Nerve Injury Alters Restraint-induced Activation of the Basolateral Amygdala in Male Rats

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Abstract

The amygdala is critical for the production of appropriate responses towards emotional or stressful stimuli. It has a characteristic neuronal activation pattern to acute stressors. Chronic pain and acute stress have each been shown to independently modulate the activity of the amygdala. Few studies have investigated the effect of pain or injury, on amygdala activation to acute stress. This study investigated the effects of a neuropathic injury on the activation response of the amygdala to an acute restraint stress. Chronic constriction injury of the right sciatic nerve (CCI) was used to create neuropathic injury and a single brief 15-minute acute restraint was used as an emotional/psychological stressor. All rats received cholera toxin B (CTB) retrograde tracer injections into the medial prefrontal cortex (mPFC) to assess if the amygdala to mPFC pathway was specifically regulated by the combination of neuropathic injury and acute stress. To assess differential patterns of activity in amygdala subregions, cFos expression was used as a marker for “acute”, restraint triggered neuronal activation, and FosB/DFosB expression was used to reveal prolonged neuronal activation / sensitisation triggered by CCI. Restraint resulted in a characteristic increase in cFos expression in the medial amygdala, which was not altered by CCI. Rats with a CCI showed increased cFos expression in the basolateral amygdala (BLA), in response to an acute restraint stress, but not in neurons projecting to the prefrontal cortex. Further, CCI rats showed an increase in FosB/ΔFosB expression which was exclusive to the BLA. This increase likely reflects sensitisation of the BLA as a consequence of nerve injury which may contribute to heightened sensitivity of BLA neurons to acute emotional/ psychological stressors.

Introduction

The amygdala plays a critical role in the integration of sensory and emotional information to co-ordinate an individual’s response to stress (LeDoux, 2000). Systematic characterisation of neuronal activation patterns in the central nervous system in response to different types of stressors, either “physical” (ie., haemorrhage or immune challenges); or “psychological” have shown that specific sub-nuclei of the amygdala are selectively activated by each of these sub-classes of stressor. The amygdala regions that are activated include both the central nucleus (CeA) and the medial nucleus (MeA) (Dayas et al., 2001a, Dayas et al., 2004), each of which shows differential patterns of activation. The MeA is selectively activated by stressors that have a psychological/emotional component such as: restraint (Dayas et al., 2001b, Dayas et al., 1999); immobilisation (Ma and Morilak, 2005); forced swim (Cullinan et al., 1995, Dayas et al., 2001b); social defeat (Chung et al., 1999, Nikulina et al., 2004); and inescapable foot shock (Rosen et al., 1998).

The amygdala is also known to play critical roles in the sensory hypersensitivity and altered affective and motivational behaviours associated with the chronic pain state. Following the induction of neuropathic pain, the CeA and the basolateral nuclei of the amygdala (BLA) undergo neuroplastic changes and show enhanced stimulus-evoked activity that is correlated with tactile allodynic responses (Ikeda et al., 2007, Goncalves and Dickenson, 2012). Furthermore, bilateral ablation of the CeA prior to the induction of neuropathic injury/pain abolishes the development of mechanical allodynia (Li et al., 2013). It has been

suggested that disruptions of the excitatory state of amygdala neurons can facilitate the transition from the acute to chronic pain state (Usdin and Dimitrov, 2016) and a number of studies have provided evidence of significant changes in the activity of CeA and BLA neurons following sciatic nerve injury (Jiang et al., 2014), and inflammatory pain (Ji and Neugebauer, 2011, Ji et al., 2010). These changes in activity have been shown to correspond with changes in both affective and cognitive behaviours. The observations that neurons of the amygdala undergo functional changes and sensitisation in experimental models of chronic pain, raises the possibility that the normal amygdala-dependent processes that integrate and coordinate the responses to "*acute*" stressors may be altered in individuals with chronic pain. The ability to deal effectively with stress is certainly impacted in people with chronic pain conditions (Jensen et al., 1991) . A major output target of BLA neurons is the medial prefrontal cortex (mPFC) (Kita and Kitai, 1990, Gabbott et al., 2006), and the BLA to mPFC connections are recognised as an important pathway for the regulation of cortical functions critical for emotional and cognitive behaviours (Sharp, 2017). Nerve-injury evoked alterations of activity in this specific pathway may well contribute to the changes in affective and cognitive behaviours that characterise the chronic pain state.

The Fos family proteins cFos, FosB and ΔFosB are inducible transcription factors that accumulate in the nuclei of neurons as a consequence of functional activity, including action potential production. In this context, these proteins have been used widely as indirect markers of neuronal activity (Yap & Greenberg, 2018; Okuno, 2011). It is widely accepted that the immunohistochemical detection of cFos expression can be used as a marker for recent neuronal activity (usually up to 2 hours), and the immunohistochemical detection of FosB and ΔFosB expression can be used to identify longer-term or chronic changes to neural activity potentially reflecting neuronal sensitization and/or functional change. Fos B is expressed for up to 12 hours after a stimulus, generally peaking at around 12 hours, and ΔFosB accumulating incrementally over periods of days (Nestler et al 2001).

The aims of this study were to use the immunohistochemical detection of the expression of the protein products of the Fos-family immediate-early genes, and to specifically use their different temporal expression profiles to investigate whether peripheral nerve injury using chronic constriction injury of the sciatic nerve (CCI), a model of neuropathic pain, can alter the neuronal activation of the amygdala triggered by acute stress. We hypothesised that neuropathic injury will: (i) alter the patterns of cFos activity in the amygdala in response to acute restraint stress and, (ii) increase the acute restraint stress triggered cFos expression in amygdala cells projecting to the mPFC, therefore, we quantified the expression of c-Fos to identify the effect of acute restraint stress on the activity of amygdala neurons. In addition, to evaluate the degree to which nerve injury might sensitize neurons in the amygdala, we also quantified FosB/ΔFosB expression in rats following CCI, sham surgery and anaesthesia exposure, we hypothesised that injury would increase FosB/ΔFosB expression in injured but not uninjured rats, independent of acute stressor exposure. It is important to note however that because the polyclonal FosB antibody we used, identifies both FosB (46-50 KDa) and its truncated fragment ΔFosB isoform (33 and 35-37 KDa) restraint stress could alter FosB/ΔFosB immunoreactivity in amygdala neurons due to small increases in FosB expression during the two hours post-stress period (Nestler et al., 2001).

