The prefrontal cortical endocannabinoid system modulates fear-pain interactions in a subregion-specific manner

Running title: *mPFC endocannabinoids and fear-pain interactions*

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Abstract

Background and purpose: The emotional processing and coordination of top-down responses to noxious and conditioned aversive stimuli involves the medial prefrontal cortex (mPFC). Evidence suggests that subregions of the mPFC, (infralimbic (IL), prelimbic (PrL), anterior cingulate (ACC) cortices), differentially alter the expression of contextually-induced fear and nociceptive behaviour. We investigated the role of the endocannabinoid system in the IL, PrL and ACC in formalin-evoked nociceptive behaviour, fear-conditioned analgesia (FCA) and conditioned fear in the presence of nociceptive tone. **Experimental Approach:** FCA was modelled in male Lister-hooded rats by assessing formalin-evoked nociceptive behaviour in an arena previously paired with footshock. The effects of intra-mPFC administration of AM251 (CB_1 receptor antagonist/inverse agonist), URB597 (fatty acid amide hydrolase inhibitor) or URB597 + AM251 on FCA and freezing behaviour were assessed. **Key Results:** AM251 attenuated FCA when injected into the IL or PrL and reduced contextually induced freezing behaviour when injected intra-IL, but not intra-PrL or intra-ACC. Intra-ACC administration of AM251 alone or in combination with URB597 had no effect on FCA or freezing. URB597 attenuated FCA and freezing behaviour when injected intra-IL, prolonged the expression of FCA when injected intra-PrL and had no effect on these behaviours when injected intra-ACC. **Conclusion and Implications:** These results suggest important and differential roles for

FAAH substrates or CB_1 receptors in the PrL, IL and ACC in the expression of FCA and conditioned fear in the presence of nociceptive tone.

Abbreviations: mPFC, medial prefrontal cortex; IL, infralimbic cortex; PrL, prelimbic cortex; ACC, anterior cingulate cortex; FCA, fear-conditioned analgesia; CB_1, cannabinoid$_1$ receptor; FAAH, fatty acid amide hydrolase; CPS, composite pain score; ANOVA, analysis of variance; FC, fear-conditioned; NFC, non-fear-conditioned; VEH, vehicle;

Keywords: pain; fear; anxiety; cannabinoid$_1$ (CB_1) receptor; anandamide; fatty acid amide hydrolase (FAAH); formalin; prefrontal cortex; stress-induced analgesia

Introduction

The medial prefrontal cortex (mPFC) is strongly implicated in cognitive, emotional and motivational processes, and in regulation of responses to aversion (Gilmartin et al., 2014, Gilmartin et al., 2013, Laviolette et al., 2005, Jiang et al., 2014, Wang et al., 2009, Resstel et al., 2006) and pain (Luongo et al., 2013, Baulmann et al., 2000, Okine et al., 2016). The mPFC is comprised of a number of subregions that can be differentiated by anatomical connectivity, cytoarchitecture and function. In rodents, the infralimbic (IL) and prelimbic (PrL) subregions of the mPFC have been shown to differentially affect acquisition, consolidation and expression of contextually conditioned fear (Corcoran and Quirk, 2007, Sharpe and Killcross, 2014, Vidal-Gonzalez et al., 2006, Almada et al., 2015, Sierra-Mercado et al., 2011). Roles for the PrL in fear-induced antinociception (Freitas et al., 2013) and formalin-induced conditioned place avoidance (Jiang et al., 2014) have also been demonstrated. The anterior cingulate cortex

(ACC) is a subregion of the mPFC involved in the modulation of fear behaviour (Einarsson et al., 2015), cognitive-affective component of pain (Johansen et al., 2001) and in top-down descending modulation of pain (Fuchs et al., 2014). The ACC is connected reciprocally with both the PrL and IL and may play a role in modulating their output (Vertes, 2002). Despite the evidence for a role of the IL, PrL and ACC in fear- and pain-related behaviour, there is a paucity of studies comparing these three subregions of the mPFC with respect to their role in fear-pain interactions which we sought to address here.

Specifically, we investigated the role of the endogenous cannabinoid (endocannabinoid) system within subregions of the mPFC in the expression of formalin-evoked nociceptive tone, fear-conditioned analgesia (FCA) and conditioned fear in the presence of nociceptive tone. The endocannabinoid system is comprised of cannabinoid type 1 (CB_1) (Devane et al., 1988, Matsuda et al., 1990) and cannabinoid type 2 (CB_2) (Munro et al., 1993) receptors, their endogenous ligands (or endocannabinoids), the two best characterised being *N*-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995), and the enzymes responsible for the synthesis and degradation of the endocannabinoids. FCA is the robust suppression of nociceptive behaviour during or following expression of classical Pavlovian conditioned fear (Butler and Finn, 2009, Ford and Finn, 2008). Our previous research has implicated the PFC in the expression of FCA in rats (Butler et al., 2011). Moreover, we and others have demonstrated a key role for the endocannabinoid system in FCA (Finn et al., 2004, Finn et al., 2003, Butler and Finn, 2009, Butler et al., 2008, Roche et al., 2007, Ford and Finn, 2008, Ford et al., 2011, Olango et al., 2014, Olango et al., 2012, Rea et al., 2013, Corcoran et al., 2015) and unconditioned stress-induced analgesia (Connell et al., 2006, Guindon and Hohmann, 2009, Hohmann et al., 2005, Suplita et al., 2005). These studies have highlighted the

importance of the endocannabinoid system in discrete brain regions including the amygdala (Rea et al., 2013), hippocampus (Ford et al., 2011) and periaqueductal grey (Olango et al., 2012), all of which are connected anatomically to the mPFC. Components of the endocannabinoid system, including CB_1 receptors and the anandamide-catabolising enzyme fatty acid amide hydrolase (FAAH), are highly expressed in the mPFC (Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Tsou et al., 1998, Egertova et al., 2003). Interestingly, de Freitas and colleagues have demonstrated that innate, unconditioned fear-induced antinociception arising from blockade of $GABA_A$ receptors in the ventromedial and dorsomedial hypothalamus is attenuated by microinjection of the CB_1 receptor antagonist/inverse agonist AM251 into the PrL (Freitas et al., 2013). However, no studies to date have compared the role of the endocannabinoid system in the PrL, IL and ACC in pain suppression arising from conditioned fear (FCA) and this was the primary aim of the present study.

We tested the hypothesis that the endocannabinoid system in the PrL, IL and ACC differentially modulates FCA and expression of fear in the presence of formalin-evoked nociceptive tone. To this end, we investigated the effects of local intra-PrL, intra-IL and intra-ACC microinjections of the FAAH inhibitor, URB597 and the CB_1 receptor antagonist/inverse agonist, AM251, alone or in combination, on formalin-evoked nociceptive behaviour, FCA and expression of fear in the presence of formalin-evoked nociceptive tone in rats. Elucidation of the role of the endocannabinoid system in different subregions of the mPFC in fear-pain interactions may facilitate increased understanding and improved treatment of pain- and fear-related disorders and their comorbidity.

Materials and Methods

Animals

A total of 280 male Lister-Hooded rats (260–350 g on day of behavioural testing; Charles River, Margate, Kent, UK) were used. Animals were housed 3-4 per cage before surgery and singly thereafter in plastic bottomed cages (L: 45 x H: 20 x W: 20 cm) with wood shavings as bedding. They were maintained at a constant temperature (22 ± 2^{o}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 (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, and the work carried out under license from the Irish Department of Health and Children, in compliance with the European Communities Council directives 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

Cannulae Implantation

Animals were left to acclimatize for 4-8 days after delivery before surgery. Stainless steel guide cannulae (5mm length, 22G, Plastics One Inc., Roanoke, VA, USA) were stereotaxically implanted bilaterally 1 mm above the right and left IL (AP + 2 mm relative to bregma, ML ± 1.5 mm relative to bregma and at a 12^{o} angle, DV – 3.6 mm from dura, toothbar set at -3 mm), or PrL (AP + 2.4 mm relative to bregma, ML ± 1.5 mm relative to bregma and at a 12^{o} angle and DV – 2.3 mm from dura, toothbar set at -3 mm) or ACC (AP + 1 mm relative to bregma, ML ± 1.3 mm at a 12^{o} angle, DV – 1.3 mm from dura, toothbar set at -3 mm) (Paxinos and Watson, 1998) under isoflurane anaesthesia (2–3% in O_2; 0.5 L/ min). Rats were deemed to be

sufficiently anaesthetised when the pedal withdrawal reflex was absent. The cannulae were permanently fixed to the skull using stainless-steel screws and carboxylate cement. A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, Virginia, USA; 0.356 mm diameter) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (5 mg/kg s.c.; Rimadyl, Pfizer, Kent, UK), and the broad spectrum antibiotic, enrofloxacin (2.5 mg/kg s.c.; Baytril, Bayer Ltd., Dublin, Ireland), were administered before surgery to manage post-operative pain and to prevent infection, respectively. Following cannulae implantation, the rats were housed singly and administered enrofloxacin (2.5 mg/kg s.c.) for a further 3 days. Rats were allowed to recover for at least 6 days prior to experimentation. During this period, the rats were handled daily, stylets checked, and their body weight and general health monitored on a daily basis.

