Response of the Tail of the Ventral Tegmental Area to Aversive Stimuli

María-José Sánchez-Catalán^{*,1,2,5}, Fanny Faivre^{1,3,5}, Ipek Yalcin¹, Marc-Antoine Muller^{3,4}, Dominique Massotte¹, Monique Majchrzak^{3,4} and Michel Barrot¹

¹Institut des Neurosciences Cellulaires et Intégratives, Centre National de la Recherche Scientifique, Strasbourg, France; ²Unitat Predepartamental de Medicina, Universitat Jaume I, Castelló de la Plana, Spain; ³Université de Strasbourg, Strasbourg, France; ⁴Laboratoire de Neurosciences Cognitives et Adaptatives, Faculté de Psychologie, Centre National de la Recherche Scientifique, Strasbourg, France

The GABAergic tail of the ventral tegmental area (tVTA), also named rostromedial tegmental nucleus (RMTg), exerts an inhibitory control on dopamine neurons of the VTA and substantia nigra. The tVTA has been implicated in avoidance behaviors, response to drugs of abuse, reward prediction error, and motor functions. Stimulation of the lateral habenula (LHb) inputs to the tVTA, or of the tVTA itself, induces avoidance behaviors, which suggests a role of the tVTA in processing aversive information. Our aim was to test the impact of aversive stimuli on the molecular recruitment of the tVTA, and the behavioral consequences of tVTA lesions. In rats, we assessed Fos response to lithium chloride (LiCl), β -carboline, naloxone, lipopolysaccharide (LPS), inflammatory pain, neuropathic pain, foot-shock, restraint stress, forced swimming, predator odor, and opiate withdrawal. We also determined the effect of tVTA bilateral ablation on physical signs of opiate withdrawal, and on LPS- and LiCl-induced conditioned taste aversion (CTA). Naloxone-precipitated opiate withdrawal induced Fos in μ -opioid receptor-positive (15%) and -negative (85%) tVTA cells, suggesting the presence of both direct and indirect mechanisms in tVTA recruitment during withdrawal. However, tVTA lesion did not impact physical signs of opiate withdrawal. Fos induction was also present with repeated, but not single, foot-shock delivery. However, such induction was mostly absent with other aversive stimuli. Moreover, tVTA ablation had no impact on CTA. Although stimulation of the tVTA favors avoidance behaviors, present findings suggest that this structure may be important to the response to some, but not all, aversive stimuli.

Neuropsychopharmacology (2017) **42**, 638–648; doi:10.1038/npp.2016.139; published online 31 August 2016

INTRODUCTION

Midbrain dopamine neurons have a crucial role in motor functions, motivated behaviors, reward-related learning, and in processing salient signals (Bromberg-Martin *et al*, 2010; Wise, 2004), as well as in associated pathologies. The GABAergic tail of the ventral tegmental area (tVTA) (Kaufling *et al*, 2009, 2010a; Perroti *et al*, 2005), or rostromedial tegmental nucleus (RMTg) (Jhou *et al*, 2009a, 2009b), is a mesopontine structure, which exerts a major inhibitory control over dopamine cells of the VTA and substantia nigra *pars compacta* (SNc) (Bourdy and Barrot, 2012; Matsui *et al*, 2014; Sánchez-Catalán *et al*, 2014). The tVTA has been implicated in motor functions (Bourdy *et al*, 2014; Lavezzi *et al*, 2015), responses to drugs of abuse (Jalabert *et al*, 2011; Jhou *et al*, 2012, 2013; Kaufling *et al*, 2010b; Lavezzi *et al*, 2012; Lecca *et al*, 2011, 2012; Matsui and

Williams, 2011; Matsui *et al*, 2014; Melis *et al*, 2014; Rotllant *et al*, 2010), and reward prediction error (Hong *et al*, 2011). In addition, stimulation of the habenulo-tVTA pathway favors avoidance behaviors (Stamatakis and Stuber, 2012).

The tVTA receives inputs from the lateral habenula (LHb) (Balcita-Pedicino *et al*, 2011; Jhou *et al*, 2009a; Kaufling *et al*, 2009), a glutamatergic diencephalic structure involved in processing aversive information and reward prediction error (Matsumoto and Hikosaka, 2009). Thus, the tVTA has been proposed as an intermediate between the LHb and the VTA dopamine neurons, and to be important in promoting behavioral avoidance. Indeed, optogenetic stimulation of LHb terminals in the tVTA induces active, passive, and conditioned behavioral avoidance (Lammel *et al*, 2012; Stamatakis and Stuber, 2012). Moreover, pharmacological stimulation of the tVTA induces conditioned place aversion (Jhou *et al*, 2013). Otherwise, ablation of the tVTA inhibits fear-conditioned freezing, passive response to a predator odor, and anxiety-like behavior in an elevated plus maze (Jhou *et al*, 2009b). It also suppresses cocaine-induced avoidance behavior in a runway operant paradigm (Jhou *et al*, 2013), and cocaine excitation of LHb-tVTA neurons contributes to cocaine-induced depressive-like behaviors (Meye *et al*, 2015).

Various psychostimulants recruit the tVTA, as indicated by Fos induction in rats, suggesting that the tVTA is a

*Correspondence: Dr M-J Sánchez-Catalán, Unitat Predepartamental de Medicina, Universitat Jaume I, Avenue Vicent Sos Baynat, s/n, 13071 Castelló de la Plana, Spain, Tel: +34 964 38 74 40, Fax: +34 964 72 90 16, E-mail: macatala@uji.es

⁵The first two authors contributed equally to this work.

