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Hippocampal monoamine changes in the Flinders sensitive line rat: A case for the possible use of selective α_{2C}-AR-antagonists in stress and anxiety disorders in companion animals

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ABSTRACT

Non-selective α_2-adrenoreceptor (AR) stimulation delivers favourable sedative, analgesic, muscle relaxant and anxiolytic actions in companion animals, but is associated with cardiovascular and respiratory side effects. Anxiety conditions underscore monoamine disturbances amenable to α_2-AR modulation. We investigated sub-chronic (14 day s.c.) treatment with the selective α_{2C}-AR antagonist, ORM-10921 (0.03, 0.1, 0.3 mg/kg/d) on hippocampal noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their turnover levels in stress sensitive Flinders Sensitive Line (FSL) rats versus Flinders Resistant Line (FRL) controls, using high performance liquid chromatography. The effects of ORM-10921 were compared to the non-selective α_2-AR antagonist, idazoxan (IDAZ; 3 mg/kg/d), and to imipramine (IMI; 15 mg/kg/d), a reference antidepressant in this model. FSL rats displayed significantly reduced 5-HT ($p = 0.03$) and DA ($p = 0.02$) levels vs. FRL controls, while NA levels showed a similar trend. ORM-10921 significantly increased NA (all doses $p \leq 0.02$), 5-HT (0.1 and 0.3 mg/kg $p \leq 0.03$) and DA levels (all doses $p \leq 0.03$), which correlated with decreased monoamine turnover. In contrast, IDAZ significantly elevated NA ($p < 0.005$) and DA ($p < 0.004$) but not 5-HT levels. IMI also significantly increased 5-HT ($p < 0.009$), with a tendency to increase NA ($p = 0.09$) but not DA. ORM-10921 exerts similar albeit broader effects on hippocampal monoamines than IDAZ, explaining earlier established efficacy associated with α_{2C}-AR antagonism in animal models of depression and cognitive dysfunction. These and the current studies encourage application of ORM-10921 in depression in humans, as well as raise the intriguing possibility that selective α_{2C}-AR antagonists may be beneficial in anxiety and stress-related disorders in companion animals. Both warrant further study.

1. Introduction

Early studies indicate that non-selective α_2-adrenoreceptor (αAR) antagonists like idazoxan (IDAZ) have antidepressant-like effects in animals (Osman et al. 1989). Similarly, multifunctional α_2-AR antagonists like mirtazapine have well-established antidepressant efficacy in humans (Anttila and Leinonen 2001; Blier 2003) while α_2-agonists such as dexmedetomidine are used in human as well as veterinary medicine to produce dose-dependent sedation, analgesia and muscle relaxation (Sinclair 2003). Anxiety and an altered stress-response are prevalent in companion animals, manifested as inappropriate aggression, excessive vocalisation, hiding, escape behaviour, destructive behaviour and obsessive behaviours, and loss of interest/enthusiasm in normally enjoyable activities (Watson et al. 2018; Gilbert-Gregory et al. 2016; Sonntag and Overall 2014; Pineda et al. 2014; Wrzosek et al. 2015). Dogs are especially prone to anxiety disorders such as separation anxiety, social and noise phobia (Sherman and Mills 2008).

Behavioural pharmacology plays an important role in veterinary

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behavioural medicine (Simpson and Papich 2003; Overall 2013). Antidepressants like serotonin selective reuptake inhibitors (SSRIs e.g. fluoxetine) and tricyclic antidepressants (TCAs e.g. clomipramine) are widely used in veterinary medicine to treat pain, anxiety and other behavioural maladies (Dharmshaktu et al. 2012; Overall 2013; Kaur et al. 2016). However, a slow onset of action (6–8 weeks) (Overall 2013) and dose-related side-effects (Fitzgerald and Bronstein 2013; Overall 2013) are problematic. As noted, α_2-AR agonists are widely used in veterinary medicine for their sedative effects (Paddleford and Harvey 1999), and to control peri-operative pain (Lemke 2004). However, cardiovascular, respiratory and sedative effects may complicate their use (Sinclair 2003; Valverde and Skelding 2019; van Oostrom et al. 2011). Similarly, non-selective α_2-AR antagonists (to antagonise the effects of α_2-AR agonists) also cause dose-dependent cardiovascular side effects (Lemke 2004; Delaunois et al. 2014). There is therefore a need for developing improved therapeutic agents for anxiety-related states in companion and other animals with a low side effect profile. In this regard, sub-sedative doses of the α_2-AR agonist, dexmedetomidine, is an effective anxiolytic in dogs without inducing sedation (Korpivaara et al. 2017), prompting a closer look at targeting the α_2-AR for veterinary application.

The α_{2A}-AR and α_{2C}-AR mediates presynaptic inhibition on noradrenaline (NA) and serotonin (5-HT) release in the brain (Bücheler et al. 2002; Hein et al. 1999). Up-regulated α_2-ARs are noted in platelets of depressed patients and in the frontal cortex and hippocampus of depressed suicide completers (Cottingham and Wang 2012), suggesting bolstered inhibition of transmitter release (Blier 2003; Cottingham and Wang 2012) that drive the behavioural and neuropathological changes of the illness (Brand et al. 2015). Chronic antidepressant treatments down-regulate these receptors (Cottingham and Wang 2012), while α_2-AR antagonists increase NA and 5-HT release (Blier 2003).