Material And Methods

Animals

Experimental procedures were performed on 61 outbred male Sprague-Dawley rats weighing 220-240g on arrival (ARC, Perth, W.A. Australia). Rats were group housed (4 per cage) and were allowed to habituate to their new environment on a reverse dark-light cycle (12:12 hrs) for 7 days with *ad libitum* access to food (standard laboratory chow) and water (tap water). Environmental enrichment consisted of cardboard boxes and tubes, PVC piping, nesting materials, paper tissues and towels, and novel food and seeds were offered 2-3 times per week. All experimental procedures were carried out with the approval of the Animal Care and Ethics Committee of the University of Sydney and in accordance with the guidelines of the Code for the Care and Use of Animals in Research Australia, and the Ethical Guidelines for Investigation Association for the Study of Pain (Zimmermann, 1983).

Experimental Design

Following the 7-day habituation period, rats received bilateral retrograde tracer injections. Rats weighed approximately 250-300g at the time of surgery. Retrograde tracer injections were followed by a 7-day post-operative recovery period. Following this period, all rats in the same cage (n=4) received the same surgical procedure, either: (i) isoflurane anaesthesia only (Anaesthetised, n=19); (ii) sham chronic constriction injury of the right sciatic nerve (Sham-injured, n=20); or (iii) chronic constriction injury of the right sciatic nerve (CCI, n=22). Rats were left uninterrupted for 11 days, other than for routine husbandry needs. On the twelfth day post-CCI, rats in each cage (n=4) were then randomly assigned to pairs, one rat received acute restraint stress for 15 minutes (n=34), its counterpart received identical handling procedures, but no restraint (n=27). Rats were perfused two hours after the restraint or handling procedure and their brains were removed and were processed for cFos immunoreactivity with specific focus on three sub-nuclei of the amygdala; the medial amygdala (MeA), central amygdala (CeA) and basolateral amygdala (BLA).

Retrograde Tracer Injections

Bilateral injections of retrograde tracer were given to 61 rats, anaesthetised with an i.m. injection of ketamine (40mg/kg) and xylazine (2.5mg/kg) before being placed in a stereotaxic frame in the ‘flat-skull’ position. Under local anaesthesia (2% lignocaine) applied subcutaneously, a midline incision was made in the scalp and the skin and muscles were reflected to reveal the frontal and parietal bone. A small craniotomy was made ~3mm rostral from bregma to reveal the dorsal surface of the frontal cortices.

A single barrel glass micropipette was lowered 2.75mm from the dorsal surface of the frontal cortex at an AP of +3.2mm from bregma and a laterality of 0.45mm left and right of the midline (determined by the presence of the superior sagittal sinus). Each rat received an iontophoretic injection of fluorogold into the prelimbic cortex on one side of the brain, and cholera toxin B (CTB) into the prelimbic cortex on the other side of the brain (20min, 2s ON/ 2s OFF, +5-10mA). Each rat received only one tracer per hemisphere, and

the sides the tracers were injected into were counterbalanced across rats. Each injection was followed by a 10-minute rest period where no current was applied, to prevent the formation of dye tracks being left on the removal of the glass pipette. These tracers were selected on the basis of their successful use in earlier double-label studies in our laboratory, showing no evidence of functional impairment of retrogradely labelled neurons, as significant numbers of double-labelled cFos and tracer containing cells were reported (Vagg et al., 2008).

Following the removal of the micropipette, the rat was removed from the stereotaxic frame, the craniotomy was sealed with a small piece of sterile gel foam, the scalp was closed and sutured and a topical antibiotic powder was applied. Each rat subsequently received 2.0ml saline i.p. before being placed in a temperature regulated recovery cage. Each rat was monitored closely during recovery and once rats were ambulatory, eating and drinking, they were returned to their home cage.

Chronic Constriction Injury Surgery

Anaesthesia was induced with isoflurane (5% in 100% oxygen), delivered via an airtight induction chamber, surgical anaesthesia was maintained with 2-3% isoflurane in 100% oxygen administered via a custom-made facemask for a period of approximately 20 minutes. As described by Bennett and Xie (1988) the right sciatic nerve was exposed via blunt dissection through the biceps femoris muscle and four chromic gut ligatures (chromic gut 5-0 Ethicon, Johnson & Johnson Medical) were tied loosely ~1mm apart, just proximal to the trifurcation of the sciatic nerve. Following ligation, the nerve was repositioned and the skin was closed and sutured, and topical antibiotic powder applied to the site. Sham surgical procedures included the blunt dissection and exposure of the right sciatic nerve, but no ligatures were applied. Anaesthetic-only control procedures required each rat to be placed under isoflurane anaesthesia for 20 minutes.

Acute Restraint Procedures

Acute Restraint Stress: Each rat was removed from its home cage and placed in a commercially available small animal restraint jacket, made from flexible canvas, for 15 minutes (Lomir Biomedical Inc, Malone NY). The entire procedure was video-recorded for assessment and quantification of each rats behaviour during the restraint. Rats were returned to their home cage following restraint stress procedures.

Handling only: Each rat was removed from their home cage and gently placed on top of the restraint jacket and table for approximately 30 seconds and then immediately returned to their home cage.

Perfusion

Two hours after the restraint or control handling procedures, each rat was gently removed from its cage and quickly and deeply anaesthetised with an intra-peritoneal injection of sodium pentabarbitone (Lethabarb, 120mg/kg). Once deep anaesthesia was achieved, each rat was perfused intra-cardially with ice-cold saline (0.9% NaCl), followed by perfusion with ice-cold fixative (4% paraformaldehyde acetate-

borate buffer, pH 9.6, 4°C (PFA)). Following fixation, brains were removed and post-fixed in a 50% (v/v) solution of PFA in 10% (w/v) sucrose dissolved in 0.1M phosphate buffered saline, pH 7.4 (PBS).

