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Molecular and electrophysiological changes in the prefrontal cortex-amygdala-dorsal periaqueductal grey pathway during persistent pain state and fear-conditioned analgesia

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Fear-conditioned analgesia (FCA) is the reduction in pain responding which is expressed upon re-exposure to a context previously paired with an aversive stimulus. Projections along the prefrontal cortex (PFC)-amygdala-dorsal periaqueductal grey (dPAG) pathway may mediate FCA. However, no studies have measured both molecular and electrophysiological changes in this pathway in rats expressing persistent pain-related behaviour or FCA. Male Lister-hooded rats, with stimulating and recording electrodes implanted in the amygdala and dPAG, respectively, either received or did not receive footshock (0.4 mA) paired with context, followed 23.5 hours later by an intraplantar injection of saline or formalin (50 µL, 2.5%) into the right hindpaw. Thirty minutes post-formalin/saline, rats were re-exposed to the context for 15 minutes, during which fear- and pain-related behaviours were assessed in addition to evoked field potential recordings in the amygdala-dPAG pathway. Immediately after the 15-minute trial, PFC tissue was isolated for measurement of total and phosphorylated extracellular-signal regulated kinase (ERK) by western blotting. Formalin-evoked nociceptive behaviour in non-fear-conditioned rats was associated with increased field potential amplitude in the dPAG and increased relative expression of phospho-ERK in the PFC. These effects were abolished in rats expressing FCA. Fear conditioning in non-formalin treated rats was associated with increased phospho-ERK in the PFC but no change in field potential amplitude in the dPAG. Together, these data suggest differential, state-dependent alterations in electrophysiological activity and ERK phosphorylation along the PFC-amygdala-dPAG pathway during pain, conditioned fear, and FCA.
Neuronal projections along the prefrontal cortex (PFC)-amygdala-periaqueductal grey (PAG) pathway play a central role in mediating fear-related behaviour and modulating activity of the descending inhibitory pain pathway in a top-down manner. The PFC processes nociceptive and aversive stimuli and neuronal projections to and from the amygdala and PAG mediate associated emotional, cognitive and behavioural responding [1-5]. Study of fear-conditioned analgesia (FCA), which can be defined as the reduction in pain responding upon re-exposure to a conditioned aversive stimulus, provides an opportunity to explore the neural inputs and molecular mechanisms underpinning endogenous pain suppression as well as pain- and fear-related behaviour.

The PAG has been shown to mediate unconditioned [6] and conditioned [7-9] fear. Endogenous analgesia which is facilitated by activation of the descending inhibitory pain pathway, such as FCA or unconditioned stress-induced analgesia (SIA), involves the dorsal (d) PAG [10, for review see 11]. Modulation of the descending inhibitory pain pathway by the dPAG is regulated by neural input from the amygdala [for review see 12]. The basolateral and central nuclei of the amygdala receive input from the prefrontal cortex [13], and the central nucleus of the amygdala, which also receives input from the basolateral amygdala [for review see 14], is the major output nucleus for projections to the dPAG [15, 16]. Electrophysiological studies have shown that potentiation of the amygdala-PAG pathway mediates anxiety-like behaviour in cats [17]. To date, there has been a paucity of studies investigating the electrophysiological correlates of pain, conditioned fear, or FCA in the dPAG which we sought to address here.
The mitogen-activated protein kinase (MAPK) pathway plays an important role in neuronal plasticity. The extent to which the MAPK pathway in the PFC mediates the processing of painful and fearful stimuli has not been fully investigated. MAPK activation in the PFC has been shown to play a role in fear extinction [18, 19]. Furthermore, previous studies by Carrasquillo and Gereau [20, 21] have shown that activation of the MAPK pathway in the amygdala mediates formalin-induced persistent pain state. In the current study, we measured phosphorylation of extracellular-signal regulated kinase (ERK) in the PFC as a measure of MAPK activation and investigated the extent to which alterations in phospho-ERK correlated with behavioural indices of pain and FCA, as well as with activity of the amygdala-dPAG pathway as measured by field potential electrophysiological recordings.

