Locomotor and anti-immobility effects of buprenorphine in combination with the opioid receptor modulator samidorphan in rats

Running title: Behavioural effects of buprenorphine and samidorphan in rats

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Abstract

Modulation of the opioid system has re-emerged as a potential therapeutic avenue for treating depression, with efficacy of a fixed-dose combination of buprenorphine (BUP), a partial μ-opioid receptor (MOR) agonist and κ-opioid receptor (KOR) antagonist, and samidorphan (SAM), a potent MOR antagonist, as an adjuvant treatment in patients with major depressive disorder (MDD). To advance understanding of the mechanism of action underlying this combination, we examined BUP, SAM and their combination in a series of rat behavioural assays. We examined effects on locomotor activity in Sprague Dawley (SD) rats over an extended period of time in a home-cage tracking system, and behavioural despair (immobility) in the forced swim test (FST), a commonly-used test to study antidepressants, in SD and Wistar-Kyoto (WKY) rats. Strain differences in opioid receptor and prepropeptide mRNA expression in the brain (prefrontal cortex, amygdala, hippocampus and striatum) were examined using qRT-PCR. BUP produced locomotor hyperactivity in SD rats from 2-6 h following administration, which was attenuated by SAM. In SD rats, a low, but not a high, dose of SAM in combination with BUP counteracted swim-stress induced immobility. This effect was not seen with BUP alone. In contrast, BUP alone reduced immobility in WKY rats, and this effect was enhanced by a low, but not high, dose of SAM. In WKY rats, MOR mRNA expression was higher in the hippocampus and lower in the striatum *vs.* SD rats. KOR mRNA expression was higher in the amygdala and nociceptin receptor (NOP) mRNA expression was lower in the hippocampus *vs.* SD rats. Differences in opioid receptor expression may account for the differential behavioural profile of WKY and SD rats. In summary, administration of BUP, a MOR receptor agonist together with a MOR opioid-receptor antagonist, SAM, reduces behavioural despair in animal models traditionally used to study effects of antidepressants.

Introduction

The opioid system, comprising of the opioid receptors μ- (MOR), κ- (KOR), δ- (DOR), and nociception/orphanin FQ (NOP) and their endogenous ligands, is implicated in a host of physiological responses and behaviours including the stress response, immune modulation, nociception and emotion. Its effects on emotion have led to a body of clinical and non-clinical studies exploring the role of the opioid system in depression. Post-mortem clinical studies have shown that individuals who died by suicide exhibit increased MOR density and reduced levels of endorphins in frontal and temporal cortices and caudate nuclei (Gross-Isseroff et al., 1990, Gabilondo et al., 1995, Scarone et al., 1990), suggesting altered endogenous opioid tone. Pharmacological studies have revealed interactions of monoaminergic antidepressants with the opioid system in mice (Berrocoso et al., 2004, Devoize et al., 1984) and MOR agonists exhibit efficacy in antidepressant screening tests in animal models (Rojas-Corrales et al., 2004). Over 100 years ago, repeated low doses of opium were routinely used to treat depression; however, due to abuse liability this approach fell out of favour with the introduction of monoaminergic antidepressants in the 1950s (Tenore, 2008). The limitations of monoaminergic antidepressants are well documented, with 2 – 4 weeks of a therapeutic delay and 30 – 50% of patients failing to achieve remission (Trivedi et al., 2006). The opioid system is therefore being revisited as a possible therapeutic target by attempting to harness the mood-improving qualities whilst mitigating the risk of unwanted effects such as addiction and dependence (Ehrich et al., 2014, Almatroudi et al., 2015).

Buprenorphine (BUP) is a semi-synthetic opioid approved for treating opioid addiction and pain. It has a long elimination half-life, reduced addiction potential and an improved safety profile compared to other opioids (Lutfy and Cowan, 2004). Clinical studies have reported its

antidepressant efficacy in depressed and treatment-resistant patients (Kosten et al., 1990, Bodkin et al., 1995, Nyhuis et al., 2008, Callaway, 1996, Karp et al., 2014, Striebel and Kalapatapu, 2014). This treatment option has not reached mainstream medicine due to the associated potential for addiction and dependence. BUP has a complex pharmacological profile; it is a partial agonist at MOR and displays mixed but primarily antagonistic actions at KOR and DOR receptors, acting as a partial agonist *in vitro*, and as an antagonist *in vivo* (Lutfy and Cowan, 2004, Huang et al., 2001, Leander, 1987, Romero et al., 1999). Samidorphan (SAM, also known as ALKS-33) is an opioid receptor modulator that has potent MOR-antagonistic properties and low intrinsic activity at KOR and DOR, high bioavailability, a long half-life and is minimally metabolised (Wentland et al., 2009a, Wentland et al., 2005, Wentland et al., 2009b, Shram et al., 2015). Ehrich and colleagues recently showed that the combination of BUP with SAM was superior to placebo for reducing depressive symptoms in treatment-resistant patients, when administered as an adjunct treatment to standard monoaminergic antidepressant therapy (Ehrich et al., 2014).

To advance our understanding of the mechanism of action of the combination of BUP and SAM, we examined the effect of these drugs in rats. Changes in locomotor activity can serve as a useful objective assay for assessing behavioural effects of pharmacological compounds. Agonists of MOR such as morphine and BUP elicit complex biphasic effects on locomotor activity (Babbini and Davis, 1972, Marquez et al., 2007). An understanding of drug-induced locomotor responses is essential as effects on general activity are a potential confounding factor in many behavioural tests including the forced swim test (FST), the most widely used non-clinical screening test for detecting efficacy of antidepressant compounds. Thus, our first aim was to characterise the locomotor profile following subacute administration of BUP alone, and in combination with SAM, in a home-cage tracking system that allows sensitive detection of changes in activity over an extended period of time, something that is not

achievable when using novel arenas such as the open field. Assessing behaviour in the animal's home-cage environment also allows for drug-induced locomotor effects to be separated from effects seen due to the arena itself.

Our second aim was to examine the effect of BUP, alone and in combination with SAM, in two rat strains using the FST. BUP and SAM doses were selected from dose-response studies (Smith et al., unpublished data). We used the Sprague-Dawley (SD) rat, a commonly used strain for behavioural experiments, and the Wistar-Kyoto (WKY) rat, which displays increased immobility in the FST and a blunted response to SSRIs (Burke et al., 2010, Lopez-Rubalcava and Lucki, 2000, Tejani-Butt et al., 2003). The WKY rat models certain behavioural and physiological characteristics of depression, including anhedonia and reduced weight gain (Burke et al., 2016, Pare, 2000), and neurobiological aspects such as altered levels of monoamines (De La Garza and Mahoney, 2004). WKY rats also exhibit an augmented stress response, including alterations of the hypothalamic–pituitary–adrenal (HPA) axis (Rittenhouse et al., 2002, Steimer et al., 2007), increased susceptibility to stress-related ulcers (Pare and Redei, 1993), anxiety-like behaviour (Burke et al., 2016) and disrupted sleep patterns (Dugovic et al., 2000). WKY rats reliably display greater levels of learned helplessness in the FST when compared to other strains (Tejani-Butt et al., 2003, Pare, 1989, Rittenhouse et al., 2002, Lahmame et al., 1997).

As the opioid system may contribute to the pathophysiology of depression, we also examined differences in gene expression (mRNA) of the opioid receptors and prepropeptides to their endogenous ligands in the prefrontal cortex (PFC), amygdala, hippocampus and striatum, key brain regions involved in processing mood and reward between WKY and SD rats.

