

The μ -opioid system in midline thalamic nuclei modulates defence strategies towards a conditioned fear stimulus in male mice

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Abstract

Background: Nuclei located in the dorsal midline thalamus, such as the paraventricular nucleus of the thalamus (PVT), are crucial to modulate fear and aversive behaviour. In addition, the PVT shows a dense expression of μ -opioid receptors (MORs) and could mediate the anxiolytic effects of opioids.

Methods: We analysed the contribution of MORs in the dorsal midline thalamus (i.e. the PVT) to the performance of mice in a classical fear conditioning paradigm. We locally injected a specific agonist (DAMGO), an antagonist (CTAP) of MOR or saline as a control into the dorsal midline thalamus of male mice, prior to fear extinction training. We assessed freezing as a typical measure of fear and extended our analysis by evaluation of aversive, non-aversive and neutral behavioural features using compositional data analysis.

Results: Pharmacological blockade of MORs through CTAP in the dorsal midline thalamus induced a fear memory extinction deficit, as evidenced by maintained freezing during extinction sessions. Stimulation of MORs by DAMGO resulted in an overall increase in locomotor activity, associated with decreased freezing during recall of extinction. Compositional data analysis confirmed the freezing-related pharmacological effects and revealed specific differences in basic behavioural states. CTAP-treated mice remained in an aversive state, whereas DAMGO-treated mice displayed predominantly neutral behaviour.

Conclusions: Fear extinction requires the integrity of the μ -opioid system in the dorsal midline thalamus. Pharmacological stimulation of MOR and associated facilitation of fear extinction recall suggest a potential therapeutic avenue for stress-related or anxiety disorders.

Keywords

Fear extinction, dorsal midline thalamus, μ -opioid receptor, opioids

Introduction

Organisms have to discriminate continuously noticeable (i.e. salient) stimuli in their environment and assign them a relevant predictive value (i.e. negative or positive valence). From this associative process animals elaborate an appropriate response that manifests in the behaviour of the animal. Nuclei located in the dorsal midline thalamus (dMT), such as the paraventricular nucleus of the thalamus (PVT), play a critical role in this task. The PVT seems to modulate a variety of aspects, such as arousal, awareness and stress coping (Kirouac, 2015), and PVT neurons have recently been shown to code for stimulus saliency (Zhu et al., 2018). The PVT also regulates emotional and behavioural responses (Kirouac, 2015), likely due to its projections to emotional valence-related structures, such as the nucleus accumbens, the extended amygdala or the prefrontal cortex (Goedecke et al., 2019; Haight and Flagel, 2014). Thus, the dMT is a critical hub for information processing in valence-coding circuits and the coordination of the behavioural response.

The dMT is rich in various opioid receptors (Brunton and Charkpak, 1998) and the opioid system modulates aversiveness associated with opioid withdrawal (Zhu et al., 2016). In particular, μ -opioid receptors (MORs) regulate opioid-mediated aversive behaviour during fear memory retrieval, particularly in some brain areas such as the ventrolateral periaqueductal gray (vlPAG) (McNally et al., 2005). Opioids are also involved in valence and

emotional circuits, and in fear memory retrieval and extinction processes, with implications for diseases such as anxiety disorders (Colasanti et al., 2011). However, the specific contribution of MORs in the dMT to the modulation of aversive behaviour has not been established so far.

Classical (Pavlovian) fear conditioning is a well-established procedure to evaluate aversive behavioural changes, and freezing is usually monitored as one behavioural output variable. This variable largely reflects passive coping behaviour, which can be complemented in several ways. First, activity scores can be used to capture the active component of the behavioural coping strategy (Fucich et al., 2016). In addition, it is mandatory that a broader spectrum of behavioural responses is analysed (i.e. including rearing, grooming, flight responses, etc.) in an attempt to study environmental or pharmacological influences (Laxmi et al., 2003; Perusini and Fanselow, 2015; Remmes et al., 2016). A more general concern is that behavioural traits often mutually exclude and complement each other, resulting in intrinsic negative correlations, which in turn require the implementation of

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compositional data analysis (CoDA) (Schilling et al., 2012; Smith et al., 2016).

Here we hypothesized that MORs in the dMT alter fear behaviour in a fear conditioning paradigm. We injected a MOR-selective agonist ([D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO)), an antagonist (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP)) or saline (Control) into the dMT of mice prior to extinction sessions. We analysed behavioural responses such as freezing to a conditioned stimulus (CS⁺) and to a neutral stimulus (CS⁻), and complemented it with an active coping behaviour analysis and with a CoDA of aversive, non-aversive and neutral behavioural features. Our experiments suggest that the μ -opioid system in the dMT participates in fear memory extinction processes.

Material and methods

Animals and housing

All procedures were performed according to European regulations on animal experimentation (Directive 2010/63/EU of the European parliament and the council), and under the surveillance of the local authorities (Bezirksregierung, Münster), and approved by the 'Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen'. Twenty-seven male mice aged between eight and 14 weeks (C57Bl6/J; Charles River, Germany) were maintained under standard housing conditions (12:12 h light:dark cycle with lights on at 06:00 h, ~21°C temperature, 50–60% relative humidity), with ad libitum access to water and food.

