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MODULATION OF CONDITIONED FEAR, FEAR-CONDITIONED ANALGESIA AND BRAIN REGIONAL C-FOS EXPRESSION FOLLOWING ADMINISTRATION OF MUSCIMOL INTO THE RAT BASOLATERAL AMYGDALA

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Running Title: Intra-BLA muscimol affects FCA and c-fos expression

Keywords: amygdala; muscimol; $GABA_A$ receptor; fear; pain; analgesia; rat; c-Fos

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Abstract

Evidence suggests that gamma-aminobutyric acid (GABA) signalling in the basolateral amygdala (BLA) is involved in pain, fear and fear-conditioned analgesia (FCA). In this study, we investigated the effects of intra-BLA administration of the $GABA_A$ receptor agonist, muscimol, on the expression of conditioned-fear, formalin-evoked nociception and fear-conditioned analgesia in rats, and the associated alterations in brain regional expression of the immediate early gene product and marker of neuronal activity, c-Fos. Formalin-evoked nociceptive behaviour, conditioned-fear and fear-conditioned analgesia were apparent in animals receiving intra-BLA saline. Intra-BLA muscimol suppressed fear behaviour and prevented fear-conditioned analgesia, but had no significant effect on the expression of formalin-evoked nociception. The suppression of fear behaviour by intra-BLA muscimol was associated with increased c-Fos expression in the central nucleus of the amygdala (CeA) and throughout the periaqueductal grey (PAG). These intra-BLA muscimol-induced increases in c-Fos expression were abolished in rats receiving intra-plantar formalin injection. These data suggest that alterations in neuronal activity in the CeA and PAG as a result of altered GABAergic signalling in the BLA may be involved in the behavioural expression of fear and associated analgesia. Furthermore, these alterations in neuronal activity are susceptible to modulation by formalin-evoked nociceptive input in a state-dependent manner.

Perspective: The expression of learned fear and associated analgesia are under the control of $GABA_A$ receptors in the basolateral amygdala, through a mechanism which may involve altered neuronal activity in key components of the descending inhibitory pain pathway. The

results enhance our understanding of the neural mechanisms subserving fear-pain

interactions.

Introduction

Fear can illicit complex changes in neuronal processing in the descending inhibitory pain pathway. Brain regions critically involved in the descending inhibitory pain pathway include the basolateral amygdaloid complex (BLA), central nucleus of the amygdala (CeA), periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM), and neuronal activity in these regions also subserves expression of fear. A large proportion of patients suffering from persistent pain often report with co-morbid anxiety disorders [1], and evidence suggests altered pain processing in patients suffering from anxiety disorders such as post-traumatic stress disorder [17]. Thus, studies investigating neuronal mechanisms in brain regions commonly implicated in both pain and fear may provide useful information that could be exploited for therapeutic gain.

Fear-conditioned analgesia is a phenomenon whereby animals re-exposed to a neutral context (e.g. conditioning arena) which has previously paired with an aversive stimulus (e.g. footshock) display conditioned analgesia [14,15,20,24]. Information relating to context or aversion/nociception which is processed in brain regions including the thalamus and cortex converges at the level of the BLA and CeA[46]. During fear conditioning, it is postulated that the convergence of information is reinforced after each footshock. Subsequent re-exposure to the context where the aversive events occurred results in hyperexcitability and increased plasticity of neurons in the BLA [55], and these neurons further relay the information through GABAergic intercalated cells [57] to the CeA. The medial sector of the CeA is thought to be the main source of amygdalar outputs to the PAG and hypothalamic sites responsible for fear behaviour [4,12,31]. Indeed, neuronal projections from the CeA to the PAG [52] are strongly involved in the endogenous aversive and analgesic systems. The activation of this pathway

results in both the expression of fear-related behaviour (e.g. freezing and 22-kiloHertz ultrasonic vocalisations) [10,49] and robust analgesia - as a consequence of activating the descending inhibitory pain pathway [27,32,37,43].

GABAergic neurotransmission in the BLA, CeA and PAG plays a key role in supraspinal modulation of pain [13,50], fear [5,9,51,55,58] and fear-conditioned analgesia [19-21]. A previous microdialysis study from our laboratory reported a significant suppression of GABA levels in the BLA of fear-conditioned rats compared with non fear-conditioned controls, suggesting that reduced GABAergic signalling in the BLA may facilitate the expression of conditioned fear [51]. The present study tested the hypothesis that $GABA_A$ receptor activation in the BLA attenuates the expression of conditioned fear and fear-conditioned analgesia in rats. A pharmacological approach was adopted, employing microinjection of the $GABA_A$ receptor agonist, muscimol, bilaterally into the BLA. Furthermore, we investigated associated alterations in the expression of the immediate early gene product, c-Fos, as an index of altered neuronal activity in the CeA and PAG.