A coronal forebrain block was made with a coronal cut at the anterior border of the optic chiasm this block was cryosectioned (Leica) in a 1:5 serial sections at 50mm thickness to assess the retrograde tracer injection sites. A similar coronal block of tissue containing the amygdala nuclei was also made and this block was cryosectioned in a 1:6 serial sections at 40mm thickness.

Immunohistochemistry

Free-floating sections containing the amygdala were washed in PBS. The sections were then incubated in 50% (v/v) ethanol for 30 mins, followed by a 30 mins incubation in 3% hydrogen peroxide (v/v) in 50% ethanol. Sections were washed in PBS, blocked in 10% normal horse serum (NHS, Sigma-Aldrich) in PBS (v/v) and incubated in polyclonal rabbit anti-cFos or anti-FosB/DFosB in 2% NHS in PBS (cFos (1:1000), 4°C overnight, Santa Cruz, Sc-52; FosB/DFosB (1:1000) 4°C overnight, Santa Cruz, Sc-48). Sections were washed in PBS and incubated in biotinylated goat anti-rabbit in 2% NHS in PBS (1:500, 2h at room temperature, Vector Labs). Sections were washed in PBS again and incubated in ExtrAvidin Peroxidase diluted in PBS (1:1000, Sigma Aldrich, 2.5h room temperature). Following a final wash in PBS, Nickel sulfate and cobalt chloride enhanced chromogenic detection of cFos with 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) was performed. The sections were incubated at room temperature in a ‘DAB mix’ (10mg DAB, 0.2ml 0.4% ammonium chloride (w/v) in PBS, 0.2ml of 20% D-glucose (w/v) in PBS made up to a total volume of 20ml). ‘DAB mix’ was filtered and 0.025% (w/v) cobalt chloride and 0.025% (w/v) nickel sulphate was added (Ni/Co-DAB). The sections were transferred to fresh ‘DAB mix’ (10ml) and a 1.6ml of glucose oxidase (Sigma: 10,000 U/ml) was added to the solution to initiate the chromogenic reaction. The reaction was stopped when the background staining became visible by rinsing the sections in several PBS washes.

Double labelling for retrogradely labelled CTB cells was performed immediately after the DAB chromogenesis was terminated in sections containing cFos (Ni/Co-DAB) or FosB/DFosB (Ni/Co-DAB) labelled cells. Sections were blocked in 10% NHS in PBS and incubated in goat anti-Cholera Toxin B (anti-CTB) (1:20000, 4°C overnight, List Pharmaceuticals) and then underwent the same antibody incubation procedures as detailed above. Anti-CTB incubated sections underwent a DAB reaction described above in a ‘DAB mix’ containing 10mg DAB, 0.2ml 0.4% ammonium chloride (w/v) in PBS, 0.2ml of 20% D-glucose (w/v) in PBS made up to a total volume of 20ml. Double-labelled cells therefore contained the amber/brown reaction product of DAB in the cytoplasm (CTB) and the blue-black reaction product of Ni/Co-DAB in the nucleus (cFos or FosB/DFosB)

Sections were mounted onto glass slides double-subbed in 1% (w/v) gelatine in distilled water. Sections were left to air dry and were dehydrated using a series of ascending ethanol washes. Sections were de-fatted in histosol (Sigma, St Louis, MO), and immediately coverslipped with DPX mounting media (Sigma, St Louis, MO).

Analysis

Rats in which the retrograde tracer injections on both sides of the brain were comparable in symmetry and size, and located within the medial wall boundaries of the PFC were processed for further analysis (40/61). The remaining 21 brains have been stored for later analysis. Fos-immunoreactive cells and retrogradely labelled cells were quantified independently by two investigators (JK (investigator) and NO (expert, non-author)), each of whom were blinded to the conditions of each rat. The number of single-labelled, Fos-IR, and double-labelled (Fos-IR and retrogradely labelled) cells were counted in five equidistant coronal sections containing the amygdala, spaced approximately 0.5mm apart and illustrated in the stereotaxic atlas of Paxinos and Watson (2005). All Fos-immunoreactive cells and retrogradely labelled cells were counted in each section and are reported here. These sections were identical to those illustrated at -1.56mm, -2.04mm, -2.52mm, -3.00mm and -3.36mm caudal to the skull-marking bregma (Paxinos and Watson, 2005). The boundaries of the sub-nuclei of the amygdala were identified from a second series of adjacent sections stained with cresyl-violet and images of these sections were overlaid with corresponding images of the sections containing Fos-IR to allow the precise sub-nuclear localisation of patterns of neuronal activation (see Figure 1).

Objective criteria for the determination of positive staining defined in our previous work were established prior to quantification (Clement et al, 1996; Vagg et al, 2008). Fos-IR cells were identified under the light microscope (Olympus BX51) by the presence of Ni/Co-DAB reaction product which was clearly visible in the nucleus as a blue-black product at 400x magnification which could also be distinguished against background at 100x and 200x magnification. CTB immunoreactive, retrogradely labelled cells were identified by the presence of the amber/brown DAB reaction product which was restricted to the cytoplasm of each cell and clearly visible at 400x magnification which could also be distinguished against background at 200x magnification. Double-labelled cells (DAB in the cytoplasm and Ni/Co-DAB in the nucleus) were identified at 400x magnification and confirmed at 1000x magnification. Images of labelled cells were captured using a digital camera (DP70 CCD LiveView Camera) (see Figure 2).

