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Activation of MORs in the VTA induces changes on cFos expression in different projecting regions: Effect of inflammatory pain

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ABSTRACT

Chronic pain is a worldwide major health problem and many pain-suffering patients are under opioid based therapy. Epidemiological data show that pain intensity correlates with the risk of misuse of prescription opioids, and other drugs of abuse including alcohol. This increased vulnerability to suffer Substance Use Disorders could be, in part, caused by functional changes that occur over the mesocorticolimbic system, a brain pathway involved in reward processing and addiction. Previous data in rats revealed that inflammatory pain desensitizes mu opioid receptors (MORs) in the ventral tegmental area (VTA). As a consequence, pain alters dopamine release in the nucleus accumbens (NAc) derived from MOR activation in the VTA and also increases intake of high doses of heroine. Given that the VTA neurons target different brain regions, in the present study we first analyzed changes induced by inflammatory pain in the MOR dependent activation pattern of the main VTA projecting areas. To do that, we administered two doses (7 or 14 ng) of DAMGO (MORs agonist) or artificial cerebrospinal fluid (aCSF) focally into the VTA of rats and measured the activation in projection areas by cFos immunohistochemistry. Our results show that focal injections of DAMGO in the VTA increases cFos expression in the majority of its projecting areas, namely NAc, basolateral amygdala (BLA), cingulate cortex (ACC) and bed nucleus of the stria terminalis (BNST), as compared to aCSF. Second, we analyzed whether inflammatory pain would affect to cFos expression using a group of rats injected with CFA in the hind paw. In this case, we found that cFos expression was not significantly different between DAMGO and aCSF administered rats in BLA, ACC and BNST. Our results confirm that inflammatory pain induces desensitization of VTA MORs in a region dependent manner which can be very relevant for addictive behaviours.

1. Introduction

Chronic pain is a worldwide major health problem, affecting up to 30% of the population in the United States (Volkow et al., 2018). Much of the prescriptions for pain-suffering patients are based in opioid prescriptions, which can ultimately drive to tolerance, overdose or even addiction (Volkow et al., 2018). New evidences as to how pain alters opioid reward are needed to pave new avenues for designing better and safer therapeutic schemes.

The activation of mu opioid receptors (MORs) located in the ventral tegmental area (VTA) increases dopamine release in its projecting regions, a mechanism that has been traditionally implicated in the reinforcing properties of drugs, especially in the case of opioids (Xiao and Ye, 2008). Pain induces plastic changes in the mesocorticolimbic system that might underlie the increased vulnerability to suffer addiction. Thus, previous studies have shown that the presence of inflammatory pain decreases MORs function in the VTA leading to (i) a dose-dependent alteration of dopamine release in the nucleus accumbens (NAc) and, (ii) an increased intake of high doses of heroin in rats, presumably as a consequence of the VTA MORs desensitization (Hipolito et al., 2015). The VTA sends dense projections to the NAc and to the prefrontal cortex, as well as to other brain areas such as the ventral pallidum (VP), the basolateral amygdala (BLA), and the bed nucleus of the stria terminalis (BNTS) (Badiani et al., 2011; Lammel et al., 2014). Given this widespread pattern of efferences, it is likely that the activation of MORs in the VTA could have different consequences in the target regions. Indeed, to our knowledge few studies have analyzed systematically the effect of intra-VTA administration of different doses

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of DAMGO, a MORs agonist, on neuronal activity in its projection areas. Additionally, we hypothesize that inflammatory pain might induce a region-dependent decrease of the neural activity induced by intra-VTA DAMGO in the different brain areas targeted by VTA neurons. Therefore, in the present study we first analyze the MOR dependent activation pattern, as measured by cFos induction, of the main VTA projecting areas and, as a first approach, the changes induced by inflammatory pain in this pattern.

2. Materials and methods

2.1. Animals

We used 34 male Wistar rats (300–340 g) housed in plastic cages ($48 \times 38 \times 21\ cm^3$) with controlled humidity and temperature (22 °C), a 12:12-h light/dark cycle and free access to food and water. All the procedures were carried out in accordance with Spanish laws (RD 53/2013) and were approved by the Animal Care Committee of the University of Valencia and Regional Government.