Drug preparation and dosage

DAMGO (Cat No 1171; Tocris-Biotechnne, Germany) was dissolved in 0.9% saline solution, in a stock concentration of 2 mM. For injections, the stock was freshly diluted to 80 μ M in saline. CTAP (Cat. No. 1560; Tocris-Biotechnne, Germany) was diluted in 0.9% saline to 500 μ M. Aliquots were kept frozen and thawed freshly before use. The final amount of substance injected into the dMT of the animals was 40 pmol for DAMGO and 250 pmol for CTAP, both delivered in 500 nL saline solution. DAMGO and CTAP doses were adapted from McNally and Cole (2006) and Meyer et al. (2007), respectively.

Surgical procedures

Animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally) after isoflurane induction (2.5% in O₂; CP-Pharma, Germany). One animal, whose cannula was misplaced, was excluded from the analysis. After reaching surgical tolerance, pain was ameliorated with carprofen (Rimadyl: 10 mg/kg subcutaneously (s.c.); Zoetis, Germany) and a local anaesthetic was applied to the incision site on the head (xylocaine 2% s.c., 0.1 mL; AstraZeneca, Germany). The animals were placed in a stereotactic device (Model 692; Kopf, Germany) and a single guiding cannula (26GA 4 mm length; PlasticsOne, VA, USA) was implanted into the PVT (anteroposterior: -0.7 mm; mediolateral: +0.3 mm; dorsoventral: +3.0 mm, angle: +5°). Acrylic cement (Pulpdent; Glasslute, MA, USA) was used to fix the cannula to the skull. The body temperature of the animals was measured using a rectal

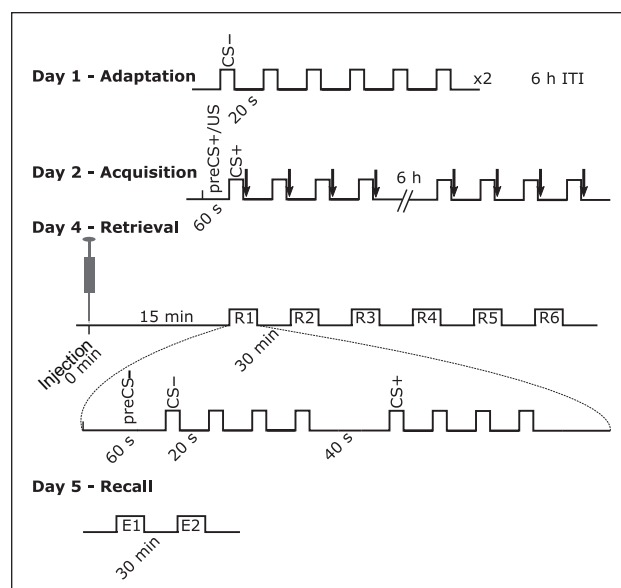


Figure 1. Timeline of the experiments. Mice were subjected to a classical (Pavlovian) fear conditioning paradigm. Conditioned stimulus – (CS⁻) consisted of a tone of 2.5 kHz (80 dB intensity; 10 s) and conditioned stimulus + (CS⁺) consisted of a tone of 10 kHz (85 dB intensity; 10 s). The unconditioned stimulus (US; black arrows) was a foot shock of 1 s duration (0.4 mA) co-terminating with the CS⁺ on day 2. μ -opioid receptor antagonist (CTAP), agonist (DAMGO) or saline (Control) was administered in a single dose on day 4, 15 min before the first R1 session started. Each R (or E) session consisted of four CS⁻ and four CS⁺ presentations regularly spaced as described. CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO: [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin; ITI: inter-train interval

probe and maintained at 37°C throughout the procedure. Behavioural experiments were performed after 7 days of post-surgical recovery.

Fear conditioning

The mice were subjected to classical (Pavlovian) fear conditioning as previously described, with some modifications (Figure 1; Jüngling et al., 2008). On day 1 (adaptation phase), the animals were placed in the conditioning arena (24.5 cm \times 19.5 cm chamber; FearConditioning System; TSE Systems; Germany) and two trains (inter-train interval (ITI), 6 h) of six neutral tones were delivered (CS⁻; sine tone 2.5 kHz, 85 dB, 10 s). On day 2 (acquisition phase), two trains (ITI 6 h) of four conditioning tones were delivered (CS⁺; sine tone 10 kHz, 85 dB, 10 s), each of which co-terminated with a 0.4 mA scrambled electric foot-shock (unconditioned stimulus (US)). During ITIs animals were kept in their home cage.

On day 4 (retrieval phase), the animals received a 500 nL injection of either DAMGO, CTAP or saline (Control) into the dMT. The animals were randomly assigned to one of the three groups. The injections were performed under isoflurane anaesthesia (1.5% in O₂) and the substances were administered at a flow rate of 0.1 μ L/min (CR700-20; Hamilton Company, NV, USA) through a cannula (33GA 5 mm length; PlasticsOne, VA, USA). Fifteen minutes after administration, animals were

exposed to six sessions (R1 to R6; ITI 30 min) of tone presentations in a new context (31 cm × 15 cm chamber). Each session consisted of four CS– tones to evaluate potential fear generalization effects followed by four CS+ tone presentations. On day 5 (recall phase), another two sessions of tone presentation were delivered (E1 and E2; ITI 30 min) in the same context as on day 4. All the behavioural sessions were video recorded and analysed offline.

After the behavioural experiments, animals were sedated with isoflurane (2.5% in O₂; CP-Pharma, Germany) and killed by decapitation and 500 nL of methylene blue (4 mg/mL) were administered using the same cannula system as on day 4. The brains were immediately extracted, frozen and cut into 50 µm thick coronal slices on a cryostat (CM3050S; Leica, Germany). Images were taken using a transmitted light microscope (Zeiss Axioscope; Zeiss, Germany). The *Allen Mouse Reference Atlas* served as reference to assess cannula placements (Figure 2(a); Allen Institute for Brain Science, 2011). In addition, histological images were compared with the Paxinos atlas (Paxinos and Franklin, 2001) to assess the anteroposterior distance of the tip to bregma, based on the relative size of adjacent brain structures in the atlas (i.e. ventricles and fimbria).

