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Enhanced nociceptive responding in two rat models of depression is associated with alterations in monoamine levels in discrete brain regions

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**Abbreviations:** 5-HT serotonin; 5-HIAA 5-hydroxyindoleacetic acid; NA Noradrenaline; OB Olfactory Bulbectomy; PFC prefrontal cortex; SD Sprague Dawley; WKY Wistar-Kyoto rat
Abstract
Altered pain responding in depression is a widely recognized but poorly understood phenomenon. The present study investigated nociceptive responding to acute (thermal and mechanical) and persistent (inflammatory) noxious stimuli in two animal models of depression, the olfactory bulbectomised (OB) and the Wistar-Kyoto (WKY) rat. In addition, this study examined if altered nociceptive behaviour was associated with changes in monoamine levels in discrete brain regions. OB rats exhibited mechanical allodynia (von Frey test) but not thermal hyperalgesia (hot plate and tail-flick tests) when compared to sham-operated counterparts. Formalin-induced nociceptive behaviour was both heightened and prolonged in OB versus sham-operated controls. An inverse correlation was observed between 5-hydroxyindoleacetic acid (5-HIAA) concentration in the hippocampus and amygdaloid cortex and nociceptive behaviour in the formalin test. In comparison, WKY rats exhibited thermal hyperalgesia in the hot plate test, while behaviour in the tail-flick and von Frey tests did not differ between WKY and Sprague-Dawley rats. Furthermore, WKY rats exhibited enhanced formalin-evoked nociceptive responding up to 40 minutes post administration, an effect inversely correlated with serotonin and 5-HIAA levels in the hypothalamus. In conclusion, these findings demonstrate that altered pain responding observed in clinically depressed patients can be modelled pre-clinically, providing a means of investigating the neurochemical basis of, and possible treatments for, this phenomenon.

Keyword: olfactory bulbectomy, Wistar-Kyoto rat, hot plate, formalin test, mechanical allodynia, serotonin
The clinical connection between pain and depression has long been recognised. Epidemiological studies indicate that up to 66% of major depressed patients report co-morbid chronic pain (Bair et al., 2003; Bair et al., 2004; Arnow et al., 2006) and chronic pain patients are up to 4 times more likely to develop major depressive disorder when compared to the general population (Von Korff and Simon, 1996; Arnold, 2006; Twillman, 2007). The co-existence of depression and chronic pain is associated with increased severity and duration of depressive and physical symptoms, poor treatment response, and is a significant risk factor for relapse (Bair et al., 2004; Ohayon, 2004; Arnow et al., 2006). Although depressed patients report significantly more frequent and intense pain, pain thresholds to pressure and cold stimuli may be increased (Lautenbacher et al., 1999). In contrast, depressed patients report a non-painful warm stimulus as more unpleasant compared with healthy control subjects (Strigo et al., 2008) and reduced pain perception threshold to a high frequency electrical skin stimulation (Adler and Gattaz, 1993). Due to the complex nature of the association between depression and pain, there is an increasing need to investigate the common neurobiological mechanisms that underlie this co-morbidity.

Although depressive-like behaviours have been examined in preclinical models of chronic pain (Kontinen et al., 1999; Goncalves et al., 2008; Hu et al., 2009; Norman et al., 2009), there have been relatively few studies investigating nociceptive responding in animal models of depression. Increased nociceptive threshold to thermal and mechanical stimuli (Pinto-Ribeiro et al., 2004; Shi et al., 2010b), hyperalgesia in response to persistent inflammatory pain (Gameiro et al., 2006; Shi et al., 2010a) and reduced mechanical allodynia following nerve injury (Shi et al., 2010b) have been reported following exposure to chronic unpredictable stress. Maternal deprivation results in enhanced nociceptive responding to thermal stimulation during adulthood, an effect attenuated by chronic imipramine treatment.
The olfactory bulbectomised (OB) model is a well validated preclinical model of depression exhibiting dysfunction in the cortical-hippocampal-amygdala circuit, leading to anatomical, behavioural, neurochemical, neuroendocrine and immune changes reflecting those observed in major depressed patients [for reviews see (Kelly et al., 1997; Song and Leonard, 2005)]. Conflicting data have been presented on nociceptive responding to acute thermal and mechanical stimuli in the OB model, with reports of both enhanced (Belcheva et al., 2009; Wang et al., 2010) and reduced (Rodríguez-Gaztelumendi et al., 2006) pain thresholds. In addition, a recent study has demonstrated that OB rats exhibit enhanced nociceptive behaviour in the formalin test of persistent inflammatory pain (Wang et al., 2010). However, studies to date have not investigated the potential neurochemical changes that may mediate altered nociceptive responding in the model. The Wistar-Kyoto (WKY) rat, a genetic animal model of depression, exhibits heightened stress-induced behavioural responses (Tejani-Butt et al., 1994; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004; Malkesman et al., 2005) and exaggerated activation of the hypothalamic-pituitary-adrenal axis to stress (Solberg et al., 2001; Rittenhouse et al., 2002), effects also observed in clinically depressed patients. WKY rats have been reported to display mechanical allodynia but no behavioural changes to acute noxious thermal stimuli (Taylor et al., 2001; Robbins et al., 2007). Enhanced nociceptive responding to visceral stimulation (Gunter et al., 2000; Gibney et al., 2010) and exacerbated mechanical allodynia following peripheral nerve injury (Zeng et al., 2008) have also been demonstrated in the WKY rat model. Only one study to date has examined nociceptive responding to a noxious inflammatory stimulus in WKY rats. Taylor and colleagues (2001) demonstrated that intraplantar zymosan administration induces a pronounced reduction in thermal latency and mechanical threshold in Sprague Dawley rat, an effect blunted in WKY rats. No significant difference in nociceptive responding to formalin administration was observed between these
strains of rats (Taylor et al., 2001). The present study aims to further evaluate and compare nociceptive responding to acute (thermal and mechanical) and persistent (inflammatory) noxious stimuli in these two animal models of depression, the OB and WKY rat.