Received 22 March 2016; revised 19 July 2016; accepted 22 July 2016; accepted article preview online 29 July 2016

common target for those drugs (Kaufling *et al*, 2010b). Similarly, the tVTA displays Fos induction following exposure to series of foot shock (Brown and Shepard, 2013; Jhou *et al*, 2009b). However, to what extent other aversive stimuli similarly recruit the tVTA remains to be determined. As the tVTA might be part of the brain circuitry implicated in avoidance behaviors, it is important to further explore its response to aversive stimuli of different nature, and more particularly to determine whether the response observed with foot shocks may be generalized and constitutes a hallmark of the structure. Here, we assessed c-Fos expression in the rat tVTA in response to lithium chloride (LiCl), β -carboline, naloxone, lipopolysaccharide (LPS), inflammatory pain, neuropathic pain, foot-shock, loud tone, restraint stress, forced swimming, fox and cat odors, and opiate withdrawal. Moreover, we also assessed the effect of tVTA ablation on physical signs of opiate withdrawal and on LiCl and LPS-induced conditioned taste aversion (CTA).

MATERIALS AND METHODS

Animals

Experiments were performed in adult male Sprague–Dawley rats (Janvier, France) housed under standard conditions ($22 \pm 1^\circ\text{C}$, 12-h light–dark cycle). Rats were habituated to the facilities for at least 1 week and daily handled the week preceding experimentation. They were 7–9 weeks old at testing or surgery time. Experiments were approved by the regional ethical committee (CREMEAS).

Drugs

Drugs (Sigma-Aldrich, France; unless otherwise stated) were prepared in 0.9% NaCl (unless otherwise stated) and injected (1 ml/kg, except for λ -carrageenan) at the following doses: D-amphetamine sulfate, 0.25, 0.5, and 1 mg/kg intraperitoneally; LiCl, 60 mg/kg intraperitoneally; β -carboline (FG7142), 15 mg/kg intraperitoneally in 45% cyclodextrin in water; naloxone hydrochloride, 10 mg/kg intraperitoneally or 1 mg/kg subcutaneously; LPS from *Escherichia coli* 0127-B8, 250 $\mu\text{g/kg}$ intraperitoneally; λ -carrageenan type IV (lot. No. 80K133h), 150 μl of 3% solution, intraplantar; morphine hydrochloride, 10, 15, 20, 30, 40, 60, and 80 mg/kg intraperitoneally (Cooper, France). 2,4,5-trimethylthiazoline (TMT) (PheroTech, Delta, BC, Canada) was dissolved in diethylphthalate.

Aversive Procedures

Drugs producing aversive state were injected in animals: LiCl, β -carboline, naloxone, and LPS. Amphetamine was used as positive control for tVTA Fos induction. Rats were perfused 2 h postinjection, except for LPS (time course: 2, 4, and 8 h).

Inflammatory pain was induced by injection of λ -carrageenan in the right hind paw (Luis-Delgado *et al*, 2006a), with perfusion being carried out 2, 8, and 24 h later. To induce neuropathic pain, rats were anesthetized (ketamine, 87 mg/kg; xylazine, 13 mg/kg), and a 2 mm cuff of PE-90 polyethylene tubing was placed around the main branch of the right sciatic nerve (Yalcin *et al*, 2014;

Benbouzid *et al*, 2008). The sham group underwent the same surgery without cuff implantation. The presence of mechanical hypersensitivity was tested using calibrated forceps (Bioseb, France) (Luis-Delgado *et al*, 2006b). On day 18 after surgery, rats were perfused 2 h after the last measure of mechanical thresholds.

For exposure to predator odors, rats were first habituated to the exposure cages ($25 \times 16 \times 22$ cm) for 15 min. On the following day, rats were exposed to the odor for 20 min, starting 3 min after beginning the session. One group was exposed to TMT, a component of red fox feces, by introducing a gauze pad with 20 μl of 10% TMT into the cage (Jhou *et al*, 2009b). Controls were exposed to the vehicle. Another group was exposed to a 25 mm piece of cat collar previously worn by a domestic cat for 2 weeks (McGregor *et al*, 2004). Controls were exposed to an unworn collar. Perfusion was carried out 2 h after starting the session.

One hour restraint stress was carried out in transparent cylinders (21×6 cm). Forced swimming was carried out by placing rats for 15 min in cylinders (50×30 cm diameter) filled to 30 cm with 25°C water, rats were then removed, dried with towels, and placed in a warmed cage (Pliakas *et al*, 2001). Perfusion was carried out 2 h after the onset of stress procedure.

As repeated foot shocks have been shown to recruit the tVTA (Brown and Shepard, 2013; Jhou *et al*, 2009b), we tested them under different procedures. Rats were habituated for 30 min to behavioral chambers ($25 \times 27 \times 18$ cm; Med Associates, Saint Albans, Vermont) with a loudspeaker, camera, and grid floor. The following day, rats were exposed to these chambers in a 38 min session. The tone and tone/shock groups received, respectively, five pseudorandom tones (4000 Hz, 15 s) or five pseudorandom tones followed by foot shock (0.5 mA, 0.8 s). The foot-shock group received five pseudorandom shocks with no tone. Freezing behavior was assessed as aversive reaction (Marchand *et al*, 2003). Rats were perfused 2 h after starting the session. In an independent experiment, we tested exposure to a single foot shock or single tone. Rats were habituated to the chamber (sound-proof, $51 \times 25 \times 24$ cm) for 2 min, and tested the following day in a 2-min session. One minute after beginning the session, rats were exposed to a single foot shock (0.5 mA, 0.8 s or 4 s; or 0.8 mA, 0.8 s) or a loud tone (3000 Hz, 1 s), placed in their home cages and perfused 1h30 after starting the test.

Morphine dependence was induced by escalating doses of morphine (2 ml/kg), two times a day for 7 days: 10, 15, 20, 30, 40, 60, and 80 mg/kg. On day 8, 1 h after the morning morphine injection (80 mg/kg), rats received naloxone (1 mg/kg, s.c.) or saline. Opiate withdrawal was assessed in transparent cages ($37 \times 25 \times 30$ cm), scoring behavioral and physiological variables over 30 min. Rats were perfused 2 h after naloxone or saline administration.