Behavioural analysis

Mouse behaviour was video-recorded and videos were analysed using the Solomon coder software (v.17.03.22). Freezing was used as readout for passive fear-related behaviour and was defined as 'body immobility and the absence of vibrissae movement associated with sniffing' (Bouton and Bolles, 1980), excluding physiologic respiration, with a minimum duration of 1 s. In the retrieval sessions on days 4 and 5, behavioural reactions were evaluated during 1 min before the first tone presentation (preCS–) and during the tone presentations (CS– and CS+). In addition to freezing, a more elaborate behavioural profile was scored, which included the estimation of six other behavioural parameters: rattling was defined as brief and fast tail movements; flight as fast active escape movements; grooming as all movements involving self-care licking; rearing as the detachment of the forepaws from the floor; exploring as the movement of the animal aiming at the surroundings, including only head movements; and quiet as the lack of movement or sleep, without any reaction to the tone. These behavioural categories were adapted from previous work (Remmes et al., 2016).

We further evaluated the average velocity of the animals during preCS–, CS+ and CS– using Ethovision software (v.14.0; Noldus Information Technology, USA). This parameter was used to evaluate active coping strategies (Pliota et al., 2018).

Statistical procedures

All statistical procedures were implemented in either GraphPad Prism software (v.6.07; GraphPad Software, CA, USA) or R software (v.3.5.1) implemented in RStudio (v.1.0.456).

To evaluate the passive coping strategies, the percentage of time freezing was used. During acquisition, freezing during single CS/US presentations was evaluated. During retrieval (R, day 4) and recall sessions (E, day 5), the four CS+ and the four CS– presentations were averaged for each R and E session. Data were

evaluated using a two-way repeated measures analysis of variance. If only the main effect of time was detected, treatments were pooled and differences over time were evaluated with a Dunnett's post-hoc test. When significant interaction was present, two different Dunnett's post-hoc tests were performed: a time course analysis, to evaluate changes with respect to values in R1 in each treatment, and a treatment comparison, to evaluate differences with respect to Control at a given time. When many *p*-values are reported at the same time, the most conservative value is given (i.e. the smallest for non-significant values and the highest for significant values). PreCS– behavioural measurements were used to verify standard baseline conditions in the animals, but excluded from the global statistical analysis.

To evaluate the active coping strategies, mean velocity during the tone presentations was used. During the acquisition average velocities for each tone were scored. The average velocity 1 min before the first CS+/US event (preCS+/US) was scored to define the basal locomotor state, but excluded from the statistical analysis. During retrieval and recall sessions, in preCS– time, the average velocity during four CS– and four CS+ presentations was measured and averaged for each R and E session. Analogous statistical testing as for the freezing behaviour was performed.

In order to extract an overall behavioural profile for each animal, its freezing behaviour and six additional behavioural expressions were evaluated in a CoDA. The seven behavioural features were grouped into three categories: the non-aversive category included rearing, grooming and quiet periods; the neutral category included exploring; and the aversive category combined freezing, flight and rattling. The compositional nature of the data is reflected in the way that all seven behavioural features add up to a constant value for each animal and session. To deal with zero values, they were replaced with the minimal amount of scorable freezing in the Solomon Coder (0.2 seconds), and an isometric log-ratio (ilr) transformation was applied to the three behavioural categories (Egozcue et al., 2003; Supplementary material, Material and methods online) with the *compositions* R package (v.1.40-2). ilr transformation can be understood as a centred log-ratio (clr) transformation followed by an isometric rotation (Supplementary Material and methods, Figure S4(c) to (e)). In a spatial representation, clr transformation shifts the origin of the coordinate system into the centre of the plane with a logarithmic transformation (Supplementary Figure S4(d)). Following isometric rotation constrains the representation of the data to a 2D plane, keeping the distances between the data points (Supplementary Figure S4(e)). Thereafter multivariate analysis of variance with time session as a within factor, and Hotelling's comparison corrected by Bonferroni method were performed.

Results

Activation of the μ -opioid system in the dMT modulates passive behaviour

As previous work has demonstrated that the dMT participates in fear memory retrieval (Padilla-Coreano et al., 2012), we evaluated whether MORs in the dMT alter freezing behaviour in our auditory fear conditioning paradigm. To do so, we divided mice into three treatment groups (DAMGO, CTAP, Control; Figures 1 and 2). To assess passive behavioural coping strategies associated with fear expression of the animals, we analysed freezing