Common neurobiological mechanisms have been proposed to underlie the co-morbidity of depression and pain, which is exemplified by the current use of antidepressant agents as first-line treatment for neuropathic pain (Mico et al., 2006). Dysregulation of central monoaminergic function, as occurs in major depressive disorder (Schildkraut and Kety, 1967), may incite or enhance pain perception and sensitivity in these patients. Thus, modulating brain monoamine levels may act concurrently to relieve depressive symptoms and facilitate the descending inhibitory pain pathways thereby inducing analgesia. Alterations in the functioning of the serotonergic and noradrenergic systems have been demonstrated in both OB (Lumia et al., 1992; Song and Leonard, 1995; Redmond et al., 1997; Connor et al., 1999; Hellweg et al., 2007) and WKY (Pare and Tejani-Butt, 1996; Durand et al., 2003; De La Garza and Mahoney, 2004; Pearson et al., 2006) rats, effects which may underlie altered nociceptive responding in these models. As such, a further aim of this study is to investigate if persistent pain behaviour following an inflammatory noxious stimulus is associated with alterations in monoamine levels in discrete brain regions implicated in modulation of emotional and nociceptive responding.
EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out on singly housed male Lister-Hooded (Charles River, UK), Sprague Dawley (SD) and Wistar-Kyoto (WKY) rats (Harlan, UK) (weight 220-250g at the beginning of the study). The choice of Lister-Hooded rats for assessing bulbectomy-induced changes in nociceptive behaviour was based on our previous characterization of the formalin response in this strain of rat (Finn et al., 2003; Roche et al., 2007b; Roche et al., 2010). The most widely used comparator rat strain for the WKY is the SD rat and, therefore, nociceptive behaviour was also assessed in these two strains. All animals were maintained at a constant temperature (20 ± 2°C) under standard lighting conditions (12:12h light–dark, lights on from 0800-2000h). Experiments were carried out during the light phase between 0900h and 1700h. Food and water were available *ad libitum*. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609 and all efforts were made to minimise the number of animals used and their suffering.

Bilateral Olfactory Bulbectomy (OB) Surgery

Bilateral olfactory bulbectomy was performed on Lister-hooded rats anaesthetised with isoflurane anaesthesia (3% in O₂), essentially as outlined previously (Roche et al., 2007a; Roche et al., 2008). In brief, the head was shaven and a midline sagittal incision was made in the skin overlying the skull. Two burr holes of 2mm diameter were drilled into the skull, 5mm rostral to bregma and 2mm lateral to the midline. The olfactory bulbs were removed by gentle aspiration with a water vacuum pump and care was taken not to damage the frontal cortex. The burr holes were then plugged with a haemostatic sponge to control bleeding.
Sham-operated animals were treated in the same manner but the bulbs were left intact. Animals were handled daily following surgery to reduce aggression associated with OB surgery. Lesions were verified by gross inspection after completion of the study. Animals were eliminated from the analysis if the bulbs were not completely removed or if damage extended to the frontal cortex. Sham-operated animals were removed if there was any damage to the bulbs or the frontal cortex.

**Behavioural Testing**

*Open Field Test*

Open field behavioural testing was carried out the day prior to sham or OB surgery (Day 0) and again on days 3, 7 and 14 post surgery, in order to confirm OB-induced hyperactivity, a hallmark of depressive-like behaviour in the model. Behaviour in the open field was assessed once for SD and WKY rats (day 8 post arrival). On the experimental day, each animal was removed from the home cage during the light phase between 1000h and 1200h and placed singly into a brightly lit (lux 300-400) novel open field environment (diameter 75cm) where behaviour was assessed using a computerised video tracking system (EthoVision®, Version 3.1, Noldus Netherlands) for a 5 minute period. Behaviours assessed included locomotor activity (distance moved: cm) and duration of time spent (seconds; s) in the centre zone (45cm diameter), an indication of anxiety-related behaviour.

*Forced Swim Test*

Depressive-like behaviour was assessed using the forced swim test (Porsolt et al., 2001) for both WKY and SD counterparts (day 9-10 post arrival). In brief, rats were placed individually into inescapable glass cylinders (24cm diameter), containing 30cm of water at 25°C for 15 minutes. Rats were then re-exposed to the swim arena 24h later (Day 2) for a 5
minute period during which time behaviour were recorded onto DVD. Behaviours were assessed with the aid of EthoVision® software and included time spent immobile (floating and movements necessary to keep its head above water), swimming (horizontal movements) and climbing (vigorous, upward directed movements) by an experimenter blinded to group identity.

**Nociceptive Responding**

*Hot Plate test*

Nociceptive responding in the hot plate test is predominately supraspinally mediated, requiring activation of the medial prefrontal cortex in order to elicit a response (Pastoriza et al., 1996). Hot plate testing was carried out on Day 0 before sham or OB surgery, and again on days 4, 8 and 15 post surgery. Testing was carried out once for SD and WKY rats (day 14 post arrival). On the test day, the animal was taken from its home cage and placed directly onto a hot plate (IITC Life Science Inc, California, US) heated to $55 \pm 1\, ^\circ C$. Thermal nociception was measured as the time elapsed (i.e. latency to respond (seconds)) between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind paws, with a cut-off time of 40 sec to avoid tissue damage.

*Tail-flick test*

The tail-flick test is a spinally mediated reflex response (Grossman et al., 1982; Ramabadran and Bansinath, 1986; Pastoriza et al., 1996). Testing was carried out once for SD and WKY rats (day 14 post arrival) or on Day 0, 4, 8 and 15 for sham and OB animals. In brief, animals were acclimatized for 10 minutes prior to testing to a plastic restraint which allowed their tails to project out. A light source (IITC Life Science Inc, California, US) was positioned 7cm above the tail from which heat could be localised 2cm from the end of the tail and...
latency to withdraw/flinch the tail was measured (seconds), with a cut off of 10 seconds. This was repeated for each animal at 3 different light intensities (25%, 35%, & 45% of apparatus’ maximum output, 230 Voltage Alternating Current). Each heat application was carried out in triplicate for each animal with an interval of 15 seconds between each test. Tail temperature was comparable between all animals at the time of heat application.