Methods

Animals

Male Lister-hooded rats (280–350 g; Charles River, Margate, Kent, UK) were used. Animals were housed 4 per cage before surgery and were maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12 hour light–dark, lights on from 0800-2000h). Experiments were carried out during the light phase between 0800 and 1700h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

Cannulae Implantation

Stainless steel guide cannulae (Plastics One Inc., Roanoke, Virginia, USA) were stereotaxically implanted 1 mm above the right and left basolateral amygdala (AP - 0.25 cm, ML ± 0.48 cm relative to bregma, DV - 0.71 cm from skull surface; [48] under isoflurane anaesthesia (2–3% in O_2; 0.5 L/ min). The cannulae were permanently fixed to the skull using stainless-steel screws and carboxylate cement. The duration of surgery from initial anaesthesia until recovery was 50-70 minutes. A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, Virginia, USA) was inserted into the guide cannula to prevent blockage by debris. 250µL of the non-steroidal anti-inflammatory agent, carprofen (0.5% s.c.) (Rimadyl, Pfizer, Kent, UK), and 250µL of the broad spectrum antibiotic, enrofloxacin (0.5% s.c.) (Baytril, Bayer Ltd., Dublin, Ireland) were administered before the surgery to manage post-operative analgesia and to prevent infection respectively. Following cannulae implantation, the rats were singly housed and administered enrofloxacin (Baytril) for a further 3 days. At least 6 days post-surgery were allowed for recovery prior to

experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis.

Drug Preparation

The GABA$_A$ receptor agonist, muscimol (Sigma-Aldrich, Dublin, Ireland), was freshly prepared on test days at a concentration of 1.0 mg/mL in sterile saline. 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) solution was also freshly prepared in sterile saline on test days.

Experimental Procedures

The experimental procedure was essentially as described previously [6,15,16,51,53,54]. In brief, it consisted of two phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomised. On the conditioning day, rats were placed in a Perspex fear-conditioning/ observation chamber (30 x 30 x 30 cm) and after 15 s they received the first of 10 footshocks (0.4 mA, 1 s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK) spaced 60 s apart. Fifteen seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 9.5 min period.

The test phase commenced 23.5 hours later when the animals were placed under brief isoflurane anaesthesia (3% in O$_2$; 0.5 L/ min). At this time, animals received intra-BLA microinjection of either muscimol (MUSC 0.5 µg/ 0.5 µL) or sterile saline (VEH 0.5 µL) into the right and left BLA 60-90 seconds prior to formalin administration. Subjects received an intra-plantar injection of 50 µL formalin (Form 2.5% in 0.89% NaCl) or saline (Sal 0.89%

NaCl) into the right hindpaw. The dose of muscimol (0.5 µg/ 0.5 µL / side) was chosen based on studies from the literature demonstrating that intra-cerebral administration of muscimol suppressed the expression of conditioned fear [34,38]. A full description of the injection procedure has been published previously [54]. This design resulted in eight experimental groups as illustrated below

Group No:	Fear Conditioning	Saline/ Formalin *i.pl.*	Intra-BLA Vehicle/Muscimol	No. rats per group (*n*)
1	No FC	Saline	Vehicle	7
2	No FC	Saline	Muscimol	6
3	No FC	Formalin	Vehicle	6
4	No FC	Formalin	Muscimol	6
5	FC	Saline	Vehicle	6
6	FC	Saline	Muscimol	6
7	FC	Formalin	Vehicle	6
8	FC	Formalin	Muscimol	6

Following intra-BLA injection of muscimol or vehicle, rats were returned to their home cage until 30-min post-formalin injection at which point they were returned to the same perspex observation chamber to which they had been exposed during the conditioning phase. A video camera located beneath the observation chamber was used to monitor animal behaviour, while a bat detector (Batbox Duet; Batbox, Steyning, West Sussex, UK) positioned over the arena was used to detect ultrasonic vocalization in the 22 kHz range. The audio feed from the bat detector and video feed from the camera were recorded onto DVD for 15 minutes. The 30–45 min post-formalin interval was chosen on the basis of previous studies demonstrating that formalin-evoked nociceptive behaviour is stable over this time period; and that fear-conditioned analgesia and conditioned-fear expressed during this period are subject to supraspinal modulation [15].