The hippocampus, central in mood and anxiety disorders (Brand et al. 2015), receives extensive monoamine input from the locus coeruleus (NA), raphe nucleus (5-HT) and ventral tegmentum (dopamine; DA). The α_{2A}-AR is broadly distributed in the brain and periphery (Rosin et al. 1996; Scheinin et al. 1994), with the α_{2C}-AR selectively localised in stress-regulatory regions of the brain, viz. hippocampus, striatum and frontal cortex (Fagerholm et al. 2008; Rosin et al. 1996; Scheinin et al. 1994). α_2-Adrenoceptors also have a wide brain distribution in companion animals, although species-dependent differences may be evident (Hellyer et al. 2003). Transgenic α_{2C}-AR-knock out mice present with enhanced startle and other anxiety-like responses, whereas α_{2C}-AR-over-expressing mice show the opposite (Sallinen et al. 1998b). The α_{2C}-AR also shows differential action in behavioural models for cognition and depression when compared to the α_{2A}-AR. Selective α_{2A}- and α_{2C}-AR stimulation mediates positive (Björklund et al. 2001) and negative effects (Björklund et al. 1998; Björklund et al. 1999a; Björklund et al. 1999b), respectively, on cognition. In other tests α_{2C}-AR antagonism, but agonism at the α_{2A}-AR, display antidepressant-like effects (Sallinen et al. 1999; Schramm et al. 2001). Therefore, selectively targeting α_2-AR subtypes may engender more specific and reliable psychotropic effects (Scheinin et al. 2001; Uys et al. 2017a). However, the neurochemical profile that may facilitate the superior antidepressant, anxiolytic and pro-cognitive effects associated with selective versus non-selective α_{2C}-AR antagonism (Uys et al. 2017b) remains unknown.

α_{2C}-ARs have important effects on neurotransmitters regulating stress and anxiety, with α_{2A}- and α_{2C}-ARs mediating different, albeit activity-dependent presynaptic inhibitory actions on NA release (Uys et al. 2017a). Furthermore, the α_{2C}-AR produces less pronounced inhibition of 5HT release in hippocampal tissue, while the α_{2A}-AR strongly inhibits 5HT release (Scheibner et al. 2001). Therefore, α_{2C}-AR modulation would provide more targeted effects on noradrenergic and serotonergic systems, with less peripheral side effects.

Flinders Sensitive Line (FSL) rats present with stress-sensitive biobehavioural disturbances akin to depression, and hence present some parity with domestic animals. These characteristics include impaired escape behaviour, anhedonia, sleep disturbances, anxiety and impaired declarative memory, reduced limbic 5-HT and limbic neurotrophins (Overstreet and Wegener 2013), disturbances in brain α_2-adrenoceptor density (Lillethorup et al. 2015; Landau et al. 2015), and disordered glutamate signalling (Wegener et al. 2010). Importantly, the above biobehavioural deficits respond preferentially to chronic antidepressant treatment (Overstreet and Wegener 2013).

We investigated the dose-related effects of sub-chronic treatment with the selective α_{2C}-AR antagonist, ORM-10921 (Sallinen et al. 2013) on hippocampal levels of NA, DA, 5-HT and their turnover rates in FSL rats, versus the non-selective α_2-AR antagonist, IDAZ, and the reference tricyclic antidepressant imipramine (IMI). We hypothesize that FSL rats will present with hippocampal monoamine changes congruent with depression, and that ORM-10921 and IMI will reverse these changes, albeit with some important differences. IDAZ and ORM-10921 will present with different actions on monoamine changes in FSL rats.

2. Methods

2.1. Animals and drug treatment

Eight week old male FSL and Flinders Resistant Line (FRL) control rats were bred and cared for at the Vivarium of the North-West University (NWU). The original colonies were obtained from Dr. David H. Overstreet, University of North Carolina, USA. The rats were reared under identical conditions: cages (230(h) x 380(w) x 380(l) mm), temperature (21 ± 5 °C), humidity (50 ± 10%), white light (350–400 lx), 12 h light/dark cycle and food and water ad libitum. Animals were bred, supplied and housed at the Vivarium (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-clinical Drug Development Platform of the NWU. All experiments were approved by the relevant animal research ethics committee (NHREC reg. Number AREC-130913-015) at the NWU. All animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation (ethics approval number: NWU-00050-13-A5).

FRL rats or out-bred Sprague Dawley are used as the control for FSL rats (Overstreet and Wegener 2013). FRLs were therefore included to confirm the depressive phenotype of the FSL rats, and received vehicle treatment but no drug treatment. FRL and FSL rats (10–11 per group) were randomly divided into a saline control group and 5 drug treatment groups (FSL). Drug or vehicle (saline) was injected subcutaneously (s.c.) once daily for 14 days. ORM-10921 (ORM), a gift from Orion Pharma (Orion Corporation, Turku, Finland), was administered at doses of 0.03, 0.1 and 0.3 mg/kg, based on earlier studies describing antidepressant dosages (Sallinen et al. 2013; Uys et al. 2017b) and dissolved in physiological saline to an injection volume of 1 ml/kg. Idazoxan hydrochloride (IDAZ) (Sigma Aldrich, South Africa) was administered at a dose of 3 mg/kg, based on earlier studies (Castagné et al. 2009; Rénéric et al. 2001; Uys et al. 2017b). Imipramine hydrochloride (IMI) (Sigma Aldrich, South Africa) was administered at a dose of 15 mg/kg, based on its well-described antidepressant-like effects at this dose (Castagné et al. 2011; Chen et al. 2010; de Morais et al., 2014). Importantly, these studies enabled us to decide on a single dose selection for IDAZ and IMI without over-exploiting the number of animals.