**Von Frey Testing**

Rats were placed in a Perspex compartment with a wire mesh bottom and allowed to acclimatise for a minimum of 20 minutes prior to testing. Mechanical sensitivity was assessed using von Frey hairs and a modification of the up–down method (Chaplan et al., 1994). Briefly, filaments of increasing weight (1.4g-100g) were applied perpendicularly to the plantar surface of the hindpaw. A positive response was recorded if the animal responded to the applied filament by flinching, licking, or sharply withdrawing the paw within the 6 s of filament application. The rat was tested 5 times with each filament with a minimum of 6 s between each test until a 5/5 or 100% response was obtained for 2 consecutive filaments on each paw of each animal. The 50% withdrawal threshold (g) was determined for each animal. Baseline responding was assessed prior to sham or OB surgery and the effect of bulbectomy assessed on day 16 post surgery. Responding was assessed once in both WKY and SD rats (day 3 post arrival).

**Formalin-induced nociceptive behaviour**

Nociceptive behaviour in the formalin test was assessed only once for each animal, at least 2 days following the last behavioural assessment. In brief, rats were placed in a Perspex observation chamber (30 x 30 x 30 cm) for 10 minutes after which time they received an intra-plantar injection of 50µL formalin (2.5% in saline (0.9% NaCl)) or saline into the right
hindpaw under brief isoflurane anaesthesia as previously described (Finn et al., 2003; Roche et al., 2007b; Roche et al., 2010). Rats were returned to their home cage for a further 3 minutes at which point they were returned to the same Perspex observation chamber to which they had been previously exposed. Behaviour was recorded for 2 hours from a video camera located beneath the observation chamber. Behaviour was analysed with the aid of EthoVision® software by a rater blind to experimental conditions. Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring technique (CPS-WST\textsubscript{0,1,2}) (Watson et al., 1997). According to this method, pain behaviours are categorised as time spent raising the formalin injected paw above the floor without contact with any other surface (C1) and holding, licking, biting, shaking or flinching the injected paw (C2) to obtain a composite pain score (CPS = (C1 + 2(C2))/(total duration of analysis period)). Formalin-induced oedema was assessed by measuring the change in the diameter of the right hind paw immediately before and 2 hrs after formalin administration using Vernier callipers.

**HPLC analysis of brain tissue monoamine concentrations**

Rats were decapitated and the brains quickly removed 2 hours following intra-plantar formalin administration, discrete brain regions (prefrontal cortex, hypothalamus, hippocampus, amygdaloid cortex, thalamus and cerebellum) dissected out on an ice cold plate, weighed and 1 ml of mobile phase (0.1 M citric acid, 0.1 M NaH\textsubscript{2}PO\textsubscript{4}, 1.4 mM 1-octane sulfonic acid, 0.01 mM EDTA, 10% methanol; pH 2.8) containing 2ng/20µl N-methyl 5-HT as an internal standard was added to each region. Samples were subsequently homogenized, centrifuged at 4°C for 15 minutes at 14000g and stored at -80°C until monoamine determination. The HPLC method used was based on that of Seyfried et al. (1986) and routinely used in our laboratory (Roche et al., 2007b). Briefly, a 20µl sample of
supernatant was injected onto Shimadzu HPLC with a reverse-phase C18 column (Licrosorb RP-18 column; Phenomenex, Macclesfield, Cheshire, UK). Electrochemical detection was used to determine peak heights of monoamines and their metabolites. The electrode was maintained at +0.8 V and the flow rate of the mobile phase through the system was 1 ml/min. Peak heights for standards of 2ng/20μl noradrenaline, serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) were obtained each day prior to injection of samples and after every 10 samples. The data were calculated as ng neurotransmitter/g of tissue.

**Statistical Analysis**

SPSS statistical package was used to analyse all data. Behavioural data were analysed using unpaired, two-tailed t-tests with comparisons between Sham vs OB or SD vs WKY. Repeated measures analysis of variance (ANOVA) was used to assess changes over time. Mechanical sensitivity and neurochemical data was assessed using two-way ANOVA with the factors of bulbectomy/strain and left/right paw or formalin. *Post-hoc* analysis was performed using Fisher’s LSD test when appropriate. Pearson’s correlation analysis was used to assess correlations between area under the curve (AUC) for formalin-evoked nociceptive behaviour and neurotransmitter levels in discrete brain regions. Data were considered significant when P<0.05. Results are expressed as group means ± standard error of the mean (SEM).
RESULTS

Behavioural evaluation of anxiety- and depressive-related phenotypes in OB and WKY rats

No significant difference on distance moved in the open field was observed between sham and OB rats prior to or 3 days post surgery (data not shown). However, OB rats displayed a characteristic increase in distance moved on day 7 (Sham: 2654 ± 318 cm vs OB: 3939 ± 315 cm; P<0.05) and day 14 (Figure 1A) post surgery when compared to sham-operated controls. The OB-induced increase in distance moved was associated with enhanced thigmotactic activity (distance moved in outer zone) and was not associated with alterations in anxiety-related behaviour as OB and sham-operated rats spent a comparable duration of time in the centre zone of the open field (Figure 1B).

In comparison, WKY rats demonstrated anxiogenic-like behaviour in the open field, exemplified by a decrease in duration of time spent in the centre zone (Figure 1B) and reduced number of entries into the centre zone (WKY: 0.2 ± 0.1 SD: 8.5 ± 2.5; P<0.01) when compared to SD counterparts. In addition, WKY rats exhibited a pronounced reduction in distance moved in the open field over the 5 minute trial (Figure 1A). An increased duration of immobility in the forced swim test and a corresponding reduction in climbing behaviour was observed for WKY rats compared with SD counterparts (Figure 1C), indicative of a depressive-like phenotype in this strain of rat.