Behavioural Analysis

Behaviour was analysed using the Observer 5 software package (Noldus, Netherlands), which allowed for continuous event recording over each 15 min trial. A rater blind to experimental conditions assessed fear behaviour (duration of freezing while emitting 22 kHz ultrasonic vocalisation), nociceptive behaviour (composite pain score (CPS)) and general behaviours (rearing, walking and grooming), as described previously [60]. Formalin-induced oedema was assessed by measuring the change in the diameter of the right hindpaw immediately before and 2 h after formalin administration using Vernier callipers.

Histology

Following behavioural testing, rats were returned to their home cage until 2 h post-formalin administration. Rats were deeply anesthetised (5% isoflurane in O_2) and transcardially perfused with 100 ml of heparinised saline solution followed by 500 ml of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 at 4^oC. At the end of perfusion, 0.5µL of 1% fast green dye was slowly injected via each cannula to mark the BLA injection site position. Brains were removed and stored in the same fixative for 90 min at 4^oC followed by immersion in 20% sucrose solution in 0.1 M PB containing 0.1% sodium azide (NaAz) for at least 24 h. Brains were later coronally sectioned (40 µm thickness) using a microtome blade and collected in 0.1 M PB. Sections containing the BLA were mounted onto glass slides and counter-stained with Cresyl Violet in order to locate the precise position of the site of microinjection using a light microscope. The remaining sections were used for immunohistochemistry.

Immunohistochemistry

Fos immunolabelling was performed using a polyclonal antibody directed against residues 4–17 of human c-Fos (Calbiochem, Merck Biosciences, Nottingham, UK) and carried out as previously described [53]. In brief, sections were quenched in 0.75% hydrogen peroxide (H_2O_2) for 20 min to remove endogenous peroxidase activity. Following a series of washes in phosphate buffer (PB), sections were incubated in phosphate buffered saline containing rabbit anti c-Fos antibody (1:20,000; Ab-5 Merck Biosciences, UK), 0.3% Triton X (TX), 0.04% bovine serum albumin (BSA) and 0.1% NaAz for 24 h. The incubated sections were then washed and incubated for 90 min in biotinylated goat-anti-rabbit antisera (1:200; Jackson ImmunoResearch Europe, UK), followed by washes in PB. The secondary antibody had minimal cross-reactivity to non-target species. Sections were then incubated in Vectastain avidin–biotin-peroxidase complex (ABC) (1:600; ABC Elite Kit; Vector Laboratories Ltd., Peterborough, UK) for a further 90 min followed by immersion in 0.02% 3,3-diaminobenzidine–4HCl (DAB) containing 0.01% H_2O_2 in PB for development of a brown reaction product. In order to visualise immunostaining, all sections were mounted on gelatin-coated glass slides and air-dried, dehydrated in graded alcohols, cleared with xylene and coverslipped with DePex mounting medium.

Quantification of immunohistochemical staining

Photomicrographs were taken with an Olympus microscope BX51 and Olympus C5060 digital camera (Mason Technology, Dublin, Ireland). Regions of interest were defined based on the extent of cellular groups comprising specific landmarks in accordance with Paxinos et al. (1985) rat brain atlas. The anterior–posterior (AP) level from bregma of the regions analysed was as follows: Central nucleus of the amygdala (CeA; AP: -1.6 to -3.2 mm),

Rostral, Intermediate and Caudal periaqueductal grey (Rostral PAG; AP: -5.2 to -5.8, Intermediate PAG; AP: -6.04 to -7.04, Caudal PAG; AP: -7.3 to -8.8 mm). Fos immunoreactive profiles were captured from a fixed area under 100–200X magnification in at least three sections per region per rat, bilaterally and quantified using 'image j' analysis software (NIH, Bethseda, Maryland, USA). Fos expression did not differ between the left and right sides in response to any of the experimental procedures and thus counts were averaged to give the mean number of Fos positive profiles per region per animal.

Statistical analysis

SPSS statistical package (SPSS v15.0 for Microsoft Windows; SPSS, Inc., Chicago, IL) was used to analyse all data. Paw oedema, behavioural data and the number of c-Fos-like immunoreactive neurons per region were analysed using 3-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test. Data were considered significant when $P < 0.05$. Results are expressed as group means ± standard error of the mean (±SEM).

Results

Histological verification of injector placement

Eighty percent of the injections were placed within the borders of the right and left BLA (Fig. 1) with the remaining 20% positioned in the central nucleus or ventral hippocampus on one or both sides. Only the results of experiments in which both injections were correctly positioned in the BLA were included in the analyses.