Drug Preparation

Formalin (37% formaldehyde solution), DMSO (dimethyl sulfoxide, 100%) and URB597 (cyclohexylcarbamic acid 3´-carbamoyl-biphenyl-3-yl ester) were purchased from Sigma-Aldrich, Dublin, Ireland. AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide was purchased from Abcam plc, Cambridge, UK.

On test days, stock solutions of 4mM AM251 (CB_1 receptor antagonist/inverse agonist) and 0.2mM URB597 (FAAH inhibitor) were prepared in 100% DMSO vehicle (VEH). For bilateral intra-PrL, intra-IL and intra-ACC microinjections, AM251 and URB597 were prepared from the stock solution to a concentration of 2mM and 0.1mM in 100% DMSO vehicle respectively, while the combination was prepared by adding equal volumes of the stock 4mM AM251 and 0.2mM URB597. These doses of URB597 and AM251 are based on previous work carried out

by our laboratory and evidence from the literature in rat models of pain and fear (Laviolette and Grace, 2006, Lisboa et al., 2010, Ford et al., 2011, Freitas et al., 2013, Ebrahimzadeh and Haghparast, de Novellis et al., 2008, Rea et al., 2014a, Olango et al., 2012). A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

Experimental Procedures

The FCA paradigm was essentially as described previously (Butler et al., 2008, Finn et al., 2004, Rea et al., 2009, Roche et al., 2007). In brief, it consisted of two phases, conditioning and testing, occurring 24 h apart. 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. Three experiments, all using a different cohort of rats, were carried out involving microinjections of URB597, AM251, URB597 + AM251 (combination) or vehicle into the IL, PrL or ACC respectively. The conditioning phase for these experiments was carried out as outlined above. The test phase commenced 23.5 hours later when the subjects received an intra-plantar injection of 50 µL formalin (2.5% formaldehyde solution prepared in sterile saline) into the right hindpaw under brief isoflurane anaesthesia (2–3% in O_2; 0.5 L/ min). 15 minutes post-formalin injection, rats received bilateral intra-IL , intra-PrL or intra-ACC microinjections of vehicle (100% DMSO), 2mM AM251, 0.1mM URB597 or the combination of 2mM AM251 with 0.1mM URB597 in an injection volume of 0.3µL over a 60 s time interval using an injection pump, a 1µL Hamilton

syringe and polyethylene tubing connected to a stainless steel injector with 1 mm protrusion beyond the guide cannula (28G, Plastics One Inc., Roanoke, VA, USA). Immediately following the intracerebral microinjections, rats were returned to their home cages for 15 minutes prior to being placed into the same Perspex arenas in which they had been conditioned. A video camera located beneath the observation chamber was used to monitor animal behaviour. The video feed was recorded onto DVD for 30 minutes. The 30–60 min post-formalin interval was chosen on the basis of previous studies demonstrating that formalin-evoked nociceptive behaviour is stable over this time period, is endocannabinoid-mediated and subject to supraspinal modulation (Finn et al., 2004, Ford et al., 2011, Olango et al., 2012, Rea et al., 2011, Roche et al., 2010,).

At the end of the test phase, (60 min post-formalin injection), rats were euthanised by decapitation, and intra-cerebral microinjection of fast-green dye (0.5µL of 1% solution) was performed following decapitation for subsequent histological confirmation of the microinjection sites. The brains were subsequently excised, snap-frozen on dry ice and stored at -80^{o}C.

Formalin-induced oedema was assessed by measuring the change in the diameter of the right hindpaw immediately before, and 60 minutes after, formalin administration, using Vernier callipers.

This design resulted in 8 experimental groups (Starting n=11-12 per group for surgery; final n after removal of outliers where the cannula placements were inaccurate or injections were sub-optimal are depicted in Table 1).

Behavioural Analysis

Ethovision XT 7.0 software package (Noldus, Wageningen, The Netherlands) was used to analyse behaviour, allowing for continuous event recording over each 30 minute trial. The behaviours assessed (by an experimenter blind to treatment) were: duration of freezing (defined as the cessation of all visible movement except that necessary for breathing) as a measure of fear-related behaviour and nociceptive behaviours (composite pain score (CPS)) as described previously (Butler et al., 2008, Finn et al., 2004, Finn et al., 2006). Nociceptive behaviours were measured using the weighted composite pain scoring technique (Watson et al., 1997). Nociceptive behaviours are divided into two types according to this method; the first (pain 1) is the time spent elevating the formalin-injected paw without contact with any other surface. The second (pain 2) is the time spent holding, licking, biting, shaking or flinching the formalin-injected paw. The composite pain score equation is given as Composite Pain Score [CPS = (duration of pain 1 + 2 x duration of pain 2)/(total duration of analysis period)] (Watson et al., 1997). The Ethovision system automatically tracked total distance moved as a measure of locomotor activity.

Histological verification of intra-cerebral microinjection sites

The sites of intra-cerebral microinjection were determined prior to data analysis. Brain sections with fast-green dye mark were collected (30µm thickness) using a cryostat (Microm, Thermo Fisher Scientific, Walldorf, Germany), mounted on gelatinised glass slides and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy.

Statistical analysis

Rats were randomly assigned to experimental groups and the sequence of testing was randomized throughout the experiment. Previous published studies and power analysis suggested that when using ANOVA the sample sizes used would yield sufficient power to reliably detect differences in the means between groups with sufficient power (i.e. >90%). The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. Paw oedema data were analysed using a paired Student's t-test. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and drug treatment as the between-subjects factors. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. Data were considered significant when $p<0.05$. Results are expressed as group means ± standard error of the mean (SEM). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a, Alexander et al., 2017b).

Results

Histological verification of microinjection sites

After histological verification, 75% of the microinjections were found to be within the borders of both the left and right IL, 84% in the PrL and 78% in the ACC. The remaining microinjections were placed in the corpus callosum, or outside the borders of the IL, PrL or ACC, respectively. Only data from rats where intra-cerebral injections were accurately placed in both the left and right IL, PrL, or ACC, were included. See figures 2(A+B), 3(A+B) and 4(A+B).

Effects of intra-mPFC administration of AM251, URB597 or URB597 + AM251 on formalin-evoked nociceptive behaviour and FCA

Intraplantar injection of formalin increased right hind paw diameter (indicative of oedema) in each of the three studies [IL (T_{54} = 41.999, P < .001), PrL (T_{71} = 32.206, P < .001) and ACC (T_{72} = 34.570, P < .001)], and produced robust licking, biting, shaking, flinching, and elevation of the injected right hind paw.

In the IL study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,47)=14.857$, $p<0.001$), treatment ($F(3,47)=3.632$, $p<0.05$) and fear-conditioning*treatment ($F(3,47)=3.774$, $p<0.05$) on CPS over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,94)=62.397$, $p<0.001$), time*fear-conditioning ($F(2,94)=23.911$, $p<0.001$), time*treatment ($F(6,94)=4.383$, $p<0.001$) and time*fear-conditioning*treatment ($F(6,94)=4.994$, $p<0.001$). Further post-hoc analysis with Tukey's test revealed that FC VEH-treated rats displayed significantly less formalin-evoked nociceptive behaviour compared with NFC VEH-treated rats in the first 10 minutes of

the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.001$), confirming the expression of FCA. This FCA was significantly attenuated by intra-IL administration of either AM251 or URB597 alone (0-10 min: FC AM251 vs. FC VEH, $p<0.001$; FC URB597 vs. FC VEH, $p<0.01$) and a trend for a similar attenuation when both drugs were co-administered (FC URB597 + AM251 vs. FC VEH, $p=0.09$). Intra-IL administration of these drugs had no significant effect on formalin-evoked nociceptive behaviour in NFC rats. See figure 5(A).