Nociceptive responding of OB and WKY rats to noxious thermal stimuli

Thermal hyperalgesia was assessed using both the hot plate and tail-flick tests (Figure 2). There was no significant difference between sham and OB rats in latency to respond in the hot plate test prior to surgery. However, 3 days post surgery, OB rats displayed a decrease in
latency to respond when compared to sham-operated counterparts (sham: 14.5 ± 1.6 sec vs. OB: 9.8 ± 0.7 sec; P<0.01). However, this decrease in hot plate test latency in OB rats was transient and was not apparent on day 8 (data not shown) or day 15 (Figure 2A) post surgery. In comparison, WKY rats exhibited a significant decreased latency to respond in the hot plate test (day 14 post arrival) when compared to SD controls (Figure 2A).

There was no significant difference between sham or OB rats with respect to latency to withdraw in the tail-flick test at any of the light intensities used (25%, 35%, 45%) during any of the test days (Day 15; Figure 2B). Similarly, no significant difference was observed between WKY and SD rats in the tail-flick test (Figure 2B).

**OB but not WKY rats display mechanical allodynia**

Prior to sham or OB surgery, mean 50% withdrawal thresholds were established for both the left and right hind paw for all animals (left: 9.35 ± 0.6 g right 10.23 ± 0.8 g). Sham surgery did not significantly alter withdrawal threshold. However, OB rats displayed a reduction in withdrawal threshold indicative of the establishment of mechanical allodynia 16 days post surgery when compared to sham-operated counterparts (Figure 3A). In contrast, there was no significant difference between WKY and SD rats in relation to threshold to respond to a mechanical stimulation (Figure 3B).

**OB and WKY rats display enhanced nociceptive responding to a persistent inflammatory stimulus**

Distance moved in the novel Perspex arena was not significantly different between sham and OB (sham: 2585 ± 146 cm OB: 2729 ± 186 cm) or SD and WKY (SD: 2974 ± 300 cm
WKY: 2885 ± 565 cm) rats during the 10 minute acclimatization period prior to formalin administration. Intra-plantar injection of formalin induced right-hind paw oedema in all animals. There was no significant difference in the post-formalin minus pre-formalin change in paw diameter between sham and OB (sham: 2.21 ± 0.33 mm  OB: 2.08 ± 0.11 mm) or SD and WKY (SD: 1.66 ± 0.20 mm  WKY: 1.91 ± 0.16 mm) rats. Intra-plantar formalin administration produced robust licking, biting, shaking and elevation of the right hind paw as indicated by the composite pain score. Sham and OB animals exhibited the classic biphasic response (Figure 4A) exemplified by a peak in nociceptive behaviour approximately 5 minutes following formalin administration, which subsides and then a second phase of nociceptive behaviour beginning 15 minutes post administration. Analysis revealed that OB rats exhibited significantly higher formalin-evoked nociceptive behaviour 30-35 minutes post-formalin. Furthermore, although nociceptive behaviour of sham-operated rats began to return to baseline levels from 70 minutes post formalin, nociceptive behaviour of OB rats remained pronounced until 90 minutes post administration. Thus, OB rats display a significant increase both the magnitude and duration of second phase formalin-evoked nociceptive behaviour. Assessment of area under the curve revealed OB rats exhibit enhanced nociceptive responding following intra-plantar formalin when compared to sham-operated controls (Figure 4A (inset)).

Although WKY rats exhibit the classic biphasic nociceptive response to intraplantar formalin administration, SD rats failed to demonstrate significant pain-related behaviour in the first 5-10 minutes (Figure 4B). WKY rats demonstrated a greater formalin-evoked nociceptive responding for the first 40 minutes post administration when compared to SD counterparts. Nociceptive behavioural responding returned to baseline levels at comparable rates for both WKY and SD following intra-plantar formalin administration. Thus, WKY rats exhibit a
significant enhancement in nociceptive responding in both the first and second phase of the formalin test when compared to SD rats. Assessment of area under the curve revealed that WKY rats exhibited greater nociceptive responding following intra-plantar formalin when compared to SD counterparts over the 2 hours (Figure 4B (inset)).

**Enhanced formalin-evoked nociceptive responding in OB and WKY rats is accompanied by differential changes in brain monoamine levels**

Although minor differences in monoamine levels were observed between sham and OB rats that did not receive formalin, an increase in noradrenaline levels in the thalamus of OB rats when compared to sham-operated counterparts was the only significant difference between the groups (Table 1). Intra-plantar formalin administration significantly increased noradrenaline levels in the prefrontal cortex, hippocampus, amygdaloid cortex and cerebellum of both sham and OB rats. In addition, increases in serotonin (5-HT), its metabolite 5-HIAA, and/or serotonin turnover (5-HIAA/5-HT) were observed in all brain regions examined in both sham and OB rats 2hrs post intra-plantar formalin administration. The formalin-induced increase in 5-HT concentration in the prefrontal cortex and 5-HIAA in the hippocampus and amygdaloid cortex was significantly blunted in OB verses sham-operated rats (Figure 5). Concurrently, OB-formalin treated rats displayed an augmentation in noradrenaline and 5-HIAA concentrations in the cerebellum, and noradrenaline and 5-HT levels in the thalamus, when compared to sham-formalin treated counterparts.

Correlation analysis determined a significant inverse correlation between formalin-evoked nociceptive behaviour (area under the curve) and 5-HIAA concentration in the hippocampus (r² = 0.3389 P<0.05) and amygdaloid cortex (r² = 0.424 P<0.01).
Noradrenaline levels in the hypothalamus, hippocampus, amygdaloid cortex and thalamus were significantly lower in WKY compared to SD rats (Table 2). In addition, WKY rats exhibited reduced 5-HIAA levels in the hypothalamus and thalamus and increased 5-HT turnover in the cerebellum compared to SD counterparts. Formalin administration significantly reduced noradrenaline levels in the prefrontal cortex of both SD and WKY rats, increased levels in the cerebellum of SD but not WKY rats and reduced levels in the hypothalamus of WKY but not SD counterparts (Table 2; Figure 6). In comparison, 5-HT levels were significantly increased and 5-HT turnover reduced in all of the brain regions examined of both SD and WKY rats 2 hours post formalin administration. The formalin-induced increase in 5-HIAA in the hypothalamus and 5-HT in the hypothalamus, amygdaloid cortex and thalamus was significantly reduced in WKY rats when compared to SD counterparts (Figure 6).