Thus, the aims of this study were to determine whether formalin-induced nociceptive behavior or FCA were associated with (1) altered electrophysiological activity of the amygdala-dPAG pathway and (2) altered phosphorylation of ERK in the PFC.
2. Materials and Methods

2.1 Animals

Male Lister-hooded rats (220-260 g; Charles River, Margate, Kent, UK) were housed in groups of three at a constant temperature (22 ± 2° C) under standard lighting conditions (12:12 h light:dark, lights on from 08.00 to 20.00 h). Access to food and water was provided ad libitum. Rats were habituated to the new environment and to handling for a minimum of four days after arrival into the unit. The Lister-hooded strain was chosen for comparison with our previous work demonstrating robust expression of fear-conditioned analgesia in this strain [22-24]. All in vivo experiments were carried out in accordance with EU Directive 86/609.

2.2 Electrode implantation

Each rat was anaesthetised with an injection of sodium pentobarbital (50 mg/kg, i.p.). Electrodes (90 μm diameter) were constructed and implanted for field potential recording as described previously [25], except that the recording and stimulating electrodes ipsilaterally (right side) targeted the dPAG (6.3 mm posterior to bregma, 1.7 mm lateral to midline, and 5.0 mm from the dura at a 28° angle from left to right side) and the amygdala (2.3 mm posterior to bregma, 4.3 mm lateral to midline, and 7.5 mm from the dura), respectively. Following the electrode implantation procedure, subjects were housed singly and allowed to recover in their home cages for a minimum of 5 days.
2.3 Experimental procedures

2.3.1 Input/output and baseline recordings

Electrophysiology methodology was similar to that described previously [26]. At least 5 days following surgery, subjects were habituated to the procedure of connecting electrodes to cables which were relayed to a multichannel rotating connector while the subjects were kept in a neutral context (plastic Tupperware box). Field potentials evoked in the dPAG by 0.1-msec rectangular monophasic pulses, applied to the amygdala, were sent to an amplifier (gain 1000x; bandpass 0.001-1 kHz) and recorded for offline analysis (Spike2 software; Cambridge Electronic Design, UK). Before the first baseline recording session, responses were measured as a function of stimulus strength (input-output curves: 100-800 μA) in the neutral context. An intensity corresponding to 70-90% of the saturation level was chosen for the test stimulus, which was applied every 5 seconds 7 times during the input/output sessions. On the second habituation day, baseline recordings were measured (7 field potentials every 5 seconds over 2 sessions spaced 1 minute apart), again in the neutral context.

2.3.2 Conditioning and testing

Approximately twenty-four hours following baseline recordings, the rats were placed in a grey plastic cubic arena (30 x 30 x 30 cm) with a grid floor composed of stainless steel rods (0.5 cm diameter) placed 1.5 cm apart. With implanted electrodes connected to the stimulating/recording system, rats received a series of 10 footshocks (0.4 mA, 1 second duration) spaced 60 seconds
apart. A camera was positioned under the arena to record behaviour. The arena and stainless steel bars were cleaned (with a solution containing a mixture of 50% ethanol and lemon scent) between each rat. Eight rats received footshocks while another 8 rats were exposed to the arena for the same amount of time without receiving footshocks. Rats received the first footshock 15 seconds after placement in the arena and were returned to their home cages 15 seconds after the last footshock.

The second phase of the behavioural experiments began 23.5 hours later. The diameter of the right hindpaw was measured with Verniers calipers followed by an intra-plantar injection of 50μL formalin (2.5% formaldehyde) or 0.9% saline into the right hindpaw while under brief isoflurane anaesthesia [modification of method originally used by 27]. Thus, there were four experimental groups in total: No fear-conditioning-Saline (No FC-Sal), No fear-conditioning-Formalin (No FC-Form), Fear-conditioning-Saline (FC-Sal), Fear-conditioning-Formalin (FC-Form). Exactly 24 hours following conditioning, or 30 minutes after the intra-plantar injection of formalin, implanted electrodes were connected to the multichannel rotating connector cables and returned to the arena. Again, behaviour was recorded as described above. Field potential recordings were taken (as described above) at minutes 1, 4, 6, 9, 11, and 14 of the 15-minute behavioural trial. The number of pellets defecated was counted. Following the 15-minute re-exposure, rats were removed from the arena and rapidly anaesthetised with CO₂. The rats were then sacrificed by cervical dislocation. The tip of the electrode placements in the dPAG and amygdala were marked by passing a 0.3-0.5 mA current for 20 seconds followed by decapitation. The brain was then removed, the PFC tissue was isolated by gross dissection, weighed, snap
frozen on dry ice and stored at -80°C prior to western immunoblotting. The right hindpaw diameter was measured post-mortem using Vernier calipers.