2.2. Chemicals

The MOR agonist DAMGO (D-Ala2, N-MePhe4, Glyol5-enkephalin, Sigma Chemical Co) powder was dissolved in distilled water to obtain a 1 mM solution. This stock solution was kept frozen at −40 °C as aliquots until use. Prior to use, aliquots were conveniently diluted with artificial cerebrospinal fluid (aCSF) to the appropriate concentration (Hipolito et al., 2015). All the other reagents used were of the highest commercially available grade.

2.3. Experimental design

This study was structured in two consecutive experiments. In experiment 1 we evaluated the effect of the activation of VTA MORs on cFos expression in projection areas. All animals included in experiment 1 were pain-free and they were divided into three groups depending on the intra-VTA treatment: aCSF (n = 6), 7 ng of DAMGO (n = 5) and 14 ng of DAMGO (n = 6). In experiment 2, we tested the impact of inflammatory pain on the effect of DAMGO injected intra-VTA on cFos expression in projection areas. All animals included in this experiment developed an inflammatory pain condition (see 2.4) and they were divided into three groups depending on the intra-VTA treatment: aCSF (n = 6), 7 ng of DAMGO (n = 5) and 14 ng of DAMGO (n = 6).

2.4. Surgery and inflammatory pain model

Surgeries were performed under ketamine/xylazine (80 mg/kg and 10 mg/kg, respectively, ip) anesthesia. Rats were fixed in a stereotaxic apparatus (Stoelting) and implanted with a cannula (26G, Plastics One) aiming at the posterior VTA anteroposterior = −6.0 mm, mediolateral = 1.9 mm, dorsoventral = −8.1 mm, angled 10° from perpendicular axes (Paxinos and Watson, 2007; Hipolito et al., 2015). Cannula was implanted in a counterbalanced fashion for hemisphere.

We selected the complete Freund adjuvant model (CFA) of inflammatory pain. CFA (Calbiochem) was prepared and injected as previously reported (Hipolito et al., 2015). The injection of CFA in the hind paw was performed at the same time of the surgery. To control the inflammation produced by the CFA, dorsoventral measurements of both hind paw of the animals were obtained right before the perfusion of the animals (Fig. 1C).

2.5. Drug microinjection procedures

Intra-VTA drug microinjections were carried out 48 h after the surgery with 33G stainless steel injectors, extending 1.0 mm below the tip of the cannulae. Microinjections were carried out as previously described (200 nL, flow rate of 0.6 mL/min) (Sánchez-Catalán et al., 2009).

2.6. Immunohistochemistry

90 min after the drug microinjection, animals were deeply anaesthetized with isoflurane and transcardially perfused with 200 mL of PBS followed by 300 mL of 4% formaldehyde in PB 0.1M. 40 mm sections were obtained as described in (Zornoza et al., 2005).

Sections selected were transferred to TBS and sequentially incubated (including TBS rising between incubations) in: (1) 1% hydrogen peroxide in TBS, (2) 5% goat serum in TBS-0.3%TX, (3) anti-cFos polyclonal antibody (1:1000, Santa Cruz) overnight at 4 °C, (4) biotinylated anti-rabbit antibody (1:200; Vector Labs), (5) avidin–biotinylated peroxidase complex (1:200; ABC Elite Kit; Vector Labs). Finally, the reaction was visualized by incubating with diaminobenzidine (SigmaFAST, Sigma). Finally, sections were mounted on slides, dehydrated in alcohols, cleared and coverslipped for microscopical examination.

2.7. Image analysis

Quantification of the cFos immunoreactive cells (cFos-IR) was performed in the following brain regions: NAc, BLA, cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL), BNST and VP (Paxinos and Watson, 2007). Two sections per each animal and area were selected and images were digitalized by using a microscope (Leica) equipped with a CCD camera (representative pictures in Fig. 1A). The 10x objective was selected to obtain frames of 1026 x 769 mm and the counting of the stained nuclei per frame was carried out using the Multipoint plugin of the software Image J (NIH). The experimenter was blind to experimental grouping throughout images acquisition and processing.