Lesion

Rats were anesthetized (sodium pentobarbital, 50 mg/kg, intraperitoneally) for stereotaxic surgery. Ibotenic acid (1% in phosphate-buffered saline (PBS) 0.1 M, 0.2 μl) was injected bilaterally into the tVTA (0.2 μl) (Bourdy *et al*, 2014). Stereotaxic coordinates relative to bregma were (in mm): anteroposteriority $-6.7/6.9$, laterality $\pm 1.4/1.5$, verticality (from dura) $-7.7/7.8$, $\pm 6^\circ$ lateral angle (Paxinos and

Watson, 2007). Sham animals underwent the same procedure without lesion. Experiments started 1 to 2 weeks after surgery. After the experiments, rats were anesthetized and perfused to control for tVTA lesion.

Conditioned Taste Aversion

LiCl- or LPS-induced CTA (Slouzkey *et al*, 2013) was carried out using 30 min daily sessions over 7 days. Following 24 h of water deprivation, rats were trained in their home cage to drink water from two bottles for 30 min during 3 days. On the conditioning day, the two bottles were then filled with 0.1% saccharin solution. Ten minutes following the end of this session, rats received LiCl (Tenk *et al*, 2006), LPS (Konsman *et al*, 2008), or saline. The following 2 days, rats were exposed to two bottles of water during the session (baseline consumption). On test day, rats were allowed to drink from one bottle with water and one bottle with 0.1% saccharin, with randomized position. The aversion index was defined as (ml water/(ml water+ml saccharin) $\times$ 100) consumed during the test session.

Immunohistochemistry

Rats were anesthetized with pentobarbital overdose and perfused with 100 ml phosphate buffer (0.1 M, pH 7.4), followed by 500 ml of 4% paraformaldehyde in phosphate buffer. Brains were postfixed overnight, cryoprotected in 20% glycerol in PBS, and coronal sections (40 μ m) were cut using a cryotome. Immunohistochemistry was performed as described previously (Bourdy *et al*, 2014; Kaufling *et al*, 2010b). Primary antibodies targeted: c-Fos (Santa-Cruz; sc-52, lot 1008, 1/10000 for DAB staining, 1/1000 for immunofluorescence), μ -opioid receptor (MOR) (Millipore; AB1724, 1/400 for immunofluorescence), tyrosine hydroxylase (TH) (Millipore-Chemicon; MAB318, 1/2500), or NeuN (Millipore; MAB377, 1/5000). Visualization was performed with biotinylated secondary antibodies and peroxidase/DAB reaction after avidin-biotin-peroxidase amplification (ABC Elite; Vector Laboratories, USA), except for multiple staining performed using Alexa488- and Cy3-conjugated secondary antibodies (1/400; Jackson ImmunoResearch) and DAPI for nuclei detection.

Coronal slices from -5.80 to -7.30 mm from bregma were used to count c-Fos-positive nuclei bilaterally in the tVTA. The tVTA is present between -6 mm and -7 mm from bregma, as identified by amphetamine-induced Fos expression. Rostrally, it starts within the VTA paranigral nucleus, and dorsolaterally to the interpeduncular nucleus. It then extends caudally and dorsally, laterally to the median raphe nuclei, and is partially embedded within the superior cerebellar peduncle fibers (Kaufling *et al*, 2010a; Sánchez-Catalán *et al*, 2014). We analyzed a section every 160 μ m along the entire tVTA extent; after correction for missing sections, data were expressed per whole unilateral tVTA (Kaufling *et al*, 2010b). Images were acquired using a microscope (Eclipse E600; Nikon Instruments) with the Neurolucida software.

Analysis of c-Fos/MOR nuclei was performed using the optical fractionator technique with optical disectors (30 μ m height, 5 μ m guard zone), under the Stereoinvestigator software (MBF Biosciences). MOR immunostaining allowed

delimiting the tVTA (eg, Figure 4e) at $\times 10$ objective, and counting was performed at $\times 40$ on 10 randomly positioned counting frames per section (7–12 sections analyzed per animal). Microphotographs were acquired on a Leica SP5 II confocal microscope.

Analyses

Data are expressed as mean $\pm$ SEM. A power analysis was performed by using G*Power3 (version 3.1.9). Other statistical analyses were performed using STATISTICA 7.1 (Statsoft, Tulsa, OK, USA), with Student's *t*-test for two-group comparison and ANOVA for multiple comparisons. *Post hoc* analyses were carried out using either Dunnett or Bonferroni tests. For CTA experiments, a factorial two-way ANOVA was followed by a Duncan test. We performed a test of comparison between the mean and a standard value (50%) to compare aversion indices. Level of significance was $p < 0.05$.

RESULTS

Aversive Drugs

As psychostimulants induce Fos proteins in the rat tVTA (Kaufling *et al*, 2010b; Perrotti *et al*, 2005), we used amphetamine as an internal positive control and showed that the amphetamine c-Fos induction is dose-dependent ($F_{3,16} = 86.7$, $p < 0.001$) (Figures 1a and g). Concerning aversive drugs ($F_{3,19} = 21.9$, $p < 0.001$), tVTA Fos induction was observed only with the anxiogenic drug β -carboline (File and Baldwin, 1987) ($p < 0.001$) (Figures 1e and g). This induction was significant, but quantitatively small compared with psychostimulants. No significant induction was present with LiCl ($p = 0.35$), a drug provoking visceral illness (Ossenkopp and Eckel, 1995), with naloxone at high dose ($p = 1$), and with LPS ($F_{3,13} = 1.3$, $p = 0.31$), a bacterial endotoxin inducing sickness behavior (Figure 1) (Castanon *et al*, 2001; Konsman *et al*, 2008). Power analysis using the saline and β -carboline groups as a reference to calculate the effect size index (considering parametric *t*-test for two independent groups with $\alpha = 0.05$ and power = 0.80) gave an estimated sample size of three animals per group, indicating that n in our groups was sufficient to detect relevant differences.

Painful Stimuli

Inhibition of the tVTA impacts nocifensive responses (Jhou *et al*, 2012). However, painful stimuli did not induce c-Fos locally. With λ -carrageenan-induced inflammatory pain (Luis-Delgado *et al*, 2006a, b), no c-Fos induction was present in the tVTA (Figures 2a–c and m). Similarly, no c-Fos was observed in the tVTA with neuropathic pain ($p = 0.6$) (Figures 2d, e and m), whereas ipsilateral paw withdrawal thresholds were chronically lowered (group, $F_{1,12} = 219.2$, $p < 0.001$; paw, $F_{1,12} = 297.1$, $p < 0.001$; time $\times$ group $\times$ paw, $F_{6,72} = 18.5$, $p < 0.001$) (Figure 2f).