2.4 Behavioural analysis

Behaviour was analysed using the Observer® 5.0 software package (Noldus Information Technology, Wageningen, The Netherlands), which allowed for continuous event recording over each 15 min trial. A trained rater blind to the experimental conditions assessed behaviour. Formalin-evoked nociceptive behaviour was scored according to the composite pain scoring weighted scores technique (CPS-WST_{0,1,2}) described by Watson et al. [28]. According to this method, pain behaviours are categorised as time spent raising the paw above the floor without contact with any other surface (pain 1) and holding, licking, biting, shaking or flinching the paw (pain 2) to obtain a composite pain score (CPS = (pain 1 + 2(pain 2))/(total duration of analysis period)). Formalin-induced oedema was assessed by calculating the difference between the post-mortem diameter of the right hind paw (measured using Verniers calipers) and that measured before formalin administration.

2.5 Histology

Electrode placement sites were determined prior to data analysis. Cryocut sections (20 μM) containing the amygdala and PAG were mounted onto glass slides, stained with cresyl violet, dehydrated, defatted and exposed to xylene then (DPX)-mounted and coverslipped for confirmation of electrode tip placement under light microscope.
2.6 Western immunoblotting

PFC tissue was lysed at a ratio of 10 µL lysis buffer (80 mM sodium β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium fluoride, pH to 7.6) containing protease inhibitor cocktail (Sigma Aldrich Ireland Ltd., Dublin, Ireland) per 1 µg tissue weight. Tissue was homogenised in a 1.5 mL microcentrifuge tube using a pellet pestle cordless motor with polypropylene attachment (Sigma Aldrich Ireland, Ltd., Dublin, Ireland) and centrifuged at 14,240 g for 15 minutes at 4°C. The supernatant was collected and Bradford assay was performed [29] to determine protein levels. Samples were diluted in ice-cold lysis buffer to give equal protein concentrations followed by addition of sample buffer (50 mM Tris-HCl, 1.84% SDS, 8% Glycerol, 2% bromophenol blue, and 5% 2-mercaptoethanol). Lysates were heated at 95°C for five minutes. The proteins (20 µg in 20 µL of each sample) were then separated under reducing conditions by SDS-PAGE using 12% polyacrylamide gels and electroblotted onto a nitrocellulose protran membrane (0.2 µm; VWR International, UK). Membranes were rocked in blocking solution (5% milk, 0.5% Tween20 in TBS) for two hours. Separate membranes were incubated overnight at 4°C in primary antibody diluent (2.5% milk, 0.05% Tween20 in TBS) containing anti-ERK1/2 (1:5000) or anti-phospho-ERK1/2 (1:2000) (Cell Signaling Technologies, Boston, Massachusetts, USA). Membranes were then rocked at room temperature in secondary antibody solution (2.5% BSA, 0.05% Tween20 in TBS) containing 1:10000; peroxidase-conjugated AffiniPure mouse anti-rabbit IgG heavy and light antibodies (Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK). Chemiluminescence and film development were performed under safe-light conditions. Membranes were exposed to chemiluminescent reagents (Thermo
Fisher Scientific Inc., Rockford, Illinois, USA) for 5 minutes followed by exposure to film (Hyperfilm ECL; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) and film development. The bands on all films were quantified using densitometric analysis on ImageJ (http://rsb.info.nih.gov/ij/) with an inverted lookup table. Background integrated density values were computed and subsequently subtracted from band integrated density values to obtain corrected integrated density values. Normalized values for the western blot data were obtained by dividing the corrected integrated density values for the phospho-ERK bands by the corrected integrated density value for the total ERK bands. The normalized values were divided by the control group (No FC-Veh) to obtain a percentage control value.