2.2. Brain homogenate preparation and monoamine analyses

Animals were sacrificed by decapitation 24 h after the final drug treatment. The brain was dissected into right and left hemispheres, where after the olfactory bulb was removed and total hippocampus dissected on an ice-cooled dissection slab. The hippocampi were snap frozen in liquid nitrogen and stored in Eppendorf™ tubes at -70 °C. On the day of the analysis, brain samples were thawed and weighed, where after 1 ml of 0.1 M perchloric acid solution was added to each tube, sonicated and left on ice for 20 min to complete perchlorate

precipitation. Samples were then centrifuged at 4 °C and 24,000 x g for 20 min. 200 μl of the supernatant was withdrawn and 20 μl of the internal standard (isoprenaline HCl) added and mixed. pH was adjusted to pH 5 with 10 M potassium acetate. Quantification of hippocampal NA, 5-HT, DA, 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were performed by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD, HPLC-ECD), according to a previously described method (Harvey et al. 2006). An Agilent 1200 series HPLC, equipped with an isocratic pump and auto-sampler and coupled to an ESA Coulochem III Electrochemical detector with Chromeleon® Chromatography Management System (version 6.8), was used. Sample monoamine concentrations were determined by the response ratio (area under the peak for each monoamine/the area under the peak of the internal standard for each sample) and calculated according to the regression curves for the response ratio of the monoamine standards (range 1.25 ng/ml – 50 ng/ml) and that of the internal standard. Linear standard regression curves (regression coefficient > 0.98) were generated. Monoamine concentrations were expressed as ng/g wet weight of hippocampal tissue (mean ± SEM). Monoamine turnover for NA and 5-HT are expressed as the ratio of MHPG (ng/g) ÷ NA (ng/g) and 5-HIAA (ng/g) ÷ 5-HT (ng/g), respectively. Due to hippocampal DA levels often being below the lower limit of detection, DA turnover is not presented (Swant and Wagner 2006). Rather DA (where possible), DOPAC and HVA are presented and interpreted separately.

2.3. Statistical analyses

Normality of data was determined using the Shapiro Wilk test, which has statistical power to detect a non-Gaussian population and is well-suited to the n value-range of data reported in this study (Ghasemi and Zahediasl 2012; Razali and Wah 2011). Differences in monoamine levels in FSL vs. FRL rats were analysed with student's unpaired *t*-tests or Mann-Whitney *U* tests in the case of non-parametric data sets. In the case of unequal standard deviations, Welch's corrected *p*-value was applied to t-tests. Comparison of the effects of drug treatments on hippocampal monoamines in FSL rats were performed using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) post hoc test was applied to indicate where treatment groups differed significantly. Where the criteria of equality of variances for ANOVA was not met as indicated by the Brown-Forsythe test, Kruskal-Wallis ANOVA and Dunn's post hoc multiple comparison test was employed. Significance was set at a 5% level ($p < 0.05$). Given the risk of the p-value being confounded by the sample size, practical significance was calculated to decrease the risk of a type II statistical error (false negative), according to Cohen (1988) and Rosnow and Rosenthal (1996). Cohen's *d*-value (effect size) was calculated to indicate the practical significance (if applicable) of results demonstrating statistical significance on a 5% ($p < 0.05$) and 10% ($p \leq 0.1$) significance level. An effect size of ~0.2 to ~0.4 is considered a small effect, ~0.5 to ~0.7 a medium effect showing a trend for practical significance, and effect sizes of ~0.8 and greater considered large and practically significant (Cohen 1988). GraphPad Prism 6 (GraphPad Software Inc., La Jolla, California, USA) was used for data representation and all statistical analyses. Analysis of effect size was performed as described by Rosnow and Rosenthal (1996).

3. Results

3.1. Hippocampal 5-HT levels and 5-HT turnover

Unpaired *t*-test indicate that FSL animals presented with significantly lower 5-HT levels vs. their FRL controls ($p = 0.03$, Cohen's $d = 0.98$) (Fig. 1A). ANOVA indicated that drug treatment in FSL animals induced significant differences in hippocampal 5-HT levels ($F(5,52) = 3.228$, $p = 0.01$). Fisher's LSD test indicated that ORM 0.1 ($p = 0.0006$, Cohen's $d = 1.6$), ORM 0.3 ($p = 0.03$, Cohen's $d = 1.1$) and IMI ($p = 0.009$, Cohen's $d = 1.4$) significantly increased hippocampal 5-HT levels vs. FSL controls. ORM 0.03 ($p = 0.11$) and IDAZ ($p = 0.24$) did not significantly affect hippocampal 5-HT levels in these animals at the 5% or 10% level (Fig. 1B).