Additionally, sections of the VTA stained with cresyl violet were used for the verification of the cannulae placement. A researcher unaware of the experimental group of the animals inspected them using optical microscopy.

2.8. Statistical methods

Data are expressed as mean ± SEM. Data from experiment 1 and experiment 2 were analyzed independently since they were performed at different timing and consequently immunohistochemistry experiment was run separately. After testing for normality with the Shapiro-Wilk test, the average number of cFos-IR under different conditions (experiment, brain area and intra-VTA treatment) was analyzed using a one-way ANOVA, followed by Tukey's test. Homogeneity of variance was tested before the ANOVA was performed, and the significance level was always set at $p = 0.05$. When the assumption of the homogeneity of variances was violated, number of cFos-IR were analyzed using Brown-Forsythe test of equality of means, followed by Games-Howell. Differences between dorsoventral measurements of both hind paw of the CFA-treated animals were analyzed using a *t*-test for paired samples. Statistical analyses were performed with IBM SPSS statistics 19 software.

3. Results

All animals included in the experiment showed a correct position of the cannula tip in the posterior VTA (between −6.72 mm and −5.64 mm from bregma, Fig. 1B) (Sánchez-Catalán et al., 2009).

3.1. Effect of DAMGO injected intra-VTA on cFos expression in projection areas

In this experiment 1 we evaluated the effect of MORs activation in

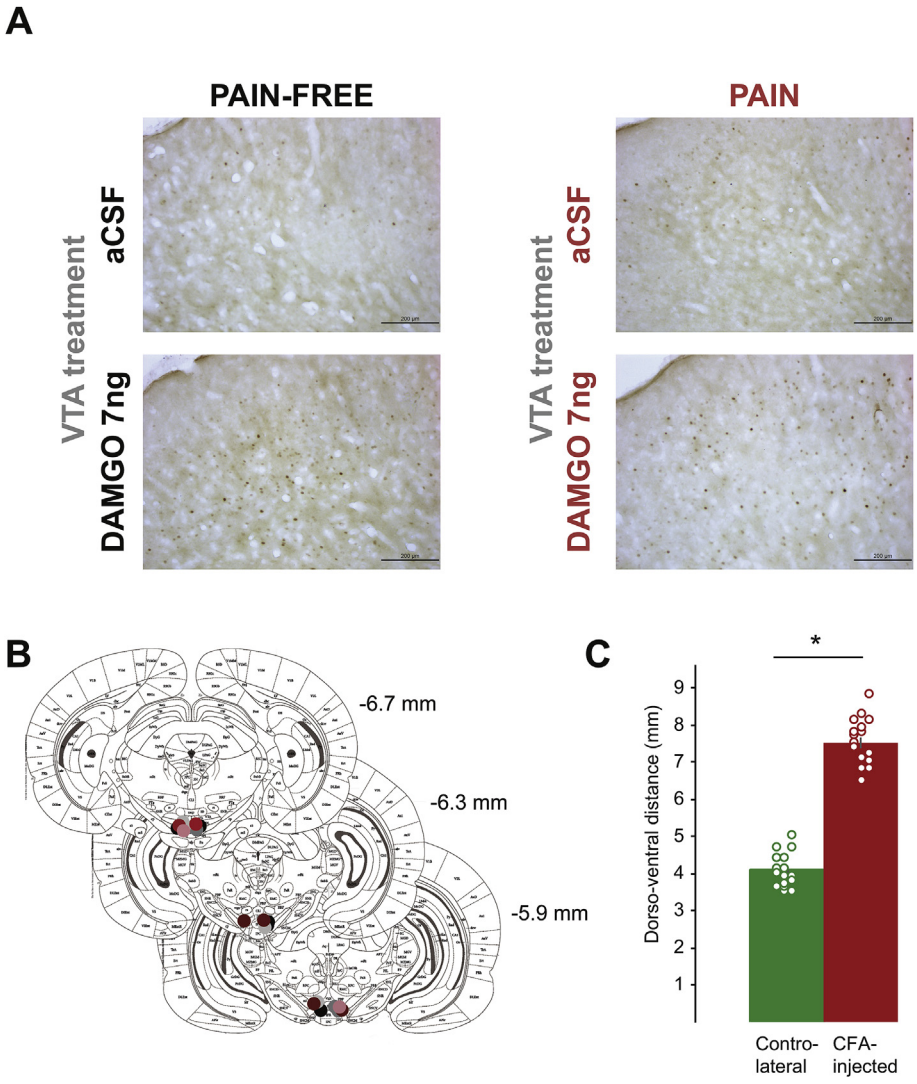

Fig. 1. A: Representative images of coronal sections after cFos staining in the ACC. B: Diagram of coronal sections indicating microinjection cannula tips in VTA for experiment 1 (light grey: aCSF treated; dark grey: 7 ng DAMGO treated; black; 14 ng DAMGO) and 2 (light red: aCSF; red: 7 ng DAMGO; dark red: 14 ng DAMGO). Dots could represent more than one cannula placement. C: Dorsoventral distance (mm) of both contralateral (green) and CFA-injected (red) hind paws of inflammatory pain animals (*: $p < 0.001$, *t*-test for paired samples). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
cFos-IR cells counting per frame after the administration of aCSF (n = 5–6), DAMGO 7 ng (n = 4–5) or DAMGO 14 ng (n = 5–6) intra-VTA in experiment 1 and 2. Data are expressed as mean ± SEM. Bold font and *: P ≤ 0.05 compared with aCSF treated group by Tukey's test.