Stressful Stimuli

Predator odors induce unconditioned fear responses in rats, and tVTA lesion changes these responses (Jhou *et al*, 2009b). However, exposure to fox or cat odors did not induce c-Fos

Figure 1 Aversive drugs barely induce c-Fos expression in the GABAergic tail of the ventral tegmental area (tVTA). (a) Amphetamine dose-response (0.25, 0.5, and 1 mg/kg intraperitoneally, $n=3-4$ per group) was used as a positive control for c-Fos expression in the tVTA. (b) Acute injection of saline ($n=5$) has no effect on c-Fos in the tVTA. (c-f) Illustrations for c-Fos in the tVTA after acute injection of naloxone 10 mg/kg ($n=4$) (c), lithium chloride (LiCl) 60 mg/kg ($n=5$) (d), β -carboline 15 mg/kg ($n=6$) (e), and lipopolysaccharide (LPS) 250 μ g/kg (f). (g) Quantification of c-Fos-positive nuclei in the tVTA. Rats were perfused 2 h after injection, except for LPS (time course: 2, 4, and 8 h, $n=3$ per time point). The squares in a_1-f_1 indicate the regions shown at higher magnification in a_2-f_2 . Scale bars, 500 μ m (a_1-f_1) and 100 μ m (a_2-f_2 , a_3-a_4 , and f_3-f_4). *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ (relative to the saline group).

Figure 2 Painful or stressful stimuli do not induce c-Fos expression in the GABAergic tail of the ventral tegmental area (tVTA). (a-c) Intraplantar injection of λ -carrageenan (Cam) does not induce c-Fos expression in the tVTA ($n=3$ per time point). (d and e) Neuropathic pain (NP) does not induce c-Fos expression in the tVTA. Sham animals underwent the same surgical procedure without cuff implantation ($n=4$ per group). (f) Cuff implantation results in a chronic ipsilateral allodynia (day 0: surgery, *** $p < 0.001$ relative to the other groups). (g and h) Fox odor exposure (2,4,5-trimethylthiazoline (TMT); control group: TMTc) do not induce c-Fos expression in the tVTA ($n=3$ per group). (i and j) Cat odor exposure (CAT; control group: CATc) do not induce c-Fos expression in the tVTA ($n=3$ per group). (k and l) Acute restraint stress ($n=5$) and forced swimming test (FST, $n=5$) do not induce c-Fos expression in the tVTA. (m) Quantification of c-Fos-positive nuclei in the tVTA. An amphetamine group (amph, 1 mg/kg) ($n=3$) was included as a positive control group. Rats were perfused 2 h after starting the behavioral test or after the last measure of mechanical thresholds for neuropathic pain. The squares in (a_1-e_1 and g_1-l_1) indicate the regions shown at higher magnification in (a_2-l_2 and g_2-l_2). Scale bars, 500 μ m (a_1-e_1 and g_1-l_1) and 100 μ m (a_2-l_2 and g_2-l_2).

Figure 2 See for caption on page 641.

in the tVTA (TMT, $p=0.69$; cat odor, $p=0.79$) (Figures 2g–j and m). Similarly, restraint stress, or forced swimming as classically used in depression research, also failed to recruit the tVTA (Figures 2k–m).

The only stress procedure that led to significant c-Fos induction in the tVTA was the repeated exposure to mild foot shocks ($F_{3,22}=23.4$, $p<0.001$) delivered in a protocol usually used to elicit fear conditioning. Freezing behavior during the

Figure 3 See for caption on page 644.

shock session was observed in foot-shock-exposed animals ($F_{3,21} = 28.7$, $p < 0.001$) (Figure 3e). This c-Fos induction was observed whether a predictive tone was present or not before each shock exposure (Figures 3a–d and f). However, a single foot shock did not evoke c-Fos in the tVTA. Indeed, no induction was observed with a single shock of intensity as used in fear conditioning (0.5 mA/0.8 s), with a single shock of intensity as used in fear conditioning but of a duration equivalent to the sum of five shocks (0.5 mA/4 s), or with a single shock of higher intensity (0.8 mA/0.8 s) (Figures 3g–l). Moreover, single salient information (arousing but non-nociceptive), such as a loud auditory stimulus, also failed to recruit the tVTA (Figure 3h). These findings support the idea that repetition of the stimulus was critical for inducing c-Fos in the tVTA as a consequence of foot shocks.

Opioid Withdrawal

The tVTA is a direct target of opiates (Jalabert *et al*, 2011), even though morphine itself does not induce Fos locally (Perrotti *et al*, 2005; Kaufling *et al*, 2010b). Here we show that precipitated opiate withdrawal (Supplementary Table S1) induces c-Fos in the tVTA ($F_{3,16} = 406.9$, $p < 0.001$) (Figures 4a and b). Morphine treatment *per se* did not affect the total number of MOR-positive neurons in the tVTA (data not shown). Withdrawal-induced c-Fos was present (15%) in MOR-expressing neurons, but mostly (85%) in neurons not expressing this receptor (Figures 4c–e). In a separate experiment, we also show that a bilateral lesion of the tVTA did not significantly change the individual physical signs of withdrawal or the global withdrawal score (Figure 4f and Supplementary Table S2, $p = 0.91$).