Unpaired *t*-test indicated that FSL controls had significantly higher 5-HT turnover ratios than FRL controls ($p = 0.04$, Cohen's $d = 1.04$) (Fig. 2A). ANOVA indicated significant differences of drug treatment on 5-HT turnover levels in FSL animals ($F(5,53) = 4.037$, $p = 0.003$). Post-hoc analysis indicated that all drug treatments decreased 5-HT turnover vs. FSL controls to a statistically significant extent, supported by large effect sizes ($d \geq 0.8$): (ORM 0.03, $p = 0.03$, Cohen's $d = 0.8$; ORM 0.1, $p = 0.0002$, Cohen's $d = 1.5$; ORM 0.3, $p = 0.001$, Cohen's $d = 1.2$; IMI, $p = 0.001$, Cohen's $d = 1.3$; IDAZ, $p = 0.01$, Cohen's $d = 0.9$ (Fig. 2B).

3.2. Hippocampal NA levels and NA turnover

Unpaired t-test showed a tendency for FSL animals to display lower hippocampal NA levels vs. FRL animals at a 10% significance level ($p = 0.07$, Fig. 3A), while the effect size ($d = 0.9$) indicates a large and practically significant effect. One-way ANOVA indicated a significant difference between FSL rats treated with either vehicle or the respective drug treatments $F(5,53) = 4.51$, $p = 0.002$, (Fig. 3B). Fisher's LSD test indicated that ORM 0.03 ($p = 0.0003$, Cohen's $d = 1.9$), ORM 0.1 ($p = 0.0001$, Cohen's $d = 1.6$), ORM 0.3 ($p = 0.02$, Cohen's $d = 2.1$) and IDAZ ($p = 0.005$, Cohen's $d = 1.9$) treatment resulted in significantly higher

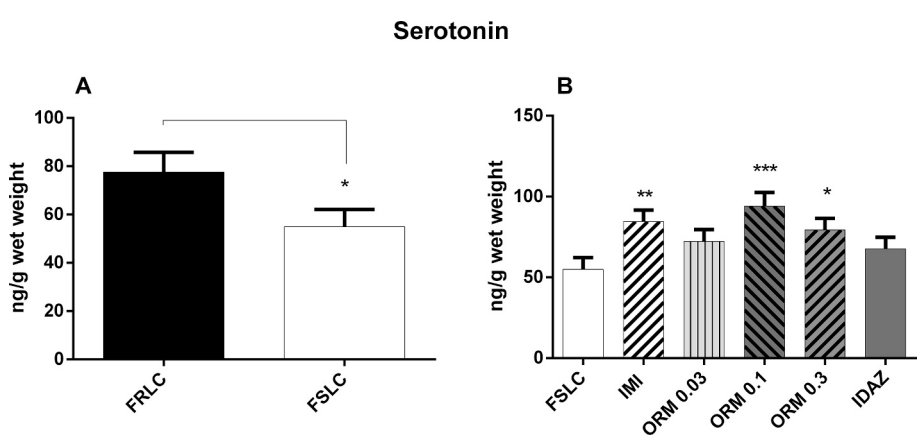

Fig. 1. Hippocampal 5-HT levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. FSL controls. $n = 9$–10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

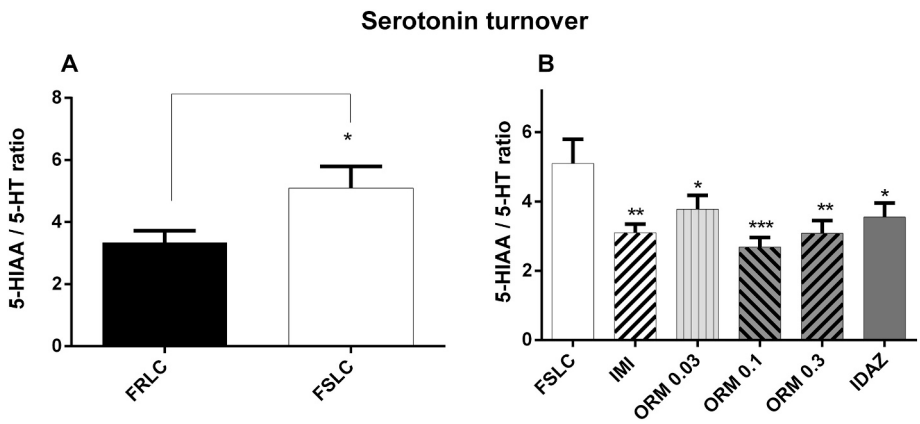

Fig. 2. Hippocampal 5-HT turnover rates in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B), and expressed as 5-HIAA (ng/g)/5-HT (ng/g). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. FSL controls. $n = 9$–10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