Bilateral muscimol microinjection into the BLA prevents the expression of conditioned fear

Re-exposure of rats receiving vehicle microinjection bilaterally into the BLA to the context paired 24 h earlier with footshock resulted in robust freezing while emitting 22 kHz ultrasonic vocalisations, compared with non-fear conditioned controls (Fig. 2; FC-Sal-Veh vs No FC-Sal-Veh, $p < 0.01$). This contextually-induced fear behaviour was of equal magnitude and duration in rats receiving intraplantar injection of either saline or formalin into the hind paw. Bilateral microinjection of muscimol into the BLA completely prevented the expression of contextually induced fear behaviour irrespective of whether they received formalin or not, as compared with vehicle-treated controls (Fig. 2; FC-Sal-Musc vs FC-Sal-Veh, FC-Form-Musc vs FC-Form-Veh $p < 0.01$).

Bilateral muscimol microinjection into the BLA prevents the expression of fear-conditioned analgesia

Intra-plantar injection of formalin significantly increased licking, biting, shaking, flinching and elevation of the injected paw, as indicated by the composite pain score (CPS) (Fig. 3; No FC-Form-Veh vs No FC-Sal-Veh, $p < 0.01$). Fear-conditioned rats displayed significantly less formalin-evoked nociceptive behaviour (i.e. CPS) during the 15-min trial, compared with

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non-fear-conditioned counterparts (Fig. 3, FC-Form-Veh vs. No FC-Form-Veh, $p < 0.01$), confirming the expression of fear-conditioned analgesia. Intra-BLA microinjection of muscimol had no effect on formalin-evoked nociceptive behaviour *per se* (Fig. 3, No FC-Form-Veh vs. No FC-Form-Musc) but prevented the expression of fear-conditioned analgesia (Fig. 3, No FC-Form-Musc vs. FC-Form-Musc). Intra-plantar injection of formalin resulted in hindpaw oedema, compared with saline-treated animals (No FC-Sal-Veh vs. No FC-Form-Veh $p = 0.001$ – data not shown). Neither fear conditioning nor drug administration had any significant effect on formalin-evoked hindpaw oedema (data not shown).

c-Fos expression in the CeA and PAG

The effects of fear-conditioning, formalin and intra-BLA muscimol microinjection, alone or in combination, on c-Fos expression in discrete rat brain regions are presented in Table 1. In rats receiving intra-BLA vehicle, contextually induced fear, formalin-evoked nociceptive behaviour or fear-conditioned analgesia were not associated with any alterations in c-Fos expression in the CeA or PAG. Conditioned fear was associated with significant increases in c-Fos expression throughout the PAG of muscimol-treated rats receiving intra-plantar injection of saline (Table 1; FC-Sal-Musc vs. NoFC-Sal-Musc, $p < 0.05$), but not those receiving intra-plantar injection of formalin (FC-Form-Musc vs NoFC-Form-Musc). There was no effect of conditioned fear on c-Fos expression in the CeA of either saline-or formalin-treated rats receiving intra-BLA muscimol (Table 1; FC-Sal-Musc vs. NoFC-Sal-Musc; FC-Form-Musc vs NoFC-Form-Musc). The muscimol-induced suppression of contextually-induced fear behaviour described above was associated with an increase in c-Fos expression, both in the CeA and throughout the different levels of the PAG, with the exception of the rostral and intermediate dorsomedial PAG. These increases in c-Fos expression in the CeA or PAG associated with the muscimol-induced suppression of conditioned fear were observed in

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rats receiving intra-plantar saline (Table 1 and Fig 4; FC-Sal-Veh vs. FC-Sal-Musc, $p < 0.05$), but not those receiving intra-plantar formalin injection (Table 1; FC-Form-Veh vs. FC-Form-Musc). Indeed, c-Fos expression was significantly lower throughout the PAG of muscimol-treated fear-conditioned rats receiving intra-plantar formalin, compared with counterparts receiving intra-plantar saline (FC-Form-Musc vs FC-Sal-Musc, $p < 0.05$).

Discussion

The results of the present study provide evidence for an important role of $GABA_A$ receptors in the BLA in the expression of conditioned-fear and fear-conditioned analgesia. Microinjection of the $GABA_A$ receptor agonist muscimol bilaterally into the BLA prevented the expression of contextually induced fear and fear-conditioned analgesia in rats. The former effect, but not the latter, was associated with an increase in c-Fos expression in the CeA and PAG. Together, the data suggest that activation of $GABA_A$ receptors in the BLA engages neurones along the amygdala-PAG pathway to effect a suppression of conditioned fear responding. The results further suggest differential muscimol-induced engagement of these amygdala-PAG neurones in the presence versus absence of formalin-evoked nociceptive tone.