Experiment	Area	VTA TREATMENT		
		aCSF	DAMGO7ng	DAMGO14ng
Experiment 1 PAIN-FREE groups	BLA	28 ± 7	44 ± 5	**47 ± 4** *
	NAc	43 ± 10	73 ± 10	**80 ± 7** *
	ACC	72 ± 20	**141 ± 16** *	103 ± 16
	PL	162 ± 32	213 ± 32	179 ± 20
	IL	157 ± 20	189 ± 23	165 ± 11
	BNST	24 ± 4	**48 ± 8** *	30 ± 3
	VP	19 ± 5	20 ± 3	19 ± 2
Experiment 2 PAIN groups	BLA	25 ± 6	38 ± 9	39 ± 5
	NAc	46 ± 13	67 ± 11	**93 ± 3** *
	ACC	99 ± 38	120 ± 19	74 ± 15
	PL	148 ± 33	209 ± 18	175 ± 33
	IL	148 ± 23	161 ± 31	150 ± 22
	BNST	29 ± 4	36 ± 7	32 ± 2
	VP	19 ± 4	21 ± 7	31 ± 4

cFos expression in VTA-recipient areas, by comparing the groups receiving a focal injection of aCSF or DAMGO in the VTA (Table 1, upper rows). In the NAc and BLA, the one-way ANOVA detected significant differences between groups ($F(2,13) = 5{,}180$, $p = 0.022$, n = 4–6 and $F(2,13) = 3.894$, $p = 0.047$, respectively). The Tukey's test showed that for both areas only 14 ng intra-VTA DAMGO significantly increased cFos expression compared to the aCSF treated group (NAc: 43 ± 10 vs 80 ± 7, $p = 0.022$; BLA: 28 ± 7 vs 47 ± 4, $p = 0.046$), although a trend to increase the cFos-IR was observed in this two areas when the 7 ng DAMGO was administered (NAc 43 ± 10 vs 73 ± 10 vs, $p = 0.108$; BLA: 28 ± 7 vs 44 ± 5 IR cells, $p = 0{,}140$). In prefrontal regions, the one-way ANOVA revealed significant differences between groups in the ACC ($F(2,14) = 3.738$, $p = 0.050$), but not in PL or IL ($F(2,14) = 0.800$, $p = 0.469$ and $F(2,14) = 0.776$, $p = 0.479$). Post-hoc analysis showed that cFos-IR was significantly higher in the 7 ng DAMGO treated animals than in aCSF treated animals (141 ± 16 vs 72 ± 20 IR cells, $p = 0.040$). However, no significant differences were found for the 14 ng DAMGO group compared to control (aCSF) or between the two different DAMGO doses ($p = 0.442$ and $p = 0.305$). Similarly, for the BNST, the one-way ANOVA showed significant differences between groups ($F(2,13) = 5.036$, $p = 0.024$) and the post-hoc analysis revealed that there was a significant difference only between the aCSF and the 7 ng DAMGO groups ($p = 0.025$). Finally, the analysis of the VP cFos counting by one-way ANOVA clearly did not show significant differences between groups in cFos IR ($F(2,13) = 0.021$, $p = 0.979$).