Correlation analysis determined a significant inverse correlation between formalin-evoked nociceptive behaviour (area under the curve) and 5-HT ($r^2 = 0.3648$ P<0.01) and 5-HIAA ($r^2 = 0.4206$ P<0.05) concentration in the hypothalamus.
DISCUSSION

The present study demonstrates that both the OB and WKY models of depression exhibit enhanced behavioural responding to acute and persistent noxious stimuli. The two models differed with respect to the specific nature of their response to the acute stimuli and the duration and time of onset of responding to the inflammatory stimulus. These behavioural responses were associated with differential alterations in monoamine levels in discrete brain regions involved in modulating emotional and pain processes.

OB rats exhibited a characteristic depressive-like, but not anxiety-like, behavioural profile characterised by enhanced hyperactivity in the open field. Although not assessed in the present study, enhanced immobility in the forced swim test (Vieyra-Reyes et al., 2008; Tasset et al., 2009) and reduced sucrose consumption (Romeas et al., 2009; Sato et al., 2010) have also been reported following bulbectomy, additional hallmarks of depressive-like behaviour. This depressive phenotype was associated with mechanical allodynia and transient thermal hyperalgesia in the hot plate test, corroborating previous findings using alternative methodologies for the assessment of nociceptive responding (Rodríguez-Gaztelumendi et al., 2006). In the latter study, thermal hyperalgesia was determined using the tail-flick test, however no significant change in latency to respond in this test was observed between sham and OB rats in the present study. It is possible that, despite efforts to habituate animals to restraint prior to testing in the present study, stress-induced analgesia may have been expressed to some extent, thereby masking the effects of bulbectomy on thermal hyperalgesia. While our results demonstrate a transient hypersensitivity of OB rats on the hot plate test, previous work has reported that OB rats exhibit decreased responding to noxious radiant heat applied to the hind paw (Wang et al., 2010) and an increased threshold to respond to a noxious mechanical pressure stimulus (Belcheva et al., 2009). Thus,
nociceptive responding of OB rats to thermal and mechanical stimuli may depend on the type and duration of stimulus applied.

In contrast to the OB model, WKY rats exhibited both a depressive- and anxiety-like behavioural phenotype exemplified by increased immobility in the forced swim test, reduced activity and decreased number of entries into the centre arena of the open field, correlating with previous studies (Tejani-Butt et al., 1994; Rittenhouse et al., 2002; Will et al., 2003). In addition, the WKY rats displayed significant thermal hyperalgesia in the hot plate, but not tail-flick test and no change in nociceptive responding to mechanical stimuli. These findings are in contrast to those previously reported, where WKY rats demonstrated mechanical allodynia but no change in responding to thermal noxious stimuli when compared to SD counterparts (Taylor et al., 2001). Methodological differences may account for the discrepancies between these studies. However, in a more recent study, no significant difference was observed in baseline mechanical threshold between WKY and Wistar rats, although WKY rats did exhibit exacerbated mechanical allodynia following the induction of chronic constriction nerve injury (Zeng et al., 2008). Enhanced pain responding observed in WKY rat may, in part, be due to genetic differences between the strains of rat used in this study. Further studies are required in order to determine if such genetic factors are related to the development of clinical depression (Lohoff). Enhanced nociceptive responding in the hot plate test is mediated, in part, by the medial prefrontal cortex (Pastoriza et al., 1996). Neurochemical alterations in the prefrontal cortex of WKY rats have been demonstrated both in the present study (discussed below) and by others (Tejani-Butt et al., 1994; Pare and Tejani-Butt, 1996; De La Garza and Mahoney, 2004; Zeng et al., 2008). In addition, WKY rats exhibit enhanced activation of the prefrontal cortex in response to visceral noxious stimulation (Gibney et al., 2010). Such alterations in the functioning of this key brain region...
may account, in part, for the enhanced nociceptive responding of WKY rats in the hot plate test when compared to SD counterparts.

In an effort to mimic the persistent nature of pain complaints reported by depressed patients, this study also evaluated nociceptive behaviour in the formalin test of persistent inflammatory pain. OB rats displayed enhanced and prolonged nociceptive responding in the formalin test, corroborating the recent work of Wang and colleagues demonstrating increased formalin-evoked pain behaviour in OB Sprague-Dawley rats (Wang et al., 2010). However, in this latter study, the increase in nociceptive responding was observed over the entire first hour post administration in contrast with the present findings of enhanced nociceptive behaviour between 30-35 minutes post formalin and prolonged responding up to 90 minutes. It is possible that the higher concentration of formalin (5%) used in the latter study (Wang et al., 2010) elicited a more pronounced response than that observed in the present study. However, as nociceptive behaviour was assessed for only one hour by Wang and colleagues, the extended responding observed in OB rats in the present study may have been overlooked in this latter study. It should also be noted that locomotor activity assessed in the 10 minute pre-formalin period did not differ between OB and sham rats, suggesting that the differences in formalin-evoked nociceptive behaviour are unlikely to be due to overt changes in locomotor activity and are more likely to relate to alterations in nociceptive processing. In addition, this further supports the finding that OB-induced hyperactivity is context-dependant, observed in a brightly lit open field but not in a non-aversive Perspex arena (Kelly et al., 1997).