Materials & Methods

Animal husbandry

Adult male SD rats (Charles River, Crl:SD, Experiment 1, 180-200g; Harlan, UK Hsd, Experiment 2, 200-250g) and WKY rats (Harlan, UK, WKY/NHsd, 200-250g) were used in the current study. Rats were group-housed on arrival to the animal unit for 4-7 days and singly-housed thereafter in plastic-bottomed cages (45 × 25 × 20 cm) containing wood shavings as bedding, in a temperature-controlled room (20 ± 2°C), with a relative humidity of 40-60% and a 12:12h light-dark cycle (lights on at 0800h). Woodchip bedding dyed black was used for home-cage tracking and animals were habituated to this bedding 24 h prior to behavioural testing. Rats were fed a standard laboratory diet of rat chow pellets (2014 14% rodent diet, Harlan, UK); food and water were available *ad libitum.* Animals were weighed and checked daily and were habituated to the injection procedure (saline) for 3 days prior to behavioural testing. Experimental protocols were 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 Health Products Regulatory Agency and in compliance with the European Union directive 2010/63/EU as well as the ARRIVE guidelines from the National Centre for the Replacement Refinement and Reduction of Animals in Research (Kilkenny et al., 2010).

Drug preparation

Buprenorphine (BUP) hydrochloride (0.3 mg/ml, Chanelle Vet Ltd., Ireland) was diluted in sterile saline to give a final concentration of 0.1 mg/mL. Samidorphan (SAM) L-malate (Alkermes Inc. Ireland) was diluted in sterile saline to give a final concentration of 0.3 mg/mL or 3 mg/mL. Combinations of these two drugs were prepared to give final concentrations of BUP (0.1 mg/mL) + SAM (0.3 mg/mL) or BUP (0.1 mg/mL) + SAM (3

mg/mL). Drugs were administered subcutaneously in an injection volume of 1 mL/kg. Doses of BUP and SAM ~~and combinations~~ were selected based on pilot studies conducted at Alkermes (~~Smith~~Alkermes, personal communication ~~*et al*~~). The half-life of a single SC injection of 0.1 mg/kg of SAM is 1.2h ± 0.2 with a time to maximal concentration (Tmax) being on average, 1 h in SD rats. The binding affinity and G protein engagement of SAM to each of the opioid receptors have been described in detail (Bidlack et al., 2018). More detail here?

Experimental design

Animals were randomised to treatment groups and an experimenter blind to treatment carried out behavioural scoring.

Experiment 1

We first characterised home-cage locomotor activity in SD rats following BUP and SAM administration. At the start of testing, rats received three subcutaneous injections mimicking the subacute dosing regimen employed for the FST, i.e., 24, 5 and 1 h prior to the usual point of swim exposure. Rats (n=6-7/group) received saline (1 mL/kg), BUP (0.1 mg/kg), SAM (0.3 mg/kg or 3 mg/kg) or a combination of BUP with either 0.3 mg/kg or 3 mg/kg SAM. The results are expressed as distance travelled (cm), and are presented either as 10 minute time bins following each injection (along with 6 time bins prior to injection to illustrate baseline activity) or as total activity over selected periods following each injection.

Experiment 2a

We then examined the efficacy of BUP and SAM drugs in the FST. SD and WKY rats received three subcutaneous injections at 24, 5 and 1 h prior to the point of behavioural testing. Rats (n=8/group) received saline (1 mL/kg), BUP (0.1 mg/kg), SAM (0.3 mg/kg or 3

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mg/kg) or a combination of BUP with either 0.3 mg/kg or 3 mg/kg SAM. Home-cage locomotor activity was recorded for 1 h prior to the second swim. In order to examine inter-strain differences in basal levels of central opioid receptor and prepropeptide gene expression, a separate group of SD and WKY rats received saline injections but were not exposed to the FST. These rats were killed by rapid decapitation immediately after the time that would correspond to the second swim exposure. The PFC, amygdala, hippocampus and striatum were gross dissected on an ice-cold plate and snap frozen on dry ice and stored at -80°C until mRNA isolation. The following gene expression levels were measured: opioid receptors (MOR, KOR, DOR and NOP) and the precursors for their endogenous ligands, pro-opiomelanocortin (POMC, precursor to endorphin); prodynorphin (PDYN, precursor to dynorphin); proenkephalin (PENK, precursor to enkephalin) and prepronociceptin (PNOC, precursor to nociceptin).

Experiment 2b

Due to the behavioural outcome of Experiment 2a, we next examined the duration of action of BUP, SAM and their combination in WKY rats exposed to the FST. We tested separate groups of rats (n=8/group) at later time points. WKY rats received vehicle, BUP (0.1 mg/kg), or a combination of BUP with either 0.3 mg/kg or 3 mg/kg SAM using the dosing schedule as described above and were re-exposed to a second swim either 24 or 48 h following the final dose. Home-cage locomotor activity was assessed for 1 h prior to the second swim as described previously.

Home-cage locomotor activity tracking

Home cage monitoring was carried out as described previously (Dunne et al., 2007), with some modifications. Briefly, each rat was placed into a cage with black bedding with a dark Perspex baseplate and the space under the food-hopper was restricted by a section of plastic

tubing to ensure the rat would be visible at all times. A camera placed above the cage recorded the footage continuously. Video files were joined using AVI Joiner (Briz Software), converted to MPEG4 using Super® Codec installer (eRightSoft) and then home-cage locomotor activity (HCLA, distance moved, cm) was tracked using EthoVision® XT video-tracking system for the entire 24 h period. The first 10 min was excluded from analysis to avoid any locomotor contribution produced by stress from the injection procedure.

Forced swim test (FST)

The modified FST (Detke and Lucki, 1996) was used for assessment of the effects of BUP and SAM in SD and WKY rats. In brief, rats were placed individually into Perspex cylinders (20 cm diameter), containing 30 cm of water at 24 ± 2 °C for 15 min, during which the rat learns of the inescapable nature of the cylinder. Rats were then re-exposed to the cylinder 24 h later (day 2) for a 5 min period. Behaviours on day 2 were manually rated using the time-sampling method (Detke and Lucki, 1996) with the aid of EthoVision XT® software. Scoring included immobility, swimming and climbing and was done by an experimenter blinded to group identity.

Gene expression analysis using quantitative RT-PCR

We examined basal levels of opioid receptor and prepropeptide mRNA expression in SD and WKY rats. mRNA was extracted from prefrontal cortical, hippocampal, amygdala and striatal tissue using NucleoSpin mRNA II total mRNA isolation kit (Macherey-Nagel, Germany) as previously described (Burke et al., 2013, Kerr et al., 2012). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. TaqMan gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB TaqMan probe were used to quantify the gene of interest and qRT-PCR was performed using a LightCycler® 480 instrument (Roche) for MOR, KOR and DOR and a Stepone Plus (Applied Biosystems) for NOP, POMC, PDYN, PENK and PNOC. Assay IDs for the genes examined were as follows: MOR (Rn01430371_m1), KOR (Rn00567737_m1), DOR (Rn00561699_m1), NOP (Oprl1, Rn00440563_m1), POMC (Rn00595020_m1), PDYN (Rn00571351_m1), PENK (Rn00567566_m1), and PNOC (Rn01637101_m1).

Polymerase chain reaction was performed using LightCycler® 480 Probes Master Mix (Roche) for MOR, KOR and DOR and Taqman Master Mix (Biosciences Ltd) for NOP, POMC, PDYN, PENK and PNOC. The cycling conditions were 90°C for 10 min and 40 cycles of 90°C for 15 min followed by 60°C for 1 min. β-Actin (Rn00667869_m1) was used as an endogenous control (house-keeping gene) to normalize gene expression data. Relative gene expression was calculated using the $^{\delta\delta}$CT method and data were expressed as % SD-vehicle non-swim controls.

Analysis of BUP levels in plasma

BUP levels in plasma (n = 6 /group) were assayed using a commercially-available ELISA kit according to manufacturer instructions (BUP 3508, Randox, UK). In brief, plasma was centrifuged at 13000 rpm for 60 seconds and diluted 1:10. These diluted samples were added in triplicate to microtitre plates that were pre-coated with BUP antibody. The plate was incubated at room temperature for 1 h in the dark, washed and the enzyme substrate was added. Following a 20 min incubation, the reaction was terminated using the stop solution. Absorbances (optical density) were read at 450 nm and sample concentrations were determined against a standard curve. The limit of detection for the kit was 0.57 ng/mL.

