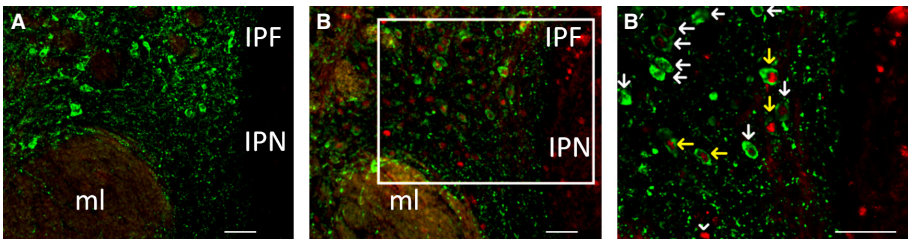

FIG. 2. Representative images of c-fos (fluorescent red staining) and TH (fluorescent green staining) expression in a control female (A) and stressed female mouse (B) following 3 consecutive days of treatment. Scale bars = 100 μm. IPF, interpeduncular fossa; ml, medial lemniscus. (B′) Magnified view of area enclosed in the white box in B. Scale bar = 100 μm. Yellow arrows designate c-fos-ir nuclei within TH-ir cell bodies, white arrows designate cells immunoreactive for TH alone, and white arrowhead designates cells immunoreactive for c-fos only.

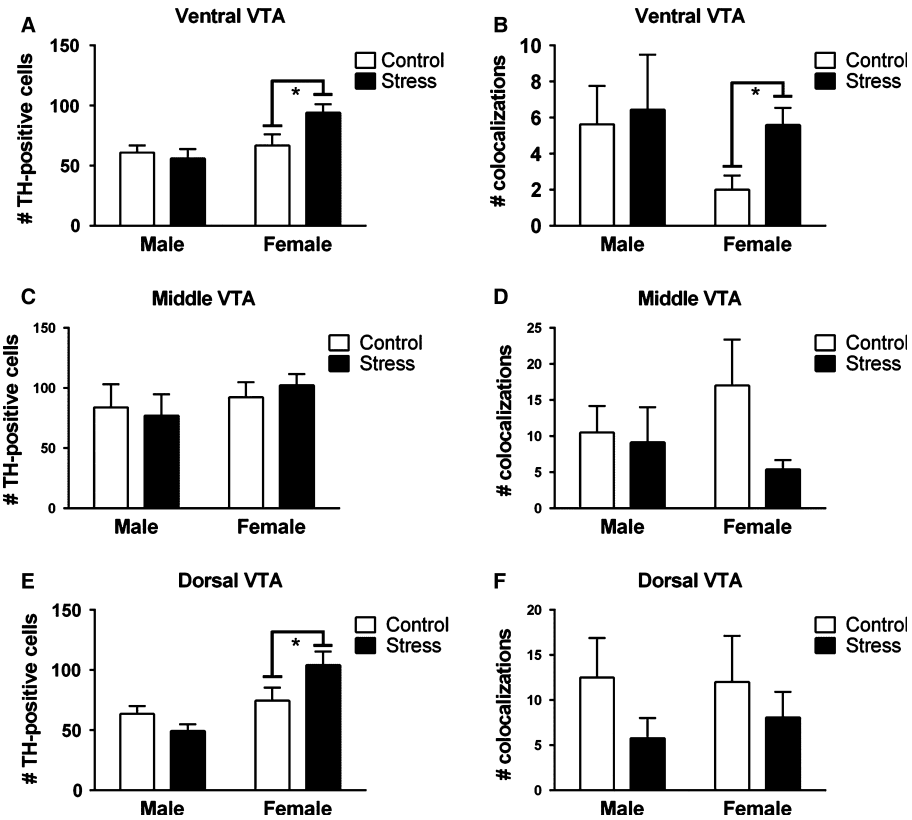

FIG. 3. Cell count data following three episodes of social defeat or control conditions (n = 7–15 per group). Females, but not males, had more TH-ir neurons (A) and more TH/c-fos colocalizations (B) in the ventral VTA after stress. There were no effects of stress on TH-positive neurons (C) or TH/c-fos colocalizations (D) in the middle sections of the VTA. Females, but not males, had more TH-ir neurons in the dorsal VTA after stress (E). There were no effects of stress on TH/c-fos colocalizations (F) in dorsal sections of the VTA. Graphs display mean + SE. *P < 0.05 effect of stress.