Our data corroborate earlier findings demonstrating that microinjection of muscimol into the rat BLA complex suppresses the expression of conditioned fear [26,34,38], and that the microinjection of benzodiazepines (allosteric modulators of the $GABA_A$ receptor) into the BLA reduces the suppression of pain-related behaviour by unconditioned [2,24,41] and conditioned [20,24] fear in rats. Previous *in vivo* microdialysis studies from our laboratory and others have shown that expression of conditioned fear is associated with a suppression of GABA release in the BLA [51,59]. Furthermore, benzodiazepine binding, mRNA and protein levels of distinct $GABA_A$ receptor subunits, GABA-synthesizing enzymes and the $GABA_A$ receptor-associated protein gephyrin are all reduced in the amygdala within hours after fear conditioning [8,22,45]. Based on these studies, we hypothesised that the reduced activation of BLA $GABA_A$ receptors that would likely result from a suppression of GABA release and a general reduction in GABAergic tone in that region may facilitate fear responding. The results of the present study, demonstrating that pharmacological activation of $GABA_A$

receptors in the BLA prevents the expression of conditioned fear and fear-conditioned analgesia, support this hypothesis. It is important to note, however, that inactivation of BLA neurons via other means, for example electrolytic or chemical lesion with non-GABA modulating agents, has also been shown to suppress the expression of contextual fear conditioning [29,30,33,44] and fear-conditioned analgesia [23,25].

To further investigate the effects of intra-BLA muscimol on neuronal activity in key components of the fear and descending inhibitory pain circuitry, we measured c-Fos expression in the CeA and PAG. The CeA is a target nucleus for neurons projecting from the BLA and the major amygdaloid output nucleus projecting to the PAG. In our study, there were no changes in c-Fos levels in the CeA or PAG associated with muscimol administration alone, or with conditioned fear, formalin-evoked nociception or fear-conditioned analgesia *per se*. The inability to detect changes in c-Fos does not necessarily mean that neurons using other transcription factors are not activated under these experimental conditions. This lack of effect is in contrast to other studies reporting an effect of formalin or conditioned fear on c-Fos levels in the CeA [28,36,56,58] or PAG [7,53]. However, the specific focus of our study was on the effects of bilateral administration of muscimol into the BLA on the expression of fear-conditioned analgesia and associated alterations in c-Fos expression, and therefore our experimental design/methodology may not have been optimal to study changes in c-Fos expression associated with conditioned fear or formalin-evoked nociceptive behaviour *per se*. Our results did reveal, however, that the suppression of conditioned fear by bilateral muscimol microinjection into the BLA was associated with a significant increase in c-Fos expression in the CeA. This finding supports previous work which has shown that the suppression of fear by the benzodiazepine diazepam, administered systemically, is also associated with increased c-Fos expression in the CeA[3]. Our data here suggest that these c-

Fos-expressing neurons in the CeA which respond during anxiolysis are subject to regulation by $GABA_A$ receptors in the BLA. Within the BLA, the majority of neurons are pyramidal-like glutamatergic projection neurons, while non-pyramidal spine-sparse GABAergic interneurons constitute 15% of the total neuronal population [36]. However, if the effects of bilateral muscimol microinjection into the BLA were mediated by $GABA_A$ receptors expressed on CeA-projecting glutamatergic neurons, then we might expect to have seen a decrease rather than the observed increase in neuronal activity (as indexed by c-Fos expression) in the CeA. Thus, these results suggest that the link between the activation of $GABA_A$ receptors in the BLA and the resulting activation of Fos-expressing neurons in the CeA is indirect and that intermediate relays exist. Evidence suggests that GABAergic interneurons in the BLA innervate pyramidal neurons and other interneurons in the basolateral nucleus [39,40] and modulate neuronal output from the BLA to regulate expression of fear [18]. Furthermore, parvalbumin-containing interneurons in the BLA have been shown to express $GABA_A$ receptors [35], reinforcing the idea that GABAergic neurotransmission plays a complex and critical role in regulating neuronal output from the BLA. However, to our knowledge, there is no evidence for GABAergic projection neurons from the BLA direct to the CeA. Paré *et al.* (2004), proposed a model whereby neurons in the BLA facilitate the activity of brainstem-projecting CeA neurons via intercalated cell masses (ITC), dense clusters of GABAergic neurons located between the basolateral amygdaloid complex and the CeA [47]. It is likely, therefore, that the intra-BLA muscimol-induced increase in c-Fos expression in the CeA in fear-conditioned rats is a consequence of $GABA_A$ receptor-mediated modulation of the complex regulatory network which subserves communication between the BLA and CeA.