In the PrL study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,64)=28.548$, $p<0.001$) but not treatment ($F(3,64)=1.498$, $p=0.224$) or fear-conditioning*treatment ($F(3,64)=2.269$, $p=0.089$) on CPS over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,128)=71.588$, $p<0.001$) and time*fear-conditioning ($F(2,128)=21.436$, $p<0.001$), but not time*treatment ($F(6,128)=1.296$, $p=0.264$) or time*fear-conditioning*treatment ($F(6,128)=1.742$, $p=0.116$). Further post-hoc analysis with Tukey's test revealed that FC VEH-treated rats displayed significantly less formalin-evoked nociceptive behaviour compared with NFC VEH-treated rats in the first 10 minutes of the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.05$), confirming the expression of FCA. This FCA was significantly attenuated by intra-PrL administration of AM251 (0-10 min: FC AM251 vs. FC VEH, $p<0.05$), but not by URB597 or co-administration of URB597 and AM251. . Indeed, FC rats that received intra-PrL URB597 (but not URB597 + AM251) had significantly lower formalin-evoked nociceptive behaviour than NFC counterparts over the first 20 min of the trial (0-10 mins: FC URB597 vs. NFC URB597, $p<0.001$; 10-20 mins: FC URB597 vs. NFC URB597, $p<0.01$), suggesting that URB597 prolonged the expression of FCA relative to VEH-treated FC rats in which significant FCA was observed in the first 10 min only. This URB597-induced prolongation of FCA was not observed in rats co-administered URB597 and AM251. Intra-PrL administration of these

drugs had no significant effect on formalin-evoked nociceptive behaviour in NFC rats. See figure 6(A).

In the ACC study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,64)=84.125$, $p<0.001$), but not treatment ($F(3,64)=1.190$, $p=0.321$) or fear-conditioning*treatment ($F(3,64)=1.429$, $p=0.243$) on CPS over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,128)=99.337$, $p<0.001$) and time*fear-conditioning ($F(2,128)=10.946$, $p<0.001$), but not time*treatment ($F(6,128)=1.744$, $p=0.116$) or time*fear-conditioning*treatment ($F(6,128)=1.173$, $p=0.325$). Further post-hoc analysis with Tukey's test revealed that FC VEH-treated rats displayed significant less formalin-evoked nociceptive behaviour compared with NFC VEH-treated rats in the first 20 minutes of the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.05$; 10-20 min: FC VEH vs. NFC VEH, $p<0.05$), confirming the expression of FCA. Intra-ACC administration of AM251 or URB597, alone or in combination, had no significant effect on the expression of formalin-evoked nociceptive behaviour *per se* or FCA. See figure 7(A).

See table 2 for a summary of results.

Effects of intra-mPFC administration of AM251, URB597 or URB597 + AM251 on the expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

In the IL study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,47)=78.621$, $p<0.001$), treatment ($F(3,47)=7.958$, $p<0.001$) and fear-

conditioning*treatment ($F(3,47)=8.063$, $p<0.001$) on the duration of freezing over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,94)=71.091$, $p<0.001$), time*fear-conditioning ($F(2,94)=69.984$, $p<0.001$), time*treatment ($F(6,94)=6.260$, $p<0.001$) and time*fear-conditioning*treatment ($F(6,94)=6.363$, $p<0.001$). Further post-hoc analysis using Tukey's test revealed that FC VEH-treated rats displayed significantly more freezing compared with NFC VEH-treated rats in the first 20 minutes of the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.001$; 10-20 min: FC VEH vs. NFC VEH, $p<0.001$). Intra-IL administration of AM251 or URB597 alone significantly attenuated this contextually induced freezing over the first 20 min of the trial (0-10 min: FC AM251 vs. FC VEH, $p<0.001$; FC URB597 vs. FC VEH, $p<0.001$; 10-20 min: FC AM251 vs. FC VEH, $p<0.001$; FC URB597 vs. FC VEH, $p<0.01$)., and during minutes 10-20 when the drugs were co-administered (FC URB597 + AM251 vs. FC VEH, $p<0.01$). See figure 5(B).

In the PrL study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,64)=37.655$, $p<0.001$) but not treatment ($F(3,64)=0.519$, $p=0.671$) or fear-conditioning*treatment ($F(3,64)=0.434$, $p=0.729$) on the duration of freezing over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,128)=39.656$, $p<0.001$) and time*fear-conditioning ($F(2,128)=22.431$, $p<0.001$) but not time*treatment ($F(6,128)=0.370$, $p=0.897$) or time*fear-conditioning*treatment ($F(6,128)=0.464$, $p=0.834$). Further post-hoc analysis using Tukey's test revealed that FC VEH-treated rats displayed significantly more freezing compared with NFC VEH-treated rats in the first 10 minutes of the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.05$). Intra-PrL administration of AM251 or URB597, alone or in combination, had no significant effect on the expression of this contextually induced freezing. See figure 6(B).

In the ACC study, 2-way repeated measures ANOVA revealed a significant effect of fear-conditioning ($F(1,64)=84.125$, $p<0.001$) but not treatment ($F(3,64)=1.190$, $p=0.321$) or fear-conditioning*treatment ($F(3,64)=1.429$, $p=0.243$) on the duration of freezing over the course of the 30 minute testing period. There was also a significant effect of time ($F(2,128)=99.337$, $p<0.001$) and time*fear-conditioning ($F(2,128)=10.946$, $p<0.001$) but not time*treatment ($F(6,128)=1.744$, $p=0.116$) or time*fear-conditioning*treatment ($F(6,128)=1.173$, $p=0.325$. Further post-hoc analysis using Tukey's test revealed that FC VEH-treated rats displayed significantly more freezing compared with NFC VEH-treated rats in the first 10 minutes of the testing period (0-10 min: FC VEH vs. NFC VEH, $p<0.05$). Intra-ACC administration of AM251 or URB597, alone or in combination, had no significant effect on the duration of freezing. See figure 7(B).

See table 3 for a summary of results.

Effects of fear-conditioning and AM251, URB597 or URB597 + AM251 on locomotor activity in formalin-treated rats

In the IL study, there was no significant effect of fear-conditioning ($F(1,47)=0.185$, $p=0.669$), treatment ($F(3,47)=0.442$, $p=0.724$) or fear-conditioning*treatment ($F(3,47)=0.206$, $p=0.892$) on distance moved. There was an effect of time ($F(2,94)=47.070$, $p<0.001$) and time*fear-conditioning ($F(2,94)=3.840$, $p<0.05$) but not time*treatment ($F(6,94)=1.626$, $p=0.148$) or time*fear-conditioning*treatment ($F(6,94)=0.811$, $p=0.564$). Further post-hoc analysis revealed no significant between group differences. See figure 5(C).

In the PrL study, there was no significant effect of fear-conditioning ($F(1,64)=0.064$, $p=0.801$), treatment ($F(3,64)=0.798$, $p=0.500$) or fear-conditioning*treatment ($F(3,64)=0.643$, $p=0.590$) on distance moved. There was an effect of time ($F(2,128)=16.590$, $p<0.001$) and time*fear-conditioning ($F(2,128)=8.287$, $p<0.001$) but not time*treatment ($F(6,128)=0.224$, $p=0.968$) or time*fear-conditioning*treatment ($F(6,128)=0.842$, $p=0.540$). Further post-hoc analysis revealed no significant between group differences. See figure 6(C).

In the ACC study, there was no significant effect of fear-conditioning ($F(1,64)=0.403$, $p=0.528$), treatment ($F(3,64)=0.428$, $p=0.734$) or fear-conditioning*treatment ($F(3,64)=0.273$, $p=0.845$) on distance moved. There was an effect of time*fear-conditioning ($F(2,128)=16.725$, $p<0.001$) but not time ($F(2,128)=1.022$, $p=0.363$), time*treatment ($F(6,128)=1.524$, $p=0.175$) or time*fear-conditioning*treatment ($F(6,128)=0.484$, $p=0.819$). Further post-hoc analysis revealed no significant between group differences. See figure 7(C).

Discussion

The data presented herein demonstrate for the first time that the endocannabinoid system in the mPFC is an important neural substrate regulating expression of FCA and fear-pain interactions. The results indicate that this regulation occurs in a subregion-specific manner, with the endocannabinoid system in the PrL, IL and ACC playing distinct and differential modulatory roles in expression of FCA and fear in the presence of nociceptive tone.