Conditioned Taste Aversion

The lack of tVTA c-Fos induction in response to most of the tested aversive stimuli raised the question of tVTA requirement for avoidance behaviors. We assessed the effect of tVTA ablation on the LiCl- and LPS-induced CTA, aversive-like behaviors not yet tested in tVTA studies. CTA being a robust conditioning, single conditioning procedures were used (Slouzkey *et al*, 2013). Only rats with lesions encompassing the tVTA were included in the results, TH immunostaining was used to control for the absence of VTA dopaminergic lesion (Figures 5a and b). Sham control rats (Sal-sham) displayed a preference for saccharin in the two-bottle choice test (Figure 5c). tVTA ablation did not abolish saccharin preference or affect the LiCl- or LPS-induced CTA (treatment, $F_{2,32} = 21.3$, $p < 0.001$; surgery, $F_{1,32} = 2.5$, $p = 0.12$; treatment $\times$ surgery, $F_{2,32} = 0.9$, $p = 0.41$). Indeed, rats showed decreased preference for the saccharin solution previously paired to LiCl or LPS injection in both

sham ($p < 0.001$ and $p < 0.01$, respectively) and lesion groups ($p < 0.001$). Moreover, the aversive index differed from chance level (50%) in LPS-lesion, LiCl-lesion and LiCl-sham groups, even though it failed to differ from chance level in the LPS-sham group, because of high variability (Figure 5c).

DISCUSSION

By exploring the impact of aversive stimuli, we found that most of them failed to significantly induce c-Fos in the tVTA. Induction was only observed with repeated foot shocks and precipitated morphine withdrawal, and mildly with β -carboline at a high dose. Opiate withdrawal Fos response in the tVTA concerned MOR-expressing and -non-expressing cells. Last, we show that bilateral ablation of the tVTA did not affect physical signs of opiate withdrawal, or LPS- and LiCl-induced CTA.

Implication of the tVTA, which receives afferents from the LHb (Balcita-Pedicino *et al*, 2011; Jhou *et al*, 2009a; Kaufling *et al*, 2009) and controls dopamine cell activity (Bourdy *et al*, 2014; Jalabert *et al*, 2011; Jhou *et al*, 2009b; Matsui and Williams, 2011), has been described in active, passive, and conditioned avoidance behaviors and in the processing of aversive information (Jhou *et al*, 2009b, 2013; Lammel *et al*, 2012; Meye *et al*, 2015; Stamatakis and Stuber, 2012). To date, however, the most striking Fos responses in this structure were observed with psychostimulants (Cornish *et al*, 2012; Jhou *et al*, 2009a, 2013; Geisler *et al*, 2008; Kaufling *et al*, 2010b; Lecca *et al*, 2011; Perrotti *et al*, 2005; Zahm *et al*, 2010) (see Supplementary Table S3 and Supplementary Data). Our first aim was thus to test whether aversive stimuli would display a common molecular impact on the tVTA, as revealed by induction of c-Fos proteins. Surprisingly, the exposure to aversive drugs such as LiCl, naloxone at high dose, or LPS did not induce c-Fos expression in the tVTA. The anxiogenic agent β -carboline, used at a high dose to model panic attacks (Thiébot *et al*, 1988), resulted in a significant induction, although quantitatively mild compared with the response observed with amphetamine. More sustained aversive experiences, such as inflammatory pain and neuropathic pain, also failed to change c-Fos expression locally, even though inhibition of the tVTA by local microinjection of endomorphin or muscimol has an analgesic action in the formalin test (Jhou *et al*, 2012). Lesion of the tVTA prevents the freezing response to TMT odor, while increasing other defensive behaviors (Jhou *et al*, 2009b), which suggests that the tVTA is important to passive defensive behavior. However, we did not evidence c-Fos induction when testing unconditioned fear through predator odor exposure, or after stress elicited by immobilization or forced swimming. Importantly, a lack of

Figure 3 Repeated foot-shock exposure induces c-Fos expression in the GABAergic tail of the ventral tegmental area (tVTA). The context (a) and the tone (b) exposure hardly induces c-Fos expression in the tVTA, whereas (c and d) repeated foot shocks (0.5 mA, 5×0.8 s) induce c-Fos expression in the tVTA, under the presence of the tone or not ($n = 5$ –6 per group). (e) Freezing behavior in the test session following repeated foot-shock exposures. (f) Quantification of c-Fos-positive nuclei in the tVTA following repeated foot-shock exposures. *** $p < 0.001$ (relative to the control group). (g–k) Single foot-shock exposure fails to induce c-Fos in the tVTA. Compared with controls (context, (g)), no c-Fos expression was present with 0.5 mA/0.8 s (i), 0.8 mA/0.8 s (j), or 0.5 mA/4 s (k) ($n = 4$ per group). The exposure to a salient loud auditory stimulus also failed to recruit the tVTA (h) ($n = 4$). (l) Quantification of c-Fos-positive nuclei in the tVTA following single foot-shock exposure or a loud auditory stimulus. Rats were perfused 2 h after the beginning of the session for repeated foot-shock experiments, or after 1 h 30 for single foot-shock experiments. The squares in (a₁–d₁ and g₁–k₁) indicate the regions shown at higher magnification in (a₂–d₂ and g₂–k₂). Scale bars, 500 μ m (a₁–d₁, g₁–k₁) and 100 μ m (a₂–d₂, g₂–k₂).

Figure 4 Opiate withdrawal induces c-Fos expression in the GABAergic tail of the ventral tegmental area (tVTA). (a) Animals receiving repeated saline injections and a saline (S/S) or naloxone (1 mg/kg) (S/N) challenge, or repeated morphine injections and a saline challenge (M/S), do not display c-Fos induction in the tVTA, whereas animals receiving repeated morphine injections and a naloxone challenge (M/N) exhibited opioid withdrawal symptoms (Supplementary Table S1) and high levels of c-Fos-positive nuclei in the tVTA. (b) Quantification of c-Fos-positive nuclei in the tVTA ($n=4-6$ per group). (c) Quantification of c-Fos-positive nuclei in MOR-positive and -negative cells of the tVTA ($n=5$ per group). *** $p < 0.001$ (relative to the control group). (d) After opiate withdrawal, c-Fos induction (in green) in the rat tVTA was present in MOR-expressing neurons (in red) (as indicated by the arrows), and in neurons not expressing them (as indicated by the arrow heads). Cell nuclei are labeled with DAPI (4',6-diamidino-2-phenylindole) (in blue). Rats were perfused 2 h after the naloxone or saline administration. Scale bars, 100 μ m (a) and 10 μ m (d). (e) Illustration of one of the sections used for stereological analysis (data in c). The square in left picture indicates the region shown at higher magnification in right pictures (top: MOR in orange; bottom: c-Fos in green). The dotted area in right pictures indicates the tVTA limits used for stereological analysis; at this anteroposterior level, the tVTA is embedded within fibers of the superior cerebellar peduncle. Scale bars, 500 μ m (left picture) and 100 μ m (right pictures). (f) The lesion of the tVTA did not significantly alter the physical signs or the global score (Gellert and Holtzman, 1978) of naloxone-precipitated opiate withdrawal (sham $n=7$; lesion $n=6$). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