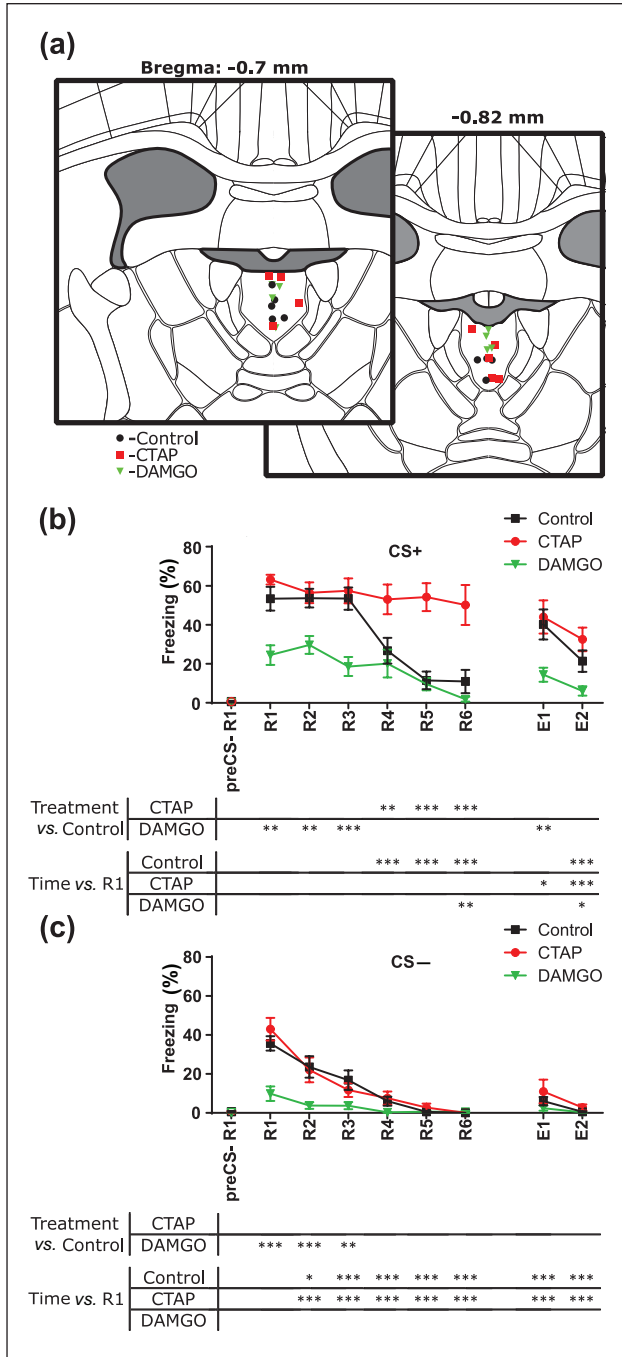


Figure 2. μ -opioid receptors in the dorsal midline thalamus modulate freezing behaviour of mice in a Pavlovian fear conditioning paradigm. (a) Schematic representation of the cannula tip positions. Image credit: Allen Institute. Mean \pm SEM of time spent freezing on the conditioned stimulus + (CS+) (b) and conditioned stimulus - (CS-) (c) on day 4 and day 5 for each animal. Asterisks represent significance with respect to time or treatment. Repeated measures two-way analysis of variance and Dunnett post-hoc test.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO: [D-Ala₂, NMe-Phe₄, Gly-ol₅]-enkephalin; E: recall; R: retrieval

behaviour during successive presentations of neutral (CS-) and aversive (CS+) conditioned stimuli. The randomly allocated DAMGO, CTAP and Control mice learned successfully during the conditioning sessions on day 2 (main effect of time: $F_{(7, 168)} = 7.2845$; $p < 0.001$; CS+/US1 vs. CS+/US2 to CS+/US8: $p_{\text{adj}} < 0.001$; Supplementary Figure S1(a)). The three groups did not differ in fear acquisition (main effect of treatment: $F_{(2, 24)} = 0.8389$; $p = 0.445$; interaction: $F_{(14, 168)} = 0.6768$; $p = 0.7948$; Supplementary Figure S1(a)). During fear memory retrieval and extinction, Control mice showed successful fear memory extinction for the CS+ from R4 on (interaction: $F_{(14, 168)} = 3.412$; $p < 0.001$; R1 vs. R4 to R6: $p_{\text{adj}} < 0.001$; R1 vs. E2: $p_{\text{adj}} < 0.001$; Figure 2(b)). In contrast, freezing behaviour decreased after R6 in DAMGO-treated mice (R6 vs. R1: $p_{\text{adj}} = 0.007$; R1 vs. E2: $p_{\text{adj}} = 0.042$) and after E1 in CTAP-treated mice (R1 vs. E1: $p_{\text{adj}} = 0.031$; R1 vs. E2: $p_{\text{adj}} < 0.001$). When we compared treatments within sessions, freezing to the CS+ remained elevated in CTAP-treated mice as compared with Control in sessions R4 to R6 (R4: Control vs. CTAP: $p_{\text{adj}} = 0.004$; R5: $p_{\text{adj}} < 0.0001$; R6: $p_{\text{adj}} < 0.0001$). DAMGO-treated animals showed significantly less freezing from R1 to R3 (Control vs. DAMGO: R1: $p_{\text{adj}} = 0.001$; R2: $p_{\text{adj}} = 0.009$; R3: $p_{\text{adj}} < 0.001$), as well as in E1 (Control vs. DAMGO: E1: $p_{\text{adj}} = 0.005$).

For the CS-, Control and CTAP-treated animals showed significantly less freezing from R2 on with respect to R1 (interaction: $F_{(14, 168)} = 5.127$; $p < 0.001$; $p_{\text{adj}} < 0.014$; Figure 2(c)). In contrast, in DAMGO-treated mice displayed low freezing values from R1 on ($p_{\text{adj}} > 0.081$). Treatment comparison within session suggests that DAMGO treatment significantly decreased freezing in R1, R2 and R3 (Control vs. DAMGO: R1: $p_{\text{adj}} < 0.001$; R2: $p_{\text{adj}} < 0.001$; R3: $p_{\text{adj}} = 0.008$). These results showed that DAMGO induced overall low freezing values, whereas CTAP-treated mice showed high freezing levels during all sessions on day 4 (R1 to R6).