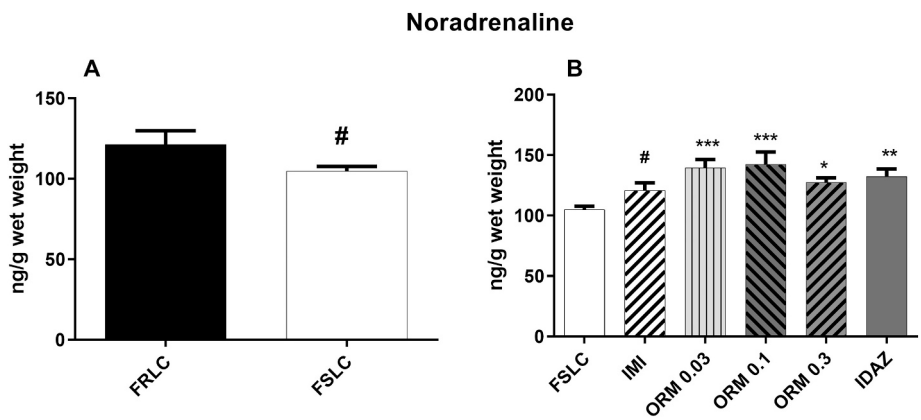

Fig. 3. Hippocampal NA levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and # practical significance vs. FRL controls (A) or FSL controls (B). $n = 9$–11. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

hippocampal NA levels vs. FSL controls, with correlating large effect sizes ($d \geq 0.8$) (Fig. 3B). Although the response to IMI treatment did not show a statistically significant increase in NA levels at the 5% level, this increase did show statistical significance at the 10% level ($p = 0.09$) along with a large Cohen's effect size ($d = 1.12$) (see Fig. 3B).

Unpaired t-test with Welch's correction indicated no statistically significant differences in NA turnover between FRL and FSL controls on a 5% significance level ($p = 0.1$), although Cohen's d-value suggested a large effect size ($d = 0.8$; Fig. 4A). ANOVA indicated that drug treatment in FSL animals induced significant differences in hippocampal NA turnover (F (5,51) = 2.446, $p = 0.04$). Fisher's LSD indicated that ORM 0.03 ($p = 0.04$, Cohen's $d = 0.95$), ORM 0.1 (p=0.004, Cohen's $d = 1.3$), ORM 0.3 ($p = 0.004$, Cohen's $d = 1.2$) and IDAZ ($p = 0.04$, Cohen's $d = 0.9$) significantly decreased hippocampal NA turnover in FSL rats (Fig. 4B). IMI also tended to decrease NA turnover in the hippocampi of FSL rats ($p = 0.052$, Cohen's $d = 0.9$), albeit missing statistical

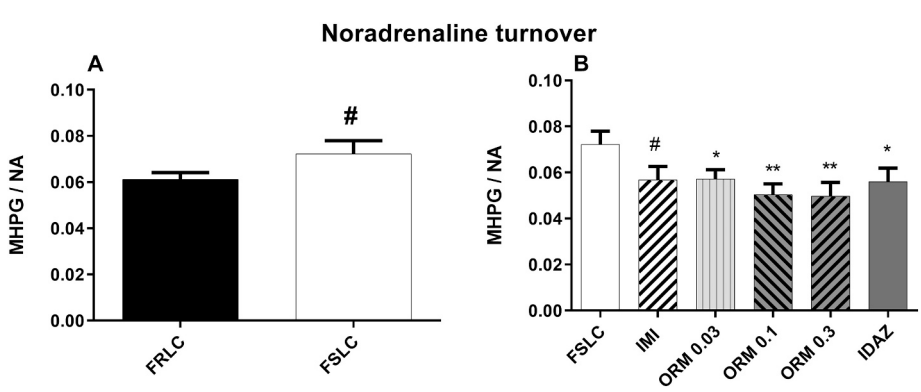

Fig. 4. Hippocampal NA turnover in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). NA turnover is expressed as MHPG (ng/g)/NA (ng/g). *$p < 0.05$, **$p < 0.01$, # practical significance vs. FRL controls (A) or FSL controls (B). $n = 8$–11. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

significance at the 5% level (Fig. 4B).

3.3. Hippocampal DA levels and DA metabolites

DA levels were below the limit of quantification (LOQ) in many of the FSL control rats, and to a lesser degree in FRL controls and all other FSL drug treatment groups ($n = 5$–9 per group). DA levels are normally low in hippocampus (Swant and Wagner 2006), while such levels are regarded as being even lower in FSL rats and commensurate with their depressive phenotype (Zangen et al. 2001). It may therefore be challenging for some analytical systems to consistently detect levels well-below the LOQ. Here we were able to detect the levels of this monoamine in approximately 50% of FSL animals. These data were then used to establish the hippocampal DA concentration using a regression formula. Non-parametric statistics was subsequently performed for the comparative analysis.

Mann-Whitney *U* test showed a significant difference between hippocampal DA levels with a large effect size in FRL vs. FSL animals ($p = 0.02$; Cohen's $d = 1.3$; Fig. 5A). Kruskal-Wallis ANOVA indicated a significant difference between FSL rats treated with the respective drug treatments (Kruskal-Wallis statistic 22.59, $p = 0.0004$). Post hoc Dunn's comparison indicated that ORM 0.03 ($p = 0.003$, Cohen's $d = 4.1$), ORM 0.1 ($p = 0.01$, Cohen's $d = 3{,}2$), ORM 0.3 ($p = 0.006$, Cohen's $d = 3.1$) and IDAZ ($p = 0.004$, Cohen's $d = 2.9$), but not IMI treatment ($p > 0.9$), significantly increased hippocampal DA levels vs. FSL controls (Fig. 5B).