Statistical analysis

Kolmogorov and Levene tests were used to determine normality and homogeneity of variance, respectively. Data were analysed using the IBM SPSS 21 statistical program. The parametric data analyses consisted of two- (BUP or SAM), or three-factor (BUP, SAM or strain) or repeated measures (time) analysis of variance (ANOVA) followed, where appropriate, by the Student Newman Keuls *post-hoc* test. Gene expression changes were analysed using unpaired t-test to detect differences between SD and WKY groups. Non-parametric data analyses consisted of the Kruskal-Wallis test followed by the Mann-Whitney

U *post-hoc* test where appropriate. P≤0.05 was deemed significant. All data are presented as the mean + SEM.

Results

Characterising the home-cage locomotor activity profile following subacute administration of BUP, SAM, and their combination, in SD rats

SD rats received three subcutaneous injections mimicking the subacute dosing regimen employed in the FST, i.e., 24, 5 and 1 h prior to the test, and locomotor activity over this time-period was examined (Fig. 1a).

Home-cage activity analysis revealed that SAM did not alter locomotor activity following the first injection, when compared to vehicle controls (Fig. 1b). In contrast, two-way ANOVA revealed a significant effect of BUP at 120-240 min ($F_{(1,34)}$ = 16.10, P<0.001) and at 240-360 min ($F_{(1,34)}$ = 19.38, P<0.001, Fig 1a) following administration; *post-hoc* analysis revealed that BUP significantly increased locomotor activity at the 120-240 min and 240-360 min timebins compared to vehicle controls. This effect was significantly attenuated by low and high doses of SAM (BUP × SAM interaction, 120-240 min: $F_{(2,34)}$ = 5.55, P=0.008; 240-360 min: $F_{(2,34)}$ = 4.89, P=0.014, Fig. 1b). Further temporal analysis using repeated measures ANOVA revealed an effect of BUP ($F_{(1,34)}$ = 16.75, P<0.001) and a time × BUP interaction ($F_{(35,1190)}$ = 1.678, P<0.001). *Post-hoc* analysis revealed that BUP elicited a hyperactive response beginning at 220 min and continuing until 360 min post-injection, an effect that was again attenuated by both doses of SAM (time × BUP × SAM, $F_{(35,1190)}$ = 1.195, P=0.03, Fig. 1c). BUP in combination with the low, but not high, dose of SAM produced an earlier increase in locomotor activity within the 10-120 min measurement period (Fig. 1b), which was significantly increased compared to the BUP alone group at the 40, 50, 60, 70, 90 and 120 min time bind following ~~from 30-120 min post-~~injection (Fig. 1c).

Following the second administration, an effect of SAM was revealed ($F_{(2,33)}$ = 3.61, P=0.38, Fig. 1d). *Post-hoc* analysis showed that both doses of SAM decreased locomotor activity

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compared to vehicle controls (10-120 min). Similarly, BUP alone reduced locomotor activity compared to vehicle controls at this time-period (BUP $F_{(2,33)} = 5.57$, P=0.024, Fig. 1d), an effect that was not altered by SAM. Similar to the first administration, BUP elicited a hyperactive response compared to vehicle controls at 120-240 min (BUP $F_{(2,34)} = 11.31$, P=0.002, Fig. 1d), an effect which was again attenuated by both doses of SAM (BUP × SAM interaction, $F_{(2,34)} = 7.74$, P=0.002). Further temporal analysis revealed that in contrast to the first administration, BUP alone elicited a time-dependent biphasic locomotor response, with a decrease in activity from 20-60 min followed by hyperactivity from 120-180 min (time × BUP, $F_{(23, 782)} = 3.38$, P<0.001, Fig. 1e); this hyperactive response was again attenuated by both doses of SAM at all time-points (time × SAM × BUP, $F_{(23, 782)} = 2.28$ P<0.001, Fig. 1e).

In the hour following the third administration, both doses of SAM reduced locomotor activity ($F_{(2,34)} = 12.78$, P<0.001. Fig 1f) when compared to vehicle controls, an effect not altered by co-administration of BUP. Furthermore, from 60-65 min following the third injection (the time-period corresponding to forced swim exposure), BUP + SAM (0.3-3 mg/kg) treated rats exhibited significantly reduced locomotor activity when compared to BUP alone (BUP × SAM interaction, $F_{(2,34)} = 4.86$, P=0.014), but not when compared to vehicle-treated controls. Therefore, the data indicate that there was no stimulatory effect of drug administration on locomotor activity at the time-period corresponding to forced swim exposure. An increase here could have influenced the interpretation of the FST data.

Effect of BUP, SAM and their combination in the forced swim test in SD rats

Examining the effect of subacute administration of BUP, SAM and their combination in SD rats exposed to the FST (Fig. 2a) revealed that SAM alone dose-dependently increased climbing behaviour when compared to vehicle controls (SAM, $F_{(2,42)} = 16.16$, P<0.001). This effect was observed in the absence of any significant effect on immobility, indicating that SAM alone has no effect on immobility in the FST in SD rats (Fig 2b). There was no

significant effect of BUP alone on immobility, swimming or climbing behaviour in SD rats. However, the combination of BUP with the low (but not high) dose of SAM resulted in a significant decrease in immobility when compared to vehicle-treated rats and those treated with SAM alone (BUP × SAM interaction, $F_{(2,42)} = 4.69$, P=0.015), indicating a dose-specific effect of the combination in SD rats (Fig. 2b).

Effect of BUP, SAM and their combination in the forced swim test in WKY rats

WKY displayed increased immobility (SD 35.13 ± 0.99 vs. WKY 48.88 ± 1.42 counts) and reduced climbing (SD 10.13 ± 2.26 vs. WKY 3.63 ± 0.84 counts) behaviour when compared to SD counterparts (effect of strain: immobility $F_{(1,84)} = 82.15$, P<0.001; climbing $F_{(1,84)} = 141.26$, P<0.001), as previously described (Tejani-Butt et al., 2003, Burke et al., 2010, Burke et al., 2016).

SAM alone increased climbing behaviour in WKY rats when compared to vehicle-treated groups ($F_{(2,42)} = 19.01$, P<0.001) without significantly reducing immobility. In contrast, BUP alone significantly reduced immobility ($F_{(1,42)} = 46.99$, P<0.001), while concurrently increasing swimming ($F_{(1,42)} = 48.13$, P<0.001) in WKY rats when compared to vehicle-treated counterparts (Fig. 2c). Moreover, the combination of BUP with the low, but not high, dose of SAM resulted in a further decrease in immobility (SAM $F_{(2,42)} = 7.36$, P=0.002) and a concomitant increase in swimming (BUP × SAM interaction $F_{(2,42)} = 5.5$, P=0.008) in WKY rats when compared to vehicle-treated, BUP alone and SAM alone groups (Fig. 2c). These data indicate a dose-specific effect of this combination.

Analysis of BUP plasma levels revealed that there were no strain differences in levels at the time of testing (Supplementary Table 1). Home-cage locomotor activity analysis confirmed that there were no stimulatory effects of drug administration in SD or WKY rats in the hour prior to the FST (Table 1). It should be noted that during the FST, we observed an unusual

phenomenon where BUP administration elicited striking “floating” type behaviour in both SD and WKY rats distinct from immobility. This behaviour occurred exclusively in the first minute and was scored separately to immobility. Both doses of SAM attenuated this behaviour (Supplemental data Fig. 2).

Long-acting effect of buprenorphine in WKY rats in the FST

As BUP alone and in combination with low dose SAM reduced immobility in WKY but not SD rats, we next examined the duration of action of this effect in WKY rats (Fig. 3a). Similar to effects observed 1 h following subacute administration, WKY rats displayed reduced duration of immobility at 24 (BUP, $F_{(1,28)} = 13.97$, P=0.001) and 48 (BUP, $F_{(1,28)} = 10.81$, P=0.003) h following the final injection of BUP when compared to vehicle-treated counterparts (Fig. 3b-c). This effect persisted up to 48 h with co-administration of both doses of SAM. Swimming was significantly increased at 24 h (Fig. 3b), but not 48 h (Fig. 3c) following the final injection of BUP (BUP, $F_{(1,28)} = 11.75$, P=0.002). Analysis of plasma revealed detectable levels of BUP at 24 h, but not at 48 h following the final injection (Supplementary Table 1), in accordance with previous reports (Chawarski et al., 1999).