TABLE 3. Mean ± SE cell counts from mice undergoing three episodes of defeat or control conditions

Region and count	Male control	Male stress	Female control	Female stress
Ventral SN TH	142.6 ± 12.6	130.1 ± 15.4	155.3 ± 35.0	174.7 ± 23.8
Middle SN TH	165.7 ± 25.3	163.4 ± 27.1	172.5 ± 28.8	177.1 ± 23.0
Dorsal SN TH	133.0 ± 27.9	151.4 ± 29.6	139.0 ± 22.7	134.0 ± 16.4
Ventral SN c-fos	10.3 ± 2.8	13.0 ± 4.5	7.0 ± 2.0	9.0 ± 1.7
Middle SN c-fos	13.0 ± 2.2	9.1 ± 2.8	13.3 ± 4.6	8.6 ± 1.9
Dorsal SN c-fos	6.6 ± 2.4	6.6 ± 2.1	7.5 ± 1.9	6.3 ± 1.9
Ventral SN TH/c-fos	1.00 ± 0.5	1.8 ± 0.6	2.8 ± 1.4	2.1 ± 0.9
Middle SN TH/c-fos	0.8 ± 0.5	0.7 ± 0.2	2.5 ± 1.0	1.0 ± 0.5
Dorsal SN TH/c-fos	1.4 ± 1.3	2.0 ± 0.9	2.0 ± 1.1	0.9 ± 0.7
Ventral VTA TH	60.9 ± 6.0	56.2 ± 7.7	66.9 ± 9.3	94.2 ± 6.9*
Middle VTA TH	83.9 ± 19.2	77.1 ± 17.9	92.4 ± 12.4	102.5 ± 9.2
Dorsal VTA TH	63.5 ± 6.5	49.3 ± 5.7	74.7 ± 10.7	104.1 ± 11.2†
Ventral VTA c-fos	9.6 ± 2.9	11.3 ± 2.9	8.0 ± 4.6	8.7 ± 1.4
Middle VTA c-fos	21.1 ± 4.2	21.9 ± 5.6	27.0 ± 6.1	8.3 ± 1.5*
Dorsal VTA c-fos	19.6 ± 5.7	31.0 ± 8.3	23.9 ± 9.3	11.1 ± 4.2
Ventral VTA TH/c-fos	5.6 ± 2.1	6.4 ± 3.1	2.0 ± 0.8	5.6 ± 1.0*
Middle VTA TH/c-fos	10.5 ± 3.7	9.1 ± 4.9	17.0 ± 6.4	5.4 ± 1.3
Dorsal VTA TH/c-fos	12.5 ± 4.4	5.8 ± 2.3	12.0 ± 5.1	8.1 ± 2.9

Data are shown through the ventral–dorsal extent of the VTA and SNc.
*$P < 0.05$ vs. female control.
†$P = 0.06$ vs. female control.

the 3 day testing period (repeated-measures ANOVA, $F_{2,50} = 3.39$, $P = 0.04$). Rearing decreased on day 3 compared with day 1 in females (paired $t_{13} = 2.83$, $P = 0.01$) but not males. There were no differences in number of backflips, number of digs, or digging duration (Table 4).

Two weeks later, during the social interaction test, there was a significant sex*stress interaction for time spent in the cage zone when a target mouse was present (Fig. 4C) ($F_{1,26} = 6.31$, $P = 0.02$). Planned comparisons showed that stressed female mice spent significantly less time in the interaction zone than control females ($F_{1,26} = 17.87$, $P < 0.001$), but this difference was not present in male mice. There were no significant effects of sex or stress on time in the cage (Fig. 4D) or corners zones during the acclimation (no target) phase. There were also no significant effects of sex or stress on distance travelled during the test, time in the centre during the open-field phase or time in the corners zone during the interaction phase (Table 4).

Following the social interaction test, there were no effects of sex or stress on SN or VTA total TH-ir neurons, or total c-fos-ir cell counts in the ventral, middle, or dorsal sections (Table 5). However, there were significantly more TH/c-fos colocalizations in the middle VTA of female mice (Fig. 5B) than male mice (Figs 5A and 6B) (Mann–Whitney $U = 149.0$, $P = 0.017$) and similar non-significant trends in the ventral VTA (Fig. 6A) (Mann–Whitney $U = 138.0$, $P = 0.07$) and dorsal VTA (Fig. 6C) (Mann–Whitney $U = 138.0$, $P = 0.07$). If all three subregions are combined, females had significantly more TH/c-fos colocalizations than males (Mann–Whitney $U = 160.5$, $P < 0.01$). There were also no effects of sex or stress on total c-fos cell counts in the posterior dorsal division of the BNST, posterior lateral division of the BNST, infralimbic cortex, prelimbic cortex, NAc core, or anterior NAc shell (Table 5). In anterior BNST subregions, average c-fos cell counts were higher in females than males but this difference was not significant (main effects of sex P-values 0.14–0.17). Female mice showed more c-fos counts in the posterior NAc shell (Fig. 5C and D) ($F_{1,24} = 10.15$, $P < 0.01$, false discovery rate corrected $P = 0.06$) than males. In correlational analyses of cell count data, a near-significant, positive correlation was detected between ventral VTA colocalizations and posterior NAc shell ($\rho = 0.702$, $P < 0.001$, false discovery rate corrected $P = 0.06$), and this correlation was stronger in females ($\rho = 0.707$, $P = 0.01$) than males ($\rho = 0.513$, $P = 0.06$), with the strongest correlation in stressed females when broken down by sex and treatment ($\rho = 0.880$, $P = 0.020$).