Effects of CTAP and DAMGO in the dMT on active behaviour

DAMGO-treated animals showed strongly reduced freezing during retrieval and extinction of conditioned fear. However, such a decrement in freezing does not necessarily reflect an attenuated fear response, since animals might simply show more activity or express more active coping strategies (i.e. fast evasive moving as flight response) under a potentially equivalent fear state.

Before testing for a potential influence of CTAP or DAMGO on locomotor activity during CS presentations, we first evaluated the basal locomotor activity of the animals and analysed their average velocity during the acquisition and retrieval before tone presentations (preCS-). No differences were found in the locomotor activity during the preCS+/US time ($F_{(2, 24)} = 2.868$; $p = 0.076$). During acquisition, velocity decreased compared with CS+/US1 presentation (main effect of time: $F_{(7, 168)} = 3.346$; $p = 0.002$; Supplementary Figure S1(b)) in CS+/US2 ($p_{\text{adj}} < 0.001$), CS+/US4 ($p_{\text{adj}} = 0.023$) and CS+/US6 ($p_{\text{adj}} = 0.038$) without differences between treatments (main effect of treatment: $F_{(2, 24)} = 1.178$; $p = 0.325$; interaction: $F_{(14, 168)} = 0.436$; $p = 0.961$). In retrieval and recall, Control and CTAP-treated mice did not change the average speed with respect to R1 during the preCS- (interaction: $F_{(14, 168)} = 7.783$; $p < 0.001$; R1 vs. R2 to

E2: $p_{\text{adj}} > 0.0810$; Figure 3(a)). In contrast, DAMGO increased preCS- activity in R2 (R1 vs. R2: $p_{\text{adj}} = 0.002$) and decreased activity from R4 on (R1 vs. R4 to E2: $p_{\text{adj}} < 0.001$). When comparing treatments within sessions, DAMGO-treated animals significantly increased their average speed in the R1 to R3 sessions during the preCS- (Control vs. DAMGO; R1 and R2: $p_{\text{adj}} < 0.001$; R3: $p_{\text{adj}} = 0.006$). These results suggest that a DAMGO injection in the dMT before R1 induced high locomotor activity in mice.

Once the basal levels of activity were assessed, we evaluated changes in active coping strategies (Pliota et al., 2018). Neither Control nor CTAP-treated mice changed their active coping strategy with respect to R1 during CS+ presentations (interaction: $F_{(14, 168)} = 7.202$; $p < 0.001$; $p_{\text{adj}} > 0.291$; Figure 3(b)) or with respect to Control values ($p_{\text{adj}} > 0.808$). Compared with Control, DAMGO increased the velocity of mice from R1 to R5 sessions (Control vs. DAMGO: R1, R2 and R3: $p_{\text{adj}} < 0.001$; R4: $p_{\text{adj}} = 0.001$; R5: $p_{\text{adj}} = 0.036$). In contrast, DAMGO-treated mice decreased their average speed with respect to R1 from R3 on (R1 vs. R3: $p_{\text{adj}} = 0.014$; R1 vs. R4 to E2: $p_{\text{adj}} < 0.001$).

During the CS- presentations, similar results were obtained. The activity of animals in the CTAP and Control groups did not change over time (interaction: $F_{(14, 168)} = 5.641$; $p < 0.001$; $p_{\text{adj}} > 0.178$; Figure 3(c)) and was indistinguishable from each other within sessions ($p_{\text{adj}} > 0.300$). However, compared with Control, DAMGO increased the activity, from R1 to R4 (Control vs. DAMGO: R1 and R2: $p_{\text{adj}} < 0.001$; R3 and R4: $p_{\text{adj}} = 0.001$), whereas they showed decreased activity with respect to R1 from R4 on (R1 vs. R4: $p_{\text{adj}} = 0.001$; R1 vs. R5 to E2: $p_{\text{adj}} < 0.001$).

Overall, these results suggest that CTAP did not change the active coping strategy of mice. Interestingly, DAMGO induced hyperlocomotion that interferes with the passive and active coping behaviour during fear retrieval and extinction learning. However, the DAMGO-induced hyperlocomotion was not detected from R4 on and velocities were indistinguishable between the treatment groups in E1 and E2 sessions. Thus, active and passive coping strategies for the DAMGO-treated mice can be evaluated in E1 and E2, indicating that DAMGO decreased freezing in these late sessions.

The μ -opioid system in the dMT affects the behavioural profile in mice

We extended the analysis to include a behavioural profile based on seven features chosen a priori (Supplementary Figures S2, S3), to assess possible effects on coping strategies. These behavioural traits were classified in three categories (aversive, neutral and non-aversive). The behaviour of each animal was characterized by the time spent in each of the three categories, and an IRT transformation was applied (Supplementary Material and methods; Figure S4). During the preCS-, behaviour did not significantly differ between CTAP-treated, DAMGO-treated and Control mice ($F_{(4, 48)} = 1.7238$; $p = 0.1602$). During the CS+, Control and DAMGO-treated animals changed their behaviour (interaction: $F_{(28, 336)} = 2.394$; $p < 0.001$; Figure 4(a)). Compared with R1, the Control mice became significantly more non-aversive in R5 ($p_{\text{adj}} < 0.001$), R6 ($p_{\text{adj}} = 0.023$) and E2 ($p_{\text{adj}} < 0.001$) and DAMGO-treated animals in R6 ($p_{\text{adj}} = 0.002$) and E2 ($p_{\text{adj}} < 0.001$). In contrast, the CTAP-treated mice failed to adapt the behaviour across the sessions ($p_{\text{adj}} > 0.084$) and remained in the