Comparison of basal expression of opioid receptor and prepropeptide genes in limbic, cortical and subcortical brain regions between SD and WKY rats

The PFC and limbic regions such as the amygdala and hippocampus are key brain regions involved in processing mood, and the striatum is implicated in reward. Analysis of gene expression revealed that WKY rats exhibited higher KOR ($t_{10} = 2.28$, P=0.045) mRNA in the amygdala; higher MOR ($t_9 = 2.43$, P=0.038) and lower NOP ($t_{14} = 2.38$, P=0.032) mRNA in the hippocampus; and lower MOR ($t_{14} = 4.60$, P<0.001) mRNA in the striatum when compared to SD counterparts (Fig. 4). There was also a numerical increase in KOR mRNA in

the hippocampus of WKY rats (t_{10} = 1.97, P=0.07). None of the prepropeptides measured were altered between SD and WKY rats.

Discussion

In the current study, we observed that BUP produces prolonged hyperactivity in the home-cage in SD rats and elicits an anti-immobility effect in the FST in WKY rats that continues for up to 48 h post-administration. We showed that BUP-induced effects on locomotor activity and immobility in the FST are subject to modulation by SAM, a novel MOR antagonist. Specifically, BUP in combination with a low (but not high) dose of SAM, elicited a unique pattern of home-cage activity in SD rats. This particular dose combination also unmasked an anti-immobility effect of BUP in SD rats and further enhanced the anti-immobility effect of BUP in WKY rats. Finally, we have shown that WKY rats have altered levels of opioid receptor mRNA expression in limbic and subcortical brain regions compared to SD rats, effects that may account for their differential behavioural profile and responsiveness to BUP.

BUP-induced locomotor hyperactivity in SD rats is attenuated by SAM

Characterising the locomotor effects of BUP and its modulation by SAM revealed that BUP alone elicits a hyperactive response following an acute administration, a finding previously shown under different experimental conditions (i.e. novel arena) in rats (Liles and Flecknell, 1992) and mice (Lelong-Boulouard et al., 2006, Kuribara and Tadokoro, 1991, Marquez et al., 2007, Hayes et al., 2000). Although the mechanisms underlying opioid-induced hyperactivity are poorly understood, BUP-induced hyperactivity has been shown to be reduced not only by the MOR antagonist naloxone, but also by haloperidol and pilocarpine, suggesting a role for the opioidergic, dopaminergic and cholinergic systems, respectively (Kuribara and Tadokoro, 1991). Increased locomotor behaviour may be precipitated by activation of MOR located on GABAergic interneurons in the ventral tegmental area resulting in dopamine release in the mesolimbic pathway (Devine et al., 1993, Garzon and

Pickel, 2001). Our data demonstrate that the MOR antagonist SAM attenuates BUP-induced hyperactivity, suggesting that the MOR may mediate the hyperactive response, supporting previous findings in MOR knockout mice (Marquez et al., 2007). The higher dose of SAM completely abolished BUP-induced hyperactivity, which correlates with microdialysis data showing complete reversal of a BUP-induced increase of extracellular dopamine in the rat nucleus accumbens shell with the same dose combination (Deaver et al., 2013). We also showed that BUP in combination with a low dose of SAM resulted in an earlier increase in activity of lesser magnitude compared to BUP alone. These data suggest an intermediate blockade of opioid receptor activity, likely the MOR, and potentially a shift of the BUP dose-response curve – correlating with microdialysis data showing that the same dose of SAM only partially blocked BUP-induced increases in extracellular dopamine in the nucleus accumbens shell (Deaver et al., 2013). Although effects at other opioid receptors cannot be ruled out, these data provide evidence for involvement of the MOR in the behavioural effects of the combination of BUP and SAM.

The effects of SAM and BUP in the forced swim test

In the FST, SAM alone increased climbing behaviour in SD and WKY rats, suggesting a potential effect of this MOR antagonist on noradrenaline and/or dopamine neurotransmission (Detke et al., 1995, Reneric and Lucki, 1998), which warrants further investigation. However, despite these increases in climbing behaviour, they did not amount to an overall impact on immobility time in either strain when SAM was administered alone. We have confined our investigations of SAM alone to a single timepoint, i.e. 1 hour following the 3rd dose. However, outside of the current experiment, we know that an acute dose of SAM (10 mg/kg) displays modest but not statistically significant increase in climbing behaviour 3h post drug dosing, which when retested after 24h had returned to control levels. Although opioid receptor antagonists usually decrease monoamine synthesis in the brain (Garcia-Sevilla et al.,

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1978), naltrexone has been shown to increase MOR expression in the brain (Tempel et al., 1985), possibly due to compensatory mechanisms, which may lead to increased monoamine release following endogenous MOR activation under stressful conditions (Chaijale et al., 2013), such as the FST.

We showed for the first time that WKY rats exhibited higher MOR and lower NOP mRNA expression in the hippocampus, lower MOR mRNA in the striatum, and in higher creased KOR mRNA expression in the amygdala when compared to SD rats. Previous studies have shown that WKY rats have higher levels of KOR gene and protein expression in the piriform cortex and locus coeruleus and higher levels of KOR mRNA and dynorphin peptide in the nucleus accumbens *versus* SD counterparts (Carr et al., 2010, Pearson et al., 2006){Dennis, 2016 #1}. Our findings advance the profile of the neurobiology of WKY rats, and may underpin some of the behavioural deficits exhibited by the model. We saw that BUP elicited a strain-dependent reduction in immobility in WKY, but not SD rats, in accordance with previous reports (Browne et al., 2015). Although it remains to be determined whether these increases in receptor expression/number result in altered function, differences in opioid receptor expression between WKY and SD rats may explain the differential behavioural response to BUP. For example, increased MOR and KOR availability and/or function may result in increased sensitivity to the effects of BUP in WKY rats. It should be noted that Browne et al (2015) showed that BUP significantly reduced immobility at doses of 0.75 and 2.25 mg/kg, but not at 0.25 mg/kg. Differences in the behavioural effects at the doses used between the studies may be due to distinct differences in experimental design. For example, Browne and colleagues tested rats 24 h after a single injection compared to the traditional three-injection paradigm used in our experiments. There is likely time-, dose- and test-dependent effects of BUP due to its complex pharmacology and the bell-shaped dose-response curve often seen with this drug (Lutfy and Cowan, 2004). Overall, the data show an

acute and long-acting effect of BUP in the FST in WKY rats, qualities comparable to other drugs that are being examined as potential fast-acting and long-lasting antidepressants such as ketamine (Tizabi et al., 2012). The WKY rat displays a blunted behavioural response to SSRI administration (Lopez-Rubalcava and Lucki, 2000, Lahmame et al., 1997), which makes it an interesting model system for the study of novel antidepressants.

In our experiments, BUP alone increased swimming (in WKY rats) in accordance with the previous literature in the WKY rat model (Browne et al., 2015) and mice (Almatroudi et al., 2015). KOR antagonists and DOR agonists also increase swimming behaviour (Mague et al., 2003, Broom et al., 2002). As drugs that increase synaptic levels of serotonin are associated with increased swimming in the FST (Detke et al., 1995), this suggests that opioid signalling is associated with increased serotonergic neurotransmission. Indeed, BUP has been shown to increase extracellular serotonin in the medial PFC (Deaver et al., 2013) and morphine has been shown to increase serotonin release in the nucleus accumbens, amygdala, frontal cortex, striatum and hippocampus (Tao and Auerbach, 1995, Fadda et al., 2005).