Blockade of CB_1 receptors in the PrL with AM251 attenuated FCA with no effects on the expression of formalin-evoked nociceptive behaviour *per se* or on contextually induced freezing in the presence of nociceptive tone. In addition, intra-PrL administration of the FAAH inhibitor URB597 prolonged the expression of FCA in the absence but not the presence of AM251. These findings together suggest that FCA is mediated by endocannabinoids acting at CB_1 receptors within the PrL, and suggest that the PrL can be considered as an additional important neural substrate for endocannabinoid-mediated FCA, alongside the ventral hippocampus (Ford et al., 2011), dorsolateral periaqueductal grey (Olango et al., 2012) and basolateral amygdala (Rea et al., 2013, Roche et al., 2007). The results of this PrL study also corroborate our previous studies (Finn et al., 2004, Rea et al., 2014b, Roche et al., 2007, Roche et al., 2010) and those of others (Helmstetter and Fanselow, 1987, Kinscheck et al., 1984), demonstrating that pain-related behaviour in fear-conditioned animals can be altered independently of the level of fear being expressed in these animals in the presence of nociceptive tone.

In contrast to the PrL, either CB_1 receptor blockade or FAAH inhibition within the IL attenuated expression of both FCA and contextually induced freezing in the presence of nociceptive tone. Thus, in contrast to the results for the PrL study where the effects of the

endocannabinoid system on FCA and fear-related freezing were dissociable, the endocannabinoid system in the IL appears to modulate FCA and contextually induced freezing in the same direction. It is also interesting that in the IL, both AM251 and URB597 attenuated FCA and freezing, given the differing mechanisms of action of these drugs (CB_1 receptor blockade versus FAAH inhibition, respectively). In both the IL and PrL, AM251 attenuated FCA, suggesting that CB_1 receptors in both of these mPFC subregions mediate FCA. While our data provide the first evidence for a role of the endocannabinoid system in the IL and PrL in mediating conditioned fear-induced analgesia, previous research has demonstrated a role for the endocannabinoid system in the PrL in the expression of unconditioned fear-induced analgesia (Freitas et al., 2013). CB_1 receptors in the PrL therefore appear to play a key role in mediating the expression of endogenous analgesia to both conditioned and unconditioned aversive stimuli. However, while the data from our PrL study are compatible with the idea that endocannabinoid-CB_1 signalling mediates FCA, the attenuation of FCA following intra-IL administration of URB597 is not compatible with the effects of AM251 in the IL and suggests that activation of a non-CB_1 receptor target by one or more FAAH substrates may instead be mediating the effects of URB597 in the IL. In this respect, FAAH substrates including AEA, PEA and OEA have been shown to activate a number of non-CB_1 targets either directly or indirectly (via substrate competition at FAAH) including CB_2 (Griffin et al., 2000, Felder et al., 1996, Petrosino and Di Marzo, 2017), TRPV1 (De Petrocellis et al., 2001, Di Marzo et al., 2001, Ross et al., 2001, Smart et al., 2000), PPARs (LoVerme et al., 2005, O'Sullivan and Kendall, 2010, Pistis and O'Sullivan, 2017) and GPR55 (Pertwee, 2007, Sharir and Abood, 2010, Ryberg et al., 2007, Kramar et al., 2017). Future studies should address the potential role of these receptors within the IL in regulation of fear, pain and FCA.

In our ACC study, in contrast to the results obtained for the PrL and IL, neither AM251 nor URB597 affected the expression of FCA or contextually-induced freezing, providing further support for the contention that the endocannabinoid system within the mPFC regulates FCA in a subregion-specific manner. In addition, the data revealed that regardless of the region injected, URB597 and AM251 and no effect on the distance moved, indicating that their effects within the IL and PrL on FCA and contextually induced freezing likely represent specific effects on nociceptive and fear-related behaviour rather than overt effects on locomotor activity.

One implication of our findings is that the endocannabinoid system within each of these mPFC subregions may be an important factor contributing to their differential regulation of fear- and pain-related behaviour which has previously been described. Alterations in endocannabinoid signalling, coupled with differences in circuitry within, and projections to and from, each of these three mPFC subregions (Vertes, 2004, Hoover and Vertes, 2007, Vertes, 2002), likely underlies their different roles in fear, pain and FCA. One of the first reports of specific but differential functions of sub-regions in the mPFC was by Vidal-Gonzalez and colleagues (2006). They found that microstimulation of the PrL enhanced the expression of conditioned fear to a tone and prevented extinction, while microstimulation of the IL reduced the expression of conditioned fear and microstimulation of the ACC had no effect on either expression or extinction of conditioned fear (Vidal-Gonzalez et al., 2006). Inactivation of the PrL but not IL depressed fear responses while inactivation of the IL but not PrL impaired the consolidation and retrieval of fear extinction in rats (Laurent and Westbrook, 2009). Lesioning (Kim et al., 2013) or pharmacological inactivation (Sierra-Mercado et al., 2011, Corcoran and Quirk, 2007) of the PrL impairs the expression of conditioned fear without affecting extinction, while inactivation of the IL has no effect on fear expression but impairs the acquisition of extinction

as well the extinction memory (Sierra-Mercado et al., 2011). Fewer studies have compared the respective roles of these mPFC regions in modulating pain. Pre- and post-conditioning muscimol-mediated inactivation of IL and PrL had no effect on expression of formalin-evoked pain *per se*, but differentially affected formalin-evoked condition place aversion which was impaired by PrL, not not IL, inactivation (Jiang et al., 2014). Similarly, in the present studies we have observed little or no effect of pharmacological modulation of the endocannabinoid system within these three mPFC subregions on formalin-evoked nociceptive behaviour in the absence of fear under the conditions of testing used herein (but see Okine et al., 2016 where test conditions differed and intra-ACC administration of AM251 reduced formalin-evoked nociceptive behaviour). Future studies should address whether this differential modulation of FCA is achieved via differential modulation of the pathways connecting these mPFC subregions to downstream components of the descending inhibitory pain pathway (e.g. the amygdala and PAG), or more locally within the three subregions themselves via alteration of incoming ascending nociceptive information.

In conclusion, the present studies provide new evidence to support a role for the endocannabinoid system within the subregions of the mPFC in the expression of FCA and conditioned fear in the presence of nociceptive tone. Furthermore, our data suggest that endocannabinoid-mediated regulation of these behaviours occurs in a mPFC subregion-specific manner. Elucidation of the role of the endocannabinoid system in different subregions of the mPFC in fear-pain interactions may facilitate increased understanding of, and development of new therapeutic approaches for, pain- and fear-related disorders and their comorbidity.

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Tables:

Group	Conditioning	Formalin *i.pl.*	Drug/Vehicle	IL (n)	PrL (n)	ACC (n)
1	FC	Formalin	100% DMSO	5	9	12
2	No FC	Formalin	100% DMSO	9	8	8
3	FC	Formalin	2mM AM251	6	8	9

4	No FC	Formalin	2mM AM251	9	8	11
5	FC	Formalin	0.1mM URB597	9	10	8
6	No FC	Formalin	0.1mM URB597	8	8	9
7	FC	Formalin	URB597 + AM251	5	9	8
8	No FC	Formalin	URB597 + AM251	8	8	7

Table 1. Summary of experimental groups and final n number per group in Experiments 1 (IL), 2 (PrL) and 3 (ACC). FC, fear conditioned; No FC, non-fear-conditioned; DMSO, dimethylsulfoxide; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; URB597, cyclohexyl-carbamic acid 3´-carbamoyl-biphenyl-3-yl ester.

	IL	PrL	ACC
Vehicle	–	–	–
AM251	↓	↓	–
URB597	↓	↑	–

URB597 + AM251	↓ (trend)	–	–

Table 2: Summary of the drug effects on the expression of FCA in rats. – = no effect, ↓ = attenuated, ↑ = enhanced or prolonged

	IL	PrL	ACC
Vehicle	–	–	–
AM251	↓	–	–
URB597	↓	–	–
URB597 + AM251	– (0-10mins) ↓ (10-20mins)	–	–

Table 3: Summary of the drug effects on the expression of contextually induced freezing in rats. – = no effect, ↓ = attenuated, ↑ = enhanced or prolonged

Figure Legends:

Figure 1. Diagrammatic depiction of the experimental paradigm, treatment groups and timeline. NFC: Non-fear-conditioned; FC: Fear-conditioned; mPFC: medial prefrontal cortex; IL: Infralimbic cortex; PrL: Prelimbic cortex; ACC: Anterior cingulate cortex.