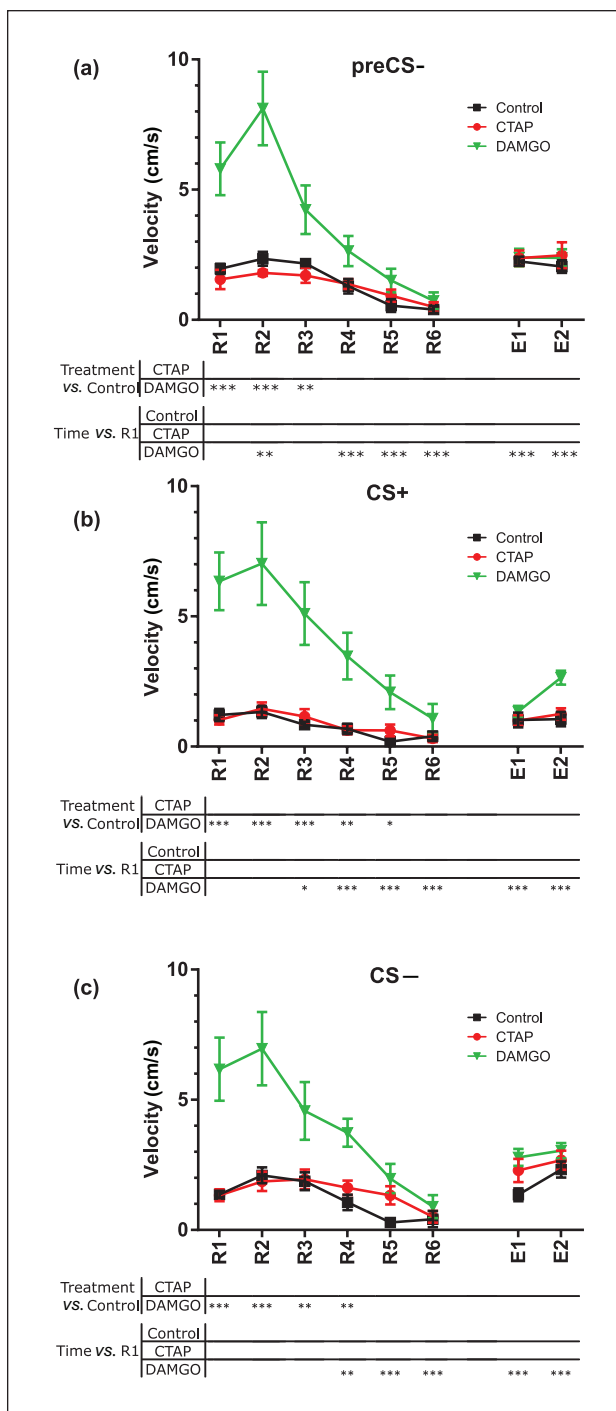


Figure 3. Effects of μ -opioid receptors in the dorsal midline thalamus on the active coping strategy during fear memory retrieval and recall. (a) Mean \pm SEM of the average speed of each animal during the 60 s before the first tone was delivered at each R and E session. (b, c) Mean \pm SEM of the active coping performance during the tone presentations in the conditioned stimulus + (CS+) (b) and conditioned stimulus - (CS-) (c) presentations. Repeated measures two-way analysis of variance and Dunnett post-hoc test.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO: [D-Ala₂, NMe-Phe₄, Gly-ol₅]-enkephalin; E: recall; R: retrieval