Unique behavioural effects of BUP in combination with a low dose of SAM

The combination of BUP with the low dose of SAM revealed interesting strain- and dose-dependent behavioural effects in the FST. Essentially, SAM enhanced the BUP-induced anti-immobility effect in WKY rats. Although BUP ~~*per se*~~ did not reduce immobility in SD rats, the addition of the low dose of SAM unmasked an anti-immobility effect. These data suggest that BUP alone is sufficient to elicit an effect in WKY rats, but in SD rats, BUP requires SAM to reveal its anti-immobility effects. This specific dose combination also revealed an earlier increase in BUP-induced locomotor activity but reduced the later BUP-induced hyperactivity seen in SD rats that received BUP alone. Previous microdialysis data have shown that 0.3 mg/kg of SAM only partially counteracts the monoamine-increasing effects of

BUP in the nucleus accumbens shell, whereas 3 mg/kg completely blocks it (Deaver et al., 2013). As such, this pattern of locomotor activity may represent an important characteristic of this combination and its effects on neurotransmission in the brain. These results suggest that the BUP and low dose SAM combination maintains a level of neurochemical release that may signify partial MOR activation resulting in the behavioural effects seen herein. Taken together, these data indicate a dose-specific anti-immobility effect of the BUP + SAM combination in two rat strains.

Combining BUP with an opioid receptor antagonist has been used for a number of years in the treatment of opioid dependence (for review see Soyka, 2015). Recent studies by Almatroudi and colleagues (2015) have shown that BUP (1 mg/kg) in combination with naltrexone (1 mg/kg), a non-selective opioid receptor antagonist, resulted in a significant reduction in immobility in the mouse FST. It should be noted that under these experimental conditions, the combination group did not differ from the BUP-alone group, which is in contrast with our results seen here in rats and with a 10-fold lower dose of BUP (i.e. 0.1 mg/kg). Almatroudi and colleagues (2015) also administered the irreversible mu-opioid receptor antagonist CCAM (3 mg/kg) in combination with BUP (1 mg/kg). The antagonist alone had no effect on immobility and did not alter the BUP-induced effect, leading the authors to the conclusion that the MOR did not contribute to the anti-immobility effect seen. These data are similar to what we have seen with the high dose of SAM (3 mg/kg). The contribution of specific receptors remains to be delineated, but it is likely that the effect of BUP alone is mediated by functional blockade of the KOR given that MOR antagonists have no effect on the BUP-induced effect in the FST. Indeed, Carr et al have shown that KOR antagonists reduced immobility in the FST in WKY rats (Carr et al., 2010). However, recent findings by the same group have revealed a role for the MOR in the effects of BUP in the

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novelty-induced hypophagia test, a paradigm that is sensitive to chronic treatment with antidepressant drugs (Robinson et al., 2017).

Together, the data presented herein support that administration of BUP, a MOR receptor agonist together with a MOR opioid-receptor antagonist, SAM, reduces behavioural despair in animal models traditionally used to study effects of antidepressants.

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Figure Legends

Figure 1. Effects of BUP (0.1 mg/kg), SAM (0.3 – 3 mg/kg), alone and in combination on home-cage locomotor activity following subacute dosing at 24, 5 and 1 h prior to the time-point corresponding to FST exposure. (a) Schematic of experimental design. (b) and (c) Locomotor activity from 10-360 min following the 1st injection. (d) and (e) Locomotor activity from 10-240 min following the 2nd injection. (f) and (g) Locomotor activity from 10-60 min following the 3rd injection. Data are presented as mean ± SEM, n = 6 – 7. *P<0.05 vs. saline. $^{+}$P<0.05 vs. BUP.

Figure 2. Effects of BUP (0.1 mg/kg), SAM (0.3 – 3 mg/kg), alone and in combination on behaviour in the FST following subacute dosing. (a) Schematic of experimental design Immobility, swimming and climbing counts as determined by the time-sampling method during the 5 min swim in (b) SD and (c) WKY rats. Data are presented as mean ± SEM, n = 8/group. *P<0.05, **P<0.01 vs. saline. $^{+}$P<0.05 vs. BUP, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. SAM.

Figure 3. Duration of action of the effect of BUP (0.1 mg/kg), SAM (0.3 – 3 mg/kg), alone and in combination in WKY rats in the FST. (a) Schematic of experimental design. (b) 24 and (c) 48 h after the final injection following subacute dosing. Data are presented as mean ± SEM, n = 8. *P<0.05 vs. saline.

Figure 4. Characterisation of basal mRNA expression of central opioid receptors and prepropeptides in the PFC (a,e), amygdala (b, f), hippocampus (c, g), and striatum (d, h) in SD vs. WKY rats not exposed to forced swim. Data are presented as mean ± SEM, n = 6-8, *P<0.05, **P<0.01, ***P<0.001 vs. SD-vehicle non-swim. PFC – prefrontal cortex; AMY – amygdala; Hippo – hippocampus; MOR – mu-opioid receptor; KOR – kappa-opioid receptor; DOR – delta-opioid receptor; NOP – nociceptin receptor; POMC – pro-opiomelanocortin PDYN – prodynorphin; PENK – proenkephalin; PNOC – prepronociceptin.

Table 1. Home cage locomotor activity 1 h prior to FST in SD and WKY rats. There was no effect of drug treatment on locomotor activity. Data are presented as mean ± SEM, n = 8/group.