2.7 Statistical analysis

Baseline recordings or test day recordings for individual rats were averaged. The field potential data were then generated by dividing the test day average by the baseline average and represented as amplitude change. SPSS statistical software was used to analyse all data. Two-way analysis of variance (ANOVA) was used to assess the effects of fear-conditioning (factor 1) and formalin (factor 2) on nociceptive behavior, field potential recordings and western immunoblotting data. *Post-hoc* pairwise comparisons were made using Student-Newman-Keul’s or Fisher’s LSD test when appropriate. Data were considered significant when $P \leq 0.05$. Results are expressed as group means ± standard error of the mean (± SEM).
3. Results

The results presented here are for rats with electrodes implanted successfully in the amygdala and dPAG and from which coherent, reliable, validated recordings were obtained (Fig. 1).

3.1 Effects of formalin and fear-conditioning on nociceptive behavior

Intraplantar injection of formalin in non-fear-conditioned rats produced robust licking, biting, shaking, flinching and elevation of the injected paw and an increase in the composite pain score (CPS), compared with corresponding saline-treated controls (Fig. 2, No FC-Form vs. No FC-Sal). Fear-conditioned rats displayed significantly less formalin-evoked nociceptive behaviour compared with non-fear-conditioned rats (Fig. 2, FC-Form vs. No FC-Form), confirming the expression of FCA. Formalin injection resulted in a significant increase in hindpaw oedema in both non-fear-conditioned (Change in paw diameter: No FC-Form: 0.165±0.005 cm vs. No FC-Sal: 0.038±0.038 cm) and fear-conditioned rats (FC-Form: 0.167±0.023 cm vs. FC-Sal: 0.025±0.025 cm).

3.2 Effects of formalin and fear-conditioning on dPAG field potentials

Non-fear-conditioned formalin-treated rats displayed significant increases in the amplitude of dPAG field potentials compared to their saline-treated counterparts (Fig. 3B, No FC-Form vs. No FC-Sal). This effect was abolished in fear-conditioned formalin-treated rats (Fig. 3B, FC-Form
vs. No FC-Form). Fear-conditioning did not alter dPAG field potentials in saline-treated rats (Fig. 3B, FC-Sal vs. No FC-Sal).

Nociceptive behaviour (CPS) in formalin-treated rats correlated positively with changes in dPAG field potential amplitude (Fig. 3C; $r^2 = 0.8082$).

3.3 Effects of formalin and fear-conditioning on relative phospho-ERK1 and phospho-ERK2 expression in the prefrontal cortex

Fear-conditioned saline-treated rats displayed significantly higher relative phospho-ERK1 expression in the PFC, compared with non-fear-conditioned counterparts (Fig. 4B, FC-Sal vs. No FC-Sal). This fear-related increase in relative PFC phospho-ERK1 expression was abolished with formalin-treatment (Fig. 4B, FC-Sal vs. FC-Form). Intra-plantar injection of formalin tended to increase relative phospho-ERK1 expression in non-fear-conditioned rats compared with saline-treated counterparts, but this effect failed to reach statistical significance. Both formalin-treated and fear-conditioned rats demonstrated significantly higher relative phospho-ERK2 expression in the PFC compared to controls (Fig. 4C, No FC-Form/FC-Sal vs. No FC-Sal). Relative PFC phospho-ERK2 expression in fear-conditioned formalin-treated rats was significantly less than either non-fear-conditioned formalin-treated rats or fear-conditioned saline-treated rats (Fig. 4C, FC-Form vs. No FC-Form/FC-Sal).

In formalin-treated rats, relative phospho-ERK1 and phospho-ERK2 expression correlated positively with the CPS (Fig. 4D and 4E). Relative phospho-ERK1 expression, but not relative
phospho-ERK2 expression, correlated positively with the percentage baseline change in dPAG field potential in formalin-treated rats (Fig. 4F and 4G).
4. Discussion

The results of the present study revealed that non-fear-conditioned rats displayed formalin-evoked nociceptive behaviour that was associated with increased dPAG field potentials, an effect which was attenuated in rats expressing FCA (i.e. in fear conditioned rats exhibiting decreased formalin-evoked nociceptive behaviour when placed back into the conditioning environment). Rats that received formalin, or which had been fear-conditioned, displayed an increase in MAPK activation in the PFC – effects which were abolished in rats expressing FCA. Thus, the data provide evidence for differential, state-dependent alterations in dPAG field potentials and PFC MAPK activation associated with (a) fear responding in the presence or absence of nociceptive tone and (b) expression of endogenous analgesia in the form of FCA.