Figure 5 GABAergic tail of the ventral tegmental area (tVTA) ablation does not affect the lithium chloride (LiCl)- and lipopolysaccharide (LPS)-induced conditioned taste aversion (CTA). (a) Histological evidence for the tVTA sham group with NeuN (a₁) and tyrosine hydroxylase (TH) (a₂) immunostaining. (b) Histological evidence for the tVTA lesion group with NeuN (b₁) and TH (b₂) immunostaining. (c) Aversion index for the LiCl and LPS-induced CTA in sham controls and lesion animals ($n=5-8$ per group). Scale bars, 200 μ m (a₁ and b₁) and 500 μ m (a₂ and b₂). ** $p<0.01$, *** $p<0.001$ (relative to their respective saline group). # $p<0.05$ and ### $p<0.001$ (referred to 50%).

c-Fos expression does not imply a lack of functional recruitment of tVTA cells. For example, there is evidence for an electrophysiological impact on tVTA activity in the absence of Fos induction with drugs that inhibit the tVTA, such as opiates and cannabinoids (Kaufling *et al*, 2010b; Lecca *et al*, 2011). However, avoidance behaviors are supposedly associated with activation, and not inhibition, of the tVTA.

Contrary to most stressful stimuli described above, c-Fos expression in the tVTA was present following exposure to repeated mild foot shocks, similarly to previous reports (Brown and Shepard, 2013; Jhou *et al*, 2009b). The mechanism underlying this expression depends on LHB inputs. Indeed, a lesion of the fasciculus retroflexus impairs this response to mild foot shocks (Brown and Shepard, 2013), and acute unpredictable foot shocks potentiate glutamate release from the LHB to the tVTA (Stamatakis and Stuber, 2012). However, these procedures relied on a series of foot shocks, that is, the repetition of identical discrete aversive events. On the contrary, exposure to a single foot shock does not induce c-Fos in the tVTA, even though the duration of the single shock is extended to 4 s (mimicking the cumulated duration of the shock series), or if the intensity is increased to reach levels usually used for

learned helplessness procedures (0.8 mA). These findings suggest that the effect of a series of foot shocks on the tVTA is unlikely related to their aversive impact *per se*, nor to the salient, arousing, or attentional properties of the shocks. These conclusions are also supported by the lack of c-Fos response following exposure to a single loud auditory tone. The present data more likely support the participation of the tVTA in learning and prediction processing associated with the repetition of discrete events. In this context, the tVTA has been shown, in monkeys, to process reward prediction error, which is mirroring the implication of the LHB in the same processing (Hong *et al*, 2011). It might be hypothesized that the response observed with a series of foot shocks may reflect attempts to predict, and/or failures in predicting, repeated occurrences of the shocks.

Present and previous data show that opiates do not induce Fos in the tVTA (Kaufling *et al*, 2010b; Perrotti *et al*, 2005). However, acute morphine withdrawal strongly induces c-Fos in this brain region. Behavioral studies support a role for the tVTA in opioid responses, as rats self-administer and develop conditioned place preference when endomorphin-1 is delivered into the tVTA (Jhou *et al*, 2012). In fact, part of the GABAergic tVTA neurons in rats express MOR (Jalabert *et al*, 2011; Jhou *et al*, 2009a, 2012; Kaufling and Aston-Jones, 2015). As a consequence, opioid administration decreases the electrophysiological activity of tVTA neurons (Jalabert *et al*, 2011; Kaufling and Aston-Jones, 2015; Lecca *et al*, 2011; Matsui and Williams, 2011), which enhances the activity of VTA dopaminergic neurons (Jalabert *et al*, 2011; Matsui and Williams, 2011). Under chronic morphine, partial tolerance develops (Matsui *et al*, 2014), and animals display altered activity of tVTA and VTA neurons, which is reversed by acute precipitated withdrawal (Kaufling and Aston-Jones, 2015). Following chronic morphine exposure, adaptive molecular changes that participate to opponent processes and were described in other brain regions (Nestler, 2015) might also be present in the tVTA and explain the c-Fos expression observed in MOR-expressing cells. This induction could thus be linked to a rebound induced by naloxone in the activity of MOR-expressing cells. On the contrary, the expression observed in MOR-non-expressing cells is more likely to be indirect, through tVTA inputs and even polysynaptic circuits. In this regard, the LHB has, for example, been shown to participate to tVTA Fos induction in response to other stimuli (Brown and Shepard, 2013; Cui *et al*, 2014). Notably, tVTA lesion had no impact on the physical signs of opiate withdrawal. However, it does not preclude its involvement in the aversiveness of opiate withdrawal.

Despite observations of c-Fos induction with a series of foot shocks or with precipitated opiate withdrawal, the lack of response to most tested conditions raises the question of the exact role of the tVTA in avoidance responses (see Supplementary Table S3 in Supplementary Data). The optogenetic stimulation of the mouse LHB recruits tVTA GABAergic neurons and induces conditioned place avoidance (Lammel *et al*, 2012). Moreover, the stimulation of terminals from the LHB in the tVTA induces passive and conditioned place avoidance, and can also decrease the reinforcing property of sucrose (Stamatakis and Stuber, 2012). Similarly, a direct S-AMPA stimulation of the tVTA induces conditioned place avoidance (Jhou *et al*, 2013). These findings from various groups converge to evidence

that stimulation of the tVTA promotes avoidance behavior. However, this does not imply that the tVTA is necessary for the physiological processing of all information leading to avoidance behavior. Indeed, we observed that the absence of the tVTA does not prevent LiCl- and LPS-induced CTAs. It shows that some forms of conditioned aversion can develop and be expressed in the absence of information processing through the tVTA.