Statistics

Our *a priori* hypotheses were that cFos expression triggered by acute restraint stress, in the amygdala, and in mPFC projecting amygdala neurons, would be altered in rats with neuropathic injury. Statistical evaluation of this hypothesis used a two-way, between group analysis of variance (ANOVA) to assess the impact of nerve injury surgery, and acute

Results

The mean (±SEM) number of cFos immunoreactive cells (cFos-IR) observed in the right and left, central (CeA); medial (MeA); and basolateral (BLA) sub-nuclei of the amygdala in; (i) non-stressed anaesthetised rats (n=6), (ii) stressed anaesthetised rats (n=7), (iii) non-stressed sham-injured rats (n=7), (iv) stressed sham-injured rats (n=6), (v) non-stressed CCI rats (n=6-7), and stressed CCI rats (n=7) are shown in Table 1.

Single Label cFos-IR Following Acute Restraint Stress and Nerve Injury

Central Amygdala (CeA): An overall comparison between the surgery and stress groups revealed no significant interaction effect in the CeA (Two-Way ANOVA, left: $F_{2, 39} = 1.225$, p=0.307, $w^2 = 0.01$; right: $F_{2, 40} = 0.515$, p=0.602, $w^2 = -0.03$). Figure 3 (a, b) shows the cFos-IR of the CeA between all experimental conditions. There was no main effect of surgery on the CeA (left: $F_{2, 39} = 0.725$, p=0.492, $w^2 = -0.01$; right: $F_{2, 40} = 0.618$, p=0.545, $w^2 = -0.02$), but there was a significant effect for stress in the left ($F_{1, 39} = 8.508$, p=0.006, $w^2 = 0.17$) and right CeA ($F_{1, 40} = 4.452$, p=0.042, $w^2 = 0.09$). A comparison of non-stressed rats to stressed rats showed a trend for increased cFos counts in the stressed rats (Table 1). *Simple Effects* Pairwise comparisons revealed acute stress increased cFos expression significantly in the left CeA of Sham-injured rats (*non-stressed sham-injured* = 3±1.23 vs *stressed sham-injured* = 11.5±1.88, p<0.01).

Medial Amygdala (MeA): An overall comparison between the surgery and stress groups revealed no significant interaction effect in the MeA (left: $F_{2, 39} = 1.959$, p=0.157, $w^2 = 0.04$; right: $F_{2, 40} = 0.395$, p=0.677, $w^2 = -0.02$). Figure 3 (c, d) shows the cFos-IR of the MeA between all experimental conditions. On the left MeA there were increased numbers of cFos-IR neurons in non-stressed anaesthetised rats compared to sham and CCI non-stressed rats. There were no differences in the numbers of cFos-IR neurons between Sham stressed, anaesthetised stressed and CCI-stressed rats on either side of the brain (Main effects of surgery, left: $F_{2, 39} = 0.079$, p=0.925, $w^2 = -0.03$; right: $F_{2, 40} = 0.301$, p=0.742, $w^2 = -0.02$). A comparison of non-stressed rats with stressed rats revealed a main effect of stress in the left ($F_{1, 39} = 18.963$, p<0.001, $w^2 = 0.33$) and right MeA ($F_{1, 40} = 26.41$, p<0.001, $w^2 = 0.43$). *Simple Effects* Pairwise comparison's revealed acute stress increased cFos-IR expression in the right, but not left MeA of anaesthetised rats (Left: *non-stressed anaesthetised* = 21.83±9.49 vs *stressed anaesthetised* = 32.14±4.32, p=0.354; Right: *non-stressed anaesthetised* = 14.5±4.56 vs *stressed anaesthetised* = 38.57±10.79, p<0.05); bilaterally in sham-injured rats (Left: *non-stressed sham* = 10.14±2.9 vs *stressed sham* = 49.83±8.792, p<0.001; Right: *non-stressed sham* = 10.14±1.9 vs *stressed sham* = 46±7.31, p<0.001); and bilaterally in CCI rats (Left: *non-stressed CCI* = 11.5±5.512 vs *stressed CCI* = 44.29±11.73, p<0.01; Right: *non-stressed CCI* = 9.57±3.86 vs *stressed CCI* = 36.29±8.14, p<0.001).

Basolateral Amygdala (BLA): An overall comparison between the surgery and stress groups revealed a significant interaction effect in the left, but not right BLA (Two-Way ANOVA, left: $F_{2, 38} = 4.768$, p=0.015, $w^2 = 0.13$; right: $F_{2, 40} = 2.608$, p=0.088, $w^2 = 0.07$). Figure 3 (e, f) shows the cFos-IR in the BLA for all experimental conditions. A comparison between the surgical conditions revealed no main effects of surgery (left: $F_{2, 38} = 2.981$, p=0.065, $w^2 = 0.07$; right: $F_{2, 40} = 1.197$, p=0.315, $w^2 = 0$). However, a main effect of stress was determined in both the left ($F_{1, 38} = 10.839$, p=0.002, $w^2 = 0.17$) and right ($F_{1, 40} = 9.436$, p=0.004, $w^2 = 0.18$) BLA. *Simple Effects Pairwise* comparisons revealed acute stress increased cFos expression bilaterally in the BLA of CCI rats (Left: *non-stressed CCI* = 1.67±0.56 vs *stressed CCI* = 23.5±7.31, p<0.001; Right: *non-stressed CCI* = 1.57±0.92 vs *stressed CCI* = 20.29±6.82, p<0.001). In both

the right and left BLA the cFos-IR was located predominantly in the caudal half of the sub-nucleus as illustrated in Figure 4.

Double Label cFos-IR and Retrograde Tracer Following Acute Restraint Stress and Nerve Injury

Cells retrogradely labelled with either CTB or fluorogold were found in the BLA, but not the CeA or the MeA, following injections centred specifically within the prelimbic area of the medial wall of the frontal cortex (between coronal sections 3.72- 4.2mm rostral to bregma in the Paxinos and Watson atlas (Paxinos and Watson, 2005). We observed that it was only injection sites that occupied >40% of this prelimbic area that resulted in significant numbers of retrogradely labelled cells in the BLA. Figure 5 shows reconstructions of the retrograde tracer injection sites.