Discussion

Here, we tested for dorsal–ventral topographical specificity of VTA dopamine neuron responses to aversive contexts by examining c-fos and TH expression immediately following a single episode or three consecutive days of social defeat stress. We also examined whether effects of social defeat were long-lasting and extended to a non-agonistic social context at 2 weeks after the final day of chronic social defeat stress. We demonstrated that a third episode of defeat stress selectively increased c-fos expression in dopamine neurons in the ventral VTA of females. This suggests that there is dorsal–ventral organization in the responsiveness of VTA dopamine neurons to aversive contexts. This was previously observed in anaesthetized rats exposed to electric shocks (Brischoux *et al.*, 2009), and we now extend this finding to social defeat in awake female California mice. Furthermore, no increases in TH/c-fos colocalizations were observed in any subregion of the adjacent SNc, which indicates that this neural response is highly localized to the ventral VTA. Intriguingly, patterns of TH/c-fos expression were very different following social interaction tests. Although no effect of defeat stress on TH/c-fos staining was observed in the VTA, females had more than twice as many TH/c-fos-stained neurons across the entire VTA compared with males. In experiments 1 and 2, there were no sex differences in TH/c-fos cell counts in control animals, which were not exposed to social stimuli. This indicates that the sex difference in TH/c-fos colocalizations observed in the social context of experiment 3 is not simply a baseline sex difference in TH/c-fos staining. This difference probably reflects the increased responsiveness of VTA dopamine neurons to social contexts in females vs. males.

Short-term effects of defeat stress in the ventral ventral tegmental area

To our knowledge, this is the first report of dorsal–ventral organization in the responsiveness of VTA dopamine neurons to a stressor

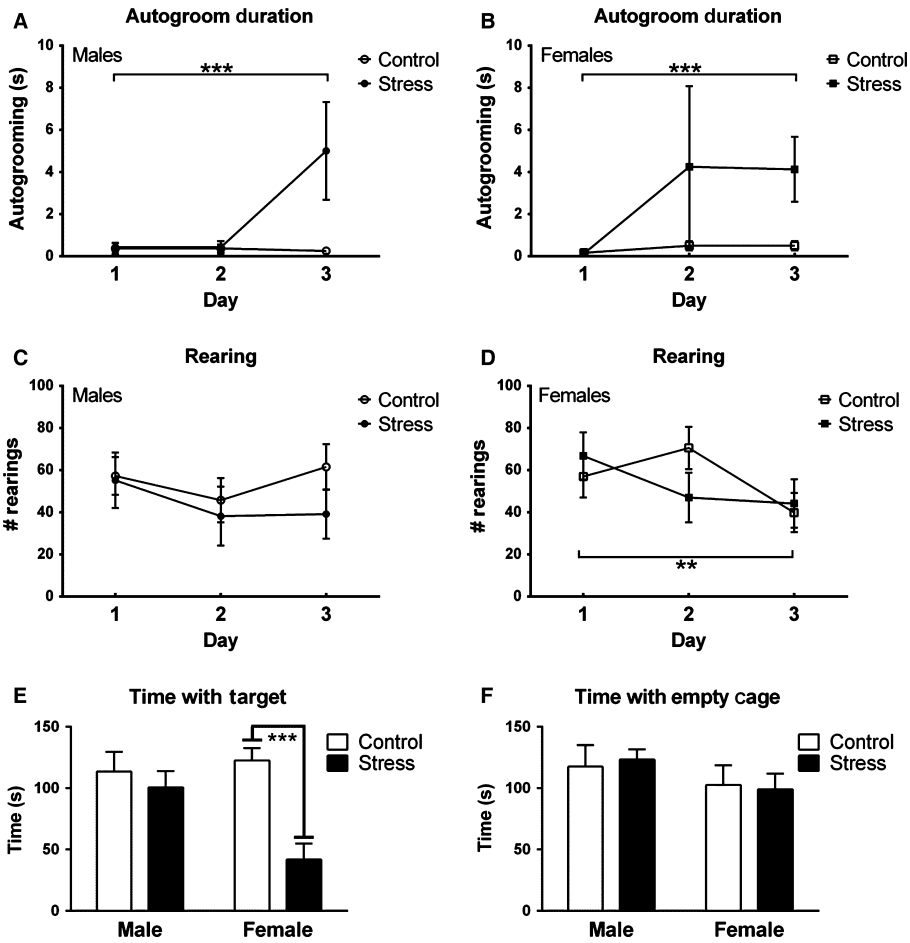

FIG. 4. Autogrooming increased in male and female mice from day 1 to day 3 during three consecutive days prior to social defeat (A and B) (n = 7–8 per group). Rearing decreased from day 1 to day 3 in females (D) but not males (C). Female mice that had been exposed to defeat stress 2 weeks prior to social interaction tests spent significantly less time in the cage zone when a target mouse was present compared with control females (E). There were no differences in time spent in the cage zone when the cage was empty (F). Graphs display mean ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ effect of stress.