The tVTA discovery was seminal, fostering research from various research groups. With evidence pointing to the tVTA as a major inhibitory control of dopamine systems, these studies converge to highlight the influence of the tVTA on dopamine-related functions, from responses to drug of abuse, to reward-related behaviors, reward prediction error, and control of motor responses. Generalization in trying to attribute simple functions to this brain region, while attractive for the sake of clarity, may however become a pitfall and mislead future research. The complexity of tVTA roles probably mirrors the complexity of dopamine systems themselves, which is not yet fully elucidated. This work participates to present efforts in understanding those functions of the tVTA. Foot-shock data suggest that the tVTA may be sensitive to the repetition of discrete emotional events, which could be related to previous evidence of reward prediction error processing by this brain region. The avoidance-inducing consequence of stimulating the tVTA reflects one of the functions of this brain region. Present data, however, suggest that all aversive situations may not be necessarily processed through the tVTA, and that some avoidance behavior can develop in the absence of the tVTA.

FUNDING AND DISCLOSURE

This research was supported by the Centre National de la Recherche Scientifique, Université de Strasbourg and its Neuropôle, and Agence Nationale de la Recherche (ANR-11-sv4-002). The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the UMS3415 Chronobiotron for animal housing, and the Neuropôle 'Plateforme imagerie *in vitro*' for confocal facilities.

REFERENCES

Balcita-Pedico JJ, Omelchenko N, Bell R, Sesack SR (2011). The inhibitory influence of the lateral habenula on midbrain dopamine cells: ultrastructural evidence for indirect mediation via the rostromedial mesopontine tegmental nucleus. *J Comp Neurol* 519: 1143–1164.

Benbouzid M, Pallage V, Rajalu M, Waltisperger E, Doridot S, Poisbeau P *et al* (2008). Sciatic nerve cuffing in mice: a model of sustained neuropathic pain. *Eur J Pain* 12: 591–599.

Bourdy R, Barrot M (2012). A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci* 35: 681–690.

Bourdy R, Sánchez-Catalán MJ, Kauffling J, Balcita-Pedico JJ, Freund-Mercier MJ, Veinante P *et al* (2014). Control of the nigrostriatal dopamine neuron activity and motor function by the tail of the ventral tegmental area. *Neuropsychopharmacology* 39: 2788–2798.

Bromberg-Martin ES, Matsumoto M, Hikosaka O (2010). Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68: 815–834.

Brown PL, Shepard PD (2013). Lesions of the fasciculus retroflexus alter foot-shock-induced cFos expression in the mesopontine rostromedial tegmental area of rats. *PLoS One* 8: e60678.

Castanon N, Bluthé RM, Dantzer R (2001). Chronic treatment with the atypical antidepressant tianeptine attenuates sickness behavior induced by peripheral but not central lipopolysaccharide and interleukin-1beta in the rat. *Psychopharmacology* 154: 50–60.

Cornish JL, Hunt GE, Robins L, McGregor IS (2012). Regional c-Fos and FosB/ΔFosB expression associated with chronic methamphetamine self-administration and methamphetamine-seeking behavior in rats. *Neuroscience* 206: 100–114.

Cui W, Mizukami H, Yanagisawa M, Aida T, Nomura M, Isomura Y *et al* (2014). Glial dysfunction in the mouse habenula causes depressive-like behaviors and sleep disturbance. *J Neurosci* 34: 16273–16285.

File SE, Baldwin HA (1987). Effects of beta-carbolines in animal models of anxiety. *Brain Res Bull* 19: 293–299.

Geisler S, Marinelli M, Degarmo B, Becker ML, Freiman AJ, Beales M *et al* (2008). Prominent activation of brainstem and pallidal afferents of the ventral tegmental area by cocaine. *Neuropsychopharmacology* 33: 2688–2700.

Gellert VF, Holtzman SG (1978). Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. *J Pharmacol Exp Ther* 205: 536–546.

Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *J Neurosci* 31: 11457–11471.

Jalabert M, Bourdy R, Courtin J, Veinante P, Manzoni OJ, Barrot M *et al* (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc Natl Acad Sci USA* 108: 16446–16450.

Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009b). The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* 61: 786–800.

Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009a). The mesopontine rostromedial tegmental nucleus: a structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. *J Comp Neurol* 513: 566–596.

Jhou TC, Xu SP, Lee MR, Gallen CL, Ikemoto S (2012). Mapping of reinforcing and analgesic effects of the mu opioid agonist endomorphin-1 in the ventral midbrain of the rat. *Psychopharmacology* 224: 303–312.

Jhou TC, Good CH, Rowley CS, Xu SP, Wang H, Burnham NW *et al* (2013). Cocaine drives aversive conditioning via delayed activation of dopamine-responsive habenular and midbrain pathways. *J Neurosci* 33: 7501–7512.

Kauffling J, Veinante P, Pawlowski SA, Freund-Mercier MJ, Barrot M (2009). Afferents to the GABAergic tail of the ventral tegmental area in the rat. *J Comp Neurol* 513: 597–621.

Kauffling J, Veinante P, Pawlowski SA, Freund-Mercier MJ, Barrot M (2010a). Gamma-aminobutyric acid cells with cocaine-induced DeltaFosB in the ventral tegmental area innervate mesolimbic neurons. *Biol Psychiatry* 67: 88–92.

Kauffling J, Waltisperger E, Bourdy R, Valera A, Veinante P, Freund-Mercier MJ *et al* (2010b). Pharmacological recruitment of the GABAergic tail of the ventral tegmental area by acute drug exposure. *Br J Pharmacol* 161: 1677–1691.

Kauffling J, Aston-Jones G (2015). Persistent adaptations in afferents to ventral tegmental dopamine neurons after opiate withdrawal. *J Neurosci* 35: 10290–10303.