Early anatomical tracing studies determined that the central nucleus of the amygdala and the PAG contain reciprocal projections to each other [15]. Later studies confirmed this reciprocity and further characterised the projections from the central amygdala to the dorsomedial and lateral PAG [30]. As mentioned previously, the amygdala-PAG pathway plays a key role in pain (including the descending inhibitory pain pathway) and fear responding [for review see 12]. The results presented here demonstrate for the first time that an increase in dPAG field potential following brief pulse stimulation of the amygdala, accompanies the expression of pain-related behaviour. Furthermore, this increase in dPAG field potential is attenuated in rats expressing FCA. Our data suggest that there is a direct correlation between the magnitude of evoked field potentials in the dPAG and the expression of pain-related behaviour, similar to the cortical somatosensory evoked potential data described by Lebrun and colleagues [32]. To our
knowledge, the present study is the first to determine electrophysiological correlates in any brain region of animals expressing FCA (or any form of stress-induced analgesia). The data corroborate classic stimulation [33] and lesion studies [10, 34] which showed the PAG to be of central importance in the descending inhibition of pain and in the mediation of FCA. Interestingly, the data suggest that FCA is associated with a decrease in dPAG field potential compared to rats expressing pain per se. Studies have shown that the projections from the central amygdala to the PAG are mostly inhibitory [35, 36] which could explain the decrease in dPAG field potential upon activation of the amygdala-dPAG pathway during descending inhibition of pain. Alternatively, the PAG neurons which make the largest contribution to the field potential recordings may in fact be GABAergic interneurones and thus a decrease in the activity of these neurons would result in increased activity of principal PAG output neurons with a consequent increase in descending inhibition of pain responding giving rise to FCA. This hypothesis is also consistent with recent work from our laboratory demonstrating that abolition of FCA by intra-amygdalar injection of muscimol is associated with increased c-Fos expression (indicative of increased neuronal activity) in the dorsal PAG [37].

The western immunoblotting data presented here reveal that MAPK activation in the PFC correlates well with the expression of pain-related behaviour and pain-related dPAG field potential amplitude. In rats expressing formalin-evoked nociceptive behaviour per se (in the absence of fear), enhanced activation of PFC MAPK was observed, an effect attenuated in rats expressing FCA. The western immunoblotting data also suggest that conditioned fear was associated with an increase in PFC MAPK activation in the absence, but not in the presence, of
formalin-evoked nociceptive tone. MAPK activation in the PFC has been implicated in the extinction of conditioned fear [19]. MAPK activation in the anterior cingulate cortex has also been shown to be required for long-term potentiation [38] and contributes to the expression of chronic pain, and in particular its affective component [39, 40]. In addition to influencing gene transcription, MAPK signalling also modulates cellular activity at the non-transcriptional level including inhibition of voltage-gated Ca$^{2+}$ [41] and K$^+$ [42] channels. There is a well-described role for neuronal projections along the PFC-amygdala-PAG pathway in both the descending modulation of pain and in conditioned fear responding [3, 4]. Thus, pain- and fear-induced elevations in MAPK signalling in the PFC could inhibit Ca$^{2+}$ or K$^+$ currents in this region and consequently alter the activity of the amygdala-PAG pathway upon exposure to a noxious stimulus or re-exposure to an aversively conditioned context. The levels of PFC ERK activation, however, were increased in fear conditioned rats that did not show an increase in dPAG field potential, suggesting some degree of dissociation with respect to the fear-related alterations in PFC ERK activation versus dPAG field potential. Thus, the correlation between PFC ERK and amygdala-PAG pathway potentiation only appears to hold true in the presence of nociceptive tone. One possible explanation is that the pathway from PFC to amygdala to PAG may be differentially engaged in the presence versus absence of nociceptive tone, with differential involvement of ERK in the PFC and/or different neuronal populations along the amygdala-PAG pathway. It should be noted, however, that the present study does not provide direct evidence for regulation of the amygdala-PAG pathway by PFC ERK and further studies are required to investigate these hypotheses.
In conclusion, the data generated demonstrate a positive correlation between the pain-related behaviour and the magnitude of the field potential in the dPAG, the latter being reduced during expression of FCA. Furthermore, the data demonstrate that while increased MAPK signalling in the PFC accompanies expression of pain-related behaviour, both FCA and fear in the presence of nociceptive tone are associated with attenuated MAPK signalling. Electrophysiological and molecular signatures of a potent form of endogenous analgesia (FCA) have been generated and the results advance our understanding of the neurobiology of pain, fear and their interaction.
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References:


Figure legends:

Fig. 1: Schematic depicting the placement of the recording and stimulating electrodes in the (A) dorsal PAG and (B) right amygdala, respectively. FC (fear conditioning); No FC (No fear conditioning); Sal (Saline); Form (formalin) [figure modified from 40].