Similar to the changes in the CeA, the suppression of conditioned fear *per se* by muscimol microinjection bilaterally into the BLA was associated with increased c-Fos expression in all regions of the PAG with the exception of the dorsomedial aspect of the rostral and intermediate PAG. Recent retrograde and anterograde tracing studies have determined that the majority of PAG-projecting CeA neurons are GAD68 mRNA positive, suggesting that these neurons are GABAergic [42]. Taken together, our data for the CeA and PAG support the supposition that activation of Fos-expressing GABAergic interneurons in the CeA [11] as a result of BLA GABA$_A$ receptor activation, may suppress PAG-projecting GABA neurons of the CeA [42] resulting in a disinhibition of neuronal firing, and thus increased neuronal activity in the PAG. Activation of the PAG (e.g. electrically or chemically) is more usually associated with increased fear responding and analgesia[8,52], however, here we have shown that increased neuronal activity in the PAG was associated with the attenuation of conditioned fear responding and fear-conditioned analgesia resulting from intra-BLA muscimol. It is possible, therefore, that the PAG neurons which make the largest contribution to the increased Fos expression observed here are in fact GABAergic interneurons, and thus an increase in the activity of these neurons would result in decreased activity of principal PAG output neurons with a consequent reduction in descending inhibition of pain responding or reduced freezing behaviour. Interestingly, the increases in CeA and PAG c-Fos expression that accompanied the suppression of fear-related behaviour by intra-BLA muscimol were only observed in rats receiving intra-plantar injection of saline and not those receiving intra-plantar injection of formalin, despite the fact that intra-BLA muscimol also suppressed fear-related behaviour in this latter group. These data suggest that formalin administration recruits neuronal processes distinct from the fear pathway that ultimately impact on common neuronal pathways downstream from the BLA, and result in a state-dependent alteration in the responsivity of the BLA-CeA-PAG pathway to intra-BLA

muscimol. Another interesting observation with respect to the PAG c-Fos expression data was that re-exposure of fear-conditioned animals to the conditioning arena significantly increased c-Fos expression in rats treated with intra-BLA muscimol, but not those treated with intra-BLA vehicle. Moreover, this fear-related increase in PAG c-Fos expression in rats receiving intra-BLA muscimol was abolished in formalin-treated rats. Roche et al. (2009) reported differential expression of c-Fos in the dPAG in fear-conditioned animals in the presence versus absence of nociceptive tone. These data suggest that $GABA_A$ receptor activity in the BLA differentially modulates fear-evoked changes in the activity of PAG-projecting neurons from the CeA in the presence versus absence of formalin-evoked nociceptive tone.

In summary, our data support and extend earlier findings implicating a role for GABAergic signalling in the BLA in the expression of conditioned fear and fear-conditioned analgesia. Moreover, the associated alterations in c-Fos expression suggest that the suppression of fear following bilateral muscimol microinjection into the BLA is associated with an increase in neuronal activity at the level of the CeA and PAG, and that these alterations in neuronal activity are susceptible to modulation by nociceptive input.

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Figure legends

Fig. 1 Schematic diagram representing the sites of microinjection of (a) Vehicle (saline) or (b) Muscimol into the left and right basolateral amygdala. (FC = fear-conditioning; No FC = no fear-conditioning; Form = formalin; Sal = saline; CeA = central nucleus of the amygdala; ic = internal capsule; LV = lateral ventricle)

Fig. 2 The effect of bilateral intra-BLA microinjection of muscimol on contextually-induced fear behaviour in rats in the presence or absence of formalin-evoked nociceptive tone, over the 15 minutes of re-exposure to the arena. Contextual fear-conditioning was associated with an increase in the duration of co-occurrence of freezing and 22kHz ultrasonic vocalisation in rats receiving either intra-plantar saline or formalin injection ($F(1,46) = 18.632$, $p < 0.01$) and intra-BLA injection of muscimol completely prevented this expression of conditioned fear-related behaviour ($F(1,46) = 18.689$, $p < 0.01$). *$p < 0.01$ vs respective non fear-conditioned saline- or formalin-treated controls *and* fear-conditioned counterparts receiving intra-BLA muscimol microinjections. Data are expressed as mean ± SEM. (No FC = non fear-conditioned; FC = fear-conditioned; Form = formalin; Sal = saline; MUSC = muscimol; VEH = vehicle (saline); US (ultrasound)

Fig. 3 The effect of bilateral muscimol microinjection into the BLA on formalin-evoked nociceptive behaviour and fear-conditioned analgesia in rats over the 15 minutes of re-exposure to the arena. Intra-plantar formalin administration significantly increased nociceptive behaviour ($F(1,46) = 18.748$, $p < 0.01$). Fear-conditioned analgesia was expressed in rats receiving intra-BLA vehicle (+$p < 0.01$ for No FC-Form-Veh vs FC-Form-