- Konsman JP, Veeneman J, Combe C, Poole S, Luheshi GN, Dantzer R (2008). Central nervous action of interleukin-1 mediates activation of limbic structures and behavioural depression in response to peripheral administration of bacterial lipopolysaccharide. *Eur J Neurosci* **28**: 2499–2510.
- Lammel S, Lim BK, Ran C, Huang KW, Betley MJ, Tye KM *et al* (2012). Input-specific control of reward and aversion in the ventral tegmental area. *Nature* **491**: 212–217.
- Lavezzi HN, Parsley KP, Zahm DS (2012). Mesopontine rostromedial tegmental nucleus neurons projecting to the dorsal raphe and pedunculopontine tegmental nucleus: psychostimulant-elicited Fos expression and collateralization. *Brain Struct Funct* **217**: 719–734.
- Lavezzi HN, Parsley KP, Zahm DS (2015). Modulation of locomotor activation by the rostromedial tegmental nucleus. *Neuropsychopharmacology* **40**: 676–687.
- Lecca S, Melis M, Luchicchi A, Ennas MG, Castelli MP, Muntoni AL *et al* (2011). Effects of drugs of abuse on putative rostromedial tegmental neurons, inhibitory afferents to midbrain dopamine cells. *Neuropsychopharmacology* **36**: 589–602.
- Lecca S, Melis M, Luchicchi A, Muntoni AL, Pistis M (2012). Inhibitory inputs from rostromedial tegmental neurons regulate spontaneous activity of midbrain dopamine cells and their responses to drugs of abuse. *Neuropsychopharmacology* **37**: 1164–1176.
- Luis-Delgado OE, Barrot M, Rodeau JL, Ulery PG, Freund-Mercier MJ, Lasbennes F (2006a). The transcription factor DeltaFosB is recruited by inflammatory pain. *J Neurochem* **98**: 1423–1431.
- Luis-Delgado OE, Barrot M, Rodeau JL, Schott G, Benbouzid M, Poisbeau P *et al* (2006b). Calibrated forceps: a sensitive and reliable tool for pain and analgesia studies. *J Pain* **7**: 32–39.
- Marchand AR, Luck D, DiScala G (2003). Evaluation of an improved automated analysis of freezing behaviour in rats and its use in trace fear conditioning. *J Neurosci Methods* **126**: 145–153.
- Matsui A, Williams JT (2011). Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons. *J Neurosci* **31**: 17729–17735.
- Matsui A, Jarvie BC, Robinson BG, Hentges ST, Williams JT (2014). Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. *Neuron* **82**: 1346–1356.
- Matsumoto M, Hikosaka O (2009). Representation of negative motivational value in the primate lateral habenula. *Nat Neurosci* **12**: 77–84.
- Melis M, Sagheddu C, De Felice M, Casti A, Madeddu C, Spiga S *et al* (2014). Enhanced endocannabinoid-mediated modulation of rostromedial tegmental nucleus drive onto dopamine neurons in Sardinian alcohol-preferring rats. *J Neurosci* **34**: 12716–12724.
- McGregor IS, Hargreaves GA, Apfelbach R, Hunt GE (2004). Neural correlates of cat odor-induced anxiety in rats: region-specific effects of the benzodiazepine midazolam. *J Neurosci* **24**: 4134–4144.
- Meye FJ, Valentinova K, Lecca S, Marion-Poll L, Maroteaux MJ, Musardo S *et al* (2015). Cocaine-evoked negative symptoms require AMPA receptor trafficking in the lateral habenula. *Nat Neurosci* **18**: 376–378.
- Nestler EJ (2015). Reflections on: 'A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function'. *Brain Res* **1645**: 71–74.
- Ossenkopp KP, Eckel LA (1995). Toxin-induced conditioned changes in taste reactivity and the role of the chemosensitive area postrema. *Neurosci Biobehav Rev* **19**: 99–108.
- Paxinos G, Watson C (2007). *The Rat Brain in Stereotaxic Coordinates* 4th edn. Academic Press: San Diego, CA.
- Perrotti LI, Bolaños CA, Choi KH, Russo SJ, Edwards S, Ulery PG *et al* (2005). Δ FosB accumulates in a GABAergic cell population in the posterior tail of the ventral tegmental area after psychostimulant treatment. *Eur J Neurosci* **21**: 2817–2824.
- Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr (2001). Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J Neurosci* **21**: 7397–7403.
- Rotlant D, Marquez C, Nadal R, Armario A (2010). The brain pattern of c-fos induction by two doses of amphetamine suggests different brain processing pathways and minor contribution of behavioural traits. *Neuroscience* **168**: 691–705.
- Sánchez-Catalán MJ, Kaufling J, Georges F, Veinante P, Barrot M (2014). The antero-posterior heterogeneity of the ventral tegmental area. *Neuroscience* **282C**: 198–216.
- Slouzkey I, Rosenblum K, Maroun M (2013). Memory of conditioned taste aversion is erased by inhibition of PI3K in the insular cortex. *Neuropsychopharmacology* **38**: 1143–1153.
- Stamatakis AM, Stuber GD (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nat Neurosci* **15**: 1105–1107.
- Tenk CM, Kavaliers M, Ossenkopp KP (2006). The effects of acute corticosterone on lithium chloride-induced conditioned place aversion and locomotor activity in rats. *Life Sci* **79**: 1069–1080.
- Thiébot MH, Soubrié P, Sanger D (1988). Anxiogenic properties of beta-CCE and FG 7142: a review of promises and pitfalls. *Psychopharmacology* **9**: 452–463.
- Wise RA (2004). Dopamine, learning and motivation. *Nat Rev Neurosci* **5**: 483–494.
- Yalcin I, Megat S, Barthas F, Waltisperger E, Kremer M, Salvat E *et al* (2014). The sciatic nerve cuffing model of neuropathic pain in mice. *J Vis Exp* **89**.
- Zahm DS, Becker ML, Freiman AJ, Strauch S, Degarmo B, Geisler S *et al* (2010). Fos after single and repeated self-administration of cocaine and saline in the rat: emphasis on the Basal forebrain and recalibration of expression. *Neuropsychopharmacology* **35**: 445–463.

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