Fig. 2: Effects of formalin and fear-conditioning on the composite pain score (CPS) over the 15-minute trial. Data are means ± S.E.M. (n=4). **P<0.01 vs. No FC-Sal, ++P<0.01 vs. No FC-Form (two-way ANOVA followed by Student-Newman-Keul’s post-hoc test: main effect of conditioning $F_{1,15}=30.274$, $P<0.0005$; formalin $F_{1,15}=38.503$, $P<0.0005$; conditioning x formalin $F_{1,15}=30.736$, $P<0.0005$). FC (fear conditioning), Form (formalin), Sal (saline).

Fig. 3: (A) Representative traces of local field potential of the dPAG following brief stimulation from the amygdala. The top traces represent the input/output curve for which a current corresponding to a 70% maximum response was determined. The traces below the input/output trace represents a baseline recording and the bottom trace represents a trace for the test day from each group. (B) Changes (mean ± SEM percentage relative to baseline) in the amplitude of dorsal PAG field potential (dPAG FP) in rats which were fear-conditioned (FC) or not fear-conditioned (No FC) and which received either a saline (Sal) or formalin (Form) injection into the right hindpaw 30 minutes prior to recording in the arena previously paired with footshock (n
\[ *=0.05 \text{ vs. No FC-Sal, } +P<0.05 \text{ vs. No FC-Form (two-way ANOVA followed by Student-Newman-Keul’s post-hoc test; main effect of conditioning } F_{1,15}=7.849, P=0.016; \text{ formalin } F_{1,15}=6.256, P=0.028; \text{ conditioning x formalin } F_{1,15}=6.648, P=0.024). \]

(C) Correlation between the composite pain score (CPS) and the dPAG field potential (dPAG FP) in formalin-treated rats. ‡ Significant correlation (Pearson production-moment correlation coefficient: \( r^2=0.8082, \) degrees of freedom=7, \( P=0.0024 \)).

**Fig. 4**: (A) Representative photomicrograph of phospho-ERK1/2, and total ERK1/2 western immunoblot. Quantitative analysis of the effects of persistent pain state, conditioned fear, and fear-conditioned analgesia on relative phospho-ERK1 (B) and phospho-ERK2 (C) expression in the rat prefrontal cortex (PFC) (\( n = 4 \)). *\( P<0.05 \) vs. No FC-Sal, +\( P<0.05 \) vs. No FC-Form, #\( P<0.05 \) vs. FC-Sal (two-way ANOVA followed by Student-Newman-Keul’s post-hoc test (A: main effect of conditioning x formalin \( F_{1,15}=13.824, P=0.003. \) B: main effect of conditioning x formalin \( F_{1,15}=25.260, P<0.0005). \) Correlation between relative-phospho-ERK1 or phospho-ERK2 expression in the PFC with the composite pain score (CPS) (D and E) and with the change in dPAG field potential (dPAG FP) in formalin-treated rats (F and G). † Significant correlation (Pearson production-moment correlation coefficient (D): \( r^2=0.5988, \) degrees of freedom=7, \( P=0.0242; \) (E): \( r^2=0.6809, \) degrees of freedom=7, \( P=0.0117; \) (F): \( r^2=0.6139, \) degrees of freedom=7, \( P=0.0214). \)
\[ \square = \text{No FC-Sal} \]
\[ \triangle = \text{No FC-Form} \]
\[ \circ = \text{FC-Sal} \]
\[ \diamond = \text{FC-Form} \]

**Dorsal PAG**

- Bregma -6.04 mm
- Bregma -6.30 mm
- Bregma -6.72 mm

**Amygdaloid Complex (right)**

- Bregma -2.12 mm
- Bregma -2.30 mm
- Bregma -2.56 mm

\[ A. \text{ Bregma -6.80 mm} \]
\[ B. \text{ Bregma -2.80 mm} \]