Veh), but not in rats receiving intra-BLA muscimol. *$p < 0.01$ vs respective non fear-conditioned or fear-conditioned saline-treated controls. Data are expressed as mean ± SEM. (No FC = non fear-conditioned; FC = fear-conditioned; Form = formalin; Sal = saline; MUSC = muscimol; VEH = vehicle (saline)

Fig. 4 Representative photomicrographs showing the effects of contextual fear conditioning and bilateral muscimol microinjection into the BLA on Fos-like immunoreactive neuron expression in the CeA (left) and ventral aspect of the PAG (right) of rats that received intra-plantar injection of saline. (No FC = non fear-conditioned; FC = fear-conditioned; MUSC = muscimol; VEH = vehicle (saline). Scale bar = 300μm

Table 1

Number of Fos-like immunoreactive neurons in the CeA and PAG

Brain Region	Number of Fos-positive nuclei							
	No FC-Sal-Veh	FC-Sal-Veh	No FC-Form-Veh	FC-Form-Veh	No FC-Sal-Musc	FC-Sal-Musc	No FC-Form-Musc	FC-Form-Musc
CeA	23 ± 9	18 ± 5 *	24 ± 6	24 ± 5	48 ± 13	50 ± 9	31 ± 6	35 ± 10
Regional analyis								
Rostral PAG								
dorsomedial	4.2±3	3.1±1	4.0±2	2.2±1	3.2±2	5.8±2	4.1±2	1.3±1
lateral	14.1±5	18.7±2 *	19.1±3	12.3±3	14.8±4 *	37.7±10	18.3±4	13.4±3 *
total	18.4±7	21.8±2 *	22.9±5	14.3±4	18.0±5 *	43.2±12	22.4±6	14.5±3 *
Intermediate PAG								
dorsomedial	1.4±0.5	1.3±0.8	1.7±0.7	2.8±0.8	2.0±0.7	3.7±1.4	1.5±0.4	1.8±0.4
dorsolateral	6.9±1	7.9±1 *	6.1±2	8.1±2	8.0±2 *	22.4±6	9.7±2	6.0±1 *
lateral	19.7±3	17.8±4 *	26.4±4	20.3±6	22.5±4 *	42.7±10	27.3±4	15.6±2 *
total	28.0±5	26.6±5 *	34.3±6	31.1±8	32.5±6 *	68.8±17	38.3±6	23.4±3 *
Caudal PAG								
dorsomedial	0.8±0.3	1.3±0.2 *	1.7±0.5	2.4±0.8	2.6±0.7	3.9±1.4	1.8±0.4	1.5±0.6 *
dorsolateral	6.9±2	5.1±1 *	8.6±3	8.6±3	8.9±2 *	21.5±7	10.9±3	7.8±2 *
lateral	16.6±7	17.4±4 *	23.1±2	20.3±4	18.8±4 *	35.5±9	26.9±5	16.8±2 *
ventrolateral	19.0±7	14.8±2 *	23.7±7	20.0±8	23.6±6 *	43.6±9	30.1±6	16.9±3 *
total	43.3±14	38.5±6 *	57.0±11	51.0±16	53.9±11 *	104.6±25	69.8±14	43.0±6 *
Columnar analysis								
Total dmPAG	1.5±0.5	1.6±0.4 *	2.1±0.7	2.6±1.0	2.7±0.7 *	5.2±1.6	2.1±0.3	1.6±0.4 *
Total dlPAG	6.7±2	6.0±1 *	8.6±2	8.3±3	8.6±14 *	24.0±6	10.3±2	6.1±1 *
Total lPAG	17.3±5	16.3±3 *	22.9±4	18.7±4	19.9±4 *	40.1±7	26.4±5	14.8±2 *
Total vlPAG	20.2±7	15.7±2 *	27.1±7	21.5±9	34.3±13	44.7±9	35.9±8	19.6±4 *