Several studies have demonstrated reduced 5-HT functioning in discrete brain regions including the frontal cortex, amygdaloid cortex and midbrain following OB surgery (Lumia et al., 1992; Redmond et al., 1997; Connor et al., 1999; van der Stelt et al., 2005; Hellweg et
al., 2007), thus the OB rat has been proposed as a model of hyposerotonergic depression.

The absence of alterations in 5-HT levels in discrete brain regions following bulbectomy is at odds with studies in the literature (Lumia et al., 1992; Redmond et al., 1997; Connor et al., 1999; van der Stelt et al., 2005; Hellweg et al., 2007) and may be a consequence of brief exposure to isoflurane anesthetic and intraplantar saline administration in the current study.

Interestingly, we found that formalin-induced increases in 5-HT or 5-HIAA in the PFC, hippocampus and amygdaloid cortex of sham rats were significantly blunted in OB rats, suggesting that OB rats display blunted 5-HT functioning in response to noxious stimulation.

In addition, a significant inverse correlation was found between nociceptive responding in the formalin test and 5-HIAA concentration in the hippocampus and amygdaloid cortex. The prefrontal cortex, hippocampus and amygdala are critical forebrain regions involved in processing both emotional and cognitive aspects of depression and pain (Ulrich-Lai et al., 2006; Matsuzawa-Yanagida et al., 2008; Strigo et al., 2008). Furthermore, the role of the serotonergic system in modulating nociceptive processing has been well documented, with 5-HT depletion (Butkevich et al., 2003; Svensson et al., 2006; Wei et al., 2010) and 5-HT$_{2C}$ antagonism in the hippocampus (Soleimannejad et al., 2006) resulting in reduced formalin-evoked nociceptive behaviour. Thus, one would expect that an attenuation of the formalin-evoked increase in central 5-HT levels as seen in OB rats in the present study would mediate a reduction in nociceptive behaviour. However, 5-HT neuronal innervation, 5-HT$_{2C}$ receptor density and 5-HT transporter sites have been demonstrated to be enhanced in the cortex of OB animals (Grecksch et al., 1997; Huether et al., 1997; Zhou et al., 1998), alterations which may also occur in the hippocampus and amygdala. Therefore, it is possible that although levels of 5-HT are blunted, 5-HT$_{2C}$ receptor activation may be enhanced thereby facilitating nociceptive processing. In addition, it should also be noted that intraplantar formalin administration induced an increase in 5HT concentration in the thalamus and noradrenaline
and 5-HIAA concentrations in the cerebellum of both sham and OB rats, with OB rats displaying significantly higher formalin-evoked levels compared with sham controls. Although it is unknown what direct effect the monoamine system in the thalamus has on formalin-evoked nociceptive behaviour, it has been demonstrated that administration of 5-HT into the medial thalamus suppressed the rat tail-flick reflex, an effect mediated by 5-HT$_{1A}$ and 5-HT$_2$ receptors (Xiao et al., 1999; Xiao et al., 2005). In addition, the cerebellum is well-positioned to modulate both emotional and nociceptive behaviours via monosynaptic connections with the amygdala, hippocampus and periaqueductal grey (Sacchetti et al., 2005). The present findings highlight that OB rats exhibit both blunted and enhanced monoaminergic responding to formalin-evoked nociception in a manner dependant on the brain region and analyte under investigation. However, the extent to which monoaminergic transmission in each of these regions contributes to the altered nociceptive processing in OB rats remains to be determined.

In comparison to the prolonged nociceptive responding observed in OB rats, WKY rats exhibited enhanced formalin-induced nociceptive behaviour during both the first and second phases of the test, up to 40 minutes post administration when compared to SD counterparts. This finding contrasts with a previous study reporting no difference in formalin-evoked nociceptive behaviour between WKY and SD rats (Taylor et al., 2001). However, the low concentration of formalin used in that study (1.25%) may in part explain this discrepancy. Our results suggest that this stress-sensitive strain of rat exhibits robust early and persistent pain behaviour in response to a noxious inflammatory stimulus, possible due to differential responding in supraspinal structures that modulate nociceptive responding in WKY and SD rats. Consistent with previous reports of altered monoaminergic functioning in the WKY versus SD rat (Tejani-Butt et al., 1994; Pare and Tejani-Butt, 1996; Durand et al., 2003; De
La Garza and Mahoney, 2004; Pearson et al., 2006), the present study demonstrated a reduction in noradrenaline levels in the hippocampus, hypothalamus, amygdaloid cortex and thalamus and concurrent reduction in 5-HIAA in the hypothalamus and thalamus of non-formalin treated WKY rats. The present study demonstrates that formalin-induced increases in monoamine levels in discrete brain regions of WKY rats were attenuated in comparison to SD counterparts. In particular, an inverse correlation was observed between formalin-evoked nociceptive behaviour and 5-HT and 5-HIAA in the hypothalamus. Increased 5-HT<sub>1A</sub> binding has previously been demonstrated in the hypothalamus of WKY rats (Pare and Tejani-Butt, 1996), an effect proposed to reflect decreased 5-HT release in this area. Anatomical connections between the hypothalamus and brain stem regions such as the rostral ventromedial medulla subserve analgesia mediated by activity of descending 5-HT neurons (Holden et al., 2005; Pinto-Ribeiro et al., 2008). In addition, bidirectional cross-talk exists between the serotonergic system and the hypothalamic-pituitary-adrenal (HPA) axis (Lanfumey et al., 2008), thus the differential nociceptive responses seen in the WKY and SD strains may be dependent upon the levels of corticosterone released during the formalin test. This may be significant since WKY rats exhibit heightened activation of the HPA axis response to stress (Solberg et al., 2001; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004). In addition, it should also be noted that WKY rats display a significantly blunted formalin-induced increase in noradrenaline concentration in the hypothalamus, hippocampus, amygdaloid cortex, thalamus and cerebellum when compared to SD controls. Further studies are required in order to elucidate the precise role of the monoaminergic system in the hypothalamus, and in other brain areas, in mediating the heightened nociceptive responding observed in WKY rats following formalin administration.
In conclusion, the present findings indicate that combining animal models of depression with persistent pain may allow for the neurobiological substrates that mediate this co-morbidity to be investigated. It is possible that alterations in central monaminergic function may, at least in part, underlie the co-morbidity of depression and pain.