Retrogradely labelled cells were found predominantly at the ventro-lateral border of the posterior part of the BLA ipsilateral to the injection site at coronal levels -2.52mm to -3.36mm from bregma represented in Paxinos and Watson (Paxinos and Watson, 2005). Additionally, small numbers of cells were found at the ventromedial border of the anterior part of the BLA ipsilateral to the injection site. Contralateral to the injection site, only small numbers of retrogradely labelled cells were observed in the BLA. Ipsilateral to the injection sites we also noted retrogradely labelled cells in the agranular insula cortex; the claustrum; the endopiriform nucleus; the midline thalamic nuclei and the perifornical hypothalamus.

There were no double labelled, cFos-immunoreactive and retrogradely labelled cells found in the BLA in any of the rats, in any of the experimental groups (Table 1).

Single Label FosB/ΔFosB-IR Following Acute Restraint Stress and Nerve Injury

The mean (±SEM) number of FosB/ΔFosB immunoreactive cells (FosB/ΔFosB-IR) observed in the BLA, CeA and MeA in each of the experimental groups is shown in Figure 6. It was hypothesised that neuropathic injury would increase the number of of FosB/ΔFosB immunoreactive cells reflecting an increase in neuronal activity in the amygdala. To investigate this non-stressed and stressed rats were combined and a one-way ANOVA with Tukey post hoc test was used to compare the effect of surgical conditions (see Table 2).

Central Amygdala: Figure 6 (a, b) shows the numbers of FosB/ΔFosB-IR neurons in the CeA of rats in all experimental conditions; a one-way ANOVA revealed no main effect surgical conditions ($F_{2, 77}$ = 0.886, p= 0.417, w^2 = 0).

Following this analysis, a two-ANOVA was also completed to show that no effects of within surgical conditions (stressed vs non-stressed) were present. The statistical test did not reveal any significant interactions between surgery X stress for the CeA (Two-way ANOVA, left: $F_{2, 36}$ = 1.009, p=0.377, w^2 = 0; right: $F_{2, 38}$ = 0.102, p=0.903, w^2 = -0.06). Within surgical group comparisons of stress did not reveal any significant effects (Left: $F_{1, 36}$ = 0.741, p=0.396, w^2 = 0; Right: $F_{1, 38}$ = 0.393, p=0.535, w^2 = -0.02).*Medial Amygdala: Figure 6 (c, d)* shows the numbers of FosB/ΔFosB-IR neurons in the MeA of rats in all

experimental conditions. A one-way ANOVA revealed no main effect surgical conditions ($F_{2, 77}$ = 0.169, P = 0.845, w^2 = -0.02).

Following this analysis, a two-ANOVA was also completed to show that no effect of within surgical conditions (stressed vs non-stressed) was present. The statistical test did not reveal any significant interactions between surgery X stress in the total MeA (Two-way ANOVA, left: $F_{2, 37}$ = 0.494, p=0.615, w^2 = -0.03; right: $F_{2, 38}$ = 0.386, p=0.683, w^2 = -0.04). Within surgical group comparisons of stress did not reveal any significant effect (Left: $F_{1, 37}$ = 0.761, p=0.39, w^2 = 0; Right: F_{1}, 38 = 0.944, p=0.339, w^2 = 0).

Basolateral Amygdala: Figure 6 (e, f) shows the number of FosB/ΔFosB-IR neurons in the BLA of rats in each experimental condition. A one-way ANOVA revealed a significant main effect of surgery was present in the BLA ($F_{2, 72}$ = 3.981, P <0.05, w^2 = 0.07). Tukey post hoc comparison further revealed CCI had significantly more FosB/ΔFosB-IR when compared to sham-injured rats (CCI = 112.56±16.36 vs sham-injured = 67.04±10.69).

Following this analysis, a two-way ANOVA was also completed to show that no effects of within surgical conditions (stressed vs non-stressed) were present. The statistical test did not reveal any significant interactions between surgery X stress in the total BLA (Two-way ANOVA, left: $F_{2, 37}$ = 0.001, p=0.999, w^2 = -0.06; right: $F_{2, 38}$ = 0.472, p=0.628, w^2 = -0.03). Within surgical group comparisons of stress did not reveal any significant effect (left: $F_{1, 37}$ = 2.111, p=0.138, w^2 = -0.03; right: $F_{1, 38}$ = 0.015, p=0.904, w^2 = -0.03).

Figure 7 shows that CCI produced an anatomically specific increase in FosB/ΔFosB-IR in the caudal BLA contralateral to the injury at -3.4mm from bregma (CCI = 60.46±9.07 vs anaesthetised = 31.33±4.78, and sham-injured = 26.83±6.25). This increase underpinned the increased total number of cells in the left BLA (shown in Figure 6 e,f) . In contrast, the significant increase in the total number of FosB/ΔFosB-IR cells in the BLA ipsilateral to the injury was due to modest increases in the number of cells across the rostro-caudal extent of the right BLA (shown in Figure 6 e,f).

Double Label FosB/ΔFosB-IR and Retrograde Tracer Following Acute Restraint Stress and Nerve Injury

Double-labelled neurons, containing FosB/ΔFosB-IR and retrograde tracer were rarely encountered in the BLA of any of the experimental groups (Table 1), despite the presence of distinct populations of single-labelled FosB/ΔFosB-IR or CTB labelled neurons.

Discussion

This study investigated the effects of neuropathic injury (CCI) on acute restraint stress evoked cFos expression in the amygdala and whether there is increased cFos expression in amygdala projections to the medial prefrontal cortex. Our data showed that in rats with CCI, acute restraint increased c-Fos

expression in neurons of the basolateral nucleus of the amygdala (BLA) suggesting increased neuronal activity in this region. This increased c-Fos expression in the BLA was not observed following restraint in either anaesthetised, or sham-injured rats suggesting that neurons in these regions were not activated. We also evaluated the effects of CCI on FosB/ΔFosB-IR expression in amygdala subnuclei to evaluate injury evoked sensitization of this region. We found that CCI triggered an almost two-fold increase in FosB/ΔFosB-IR in the BLA when compared to anaesthetised, or sham-injured rats which suggested an ongoing increase in neuronal activity specifically in the caudal BLA. In line with earlier studies of acute stressors, our data showed that, in response to acute restraint, all rats (anaesthetised, sham-injured and CCI) showed an increase in cFos-IR in the medial sub-nucleus of the amygdala (MeA). Bilateral injections of retrograde tracer into the medial prefrontal cortex (mPFC) combined with the immunohistochemical detection of Fos family proteins revealed that amygdala neurons projecting to the mPFC were not immunoreactive for either cFos or FosB/ΔFosB suggesting that this specific pathway is not activated by either acute restraint, CCI, or a combination of these stimuli. Our experiments were carried out exclusively in male rats, we are aware however that there is evidence for sex differences in the role of the amygdala in mediating changes in pain sensitivity as a result of chronic stress (Long et al., 2016), whether our findings of the effects of neuropathic injury on amygdala responses to acute stressors is also seen in female rats will be important to determine as there are many more females with chronic pain than males (Mogil, 2020).