TABLE 4. Behavioural data from experiment 3

Behaviour	Male control	Male stress	Female control	Female stress
Before social defeat				
Backflips day 1	18.9 ± 8.8	28.3 ± 14.1	2.8 ± 1.3	5.5 ± 4.4
Backflips day 2	32.8 ± 19.6	51.0 ± 21.8	19.3 ± 11.1	19.9 ± 13.3
Backflips day 3	17.9 ± 11.8	34.9 ± 14.9	15.7 ± 8.3	22.4 ± 12.6
Digs day 1	1.3 ± 0.7	2.1 ± 1.4	1.8 ± 0.8	1.0 ± 0.7
Digs day 2	1.4 ± 0.7	1.0 ± 0.7	0.8 ± 0.5	1.3 ± 0.9
Digs day 3	1.0 ± 0.8	0.7 ± 0.3	1.2 ± 0.6	1.5 ± 0.8
Digging duration day 1 (s)	2.5 ± 1.7	1.6 ± 0.9	1.8 ± 1.1	0.9 ± 0.5
Digging duration day 2 (s)	1.3 ± 0.7	0.4 ± 0.3	0.0 ± 0.2	0.8 ± 0.4
Digging duration day 3 (s)	0.9 ± 0.7	0.7 ± 0.3	0.5 ± 0.2	7.6 ± 7.3
Social interaction test				
Open-field centre time (s)	41.9 ± 11.8	34.1 ± 3.1	29.8 ± 4.6	34.8 ± 4.3
Open-field distance (m)	28.5 ± 3.0	23.5 ± 3.6	25.1 ± 3.1	24.6 ± 2.9
Time in cage zone (no target, s)	117.6 ± 17.5	123.4 ± 8.3	102.6 ± 16.0	99.2 ± 12.8
Time in cage zone (target mouse, s)	113.6 ± 16.1	100.6 ± 13.2	122.6 ± 10.0	41.8 ± 13.1***

$^{***}P < 0.001$ vs. corresponding control.

in a non-anaesthetized rodent. Although the overall number of TH/c-fos neurons in stressed females was low (11 or less), the location of this increase is consistent with a previous study on anaesthetized animals. Brischoux *et al.* (2009) demonstrated that the majority of recorded dopamine neurons were inhibited or showed no response following repeated footshocks administered to an anaesthetized male rat. However, a small number of dopaminergic neurons (5/14 neurons) had increased firing rates, and these neurons were confined to anterior medioventral parts of the VTA. In non-anaesthetized rabbits, neurons activated by an acoustic stimulus paired with an electric