Due to the low detection of DA in FSL controls, determining DA turnover by dividing the metabolites DOPAC and HVA by the values obtained for DA produced skewed data that presented evidence of elevated DA turnover in FSL controls, with all cohorts displaying disproportionately decreased DA turnover vs. FSL controls. Consequently, DA turnover was not represented as the conversion indices of the metabolites to DA, and are represented independently and interpreted accordingly.

Mann-Whitney U test indicated significantly higher HVA levels in FSL controls compared to FRL controls ($p = 0.003$; Cohen's $d = 2.1$, Fig. 6A), while ANOVA indicated significant differences between FSL treatment groups ($F(5,55) = 3.66$, $p = 0.006$). Fisher's LSD post hoc test indicates that all drug treatments, except ORM 0.03, significantly decreased HVA levels vs. FSL controls: ORM 0.1 ($p = 0.01$, Cohen's $d = 1.2$), ORM 0.3 ($p = 0.0002$, Cohen's $d = 1.9$), IMI ($p = 0.01$, Cohen's $d = 0.9$) and IDAZ ($p = 0.02$, Cohen's $d = 1$) with large effect sizes ($d \geq 0.8$) (Fig. 6B). ORM 0.03 showed significance on a 10% level ($p = 0.1$) although this treatment group showed a medium effect size ($d = 0.6$).

DOPAC levels didn't differ significantly between FSL and FRL controls ($p = 0.9$; Fig. 7A), while Cohen's test also indicated no practical significance for FSL vs. FRL controls ($d = 0.01$, small effect size). However, ANOVA indicated significant differences between FSL treatment groups ($F(5,51) = 2.95$, $p = 0.02$; Fig. 7B). Fisher's LSD indicates that ORM 0.03 ($p = 0.05$; $d = 0.9$), ORM 0.3 ($p = 0.06$; $d = 0.9$) and IDAZ ($p = 0.06$; $d = 0.9$) tended to increase DOPAC levels compared to FSL controls on a 10% significance level, with an effect size for all the aforementioned exceeding Cohen's convention for a large effect size (≥ 0.8) (Cohen 1988). Neither IMI ($p = 0.4$) nor ORM 0.1 ($p = 0.3$) indicated significant differences with the FSL controls.

4. Discussion

FSL rats displayed significantly lower levels of hippocampal 5-HT and DA vs. FRL controls, while NA levels showed a similar trend. The most important findings described here are that ORM-10921 significantly increased hippocampal NA, 5-HT and DA levels, which correlated with decreased monoamine turnover. In contrast, IDAZ elevated NA and DA but not 5-HT levels, while IMI increased 5-HT levels, but not NA or DA.

In the current study drug-naïve FSL rats showed a large effect size reduction ($p = 0.07$; $d = 0.9$) in hippocampal NA levels. This is concordant with reduced NA in depression, while noradrenergic dysregulation is also supported by evidence of decreased α_2-AR binding in these animals (Landau et al. 2015). IMI induced a large effect size increase NA ($p = 0.09$; ($d = 1.1$), which explains increased noradrenergic-mediated behaviours as described elsewhere (Detke et al. 1997; Uys et al. 2017b). The weaker effects of IMI on NA may be partly related to the ex-vivo method of analysis. While IMI-induced changes in extracellular MHPG levels have not been reported, IMI decreases pre-synaptic α_2-AR sensitivity (Sugrue 1983) that would promote NA release. Accordingly, increased extracellular NA levels and down-regulated α_2-AR sensitivity follows acute and chronic treatment with the IMI metabolite, desimipramine (Sacchetti et al. 2001), so that IMI conversion to desimipramine may explain the subtle bolstering of NA levels described here. All doses of ORM-10921 and IDAZ significantly increased hippocampal NA levels in FSL rats, with an apparent decrease in NA turnover. Thus, both selective and non-selective α_{2C}-AR antagonists increased hippocampal NA levels in FSL animals, concordant with their action as α_2-AR auto-receptor antagonists (Leonard 2003). However, with sub-chronic ORM-10921, but not IDAZ, treatment exerting antidepressant effects in FSL animals (Uys et al. 2017b), we posit that selective α_{2C}-AR antagonism has distinct pharmacological advantages which may be diminished by non-selective α_2-adrenoceptor antagonism. Whilst these primary actions on α_2-ARs support the anti-stress/anxiolytic properties of these compounds, activity at post-synaptic α_2-AR heteroreceptors on 5-HT and DA release also needs consideration (Harvey and Slabbert 2014).