Acute restraint increases cFos expression in the medial amygdala

The immunohistochemical detection of cFos has played a significant role in the systematic characterisation of the activation patterns of the brain in response to different classes of acute stressors such as physical stressors, including haemorrhage and immune challenges, and psychological/emotional stressors, including foot shock and restraint stress. These studies have provided evidence for the specific involvement of the MeA, but not the CeA or BLA to these challenges (Dayas et al., 2001a, Dayas et al., 2004). Our data are consistent with these earlier findings that following acute restraint stress there is increased cFos expression indicating that the MeA is activated in both anaesthetised, sham-injured and CCI rats. The MeA is particularly sensitive to stressors that have a psychological/emotional component, including restraint (Dayas et al., 1999, Dayas et al., 2001b), immobilisation (Ma and Morilak, 2005), forced swim (Cullinan et al., 1995, Dayas et al., 2001a), social defeat (Chung et al., 1999, Nikulina et al., 2004), and inescapable foot shock stress (Rosen et al., 1998). In addition, the MeA is responsible for initiating an integrated cardiovascular, endocrine and behavioural response that includes the activation of the paraventricular nucleus of the hypothalamus, leading to increased ACTH and corticosterone serum levels (Dayas et al., 1999, Feldman et al., 1994), hypertension and tachycardia (Fortaleza et al., 2012b, Fortaleza et al., 2012a) and anxiety-like behaviours (Liu et al., 2013).

Nerve injury increases FosB/ΔFosB expression the basolateral amygdala

The increased expression of FosB/ΔFosB-IR in the left BLA and, to a lesser extent, in the right BLA, eleven days after CCI in the right sciatic nerve indicates ongoing levels of neuronal activity in this sub-nucleus of the amygdala (Nestler et al., 1999). A role for the BLA in neuropathic pain is seldom discussed in the literature, despite reports that bilateral lesions of the BLA prior to nerve injury abolishes the development of mechanical allodynia in rats (Li et al., 2013). This is an effect that was not observed following lesions of the CeA (Li et al., 2013), although the CeA is commonly referred to as the “nociceptive amygdala”. The CeA processes acute nociceptive inputs from the parabrachial nucleus (Gauriau and Bernard, 2002). Acute electrophysiological studies following nerve injury have shown enhanced stimulus-evoked activity correlated with tactile allodynia responses (Ikeda et al., 2007, Goncalves and Dickenson, 2012); increased spontaneous firing; and a lateralised sensitivity (right) (Goncalves and Dickenson, 2012) in the CeA. Despite these observations of increased neural activity, there was no evidence of overall increased FosB/ΔFosB-IR in the CeA of our nerve-injured rats. This may reflect a specific sensitisation of CeA neurons to noxious stimuli specifically, rather than other classes of stressor. The increased spontaneous neuronal firing in the CeA of neuropathic rats may not reflect an increase in the number of active cells in the CeA, which is consistent with our data showing no differences in FosB/ΔFosB-IR. Instead, this may reflect the increased sensitivity of the CeA to producing behaviours in response to aversive stimuli.

Our data reveals that prolonged ongoing activity as a result of peripheral nerve injury, in the absence of a sensory or nociceptive input is only present in the BLA. The prolonged activation of the BLA may represent the emotional/affective component of pain. Previous studies in rodent models of persistent inflammatory pain have shown that the BLA plays a role in anxiety-like behaviours (Chen et al., 2013) as well as playing a role in value-based decision-making (Ji et al., 2010). The BLA has also been observed to have decreased arborisation in its cells (Tajerian et al., 2014) increased microglial activation during chronic pain (Taylor et al., 2017), and cell proliferation following a SNI (Goncalves et al., 2008). These data, suggest the BLA is altered during the chronic pain state and may play a significant role in the development of affective impairments observed during chronic pain.

Acute restraint increases cFos expression in the basolateral amygdala of nerve injured rats

Acute restraint (15 min) in naive rats does not result in c-Fos expression in the BLA, suggesting that BLA neurons are not activated by acute stressors (Dayas et al., 2001a). In contrast, we have shown that following CCI, cFos-IR was significantly increased in the BLA. When considered with our observation that cFos expression in the “pain”-sensitive CeA did not differ between stressed and non-stressed rats (Gauriau and Bernard, 2002), the patterns of cFos expression in the BLA indicate that the restraint jacket does not provide salient and confounding nociceptive stimulation. Rather we suggest our data shows that following a peripheral nerve injury, or whilst in a persistent neuropathic pain state, the BLA is sensitised to respond to acute psychological/ emotional stressors.

This sensitisation may be a consequence of ongoing increases in the activity of BLA neurons indicated by the significant increases in FosB/ΔFosB-IR we describe here. This increased activity may not simply reflect a continued afferent input, but rather a disruption in the balance between excitatory and inhibitory

mechanisms embedded in the amygdala. Biochemical and electrophysiological studies in brain slices of the amygdala suggest that prolonged brain changes, at least in part, are independent of continuous afferent input (Neugebauer, 2015). In neuropathic mice, inhibitory glutamate receptor mGluR7 was decreased as a consequence of spared nerve injury (SNI) in the BLA 14 days after injury (Palazzo et al, 2015) and, following inflammatory pain in the hind paw, excitatory glutamate receptor mGluR1 expression was increased in the BLA (Luongo et al., 2013). These data suggest that a shift in glutamatergic regulation via their receptors could contribute to the ongoing increases in activity in the BLA following CCI.