**Acknowledgements**

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REFERENCES


Figure Legends

Figure 1: Depressive- and anxiety-related behaviours in OB and WKY rats. OB rats exhibit (A) an increased distance moved ($t_{15} = 4.721 \ P < 0.001$) in the open field test but (B) no change in duration of time spent in the in centre zone, 14 days post surgery when compared to sham-operated controls. In comparison, WKY rats display both a reduced (A) distance moved ($t_{18} = 9.497 \ P < 0.001$) and (B) duration in the centre zone ($t_{18} = 2.969 \ P = 0.008$) when compared to SD-controls. (C) WKY rats exhibit increased immobility ($t_{18} = 2.343 \ P = 0.031$) and reduced climbing ($t_{18} = 4.954 \ P < 0.001$) behaviour in the forced swim test when compared to SD counterparts. *P<0.05 **P<0.01 vs Sham or SD counterpart. Data expressed as mean ± SEM. N = 7-10.

Figure 2: Nociceptive responding to acute thermal stimuli. No significant difference between sham and OB rats on latency to respond in both the (A) hot plate or (B) tail-flick test (light intensity 35%) 15 days post surgery. In comparison, WKY rats exhibit a reduced paw withdrawal latency compared with SD counterparts in the hot plate test ($t_{17} = 4.327 \ P < 0.001$) but not tail-flick test (light intensity 35%). **P<0.01 vs. SD counterpart. Data expressed as mean ± SEM. N = 7-10.

Figure 3: Nociceptive responding to acute mechanical stimulus. (A) OB rats display a slight reduction in withdrawal threshold to a noxious mechanical stimulus (Von Frey filament) 15 days post surgery when compared to sham-operated controls (Two way ANOVA: bulbectomy $F_{1,62} = 31.36 \ P = 0.046$). In comparison there was no significant difference on withdrawal threshold between SD and WKY rats. **P<0.05 vs. Sham-operated control. Data expressed as mean ± SEM. N = 10-19.
Figure 4: Formalin-induced nociceptive behaviour. (A) OB rats exhibited an augmentation (t30-35) and prolongation (t70-95) of nociceptive behaviour assessed as composite pain score, following intra-plantar formalin administration when compared to sham-operated controls (Repeated measures ANOVA: bulbectomy (F_{1,15} = 5.62, P = 0.032) and time (F_{23,345} = 23.594 P < 0.001)). Inset: area under the curve for formalin-evoked nociceptive behaviour (t_{15} = 2.496 P = 0.025). (B) WKY rats exhibit increased nociceptive behaviour for the first 40 minutes (t0-40) post formalin administration when compared to SD counterparts (Repeated measures ANOVA: strain (F_{1,17} = 9.324 P = 0.007); time (F_{23,391} = 40.158 P < 0.001) and strain x time interaction (F_{23,391} = 2.846 P < 0.001)). Inset: area under the curve for formalin-evoked nociceptive behaviour (t_{17} = 3.007 P = 0.008). *P<0.05 **P<0.01 vs. sham or SD counterpart. Data expressed as mean ± SEM. N = 7-10.

Figure 5: The effect of intra-plantar formalin administration on brain monoamine levels in sham and OB rats. OB rats display a significant augmentation in formalin-induced increase in (A) noradrenaline (NA) and (C) 5-HIAA in the cerebellum and (B) 5-HT (serotonin) in the thalamus when compared to sham-operated counterparts. In addition, OB rats also demonstrate a blunted formalin-induced increase in (B) 5-HT in the PFC (prefrontal cortex) and (C) 5-HIAA in the hippocampus and amygdala. **P<0.01 vs. sham-operated control. ++P<0.01 vs. OB. $P<0.05 vs. Sham-formalin treated counterparts. Data expressed as mean ± SEM. N = 7-10.

Figure 6: The effect of intra-plantar formalin administration on brain monoamine levels in SD and WKY rats. (A) Formalin administration induces a reduction in noradrenaline levels in WKY, but not SD rats. In addition, WKY rats demonstrate a blunted formalin-induced increase in (A) noradrenaline (NA) in the cerebellum, (B) 5-HIAA in the hypothalamus and
(C) 5-HT in the hypothalamus, amygdala and thalamus. *P<0.05  **P<0.01 vs. SD.  ^P<0.05 **P<0.01 vs. WKY.  ^P<0.05 **P<0.01 vs. SD-formalin treated counterparts. Data expressed as mean ± SEM.  N = 6-10.
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<td>594 ± 31</td>
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<td>0.88 ± 0.06</td>
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<td>517 ± 7**+s</td>
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<td>1.18 ± 0.06</td>
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<td>929 ± 39**+s</td>
<td>F_{1,25} = 0.11 P = 0.74</td>
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<td>5-HIIA</td>
<td>238 ± 8</td>
<td>410 ± 26**</td>
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<td>F_{1,25} = 0.37 P = 0.55</td>
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<td>NA</td>
<td>443 ± 28</td>
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<td>51 ± 9</td>
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NA Noradrenaline; 5-HT serotonin; 5-HIIA 5-hydroxyindoleacetic acid; 5-HIIA/5-HT serotonin turnover. *P < 0.05 **P < 0.01 vs. Sham-operated control.