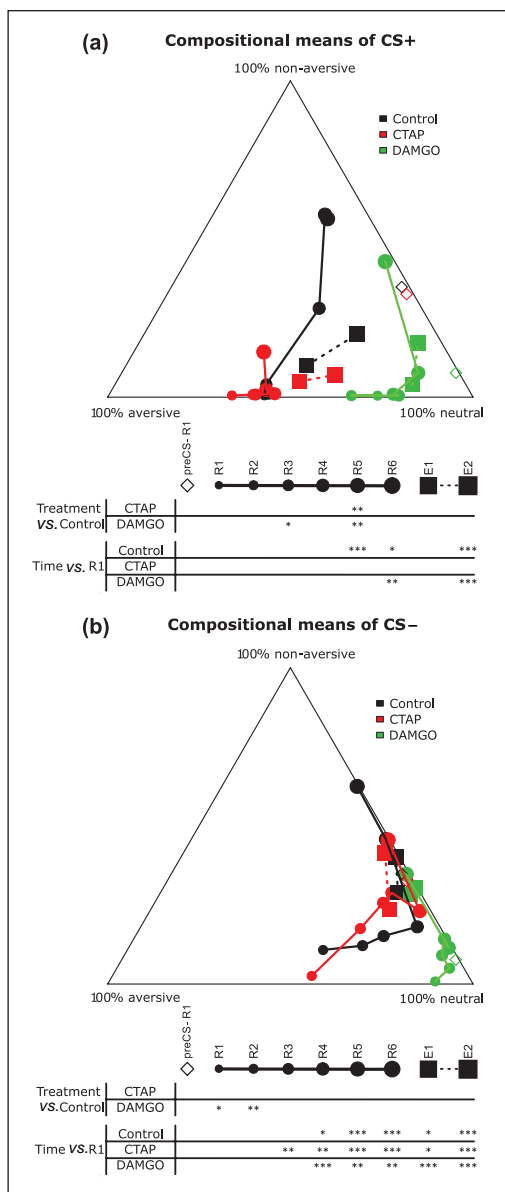


Figure 4. Compositional analysis of the seven feature based behavioural profiles for the CTAP, DAMGO and Control groups, during fear memory retrieval and recall. Compositional plots of the mean behaviour exhibited by each group across extinction sessions in conditioned stimulus + (CS+) (a) and conditioned stimulus - (CS-) (b). The proximity to each corner (aversive, neutral and non-aversive) indicates the percentage of time spent showing the corresponding behaviour. Asterisks represent significance respect time or treatment. Repeated measures two-way multivariate analysis of variance, with Hotelling's test (corrected by Bonferroni method) for pairwise comparison.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO: [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin; E: recall; R: retrieval

aversive state until the end of the experimental paradigm. Comparing treatments within sessions, DAMGO changed behaviour in R3 ($p_{adj} = 0.021$) and R5 ($p_{adj} = 0.003$) towards the

neutral category, whereas behaviour in CTAP remained aversive in R5 with respect to Control mice ($p_{adj} = 0.001$).

During CS- presentations, all treatments induced behavioural changes (interaction: $F_{(28, 336)} = 1.897$; $p = 0.005$; Figure 4(b)). CTAP-treated mice changed from neutral to non-aversive behaviours ($p_{adj}s < 0.025$), whereas for DAMGO-treated and Control animals, changes became significant from R4 on ($p_{adj}s < 0.034$), first switching from mildly aversive to neutral and later to non-aversive behaviours. Comparing treatments within sessions, DAMGO induced a shift to neutral behaviour in R1 ($p_{adj} = 0.049$) and R2 ($p_{adj} = 0.024$). These results suggest that CTAP kept the behavioural reaction to the CS+ tone invariable, not allowing behavioural changes along the multiple tone presentations.

Discussion

In the present work, we analysed the role of the μ -opioid system in the dMT in fear memory retrieval and extinction. Pharmacological blockage of MORs in the dMT impaired fear extinction and kept the behaviour in an aversive passive state. In contrast, local MOR activation by a single dose of DAMGO induced a transient increase in locomotion during fear extinction, and, more importantly, decreased freezing during recall of extinction, which was tested 24h later when direct DAMGO effects were excluded.

The blockage of the μ -opioid system by CTAP in the dMT reveals its involvement in fear extinction processes. Previous work has shown that the dMT is implicated in fear memory retrieval (Do-Monte et al., 2015; Li et al., 2014; Padilla-Coreano et al., 2012; Penzo et al., 2015), likely due to its glutamatergic projections to fear-relevant structures such as the medial prefrontal cortex and the amygdala (Haight and Flagel, 2014). Projections from the PVT to the basolateral amygdala (BLA) are, in fact, modulated by MORs in the PVT (Goedecke et al., 2019). Given that MOR activation induces a membrane hyperpolarization in neurons of the dMT (Brunton and Charpak, 1998; Goedecke et al., 2019), CTAP may facilitate neuronal activity in dMT to BLA projections. As PVT inactivation causes a fear memory retrieval deficit (Padilla-Coreano et al., 2012), CTAP should increase fear memory retrieval during R1, but instead, we detected a prominent fear extinction deficit. In line with this, local blockage of the μ -opioid system in other brain structures, such as the vIPAG (McNally et al., 2005), or systemic inhibition of the opioid system by naloxone (Kim and Richardson, 2009) affects fear extinction rather than expression. We conclude that the MOR system in the dMT participates in the fear extinction task.

In auditory Pavlovian fear conditioning, freezing is the most widely studied behavioural response in rodents. Of note, freezing is a passive expression of fear, and there are indicators of rather active coping strategies such as locomotor activity (Pliota et al., 2018). Active coping strategies during stressful events might prevent passive maladaptive responses in fear extinction paradigms (Fucich et al., 2016), which should be considered in behavioural analyses of fear. However, DAMGO application in the dMT resulted in an increase in locomotor activity, already present under baseline conditions before tone presentation, which can be assumed to interfere with active or passive behavioural response components. The persistence of this hyperlocomotion can be evaluated with the preCS- activity and seems to be relevant at least until the R4 session, and may affect the retrieval sessions

during the retrieval day. Therefore, changes in behaviour induced by DAMGO before that time point cannot be interpreted appropriately. Opioids have been previously described to induce hyperlocomotion in rodents (Vanderschuren et al., 1997), and these effects have been linked to the activation of the mesolimbic reward pathway. As DAMGO decreases the neural activity of the PVT *in vitro* (Goedecke et al., 2019), hyperlocomotion might be associated with a low activity of the dMT. However, there is no hyperlocomotion effect reported after PVT lesion (Li et al., 2014) or pharmacological application of muscimol, a GABA-A receptor agonist (Padilla-Coreano et al., 2012). Some other authors even reported a hypolocomotional effect after the injection of the GABA-A receptor and GABA-B receptor agonists muscimol and baclofen into the PVT (Barson and Leibowitz, 2015). Therefore, DAMGO-induced hyperlocomotion cannot be attributed to a simple decrease of neural activity in the PVT. Rather, a specific neuronal subpopulation expressing MOR may lead these effects.