3.2. Impact of inflammatory pain on the effect of DAMGO injected intra-VTA on cFos expression in projection areas

In this second experiment, we evaluated how inflammatory pain affected the previously described MORs activation pattern by comparing the expression of cFos-IR following the administration of DAMGO intra-VTA in the rats administered with CFA in the hind paw (Table 1, lower rows). The dorsoventral measurements of the rats hind paw injected with CFA was significantly different from the non-treated hind paw confirming the presence of inflammation (CFA treated paw 7.5 ± 0.2 mm vs. non-treated paw 4.1 ± 0.1 mm, *t*-test for paired samples $p < 0.001$, Fig. 1C).

Regarding the NAc, the Brown-Forsythe test showed significant differences for the cFos counting ($p = 0.023$) concretely between the highest dose treated group, 14 ng of DAMGO, and aCSF treated group (46 ± 13 vs 93 ± 3; $p = 0.011$).

By contrast, after the administration of 7 or 14 ng of DAMGO none of the other areas of the study showed an increase of the cFos-IR cell counting. Indeed, the statistical analysis performed for each area fail to find significant differences between groups: BLA (one-way ANOVA $F(2,13) = 0.904$, $p = 0.429$); ACC (Brown-Forsythe test $p = 0.508$); PL (one-way ANOVA $F(2,14) = 0.995$, $p = 0.394$); IL (one-way ANOVA $F(2,14) = 0.075$, $p = 0.929$); BNST (one-way ANOVA $F(2,13) = 0.458$, $p = 0.642$); VP (one-way ANOVA $F(2,13) = 0.365$, $p = 0.701$).

4. Discussion

Our results show that local MORs agonism in the VTA increases cFos expression in the majority of its projection areas (NAc, BLA, ACC and BNST), with the exception of PL, IL and VP. But more interestingly, we reveal that, inflammatory pain suppresses this increase in neural activation in the majority of regions (BLA, ACC and BNST).

The effects of focal injections of DAMGO were dose dependent, with some areas showing an increase of activation after the high dose but not after the low dose, and viceversa. These results obtained in experiment 1, reveal the complexity of the opioidergic control of the VTA projections activity. Thus, in the case of NAc and BLA the administration of 14 ng DAMGO, but not 7 ng DAMGO, significantly increased cFos-IR counting as compared to aCSF administration. This increase may be due to a higher activity of the VTA dopamine neurons projecting directly from the VTA to the NAc and BLA (Juarez and Han, 2016; Lammel et al., 2014). Following the mechanism described by Johnson and North (1992), local activation of the MORs in the VTA results in the hyperpolarization of the GABA interneurons, what decreases the spontaneous GABA-mediated synaptic input to the dopamine cells (Johnson and North, 1992). Therefore, the observed increase of cFos expression in the NAc and BLA may be the result of a disinhibition of the VTA dopaminergic neurons exerted by the activation of the MORs. Curiously, although the administration of 7 ng of DAMGO increased cFos-IR counts in both NAc and BLA up to 170 and 157%, respectively, from aCSF cFos-IR counts, this effect was not statistically different. Indeed, this increase was equal to the one observed by the administration of the 14 ng dose, since no statistical differences were detected between these two treatment groups. One possible explanation may rely in a different sensitivity to the lower dose of DAMGO between animals, which may underlie the higher dispersion degree of our results, occluding a significant effect. Although a more complete dose response curve of the effect of DAMGO administered intra-VTA would provide a better idea of the pharmacology of the MORs in the VTA, it is important to notice that our results agree with all previous data showing that the agonism of MORs in the VTA drives to the activation of projecting dopamine neurons to NAc and BLA.