TABLE 5. Mean ± SE cell counts from mice in experiment 3

Region and count	Male control	Male stress	Female control	Female stress
Ventral SN TH	100.1 ± 17.1	116.6 ± 19.6	123.4 ± 10.8	138.3 ± 16.0
Middle SN TH	134.3 ± 8.5	114.0 ± 19.6	128.6 ± 18.4	144.3 ± 11.6
Dorsal SN TH	143.9 ± 8.9	130.9 ± 16.8	135.9 ± 13.8	155.5 ± 14.6
Ventral SN c- fos	5.6 ± 1.4	3.7 ± 1.1	8.9 ± 2.4	7.2 ± 1.3
Middle SN c-fos	4.9 ± 1.0	5.1 ± 2.1	8.7 ± 3.0	7.2 ± 1.9
Dorsal SN c-fos	3.0 ± 1.0	5.9 ± 2.1	7.1 ± 2.3	7.7 ± 1.5
Ventral SN TH/c-fos	0.4 ± 0.2	0.1 ± 0.1	0.6 ± 0.4	0.8 ± 0.3
Middle SN TH/c-fos	0.8 ± 0.3	0.1 ± 0.1	0.7 ± 0.4	0.7 ± 0.3
Dorsal SN TH/c-fos	0.3 ± 0.2	0.7 ± 0.4	0.3 ± 0.2	0.5 ± 0.3
Ventral VTA TH	103.6 ± 6.4	100.4 ± 11.7	95.9 ± 8.1	120.2 ± 15.1
Middle VTA TH	120.0 ± 11.4	105.7 ± 8.1	107.6 ± 16.8	126.7 ± 10.70
Dorsal VTA TH	106.6 ± 5.1	98.9 ± 8.1	108.6 ± 9.3	129.2 ± 12.8
Ventral VTA c-fos	11.4 ± 2.2	13.6 ± 4.7	34.9 ± 11.1	19.3 ± 5.2
Middle VTA c-fos	10.5 ± 3.6	9.6 ± 4.4	31.9 ± 8.6	23.2 ± 4.9
Dorsal VTA c-fos	12.8 ± 5.3	14.3 ± 5.9	26.3 ± 5.3	24.3 ± 5.0
Ventral VTA TH/c-fos	2.1 ± 0.9	1.0 ± 0.3	4.00 ± 1.2†	3.83 ± 2.0†
Middle VTA TH/c-fos	0.9 ± 0.3	1.0 ± 0.5	3.6 ± 1.2*	5.5 ± 3.3*
Dorsal VTA TH/c-fos	2.4 ± 2.0	1.9 ± 0.8	3.0 ± 1.1†	5.5 ± 1.8†
infralimbic cortex c-fos	108.7 ± 27.2	104.3 ± 29.4	88.4 ± 14.8	93.4 ± 15.0
Prelimbic cortex c-fos	81.7 ± 28.3	46.6 ± 8.7	52.9 ± 11.1	59.8 ± 36.4
Anterior NAcC c-fos	259.6 ± 101.2	263.4 ± 100.6	210.7 ± 54.6	233.6 ± 47.4
Anterior NAcSh c-fos	234.7 ± 98.9	123.9 ± 30.7	139.3 ± 30.4	144.8 ± 38.1
Posterior NAcC c-fos	104.7 ± 23.5	85.0 ± 16.9	123.0 ± 22.2	134.5 ± 24.4
Posterior NAcSh c-fos	60.4 ± 8.4	60.7 ± 8.7	112.3 ± 18.0†	112.0 ± 22.3†
Anterolateral BNST c-fos	216.6 ± 65.0	226.3 ± 49.9	327.8 ± 95.9	333.5 ± 72.1
Anteromedial BNST c-fos	236.4 ± 67.5	227.4 ± 50.7	339.8 ± 100.0	339.4 ± 71.4
Ventrolateral BNST c-fos	161.3 ± 62.0	186.1 ± 49.1	273.7 ± 79.1	244.8 ± 54.7
Ventromedial BNST c-fos	222.1 ± 68.0	196.9 ± 40.4	308.2 ± 87.8	309.2 ± 62.9
Posterior dorsal division of the BNST c-fos	3.7 ± 1.1	6.8 ± 2.0	5.0 ± 1.5	5.9 ± 1.3
Posterior lateral division of the BNST c-fos	5.0 ± 1.3	11.7 ± 1.9	12.1 ± 3.4	13.9 ± 2.3

*$P < 0.05$ vs. corresponding male group.
†$P = 0.06$ vs. corresponding male group.

shock were mostly confined to the ventral VTA (i.e. ventral to the apex of the fossa), and neurons that showed decreased activity to the conditioned stimulus were more dorsal (Guarraci & Kapp, 1999). In this study, however, it was not clear whether these cells responding to conditioned stimuli were dopamine neurons.

In addition to the heterogeneity of VTA subpopulations that have been found to respond to aversive contexts, several studies have also reported the number of dopaminergic neurons responding to aversive contexts to be highly variable. Previous studies examining the impact of aversive contexts on dopamine neurons have examined many types of aversive stimuli and have observed variable results (Marinelli & McCutcheon, 2014). In a TH/c-fos double-label study similar to our study, 30 min of restraint stress increased the number of TH-positive neurons that displayed fos-like immunoreactivity in the VTA by sevenfold compared with controls when brains were collected at 4.5 h after restraint (Deutch *et al.*, 1991). However, when brains were collected at 2.25 h after restraint, there was no difference from controls. Similarly, a single electric shock inhibited the activity of putative dopamine neurons in anaesthetized rats but repeated footshocks generated increased activity in a population of putative dopamine neurons in the medial VTA (Valenti *et al.*, 2011). A recent study used *in vivo* calcium imaging to visualize the activity of VTA dopamine neurons during fear conditioning in freely moving mice. Calcium transients were present following the conditioned stimulus on day 2 of conditioning in a small percentage of dopaminergic neurons that were not present following the conditioned stimulus during the first day of conditioning, suggesting that fear learning induces plasticity with experience (Gore *et al.*, 2014). A common thread is that, when VTA dopamine neurons respond to noxious contexts with increased activity, it is usually after repeated exposure. Similarly, previous studies reporting defeat-induced increases in dopamine content (Mos & Van Valkenburg, 1979; Puglisi-Allegra & Cabib, 1990) or dopamine release (Tidey & Miczek, 1996) in the NAc observed these effects after multiple episodes of social defeat. In rats, about 50% of dopamine neurons in the ventral VTA project to the NAc shell (Melis *et al.*, 2007). Overall, it appears that the duration of the stressor, and also its predictability, are important factors determining the responsiveness of VTA dopamine neurons.