DAMGO shows a short half-life *in vivo* (~15 min (Szeto et al., 2001)). As a consequence, DAMGO-induced hyperlocomotion is absent on day 5 and the reduced freezing behaviour on that day cannot be masked directly by the hyperlocomotion. Interestingly, other hyperlocomotion-inducing stimulants, such as amphetamine, did not facilitate fear memory extinction consolidation on day 5 (Mueller et al., 2009). Therefore, although we cannot fully exclude that in our hands the hyperlocomotion itself is triggering the fear memory extinction consolidation in day 5, this is likely not the case. Despite this, the hyperlocomotion present on day 4 impedes a proper fear memory retrieval analysis, raising the question of whether DAMGO was able to erase the fear memory or facilitate the extinction. Some evidence points out that opioids might be able to erase fear and pain memory traces (Sandkühler and Lee, 2013). However, this is unlikely in our experimental conditions. Memory erasure procedures typically involve experimental designs affecting memory consolidation some hours (6h) after the memory acquisition, which is far from the time steps used in this work (~36h from the last acquisition trial), where the consolidation procedures have presumably finished. We, rather, suggest that the activation of the MOR in the dMT during the CS re-exposure facilitates fear extinction consolidation. This effect is likely associated with the inactivation of the dMT, as the optogenetic inhibition of the PVT to central amygdala pathway has similar effects in fear extinction consolidation (Do-Monte et al., 2015). In fact, projections from the PVT to the central amygdala might mediate the effects on freezing behaviour (Do-Monte et al., 2015; Penzo et al., 2015), which can be influenced by DAMGO treatment (Goedecke et al., 2019). Our results are, therefore, in line with previous findings, and suggest that the activation of MORs in the dMT influences fear extinction consolidation.

Finally, to evaluate changes in the behavioural pattern of the animals along the extinction sessions, we implemented a behavioural analysis that completes the aforementioned freezing with other complementary behavioural traits. As the values tend to sum to a constant value, data require special statistical handling known as CoDA (Aitchison, 1982). With this we were able to avoid spurious correlations in the statistical analysis.

Through the presentation of the successive tones, control animals showed aversive behaviours at the beginning of the day 4, developing towards neutral behaviour, and finally ended being mostly non-aversive. The behaviour of all the groups rapidly

changed in the case of the CS- presentation, but for the CS+, CTAP-treated mice remained in an aversive state. DAMGO-treated mice showed predominantly a neutral behaviour, yet they were still able to adapt their behaviour. The dMT is involved in behavioural flexibility in tasks where rodents have to adapt their behaviour to rewarding cues changing over time (Marton et al., 2018). Our results show that specifically MORs in the dMT participate in the elaboration of the behaviour, which, in turn, might influence the fear status in later sessions.

The following points have to be considered when interpreting our data. First, as only males were used, results might not be directly generalized to female subjects. Second, while correct placement of cannulas was documented and confirmed to be in the PVT, drug effects in adjacent regions like the paratenial, the mediodorsal or centromedial nucleus cannot be excluded completely, due to the small nature of this thalamic structure. Therefore, injections are expected to affect mainly PVT, with partial involvement of other adjacent dMT structures. Finally, in our experimental conditions we detected a slight increase in CS-freezing in contrast to previous results in our group (see, for instance, Chauveau et al., 2012). We consider this a consequence of delaying the retrieval one day more with respect to our previous experiments. This delay was necessary to ensure the recruitment of the PVT into the fear memory circuit (Do-Monte et al., 2015; Padilla-Coreano et al., 2012). As it has been proposed that contextual fear memory generalization increases when a delay between acquisition and retrieval is present (Wiltgen and Silva, 2007), we suggest that cued fear generalization is also affected when retrieval sessions are delayed.

Our results suggest that MORs in the dMT are a potential target for pharmacological interventions in fear- and anxiety-related disorders. Indeed, human studies have shown lower incidence of post-traumatic stress disorder (PTSD) in patients that have received morphine after a traumatic event (Bali et al., 2015; Bryant et al., 2009; Holbrook et al., 2010). In animal studies, the intervention with morphine in a PTSD model facilitates fear memory extinction (Szczytkowski-Thomson et al., 2013), indicating that a local access to the dMT is not necessary to make opioid treatments effective. In light of the addictive potential and possible misuse of medication targeting MORs, alternative pharmacological tools are studied that aim to increase endogenous opioid levels. In fact, local application of an enkefalinase inhibitor in the rodent vIPAG facilitated fear extinction (McNally, 2005), as MORs in this brain structure participate in fear extinction processes (McNally et al., 2005). These results show that pharmacologically targeting the opioid system may benefit the treatment of anxiety spectrum disorders.

Using pharmacological interventions in combination with detailed behavioural analysis, we show the involvement of the μ -opioid system in fear extinction. Extinction learning following cued Pavlovian fear conditioning in rodents can be considered a model of exposure therapy in phobic patients, where the subjects are exposed to the phobogenic object in a controlled manner. Interestingly, application of opioid antagonists during exposure therapy has been shown to decrease the tolerance to the exposure agent (Kozak et al., 2007; Merluzzi et al., 1991). Taken together, we suggest that the μ -opioid system in the dMT participates in the anxiolytic effects of the systemic application of μ -opioid agonists in humans (Bali et al., 2015; Bryant et al., 2009; Holbrook et al., 2010) and rodents (Szczytkowski-Thomson et al., 2013).

Author contribution

XB performed the experiments, the statistical analysis. LG, PB and HCP provided expert opinion, reviewed and approved the final manuscript. XB wrote the paper, with contributions by KJ and HCP. KJ supervised the study, reviewed and approved the final manuscript.

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Supplemental material

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