Fig.1

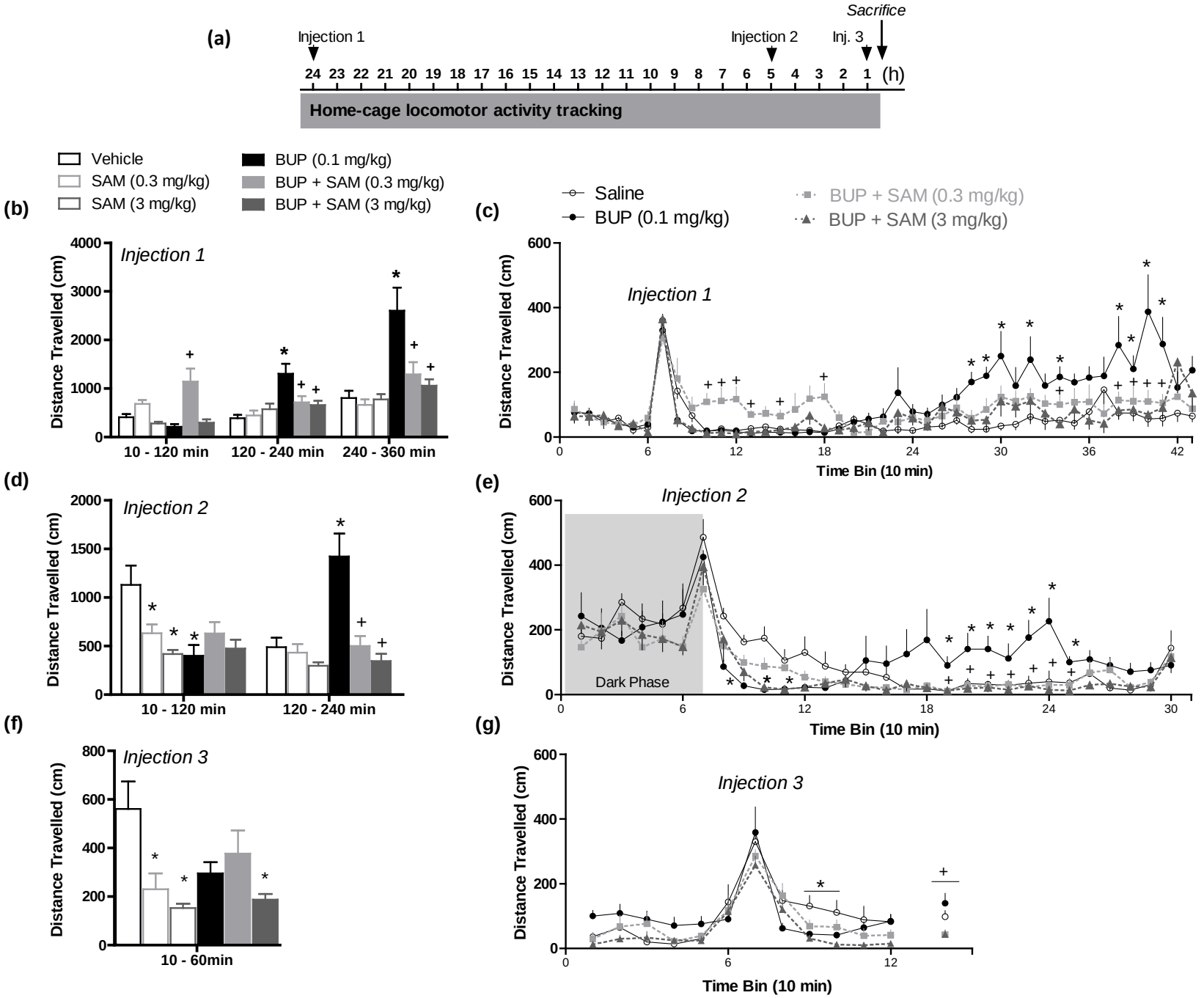

(a)
Injection 1
Injection 2
Inj. 3
Sacrifice
24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 (h)
Home-cage locomotor activity tracking
Vehicle
SAM (0.3 mg/kg)
SAM (3 mg/kg)
BUP (0.1 mg/kg)
BUP + SAM (0.3 mg/kg)
BUP + SAM (3 mg/kg)
(b)
Injection 1
Distance Travelled (cm)
10 - 120 min
120 - 240 min
240 - 360 min
(c)
Saline
BUP (0.1 mg/kg)
BUP + SAM (0.3 mg/kg)
BUP + SAM (3 mg/kg)
Injection 1
Distance Travelled (cm)
Time Bin (10 min)
(d)
Injection 2
Distance Travelled (cm)
10 - 120 min
120 - 240 min
(e)
Injection 2
Dark Phase
Distance Travelled (cm)
Time Bin (10 min)
(f)
Injection 3
Distance Travelled (cm)
10 - 60min
(g)
Injection 3
Distance Travelled (cm)
Time Bin (10 min)

Fig. 2

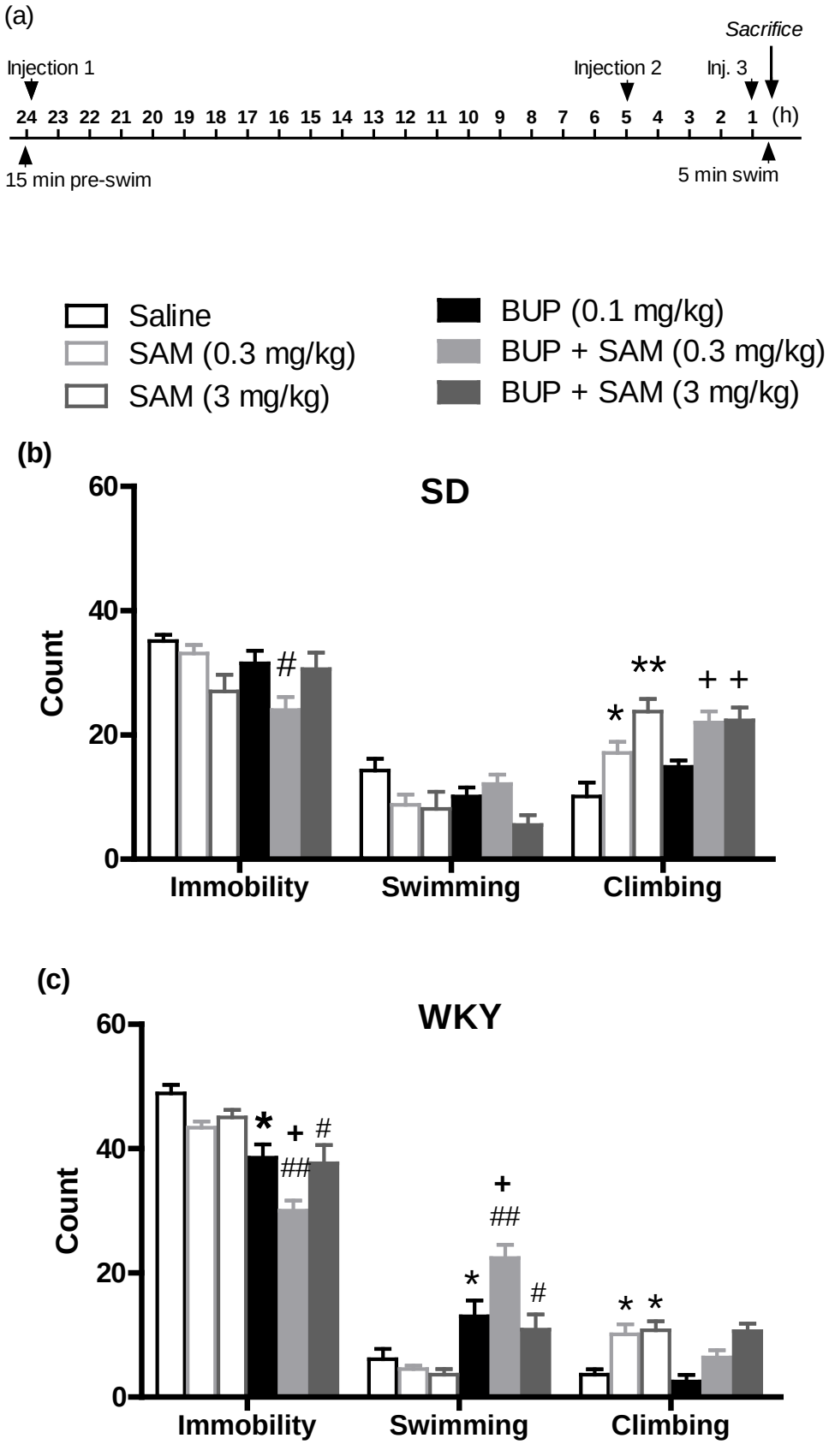

(a)
Sacrifice
Injection 1
Injection 2
Inj. 3
24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 (h)
15 min pre-swim
5 min swim
Saline
SAM (0.3 mg/kg)
SAM (3 mg/kg)
BUP (0.1 mg/kg)
BUP + SAM (0.3 mg/kg)
BUP + SAM (3 mg/kg)
(b)
SD
Count
60
40
20
0
Immobility
Swimming
Climbing
(c)
WKY
Count
60
40
20
0
Immobility
Swimming
Climbing