In male rodents, defeat-induced social withdrawal is modulated by the excitability of VTA dopamine neurons (Chaudhury *et al.*, 2013), and these changes in neurophysiology are present at 4 weeks post-stress (Krishnan *et al.*, 2008; Razzoli *et al.*, 2011). Defeat stress also increased dopamine and homovanillic acid content in the NAc of both male and female California mice at 2 weeks following defeat (Campi *et al.*, 2014). However, it appears that c-fos immunohistochemistry may not be an optimal technique for detecting these forms of long-lasting changes. Optical inhibition of VTA dopamine neurons projecting to the NAc attenuated defeat-induced social withdrawal in male mice (Chaudhury *et al.*, 2013), indicating that hyperactivity of VTA dopamine neurons plays an important role in mediating social withdrawal. Optical activation of VTA dopamine neurons has been reported to reduce behavioural despair and anhedonia in male mice exposed to chronic mild stress (Tye *et al.*, 2013). At first glance, the two optogenetic studies appear to produce contradictory results. However, social defeat stress induces chronic hyperactivity in VTA dopamine neurons, whereas chronic mild stress reduces the activity of VTA dopamine neurons (Chang & Grace, 2014). In both studies, manipulations that minimized the impact of psychosocial stress on VTA dopamine neurons had antidepressant effects. Thus, although different forms of stress may

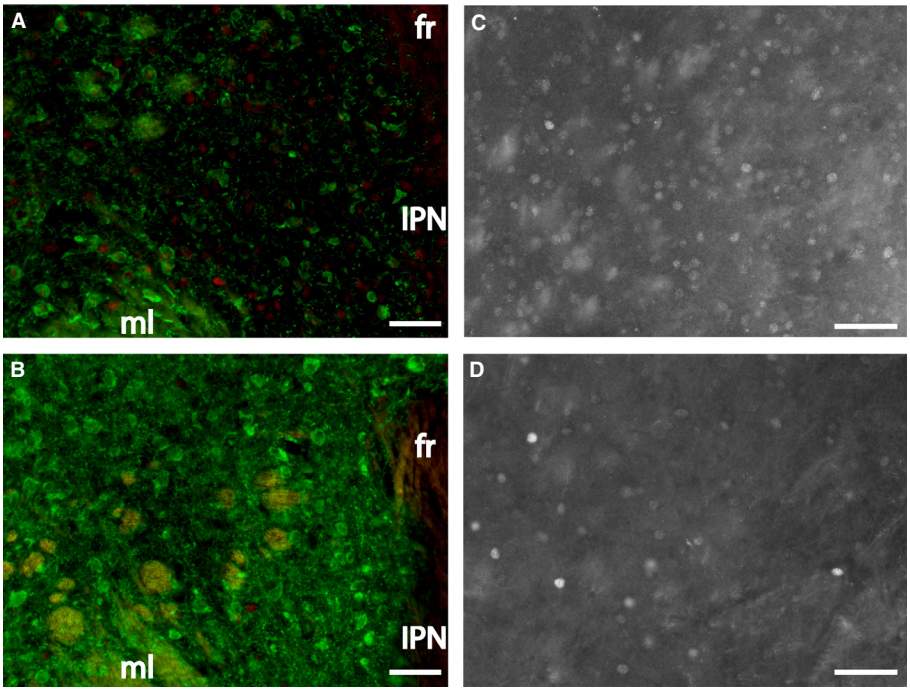

FIG. 5. Representative photomicrographs of TH (green)/c-fos (red) immunostaining in the middle sections of the VTA in control female (A) and male (B) mice following the social interaction test. fr, fasciculus retroflexus; ml, medial lemniscus. Representative photomicrographs of fos staining in the same order by sex are shown for the posterior NAc shell (C and D). There was a significant sex difference, with females having higher counts than males, in all of these regions. Scale bars = 100 μm for all images.

FIG. 6. Cell count data following social interaction with three episodes of social defeat or control conditions 2 weeks previously (n = 6–8 per group). There were no effects of stress or sex on TH/c-fos colocalizations in ventral VTA sections (A). Females had significantly more TH/c-fos colocalizations in middle VTA sections than males (B). There were no effects of stress or sex on TH/c-fos colocalizations in dorsal VTA sections (C). Graphs display mean + SE. *P < 0.05 sex difference; +non-significant trend for sex difference (P = 0.07).

have different effects on VTA dopamine neurons, interventions that restore normal activity appear to have therapeutic effects.

In addition to dorsal–ventral topographic organization, tracing studies have found medial–lateral organization of VTA dopamine neuron populations in relation to their projections to medial and lateral subdivisions of the NAc shell, NAc core, and PFC (Lammel *et al.*, 2008). An aversive hindpaw formalin injection increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid : *N*-Methyl-D-aspartic acid receptor ratios in PFC-projecting and NAc-projecting medial and lateral VTA dopamine neurons, respectively (Lammel *et al.*, 2011). The increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid : *N*-Methyl-D-aspartic acid receptor ratio in medial VTA mesocortical dopamine neurons parallelled the increase in excited dopamine neurons identified in the ventromedial VTA by Brischoux *et al.* (2009) following noxious footshock. Interestingly, NAc shell-projecting neurons in the lateral VTA, but not the medial VTA, were found to have importance for aversive signalling. Thus, these experiments suggest that increased responses of VTA dopamine neurons following aversive contexts occur across the medial–lateral extent of the VTA, and that track tracing can further refine a medial/lateral gradient of aversive signalling within VTA subdivisions. Although our approach did not allow for the refined analysis of VTA subdivisions along a medial–lateral axis, future studies that combine track tracing with dorsal/ventral divisions could further distinguish the topographical specificity of dopamine neurons that differentially respond to appetitive and aversive contexts.