Figure 2. (A+B) Diagrammatic representation of the confirmed microinjection sites of all animals that underwent surgery to place guide cannulae in the left and right IL. NFC: Non-fear-conditioned; FC: Fear-conditioned; IL: Infralimbic cortex.

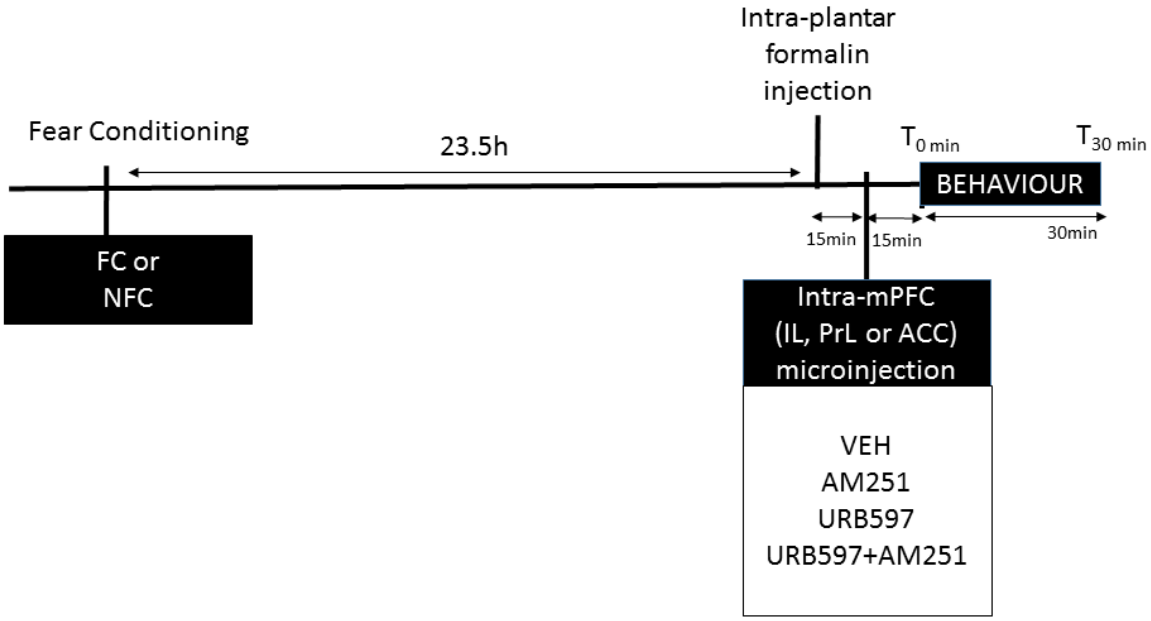
Figure 3. (A+B) Diagrammatic representation of the confirmed microinjection sites of all animals that underwent surgery to place guide cannulae in the left and right PrL. NFC: Non-fear-conditioned; FC: Fear-conditioned; PrL: Prelimbic cortex.

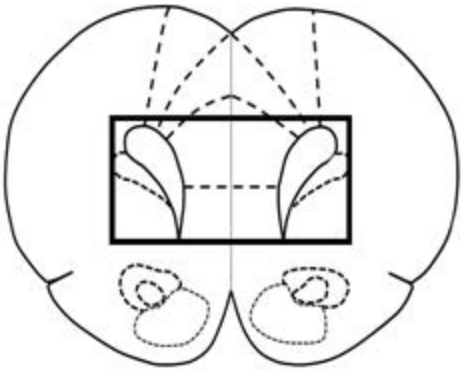
Figure 4. (A+B) Diagrammatic representation of the confirmed microinjection sites of all animals that underwent surgery to place guide cannulae in the left and right ACC. NFC: Non-fear-conditioned; FC: Fear-conditioned; ACC: Anterior cingulate cortex.

Figure 5. (A) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the IL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. (B) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the IL on the duration of freezing in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. (C) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the IL on the distance moved (cm) in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. ***$p<0.001$, FC vs. NFC; ##$p<0.01$, ###$p<0.001$ vs. FC VEH. All data are expressed as mean ± SEM. NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

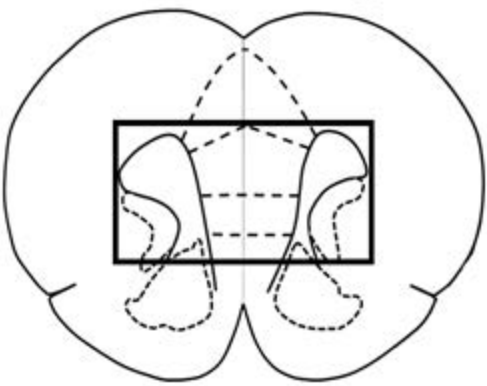
Figure 6. (A) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the PrL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. (B) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the PrL on the duration of freezing in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. (C) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the PrL on the distance moved (cm) in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. *$p<0.05$, **$p<0.01$, ***$p<0.001$ FC vs. NFC; #$p<0.05$ vs. FC VEH. All data are expressed as mean ± SEM. NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Figure 7. (A) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. (B) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the ACC on the duration of freezing in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. (C) Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597 + AM251 directly into the ACC on the distance moved (cm) in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. *$p<0.05$, **$p<0.01$, ***$p<0.001$, FC vs. NFC. All data are expressed as mean ± SEM. NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

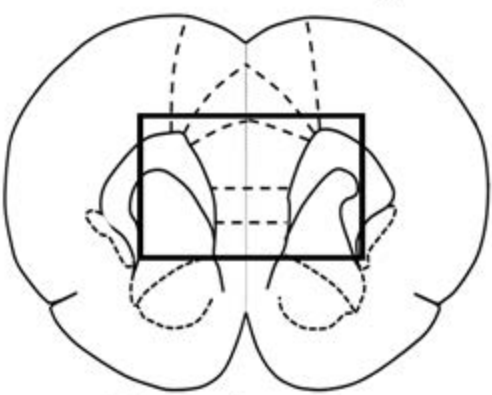

Intra-plantar
formalin
injection
Fear Conditioning
23.5h
T0 min
T30 min
BEHAVIOUR
15min
15min
30min
FC or
NFC
Intra-mPFC
(IL, PrL or ACC)
microinjection
VEH
AM251
URB597
URB597+AM251