Conversely, in the ACC, cFos-IR was increased when administering 7 ng of DAMGO in the VTA but not when 14 ng DAMGO was injected. By contrast, neither PL or IL were significantly activated by either dose. This observation is in agreement with previous data showing that the activation of MORs in the VTA increases dopamine release in the ACC but not in other prefrontal areas (Narita et al., 2010). Similarly to ACC, the low dose of DAMGO intra-VTA increased cFos-IR in the BNST. Although they have received less attention, there is also evidence that the BNTS receives dopaminergic input from the VTA (Badiani et al., 2011), that could be activated by focal injections of the MOR agonist. The lack of effect of the higher dose of DAMGO in both ACC and BNST suggest that the opioidergic control of the VTA over these projecting regions is more complex. In this sense, VTA also sends GABAergic projections onto the mPFC (Carr and Sesack, 2000), that might counteract the activation induced by dopaminergic projections, adding complexity to the opioidergic modulation of VTA efferences.

Finally, in the case of the VP, the activation of MORs in the VTA does not increase cFos expression. This could be due to the heterogeneity of the VTA projections to the VP that probably are not under MORs control (Lammel et al., 2014; Yoo et al., 2016), although lack of effect of the selected doses might also contribute.

Very interestingly, the presence of inflammatory pain impaired the above-described increase of cFos expression induced by intra-VTA DAMGO in all areas analyzed, except in NAc. Previous studies have already shown that pain-induced desensitization of VTA MORs has an effect on the NAc activity (Hipolito et al., 2015; Ozaki et al., 2002). In this framework, data from Hipolito et al. (2015) showed that higher doses of DAMGO are able to reverse inflammatory pain-induced changes, a fact that further supports that VTA MORs are desensitized in animals under inflammatory pain condition (Hipolito et al., 2015). Surprisingly, in our study, cFos-IR in the NAc was higher after the administration of 14 ng of DAMGO in both saline and CFA-treated animals. Several reasons may prevent a shift in the dose response curve in our current experiment. First of all, we may notice that we were not able to detect an effect for the lower dose of DAMGO in the experiment 1 (pain-free animals). Second, it is possible that 14 ng is already a high dose that activates the dense projections from VTA to NAc, overcoming the lack of MORs effect. In this case, it is possible that an intermediate DAMGO dose would be useful to detect an increase of cFos expression only in saline treated animals. And third, it is important to note that cFos expression is the result of several events occurring during a longer period of time than the effects on dopamine. For example, in the microdialysis experiments performed by (Hipolito et al., 2015), the inflammatory pain-induced desensitization effect was observed during the first 40 min after DAMGO treatment. After that, dopamine levels in inflammatory pain animals were not different from pain-free animals. Thus, our current results may show that in inflammatory pain animals 14 ng of DAMGO administered intra-VTA can induce similar overall activation than in pain-free animals, although behavioural outputs could radically differ.

Contrary, in the other regions studied inflammatory pain altered the patter of cFos-IR observed in experiment 1 after the administration of 7 or 14 ng of DAMGO in the other regions studied. In fact, in the presence of inflammatory pain, intra-VTA DAMGO did not induce an activation of the BLA, ACC and BNST. Therefore, the DAMGO doses that induce cFos expression in the aforementioned projecting regions in experiment 1 are insufficient to activate MORs in CFA-treated animals.

To conclude, our results show a heterogeneity of response to DAMGO administered intra-VTA depending on the target region. More interestingly, our data confirm that inflammatory pain induces desensitization of VTA MORs that affects other projecting areas apart from the NAc. The present results constitute a starting point to further study in depth the nature of the VTA projections to the different target areas. Further studies should investigate the exact mechanism as to how local MORs in the VTA control the activity of its target regions, and how inflammatory pain interferes with this process. These future studies will shed light into the interplay between pain and reward processing, which has important consequences for both behavioral responses towards opioids and natural rewards.

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