FSL rats displayed significantly reduced hippocampal 5-HT levels vs. FRL rats, with significantly increased 5-HT turnover, suggesting a

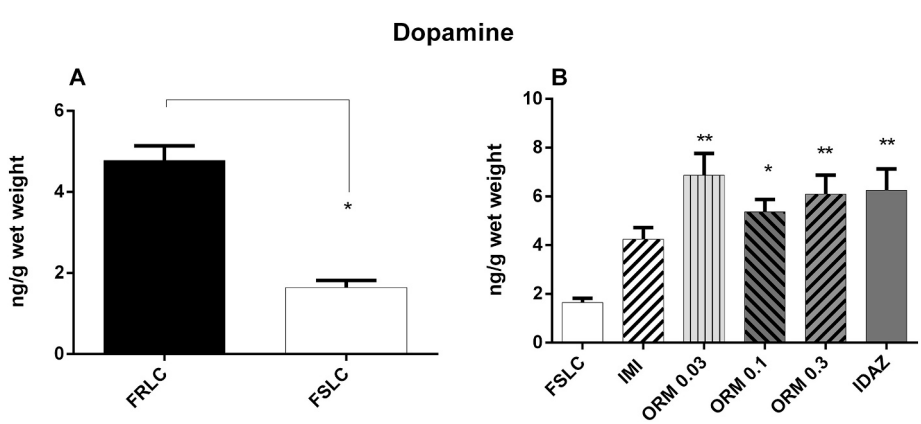

Fig. 5. Hippocampal DA levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). $*p < 0.05$, $**p < 0.01$, vs. FSL controls. $n = 5$–9. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

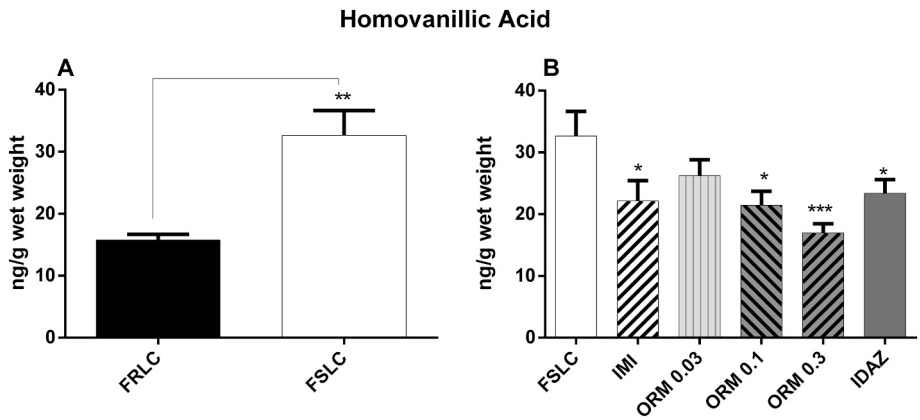

Fig. 6. Hippocampal HVA levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). $*p < 0.05$, $**p = 0.002$, $***p < 0.001$ vs. FSL controls. $n = 8–12$. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

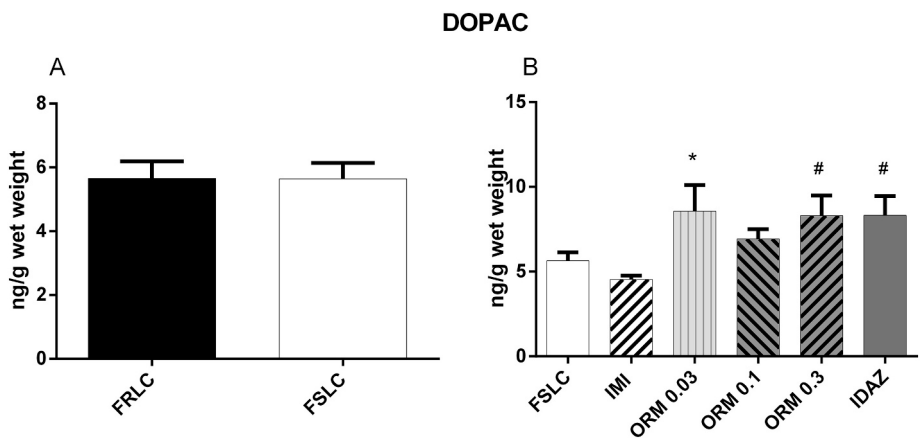

Fig. 7. Hippocampal DOPAC levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). $*p < 0.05$ and # practical significance vs. FSL controls. $n = 8–11$. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM = ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

serotonergic deficiency congruent with observations in relation to human depression (Kharade et al. 2010). Lower total 5-HT tissue levels in FSL vs. FRL controls is predictive of reduced 5-HT synthesis. Considering treatment response, chronic (not acute) antidepressant treatment normalizes altered serotonergic activity in FSL animals (Zangen et al. 2001; Zangen et al. 1997; Brand and Harvey 2017). IMI, in the present study, significantly reversed lower hippocampal 5-HT levels and significantly decreased 5-HT turnover in FSL rats, which is consistent with previous reports (Alpers and Himwich 1972; Sugrue 1983). This effect is commensurate with a serotonergic-based antidepressant response via 5-HT reuptake inhibition (Felton et al. 2003; Vetulani and Nalepa 2000). Importantly, while the α_{2C}-AR-antagonist ORM-10921 (0.1, 0.3 mg/kg) also significantly increased hippocampal 5-HT levels and significantly decreased 5-HT turnover, the non-selective α_2-AR-antagonist IDAZ significantly reduced 5-HT turnover *without* increasing 5-HT levels. While the latter data appear counterintuitive, IDAZ decreases hippocampal 5-HT synthesis in vivo (Llado et al. 1996) and does not demonstrate serotonergic driven antidepressant-like effects in FSL rats (Uys et al. 2017b).