An increased responsiveness of the BLA towards psychological/ emotional stressors in individuals with neuropathic pain may also be a reflection of a resetting of BLA sensitivity following activation of stress responsive circuitry and the production of an endocrine 'stress response' by the CCI. This 'stress signaling' usually involves increases in corticosterone releasing factor (CRF), an increase in glucocorticoids and increased noradrenergic signaling (Ulrich-Lai and Herman, 2009). In arthritic pain, hyperactivity of the BLA neurons was shown to be a consequence of CRF1 receptor activation (Ji et al., 2010). An increased CRF1 receptor density in the BLA neurons following CCI may serve as a mechanism of increased acute restraint sensitivity in the BLA. Previous studies looking at the effect of CCI on glucocorticoid receptor (GR) and CRF expression revealed increased GR and CRF mRNA expression in the CeA and MeA, but not the BLA as consequence of nerve injury (Ulrich-Lai et al., 2006), which suggests that glucocorticoids and CRF may have only an indirect effect on the BLA of neuropathic rats.

Following repeated stress, ß-adrenoreceptors activation in the BLA is important in habituating the physiological stress response, and reducing p-ERK activation in the BLA neurons (Grissom and Bhatnagar, 2011). Studies evaluating the effects of restraint stress on alpha1A adrenergic receptor mRNA showed that a single restraint increased mRNA in the midbrain, but not the hypothalamus, and repeated stress reduced levels in both midbrain and hypothalamus (Miyahara et al., 1999). A potential mechanism for the increased sensitivity of BLA neurons to acute restraint stress is the upregulation of adrenergic receptors as a consequence of a peripheral nerve injury. An increase in adrenergic receptor density may shift the threshold for neuronal activation in the BLA observed in neuropathic rats.

Functional Implications

Major outputs of the BLA whose activity may be altered by CCI include projections to the ventral hippocampus, nucleus accumbens (NAc), or bed nucleus of the stria terminalis (BNST) pathways. Our data suggest that projections to the mPFC are unlikely to be altered. Each of these pathways are important in the regulation of mood and/or affect, and the high comorbidity of affective mood disorders in chronic neuropathic pain might, in part, be regulated by altered activity in these pathways. BLA projections to each of these areas is considered below.

BLA to Ventral Hippocampus: The hippocampus and BLA are two regions of the brain that can operate independently of each other to influence cognitive and emotional behaviours. However, the synergistic function of the BLA and hippocampus can also be observed in the regulation of emotional behaviours

(Wang et al., 2011, Adhikari et al., 2010, Adhikari et al., 2011). Experiments combining pharmacological and optogenetic manipulations of the projection from the BLA to the ventral CA1 region of the hippocampus (*v*CA1) revealed a functional role for this pathway in both anxiety-like behaviours and social behaviours. Photoactivation of anterior BLA neurons projecting to the vCA1 was sufficient to produce anxiety-like behaviours, whilst photoinhibition produced robust anxiolytic effects (Felix-Ortiz et al., 2013). In addition to modulating anxiety-like behaviours, excitatory projections from the anterior BLA to the vCA1 region also decreased social behaviours in a resident-intruder social interactions test (Felix-Ortiz and Tye, 2014). In contrast to these roles in affective and social behaviours, neurons in the posterior regions of the BLA to vCA1 modulate emotional spatial memory (Yang et al., 2016). Following SNI, mice show increased anxiety-like behaviour and evidence of increased neuronal activity in the ventral hippocampus, indicated by increased pERK expression, (Mutso et al., 2012). An increase in BLA activity in SNI mice, similar to that reported in the CCI rats in this study, could contribute to the increased ventral hippocampal activity, and lead to anxiety-like behaviours. SNI also reduces neurogenesis in the hippocampus, which is thought to contribute to these changes in affective behaviours (Mutso et al., 2012). In addition, the reductions in social interactions observed following peripheral nerve injury (Keay et al., 2004, Monassi et al., 2003), which correlate with hippocampal volumetric reductions (Kalman and Keay, 2014), may be driven in part by the ongoing activity of BLA to vCA1 projections. Rats with SNI show deficits in working memory (Leite-Almeida et al., 2012, Ren et al., 2011), short-term memory (Ren et al., 2011) and recognition memory (Kodama et al, 2011), each of which are linked to deficits in hippocampal function (Kodama et al., 2011, Ren et al., 2011). Increased activity in posterior BLA neurons projecting to the vCA1 region may contribute to these impairments in neuropathic pain states.

On balance, increased activity in the BLA to vCA1 pathway can contribute to increased anxiety-like behaviours, reduced social behaviours and disrupted memory processes, each of which are components of the co-morbid changes in complex behaviours that often accompany persistent pain states.

BLA to Nucleus Accumbens: Monosynaptic glutamatergic projections from the BLA converge onto the distal dendrites and spines of medium spiny neurons of the shell of the NAcc (Johnson et al., 1994) and are involved in the estimation of reward salience (Shizgal, 1997), and directing attention to motivationally salient stimuli by integrating prior experiences (Baxter and Murray, 2002). The BLA regulates dopamine efflux within the NAcc via glutamate receptor-dependent modulation (Howland et al., 2002), as well as facilitating NAcc activity by the modulation of D1 receptor activation on medium spiny neurons (Floresco et al., 2001, Yim and Mogenson, 1982, O'Donnell and Grace, 1996, Smith-Roe and Kelley, 2000), to promote hyper-locomotion (Rouillon et al., 2008). Little is known about the influence that the BLA has on motivated and reward behaviours in the chronic neuropathic pain state.