+3.2 mm from Bregma



+2.7 mm from Bregma



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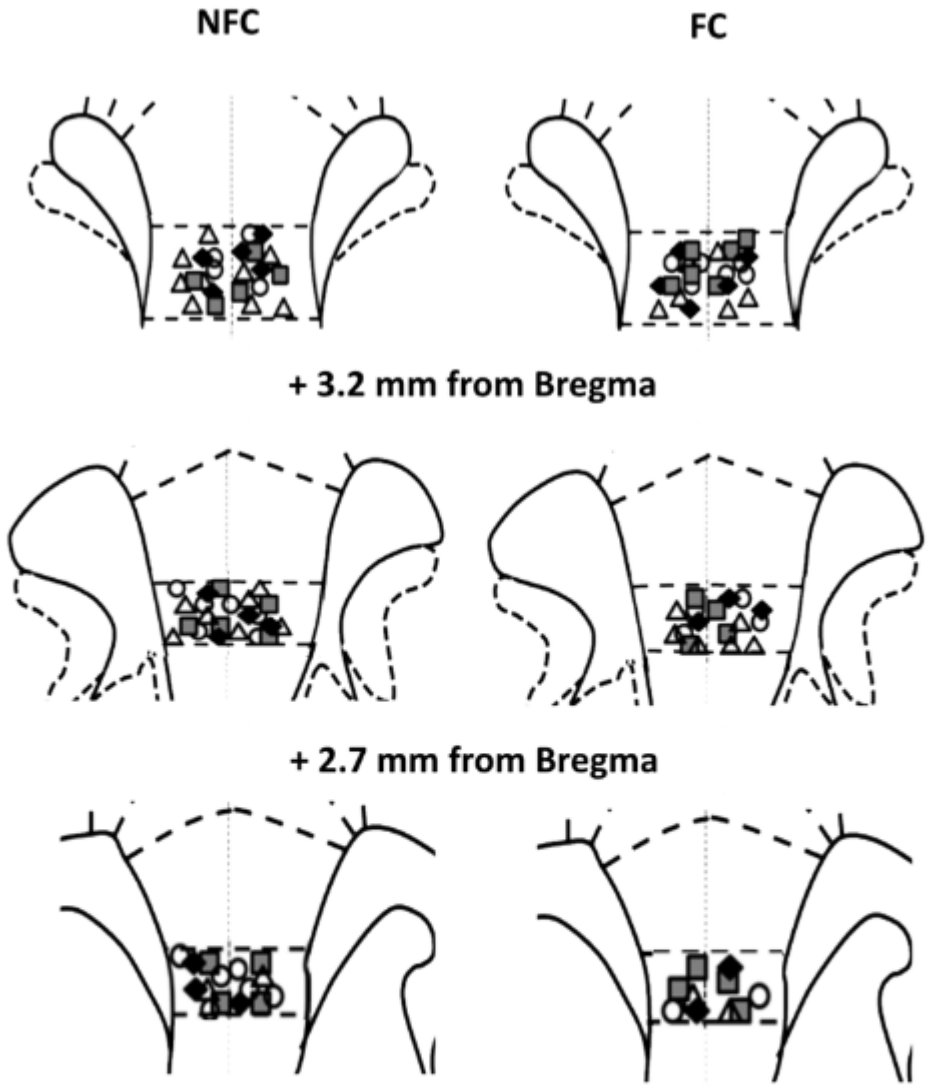
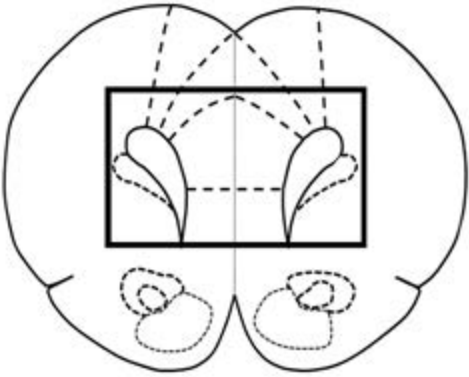
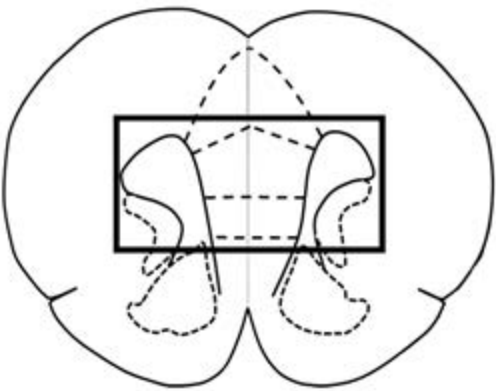

NFC
FC
+ 3.2 mm from Bregma
+ 2.7 mm from Bregma
+ 2.2 mm from Bregma
Vehicle
AM251
URB597
AM251 + URB597



+3.2 mm from Bregma



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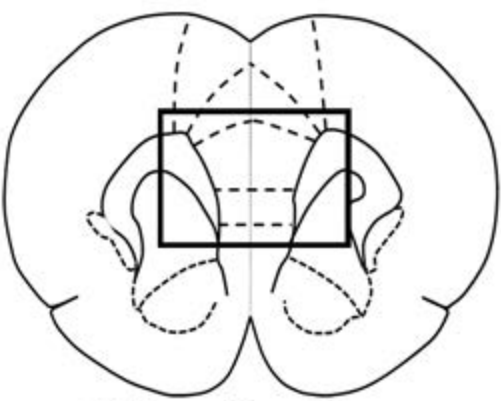
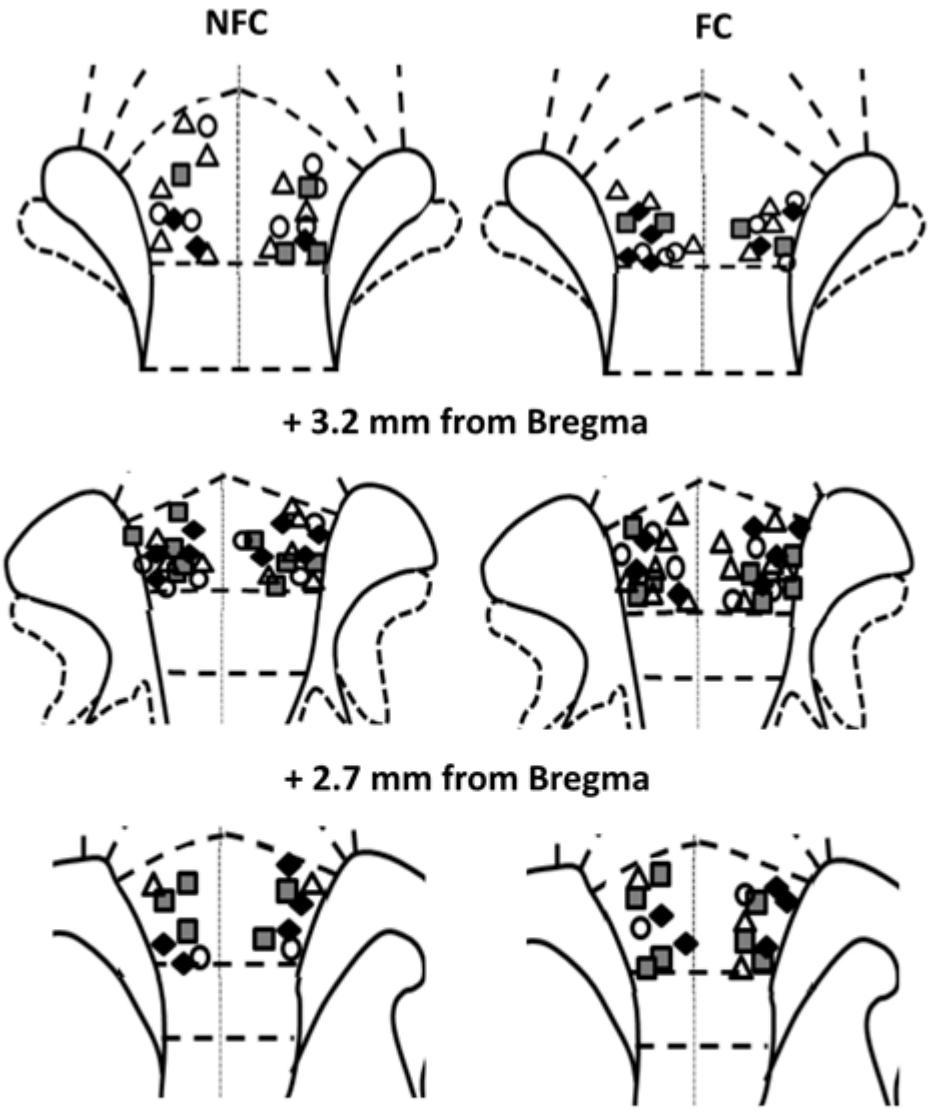
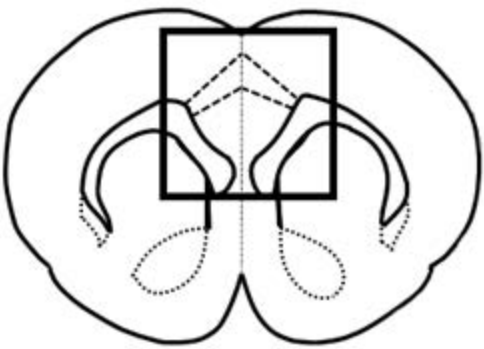


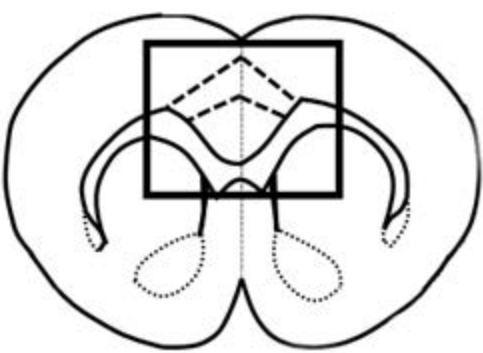
+2.2 mm from Bregma


NFC
FC
+ 3.2 mm from Bregma
+ 2.7 mm from Bregma
+ 2.2 mm from Bregma
Vehicle
AM251
URB597
AM251 + URB597