Fig. 3

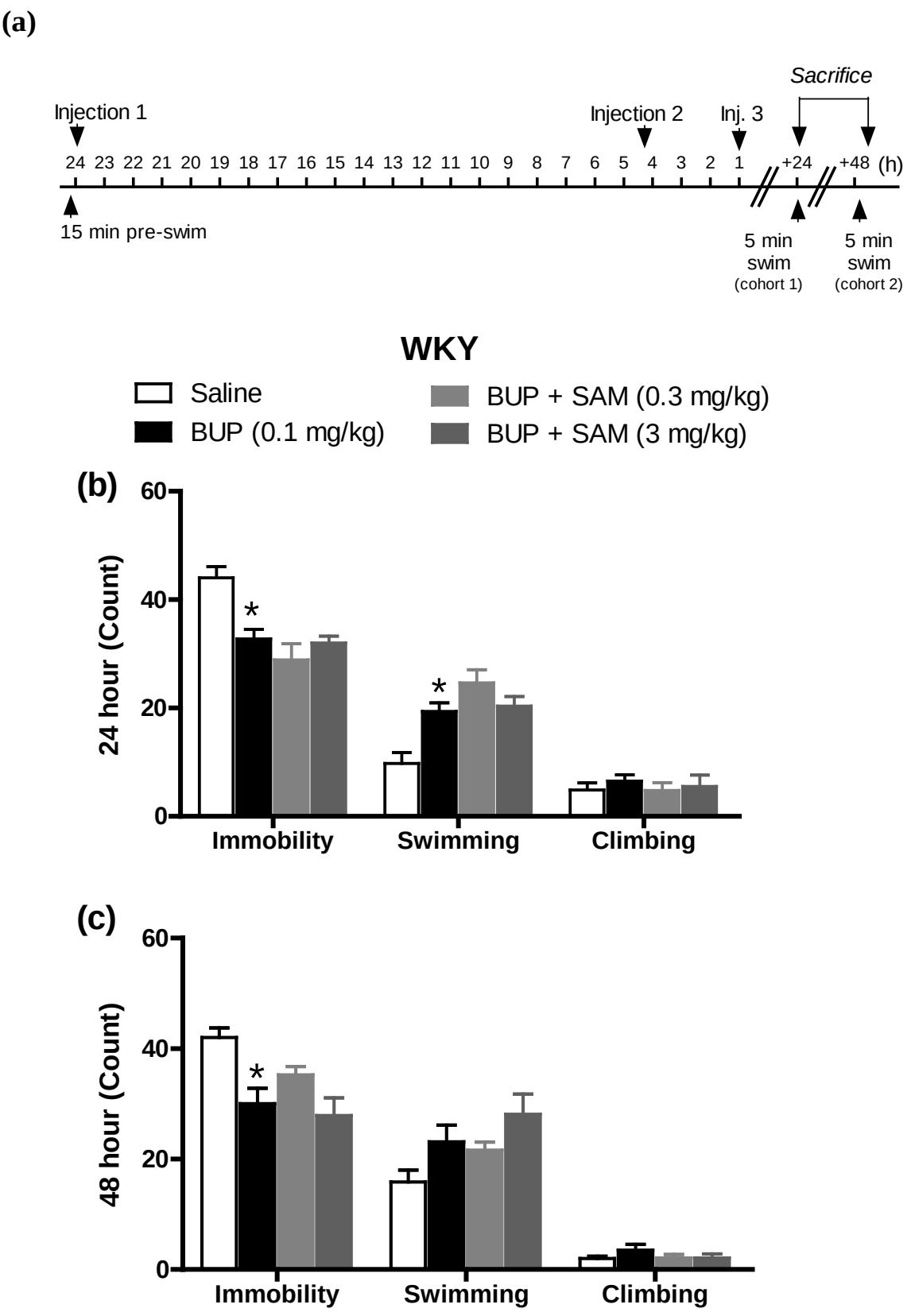
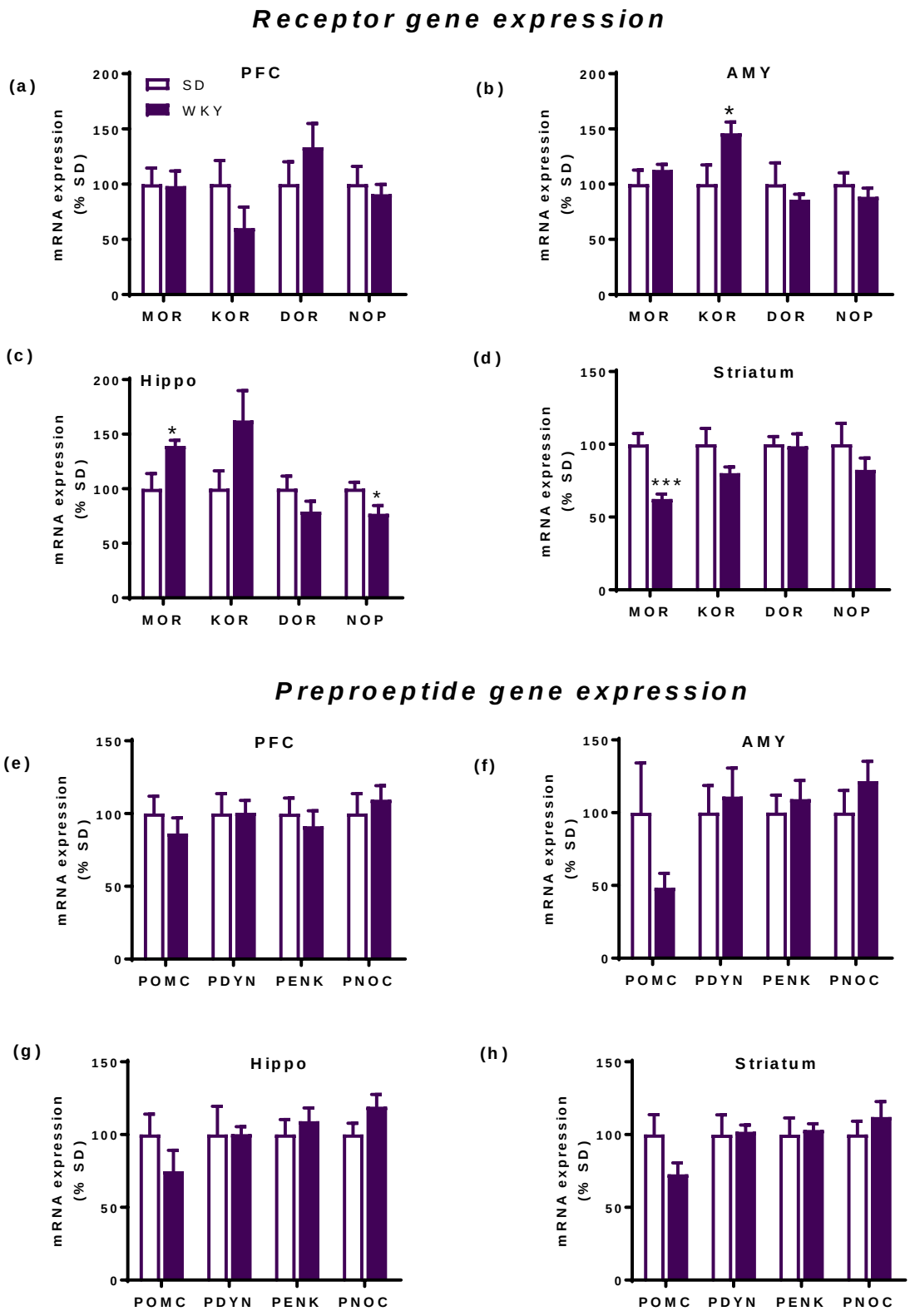

(a)
Sacrifice
Injection 1
Injection 2
Inj. 3
24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 +24 +48 (h)
15 min pre-swim
5 min swim (cohort 1)
5 min swim (cohort 2)
WKY
Saline
BUP (0.1 mg/kg)
BUP + SAM (0.3 mg/kg)
BUP + SAM (3 mg/kg)
(b)
24 hour (Count)
60
40
20
0
*
*
Immobility
Swimming
Climbing
(c)
48 hour (Count)
60
40
20
0
*
Immobility
Swimming
Climbing