Increased BLA activity is a characteristic of some rat models of addiction (Rademacher et al., 2015) and results in insensitivity to reward devaluation (Wassum and Izquierdo, 2015). Insensitivity to reward devaluation is also triggered by stress, however, certain stressors can also result in the enhancement of the motivation for reward (Sharp, 2017). Re-instatement of extinguished drug-seeking behaviours can be triggered by acute exposure to stress and this is dependent on activity in the BLA (Karimi et al., 2014).

Self-administration of nicotine is impaired by disruption of the BLA to NAcc pathway, and stress-induced increases in motivation for drug-seeking behaviours were dependent on BLA activity (Yu and Sharp, 2015). Impaired reward processing is observed in many chronic pain patients who show deficits in emotional decision-making tasks such as the IOWA Gambling Task (Apkarian et al., 2004). Chronic pain patients also demonstrate a high prevalence of substance abuse (Martel et al., 2017) These observations are consistent with alterations in BLA reward circuitry as demonstrated by several animal models of nerve injury leading to persistent neuropathic pain.

BLA to Bed Nucleus of the Stria Terminalis: The BNST is implicated in the emotional aspect of pain (Deyama et al., 2007) and plays a crucial role in the expression of anxiety-like behaviours (Davis et al., 2010). Variations in the expression of fear and anxiety are at least in part influenced by BNST and its downstream targets (Duvarci et al., 2009). Inter-individual variations in anxious temperament were predicted by presynaptic serotonin reuptake transporter binding (Oler et al., 2009) and glucose uptake (Fox et al., 2008) in the BNST. This suggests that individuals with higher anxiety-like behaviours have higher baseline BNST activity, and/or increased activity in BNST afferents. The BNST receives excitatory inputs from the BLA (Krettek and Price, 1978a, Krettek and Price, 1978b), and neurons from the caudal BLA project specifically to the lateral division of the BNST (McDonald, 1991, Dong et al., 2001, Weller and Smith, 1982), which is essential for the expression of anxiety-like behaviours (Walker and Davis, 1997). CRF containing neurons, and neurons with CRF receptors are found in high concentrations in the BNST (Swanson et al., 1983). This population of CRF-ergic neurons in the BNST have an essential role in mediating anxiety-like behaviours in response to a range of stressors (Lee and Davis, 1997, Davis et al., 2010). The BLA projections to the BNST play a major regulatory role in mediating CRF-induced anxiety-like behaviours. Inactivation of the BNST abolishes the CRF induced stress response, and CRF levels potentiate excitatory drive from glutamatergic BLA projections to the BNST. The facilitatory effect of CRF on BNST excitation is thought to be a consequence of pre-synaptic CRF receptors located within the BNST (Davis et al., 2010, Justice et al., 2008). Following CCI there is a significant increase in CRF and CRF mRNA in the BNST of rats 24 days post-injury (Rouwette et al., 2012); and the patterns of neural activity to acute noxious events are altered significantly, suggesting a substantially altered role of the BNST in the chronic pain condition (Morano et al., 2008). Increased CRF in the BNST of neuropathic rats may serve to amplify anxiety-like behaviours and excitatory inputs from the BLA may play a significant role in this exacerbation.

In summary, these data show that neuropathic injury increases ongoing activity of the basolateral amygdala, (FosB/ΔFosB expression) and results in the activation of BLA neurons (c-Fos expression) to acute restraint stress. The increased basal activity of BLA neurons following neuropathic injury may underlie the increased sensitivity of BLA neurons to acute emotional/ psychological stressors. The functional consequences of the BLA activation to acute stressors in the neuropathic pain state is yet to be elucidated, but it may contribute to altered regulation of emotional behaviours via its extensive projections to the ventral hippocampus, nucleus accumbens and the BNST.

Abbreviations

CCI - chronic constriction injury of the right sciatic nerve

CTB - cholera toxin B

mPFC - medial prefrontal cortex

BLA – Basolateral amygdala

CeA – Central amygdala

MeA – Medial amygdala

PFA – 4% paraformaldehyde acetate-borate buffer

PBS – 0.1M phosphate buffered saline

NHS – 10% normal horse serum

DAB – 3, 3-diaminobenzidine tetrahydrochloride

CRF – Corticosterone releasing factor (CRF)

GR – Glucocorticoid receptor

LC – Locus coeruleus

NTS – Nucleus of the solitary tract

NAcc - Nucleus accumbens

vCA1 – Ventral CA1

SNI – spared nerve injury

Declarations

Competing interests: The authors declare no competing interests.

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Tables

Table 1: Mean (±SEM) numbers of Fos-immunoreactive cells in the BLA, CeA and MeA *P<0.05, **P<0.01 and ***P<0.001, represent significant differences in numbers of Fos-immunoreactive cells between stressed and non-stressed rats in their corresponding surgical treatment groups (anaesthetised (n=12), sham-injury (n=12) or CCI (n=13-14)), Two-way ANOVA with pairwise comparisons.
Table 1 is missing from this version.

Table 2: Mean (±SEM) numbers of FosB/ΔFosB-immunoreactive labelled cells in the BLA, CeA and MeA

Region	Anaesthetised	Sham	CCI
CeA (Left) (n=12)	113.92±10.3	83.58±16.62	94.67±9.65
CeA (Right) (n=12-14)	98.5±11.64	90±16.09	90.0±11.51
CeA (Total) (n=24-26)	106.21±9.13	86.79±11.42	92.33±8.32
MeA (Left) (n=12-13)	87.17±15.33	79.42±16.57	79.23±10.63
MeA (Right) (n=12-14)	61.75±13.39	73.17±16.57	78.79±10.63
MeA (Total) (n=24-27)	74.46±10.29	76.29±10.96	79.0±7.49
BLA (Left) (n=12-13)	65.08±14.36	75.5±18.25	114.38±18.41
BLA (Right) (n=12-14)	74.17±14.44	58.58±11.48	110.86±27.23
BLA (Total) (n=24-27)	69.63±10.0	67.04±10.69	***112.62±16.36**

*P<0.05 represent significant differences of the total number of FosB/]FosB-immunoreactive cells between Sham and CCI rats, One-way ANOVA with Tukey post hoc test.