+ 2.2 mm from Bregma



+ 1.7 mm from Bregma



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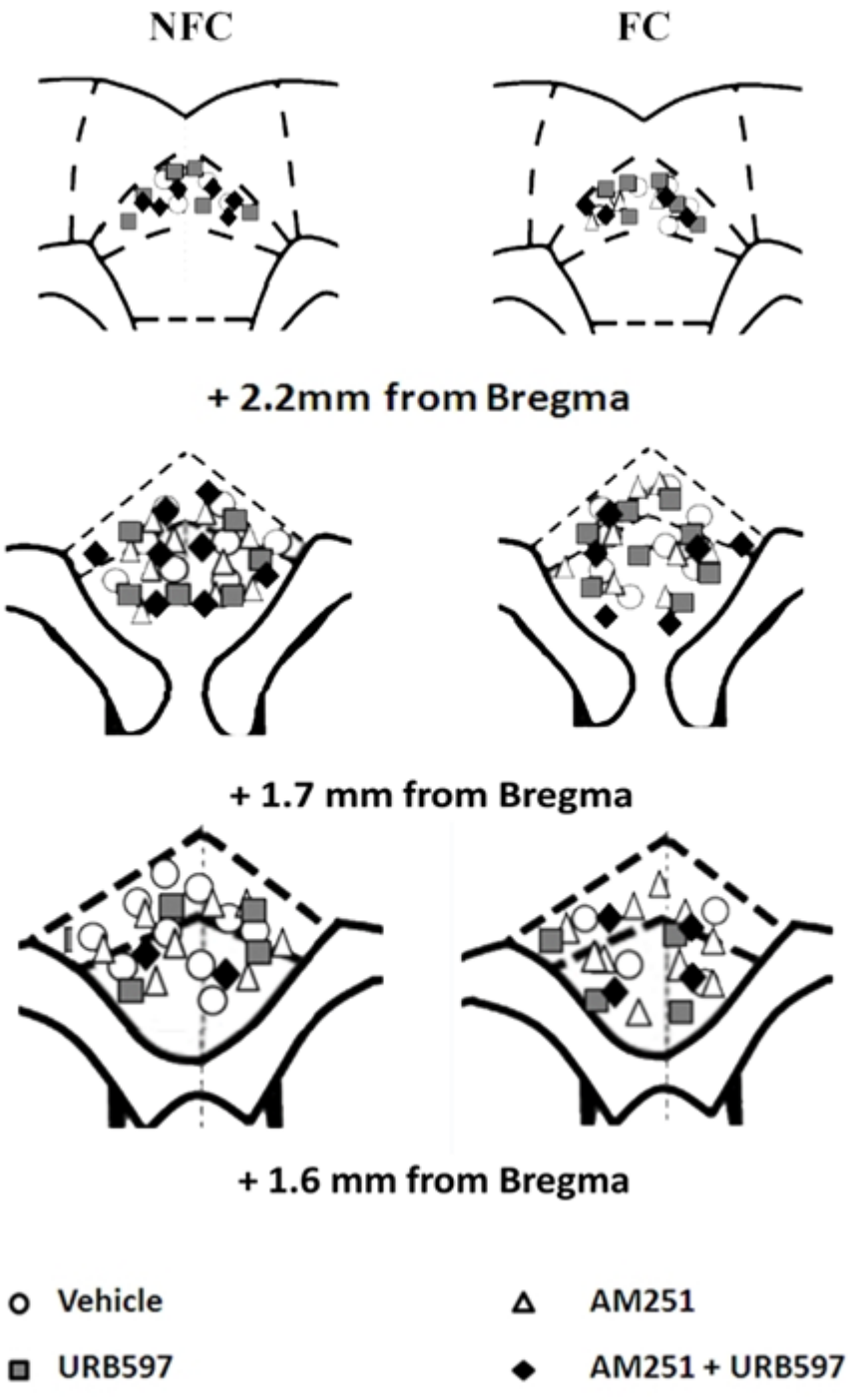
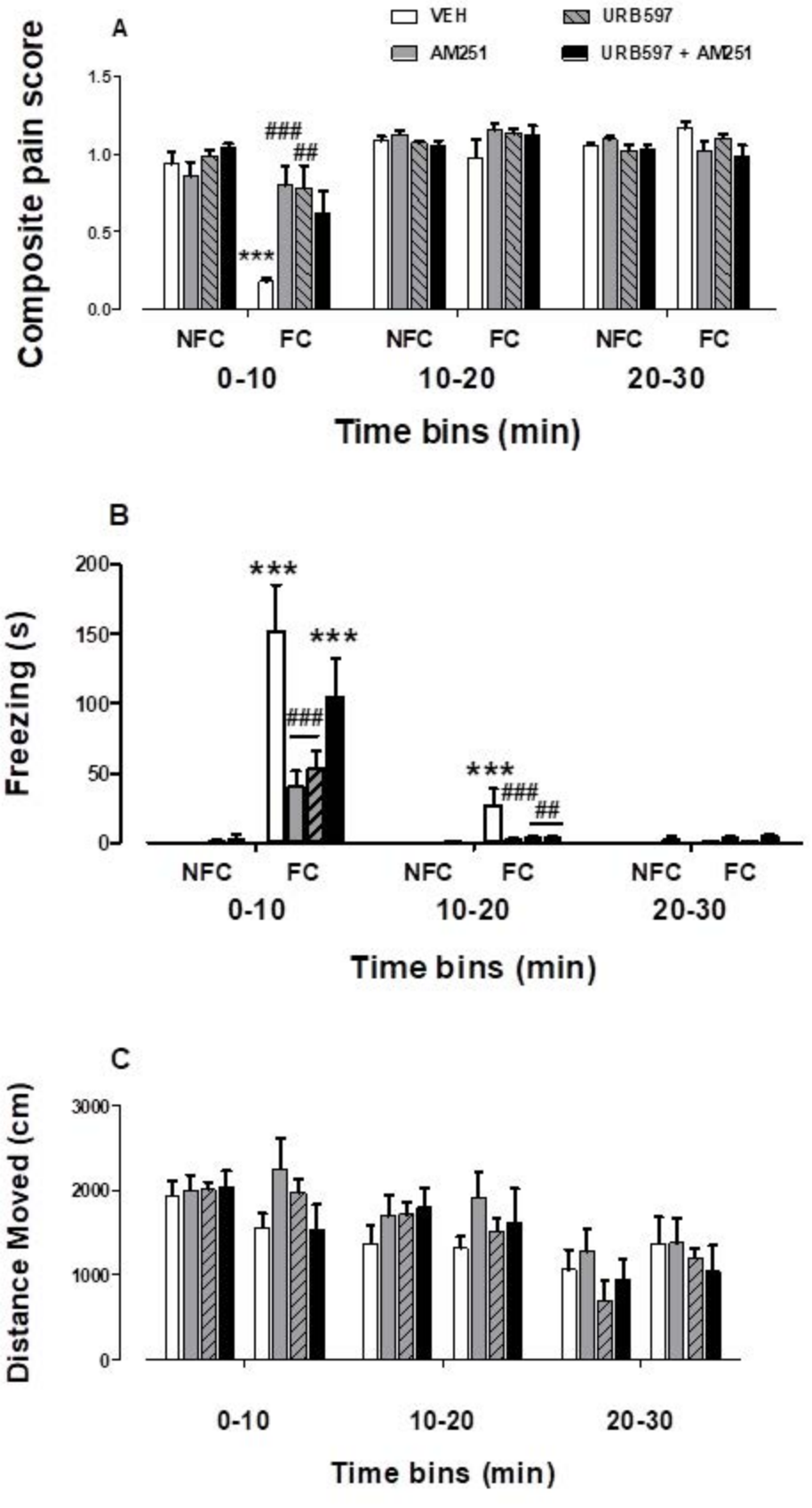
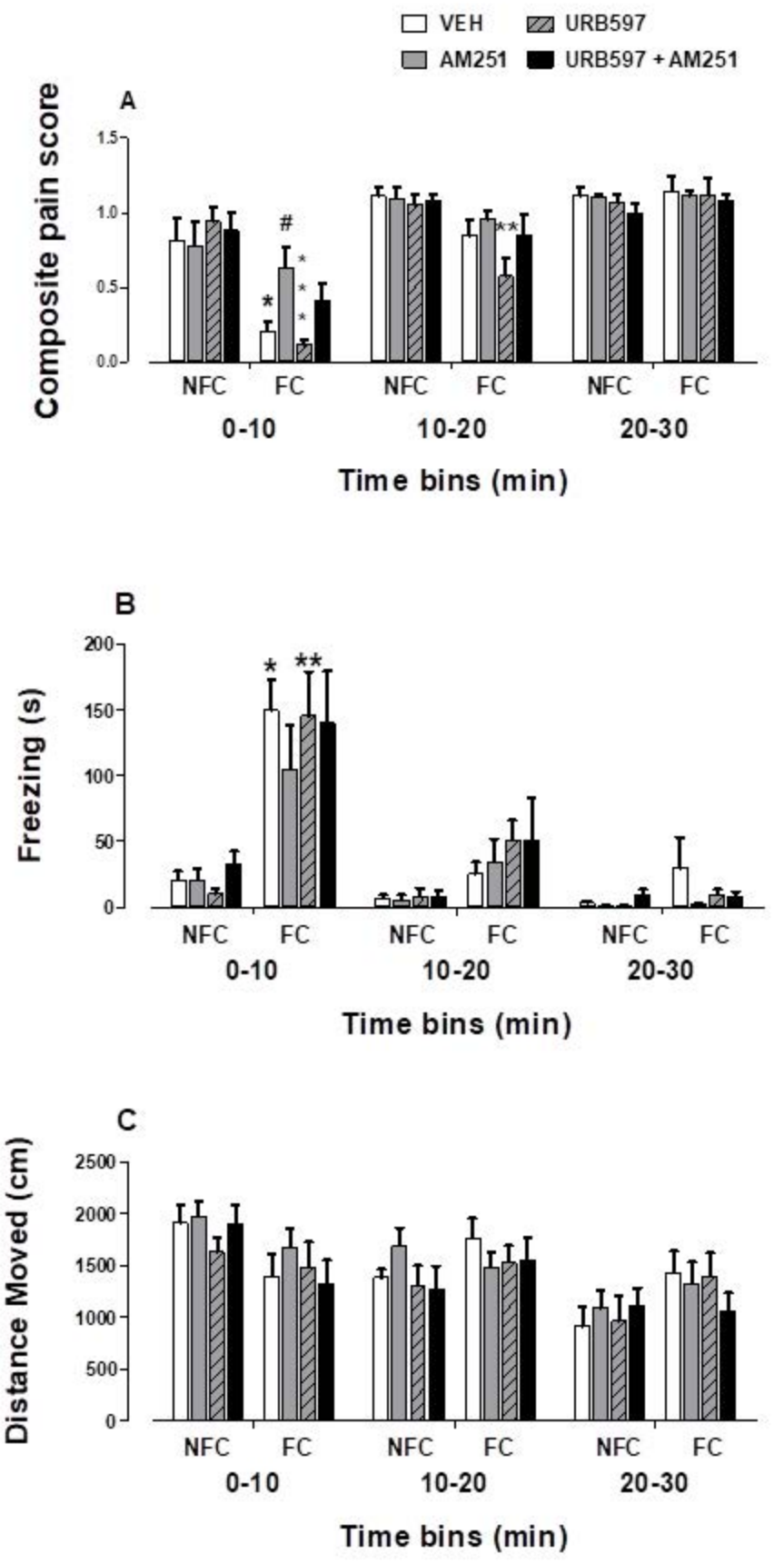
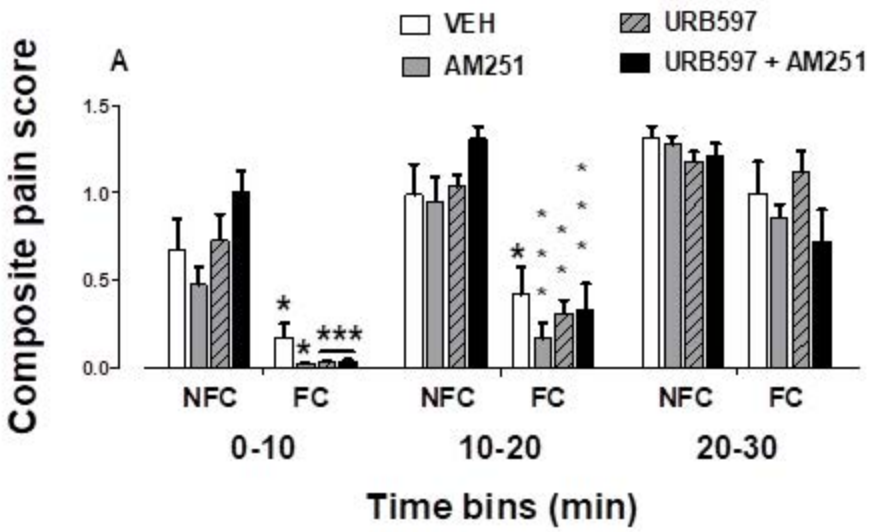