* P < 0.05 versus FC-SAL-MUSC

Fig 1a VEHICLE

Bregma -3.14mm
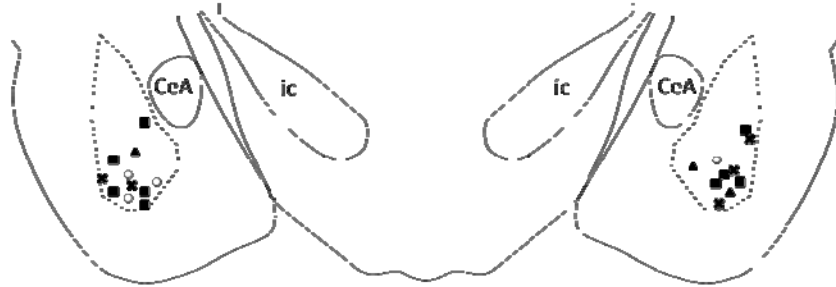
Bregma -3.60mm
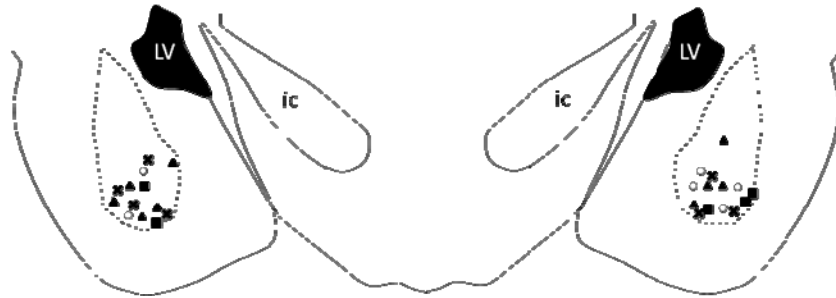
■ NoFC-Sal ▲ NoFC-Form

○ FC-Sal ✖ FC-Form

Fig 1b MUSCIMOL

Bregma -3.14mm

Bregma -3.60mm

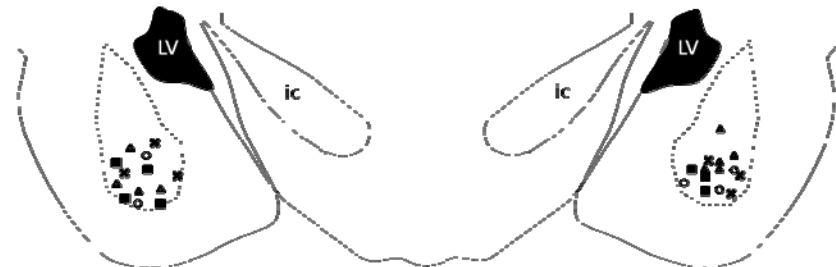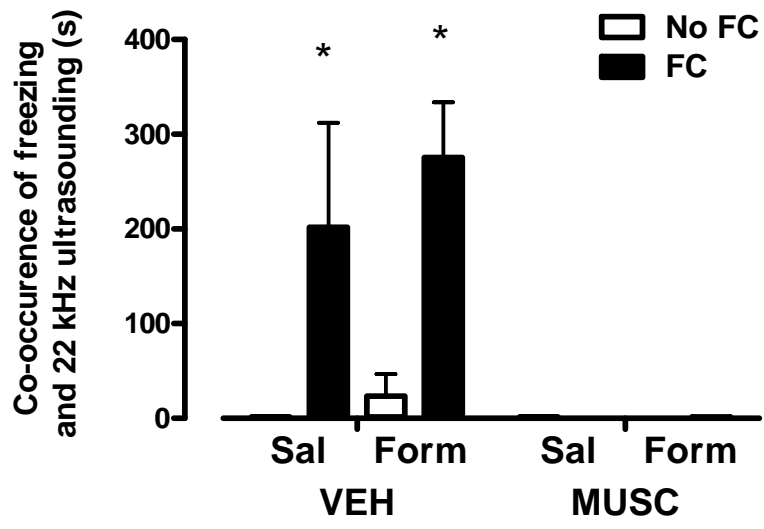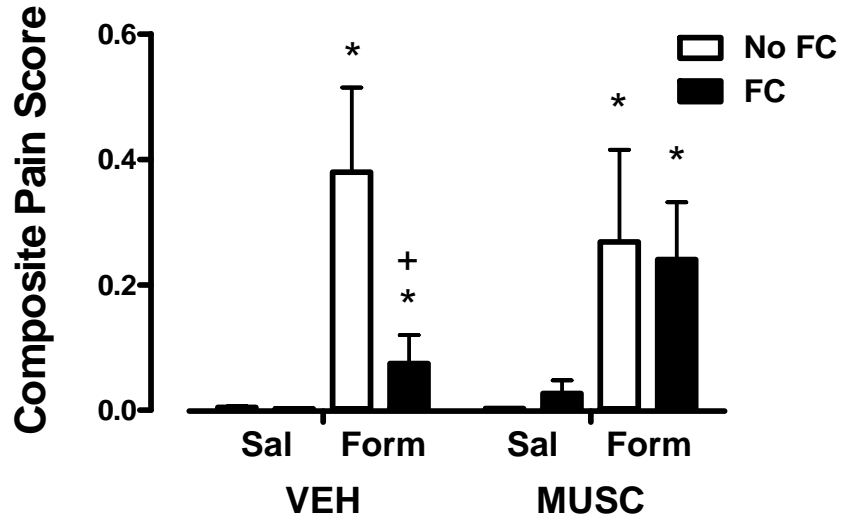Fig. 2

Fig. 3

Fig. 4