Effects of stress on tyrosine hydroxylase cell number

Intriguingly, a third episode of defeat stress increased the total number of TH-positive cells in the ventral VTA of females. A similar trend was observed in the dorsal VTA. This effect could be

mediated by sex differences in glucocorticoid levels. Dexamethasone (a synthetic glucocorticoid) rapidly increases TH protein expression but not TH mRNA (Chen *et al.*, 2008; Tank *et al.*, 2008). Interestingly, female California mice have higher corticosterone levels immediately following social conflict than males. This effect is observed in female residents (Trainor *et al.*, 2010) and intruders (Trainor *et al.*, 2013). This sex difference is dependent on gonadal hormones (Trainor *et al.*, 2013), similar to other rodent species (Viau, 2002; Handa & Weiser, 2014). Chronic mild stress increases corticosterone levels (Grippo *et al.*, 2005) and was found to increase TH protein in the VTA as measured with western blot (Ortiz *et al.*, 1996). It is possible that high corticosterone levels need to be maintained in order to promote increased TH expression in the VTA (Ortiz *et al.*, 1995). An important aspect of experiment 3 is that animals were not exposed to any stressors outside routine husbandry after the last episode of defeat. In previous studies using this design, corticosterone levels in stressed female California mice are no different from control females at 2 weeks following control or stress manipulations (Trainor *et al.*, 2011). This could explain why a change in TH cell number was observed immediately after but not at 2 weeks after a third episode of defeat.

Glucocorticoid signalling in the mesolimbic dopamine system has been previously identified as important for defeat-induced social withdrawal in male rodents. In male mice, selective deletion of glucocorticoid receptors on D1 receptor-expressing neurons in the NAc (but not VTA dopamine neurons) blocked defeat-induced social withdrawal (Barik *et al.*, 2013). In California mice, ovariectomy blocks post-defeat increases in corticosterone levels in females but does not block defeat-induced social withdrawal (Trainor *et al.*, 2013). Although this would appear to contradict the glucocorticoid receptor deletion study, the glucocorticoid receptor deletion was accomplished using a dopamine transporter regulatory element, which results in glucocorticoid receptor deletion occurring early in life. Intriguingly, developmental exposure to dexamethasone induced stronger increases in TH immunoreactivity in the VTA of female vs. male rats (McArthur *et al.*, 2006). Plasticity in TH immunostaining has also been observed in adults. In prairie voles, androgens increase TH-positive cell counts in the BNST and medial amygdala, and these differences were hypothesized to reflect different levels of TH synthesis that facilitated detection via immunohistochemistry (Northcutt *et al.*, 2007). These findings suggest that sex-specific changes in hormone responses have important effects on dopamine cells. Similarly, we speculate that repeated stress in female California mice increases TH production and therefore facilitates the detection of TH neurons via immunohistochemistry. Increased TH immunoreactivity in the VTA was observed in rats exposed to chronic unpredictable stress for 10 days (Ortiz *et al.*, 1996), and this increase was hypothesized to enhance the magnitude of dopamine synthesis. As we only observed increased TH immunoreactivity immediately after a third episode of defeat, this suggests that TH expression is affected by ongoing stressors.

Sex differences in tyrosine hydroxylase/c-fos following a social interaction test

Although we did not observe effects of social defeat on TH/c-fos colocalizations in the social interaction test, females had more TH/c-fos colocalizations across the entire extent of the VTA compared with males. There were no sex differences in TH/c-fos colocalizations in control animals in experiments 1 and 2, which were placed into a novel empty cage. This indicates that the sex differences in TH/c-fos in experiment 3 cannot solely be attributed to exposure to a novel environment and is most likely to be mediated by the social context. Several reports suggest that the mesolimbic dopamine system responds more strongly to appetitive contexts in females than males. Following i.p. injection of amphetamine, greater increases of dopamine release in the NAc were observed in female rats compared with male rats (Virdee *et al.*, 2014). Consistent with this effect, females are more likely to develop conditioned place preference to psychostimulants (Lynch *et al.*, 2002; Russo *et al.*, 2003; Carroll & Anker, 2010; Trainor, 2011). Natural rewards also generate stronger responses in VTA dopamine neurons in females. In juvenile rats, social play induces significant increases in TH/c-fos cells in the VTA of females but not males (Northcutt & Nhuyen, 2014). Interestingly, a recent study used *in vivo* calcium imaging to demonstrate increased activity of VTA dopamine neurons in female mice during social interactions with an unfamiliar female (Gunaydin *et al.*, 2014). Together, these data support the hypothesis that, across the entire VTA, dopamine neurons respond to social interactions more strongly in females compared with males.