NFC
FC
+ 2.2mm from Bregma
+ 1.7 mm from Bregma
+ 1.6 mm from Bregma
Vehicle
AM251
URB597
AM251 + URB597


A
VEH
AM251
URB597
URB597 + AM251
Composite pain score
1.5
1.0
0.5
0.0
###
##
***
NFC
FC
NFC
FC
NFC
FC
0-10
10-20
20-30
Time bins (min)
B
Freezing (s)
200
150
100
50
0
***
***
###
***
###
##
NFC
FC
NFC
FC
NFC
FC
0-10
10-20
20-30
Time bins (min)
C
Distance Moved (cm)
3000
2000
1000
0
0-10
10-20
20-30
Time bins (min)


VEH
URB597
AM251
URB597 + AM251
A
Composite pain score
1.5
1.0
0.5
0.0
#
*
**
NFC
FC
0-10
10-20
20-30
Time bins (min)
B
Freezing (s)
200
150
100
50
0
*
**
C
Distance Moved (cm)
2500
2000
1500
1000
500
0


A
VEH
AM251
URB597
URB597 + AM251
Composite pain score
1.5
1.0
0.5
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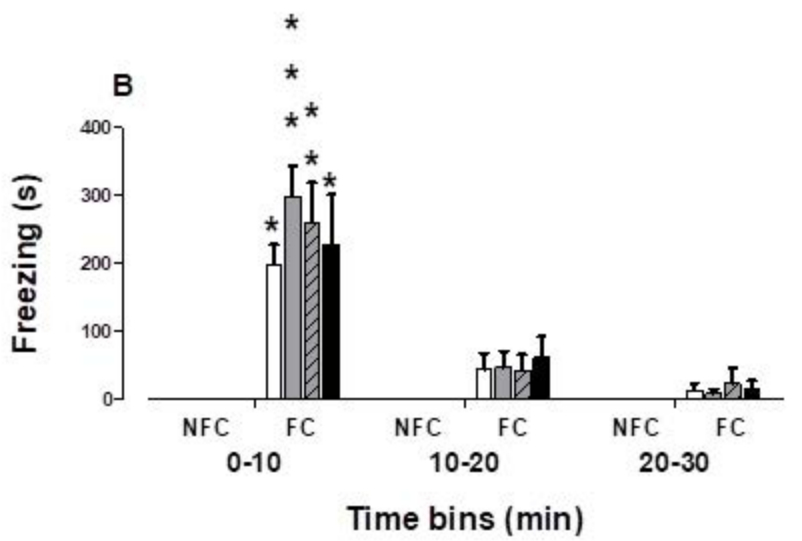
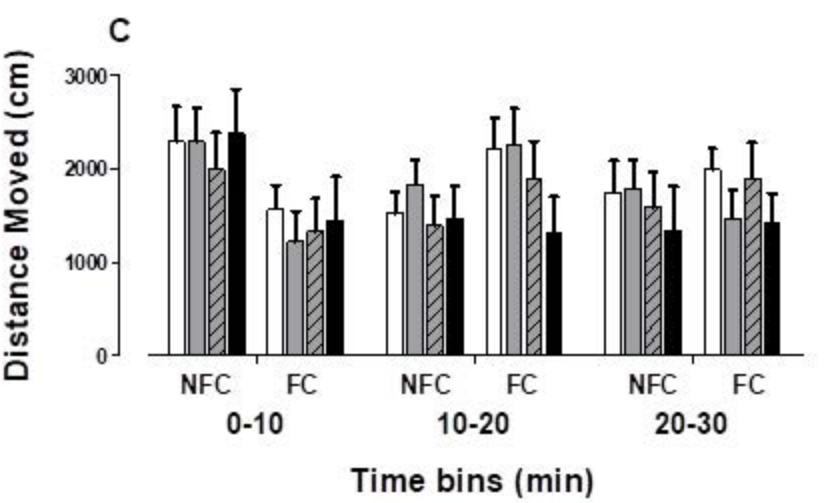
NFC
FC
0-10
10-20
20-30
Time bins (min)


B
Freezing (s)
400
300
200
100
0
NFC
FC
0-10
10-20
20-30
Time bins (min)


C
Distance Moved (cm)
3000
2000
1000
0
NFC
FC
0-10
10-20
20-30
Time bins (min)