Fig. 4

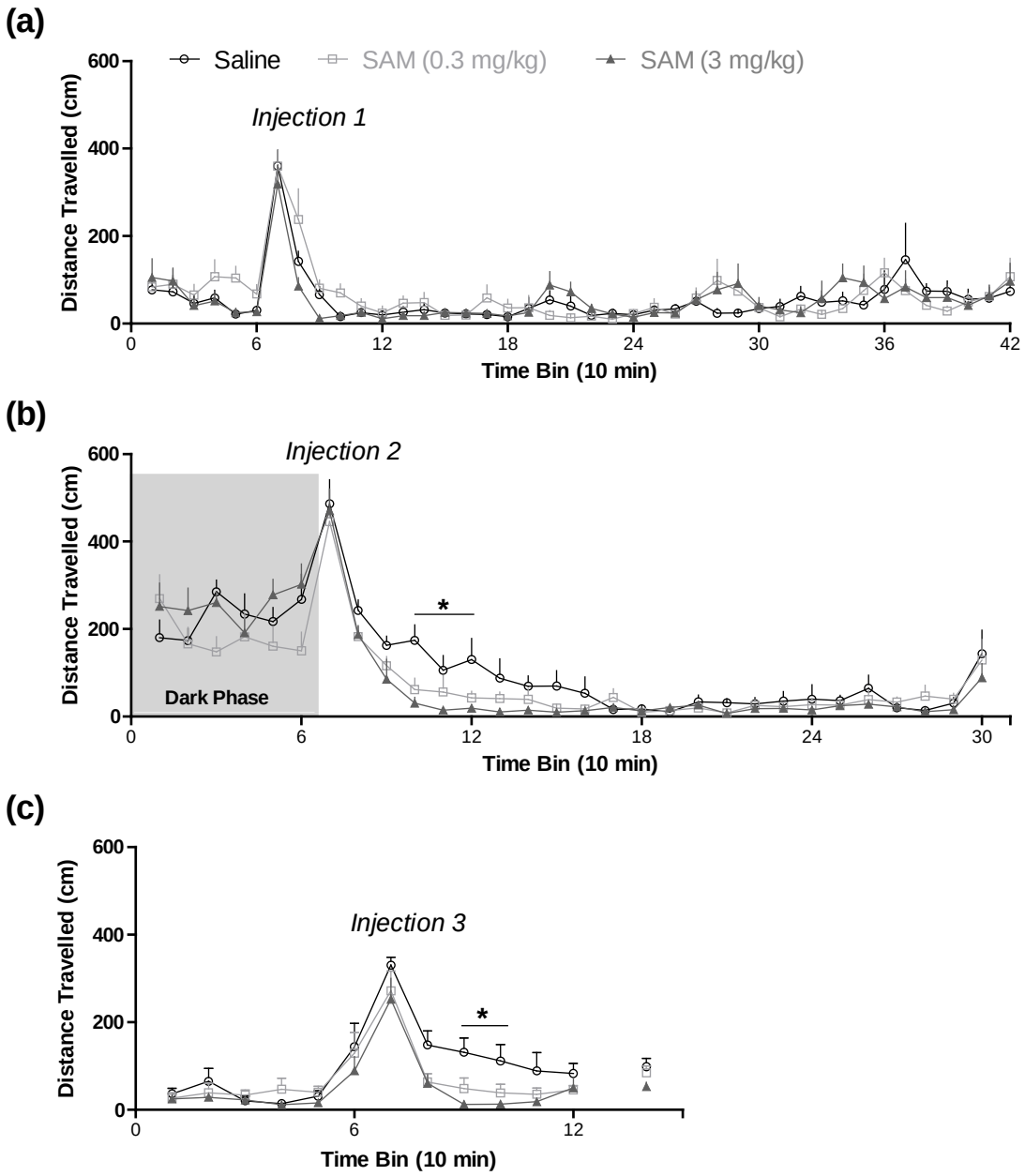

Receptor gene expression
(a)
PFC
SD
WKY
mRNA expression (% SD)
200
150
100
50
0
MOR KOR DOR NOP
(b)
AMY
*
MOR KOR DOR NOP
(c)
Hippo
*
*
MOR KOR DOR NOP
(d)
Striatum
***
MOR KOR DOR NOP
Preproeptide gene expression
(e)
PFC
150
100
50
0
POMC PDYN PENK PNOC
(f)
AMY
POMC PDYN PENK PNOC
(g)
Hippo
POMC PDYN PENK PNOC
(h)
Striatum
POMC PDYN PENK PNOC

		Distance moved in 1 hour (cm)
SD	Vehicle	1685±174
	SAM (0.3 mg/kg)	1548±134
	SAM (3 mg/kg)	1315±118
	BUP (0.1 mg/kg)	1752±291
	BUP + SAM (0.3 mg/kg)	1714±180
	BUP + SAM (3 mg/kg)	1314±79
WKY	Vehicle	1164±217
	SAM (0.3 mg/kg)	986±150
	SAM (3 mg/kg)	894±110
	BUP (0.1 mg/kg)	924±217
	BUP + SAM (0.3 mg/kg)	1027±87
	BUP + SAM (3 mg/kg)	861±115

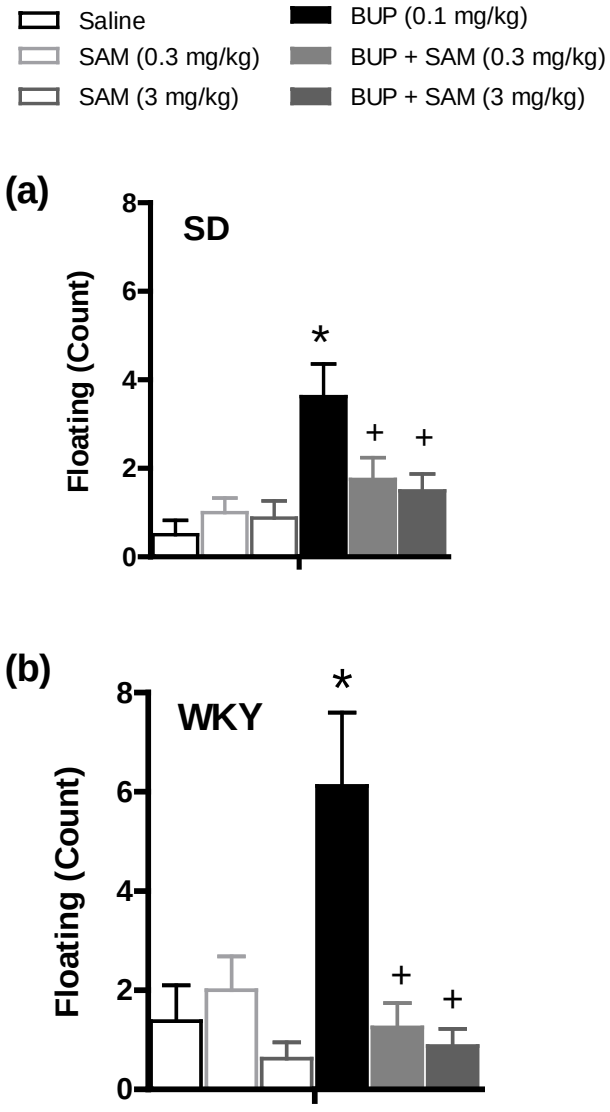
Table 1. Home cage locomotor activity 1 hour prior to FST in SD and WKY rats

Supplementary material

Supplementary Fig. 1



Supplementary Fig. 2



	SD 1h (ng/ml)	WKY 1h (ng/ml)	WKY 24h (ng/ml)	WKY 48h (ng/ml)
BUP (0.1 mg/kg)	2.93±0.45	3.08±0.21	1.32±0.41	nd
BUP + SAM (0.3 mg/kg)	3.08±0.20	3.77±0.24	0.76±0.46	nd
BUP + SAM (3 mg/kg)	3.65±0.24	3.38±0.10	0.56±0.38	nd

Supplementary Table 1. These data show that there are no differences in plasma levels of BUP in SD vs. WKY rats. Although plasma levels have decreased to non-detectable levels by 48h, the behavioural effect in the FST still persists.

		24 hour (cm)	48 hour (cm)
WKY	Vehicle	1111±177	1076±135
	BUP (0.1 mg/kg)	1443±219	875±168
	BUP + SAM (0.3 mg/kg)	1455±236	1195±255
	BUP + SAM (3 mg/kg)	1345±181	879±129

Supplementary Table 2. Home cage locomotor activity 1 hour prior to FST. Total distance moved in 1 hour prior to second swim exposure (cm)

Supplementary figure 1. Temporal effects of SAM (0.3 – 3 mg/kg) alone on home-cage locomotor activity following subacute dosing. (a) There was no effect of either dose of SAM on locomotor activity from 10-360 min following the 1st injection. (b) Both doses of SAM reduced locomotor activity from 20-40 min ($F_{(2,34)}$ = 12.78, P<0.001) following the 2nd administration. (c) Both doses of SAM reduced locomotor activity from 20-40 min following the 3rd administration ($F_{(2,34)}$ = 6.95, P=0.003). Data are presented as mean ± SEM, n =8, *P<0.05 vs. saline.

Supplementary figure 2. Floating behaviour during the first minute of the forced swim test in (a) SD and (b) WKY rats. **SD**: BUP ($F_{(1,42)}$ = 15.69, P<0.001), SAM x BUP interaction ($F_{(2,42)}$ = 4.61, P=0.015). **WKY**: SAM ($F_{(2,42)}$ = 7.87, P=0.001), BUP ($F_{(1,42)}$ =4.98, P=0.031), SAM X BUP ($F_{(2,42)}$ = 7.10 P=0.002). Data are presented as mean ± SEM, n =8, *P<0.05 vs. saline, $^{+}$P<0.05 vs. BUP.

Supplementary Table 1. BUP levels (ng/ml) as determined by ELISA in SD and WKY rats measured in plasma at 1 hour following the final dose, and in WKY rats at 24 and 48 hours following the final dose. (nd – not detected).

Supplementary Table 2. Home cage locomotor activity 1 hour prior to FST.