**P < 0.01 vs. OB.  $P < 0.05 $P < 0.01 vs. Sham-formalin treated rats. Data expressed as mean neurotransmitter concentration (ng/g tissue) ± SEM. N = 7-10.
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<td>5-HT</td>
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<td>1060 ± 47**</td>
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<tr>
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<td>5-HIAA/5-HT</td>
<td>1.87 ± 0.18</td>
<td>1.96 ± 0.21</td>
<td>0.61 ± 0.04**</td>
<td>0.63 ± 0.04**</td>
<td>F₁,₂₆ = 0.25 P = 0.62</td>
<td>F₁,₂₆ = 130.54 P &lt; 0.01</td>
</tr>
<tr>
<td>Amygdaloid</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>NA</td>
<td>744 ± 35</td>
<td>648 ± 37*</td>
<td>668 ± 26</td>
<td>566 ± 21** SS</td>
<td>F₁,₂₇ = 11.16 P &lt; 0.01</td>
<td>F₁,₂₇ = 7.09 P = 0.01</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>479 ± 25</td>
<td>388 ± 48</td>
<td>1301 ± 51**</td>
<td>1120 ± 56** SS</td>
<td>F₁,₂₇ = 5.85 P = 0.02</td>
<td>F₁,₂₇ = 192.74 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>353 ± 19</td>
<td>309 ± 16</td>
<td>414 ± 16</td>
<td>393 ± 20**</td>
<td>F₁,₂₇ = 2.77 P = 0.11</td>
<td>F₁,₂₇ = 13.60 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HIAA/5-HT</td>
<td>0.74 ± 0.05</td>
<td>0.85 ± 0.10</td>
<td>0.32 ± 0.02**</td>
<td>0.36 ± 0.02**</td>
<td>F₁,₂₇ = 2.23 P = 0.15</td>
<td>F₁,₂₇ = 97.22 P &lt; 0.01</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>NA</td>
<td>704 ± 33</td>
<td>521 ± 19*</td>
<td>768 ± 73</td>
<td>511 ± 33** SS</td>
<td>F₁,₂₈ = 17.29 P &lt; 0.01</td>
<td>F₁,₂₈ = 0.19 P = 0.67</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>518 ± 21</td>
<td>430 ± 24</td>
<td>1217 ± 80**</td>
<td>818 ± 40** SS</td>
<td>F₁,₂₇ = 12.27 P &lt; 0.01</td>
<td>F₁,₂₇ = 81.11 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>729 ± 32</td>
<td>593 ± 23*</td>
<td>721 ± 25</td>
<td>641 ± 27</td>
<td>F₁,₂₇ = 11.93 P &lt; 0.01</td>
<td>F₁,₂₇ = 0.00 P = 0.99</td>
</tr>
<tr>
<td></td>
<td>5-HIAA/5-HT</td>
<td>1.45 ± 0.09</td>
<td>1.41 ± 0.08</td>
<td>0.61 ± 0.04**</td>
<td>0.78 ± 0.05**</td>
<td>F₁,₂₇ = 0.46 P = 0.50</td>
<td>F₁,₂₇ = 123.60 P &lt; 0.01</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>NA</td>
<td>254 ± 16</td>
<td>220 ± 11</td>
<td>306 ± 14**</td>
<td>245 ± 7** SS</td>
<td>F₁,₂₈ = 14.53 P &lt; 0.01</td>
<td>F₁,₂₈ = 9.41 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>76 ± 6</td>
<td>65 ± 4</td>
<td>161 ± 12**</td>
<td>147 ± 7**</td>
<td>F₁,₂₈ = 1.68 P = 0.21</td>
<td>F₁,₂₈ = 70.61 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>91 ± 6</td>
<td>96 ± 5</td>
<td>116 ± 5**</td>
<td>123 ± 4**</td>
<td>F₁,₂₈ = 1.49 P = 0.23</td>
<td>F₁,₂₈ = 25.51 P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>5-HIAA/5-HT</td>
<td>1.20 ± 0.07</td>
<td>1.49 ± 0.06**</td>
<td>0.75 ± 0.05**</td>
<td>0.85 ± 0.04**</td>
<td>F₁,₂₈ = 13.79 P &lt; 0.01</td>
<td>F₁,₂₈ = 107.88 P &lt; 0.01</td>
</tr>
</tbody>
</table>

NA Noradrenaline; 5-HT serotonin; 5-HIAA 5-hydroxyindoleacetic acid; 5-HIAA/5-HT serotonin turnover. *P<0.05  **P<0.01 vs. SD.  +P<0.05 ++P<0.01 vs. WKY. $P<0.05 $$P<0.01 vs. SD-formalin treated rats. Data expressed as mean neurotransmitter concentration (ng/g tissue) ± SEM. N = 6-10 per group.
Figure

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A

Distance Moved (cm)

Duration (s)

Sham OB SD WKY

B

Duration in centre zone (s)

Duration (s)

Sham OB SD WKY

C

Duration (s)

Immobile Swimming Climbing

Sham OB SD WKY

Figure 1: Graphs showing

A. Distance Moved (cm) across different groups.

B. Duration in centre zone (s) across different groups.

C. Duration (s) for Immobile, Swimming, and Climbing activities.
Figure
Click here to download Figure: Figure 2.docx

A  Hot Plate Test

B  Tail-flick Test
Figure

Click here to download Figure: Figure 3.docx

A

50% Withdrawal threshold (g)

Sham
OB

Left
Right

B

50% Withdrawal threshold (g)

SD
WKY

Left
Right
Figure

A

Sham
OB

Composite Pain Score

Time (5 min bins)

0 10 20 30 40 50 60 70 80 90 100 110 120
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00

Area Under Curve

Sham OB

0
20
40
60
80
100
120

B

SD
WKY

Composite Pain Score

Time (5 min bins)

0 10 20 30 40 50 60 70 80 90 100 110 120
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00

Area Under Curve

SD WKY

0
20
40
60
80
100
120

**

Figure
Click here to download Figure: Figure 4.docx
Figure

Click here to download Figure: Figure 6.docx

A

B

C

NA conc (ng/g of tissue)

5HT conc (ng/g of tissue)

5HIAA conc (ng/g of tissue)

Hypothalamus
Cerebellum

Hypothalamus
Amygdaloid Cortex
Thalamus

SD
WKY
SD-Form
WKY-Form

$\*
$$
++$

$**$

$**$

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* $*

$**$

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$++$

$++$

$++$

Figure 6.docx