It is possible that gonadal hormones might have an effect on TH/c-fos expression in male and female California mice. In the VTA, androgen receptors are expressed on VTA dopamine neurons and non-dopamine neurons express oestrogen receptor α (Kritzer & Creutz, 2008). We did not monitor the oestrous cycle, so it is possible that the oestrous stage may have affected cell counts. However, gonadectomy has no effect on behaviour in the social interaction test and does not alter sex differences in social withdrawal (Trainor *et al.*, 2013). In addition, in both control and stressed female California mice, there were no differences in social interaction behaviour across different stages of the oestrous cycle (Trainor *et al.*, 2011). Furthermore, a recent metanalysis showed that variability in studies of females that do not control for oestrous cycle stage was no different from studies of males (Prendergast *et al.*, 2014). Thus, although the oestrous cycle could have had an impact on cell counts, it does not alter the fact that important sex differences were observed in both experiments 2 and 3.

Anxiety-like behaviour prior to a third episode of defeat stress

The behavioural data in experiment 3 showed that anxiety-like behaviours were induced immediately before a third episode of defeat, whereas mice assigned to control conditions showed no changes in behaviour. Over the course of the 3 day testing period, only mice exposed to defeat increased autogrooming behaviour immediately prior to each testing session. Autogrooming is an anxiety-like behaviour that is reduced by anxiolytic drugs such as diazepam (Dunn *et al.*, 1981; Moody *et al.*, 1988) and is induced by chronic mild stress in California mice (Harris *et al.*, 2013) and restraint stress in prairie voles (Smith & Wang, 2014). The increased autogrooming observed in mice assigned to defeat suggests that transportation to the testing room on day 3 triggers a conditioned response of anxiety-like behaviour. Although we did not directly compare mice exposed to one vs. three episodes of stress, it was only after a third episode of defeat that increases in TH/c-fos colocalizations were observed. At this point it is not clear why three episodes of defeat stress, which induced increased autogrooming behaviour, did not increase TH/c-fos colocalizations in males. Experiments 1 and 2 did not include an experimental group with a sedated resident, which raises the possibility that changes in TH/c-fos cell counts may have been caused by exposure to olfactory cues from the resident. An intriguing future experiment would be to investigate whether simply placing a socially stressed animal into the same room where it experienced previous episodes of defeat could increase TH/c-fos colocalizations.

In experiment 3, females had more c-fos-positive cells in the posterior NAc shell. Ventral VTA colocalizations were also positively correlated with c-fos in the posterior NAc shell in females but not males, and this correlation was strongest in stressed females. Posterior subregions of the NAc have been found to have stronger effects on aversive behavioural responses, whereas more anterior subregions of the NAc have important effects on appetitive behavioural responses (Richard & Berridge, 2011). Posterior regions of the NAc are activated by basolateral amygdala stimulation (Gill & Grace, 2011) and also receive stronger norepinephrine input (Berridge *et al.*, 1997). A previous study showed that females exposed to defeat had more phosphorylated cyclic AMP response element-binding protein-positive cells in the NAc compared with control females immediately following a social interaction test (Trainor *et al.*, 2011). In that study, phosphorylated cyclic AMP response element-binding protein cell counts were taken primarily in posterior regions of the NAc, but anterior–posterior subregions were not directly compared. Together, these results suggest that anterior and posterior NAc neurons have different effects on the valence generated by social stimuli, and suggest that there are sex differences in how these neurons are affected by social defeat.

Conclusions

We demonstrated important sex differences in how VTA dopamine neurons respond to social contexts generating approach and aversion. Although it is well established that social stressors have important effects on the function of VTA dopamine neurons, few studies have considered females. Our results indicate that, in the aversive context of repeated social defeat, neurons in the ventral VTA are preferentially activated in females. In contrast, in the context of a social interaction test (which normally induces strong approach responses), dopamine neurons across the entire VTA are more responsive in females. Together, these results suggest that VTA dopamine neurons are more responsive to both positive and negative social contexts in females, and that the short-term effect of negative contexts on VTA dopamine neurons is localized primarily to the ventral VTA. This suggests that further study of sex differences in VTA function is warranted, given that depression is often linked to altered function in the mesolimbic dopamine system (Russo & Nestler, 2013). Future studies looking at the effects of stressors on the mesolimbic dopamine system should consider males and females as well as topographical organization in both the NAc and VTA.

Conflict of interest statement

The authors declare no conflict of interest.

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Abbreviations

AC, anterior commissure; BNST, bed nucleus of the stria terminalis; IPN, interpeduncular nucleus; Ir, immunoreactive; NAc, nucleus accumbens; PBS, phosphate-buffered saline; PFC, prefrontal cortex; SN, substantia nigra; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; Tx, Triton X; VTA, ventral tegmental area.

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