FSL rats had significantly lower DA and significantly higher HVA levels versus FRL controls, as shown here. DA mediates hedonic and motivational behaviour (Grace 2016), although primarily through cortico-striatal actions. Noradrenergic fibres may also be the primary source of DA release in the hippocampus due to limited dopaminergic input from the ventral tegmentum (Smith and Greene 2012). FSL rats present with decreased 5-HT-mediated release of DA (Zangen et al. 2001) as well as decreased limbic DA neurotransmission (Friedman et al. 2007; Friedman et al. 2005), abrogated by antidepressants (Dremencov et al. 2004; Roth-Deri et al. 2009). Congruent with this, reduced dopaminergic activity is evident in brains of depressed suicide completers (Pitchot et al. 2001). IMI increased hippocampal DA levels albeit not significantly, possibly due to the non-parametric analysis, although significantly decreased HVA levels suggest decreased DA metabolism. Indeed, IMI has indirect dopaminergic effects, including increasing functional activity (Muscat et al. 1990), increased post-synaptic DA receptor sensitivity (Dziedzicka-Wasylewska and Rogoz 1998) and increasing extracellular mesolimbic DA output (Rossetti et al. 1993). α_{2C}-AR antagonism affects DA activity (Sallinen et al. 2013; Sallinen et al. 1999; Sallinen et al. 1998b; Sallinen et al. 1997), notably increasing DA release in the frontal cortex (Sallinen et al. 2013). Indeed, all doses of ORM-10921 significantly and with large effect sizes reversed (increased) lowered hippocampal DA levels in FSL rats, with significantly decreased HVA levels. ORM-10921 at doses of 0.03 and 0.3 mg/kg also increased DOPAC levels at a 10% significance level with a large effect size ($d = 0.9$). The forced swim-stress test has been shown to deplete mesolimbic DA in rats (Rossetti et al. 1993), while increased hippocampal DA (Perona et al. 2008; Renoir et al. 2012) may contribute to the antidepressant and pro-cognitive effects of ORM-10921 (Sallinen et al. 2007; Sallinen et al. 2013; Uys et al. 2017b). However, while IDAZ also significantly increased hippocampal DA, decreased HVA levels, and

moderately increased DOPAC levels ($p = 0.06$; $d = 0.9$), congruent with earlier studies (Borgkvist et al. 2012; Matsumoto et al. 1998), it failed to demonstrate an antidepressant or pro-cognitive effect in FSL rats (Uys et al. 2017b).

Human to animal translation (e.g. Watson et al. 2018) has prompted successful off-label use of antidepressants in pets (Sartini et al. 2019; Chutter et al. 2019; Fitzgerald and Bronstein 2013). Although deficits in 5-HT, DA and NA transmission contribute to the symptoms of major depression (Krishnan and Nestler 2008), current antidepressants are at best 65% effective (Brand and Harvey 2017). Similarly, widespread use of these compounds in companion animals (Chutter et al. 2019; Kaur et al. 2016; Watson et al. 2018; Gilbert-Gregory et al. 2016; Pineda et al. 2014; Wrzosek et al. 2015), also with variable efficacy (Overall 2013), advocates an urgent need for new therapies.

Anxiety in companion animals can be linked to reduced DA, 5-HT and NA, as well as elevated 5-HT and NA (Brand et al. 2015). Interestingly, Alzheimer's disease (AD) (Novais and Starkstein, 2015) and canine cognitive dysfunction (CCD) (Dewey et al. 2019) present with similar cognitive and mood related deficits, both of which may benefit from elevating brain monoamines (Kharade et al. 2010). While ORM-10921 (0.1, 0.3 mg/kg) increased hippocampal 5-HT, NA and DA levels, IDAZ similarly increased NA and DA but not 5-HT. With the exception of IDAZ not increasing 5-HT, these effects are concordant with antidepressant-induced antagonism of the α_2-AR auto-receptor (Leonard 2003). Although further study is required, the lack of evident serotonergic effects for IDAZ may underlie its weaker antidepressant-like response versus ORM-10921 (Uys et al. 2017b).

Clinical and pre-clinical evidence supports targeting of the α_{2C}-AR in psychiatric pharmacotherapeutics (Uys et al. 2017a). Since α_{2A}-ARs are useful as a surrogate marker of noradrenergic activity in humans (Cottingham and Wang 2012; Brand et al. 2015), these data suggest noteworthy translational validity from rat/human to companion animals. Autoradiography studies have confirmed α_2-ARs in brain of horses and dogs (Hellyer et al. 2003), while PET and ex vivo brain autoradiography studies confirm α_{2C}-AR in rodents (Arponen et al. 2014) and humans (Lehto et al. 2015). In the periphery, α_2-ARs are expressed on canine, leporine, feline, and murine platelets, with α_{2A}-AR active compounds showing similar affinity for canine and human platelet α_2-ARs (Hikasa et al. 2013). This evidence is corroborated by the successful use of α_2-agonists in veterinary medicine for their sedative, analgesic and muscle relaxing effects (Ogata and Dodman 2011), with low dose dexmeditomidine being a valuable anxiolytic in companion animals (Ogata and Dodman 2011; Korpivaara et al. 2017). Paradoxically, the α_{2A}-AR antagonist and antidepressant, mirtazapine, is beneficial in treating social fears in dogs (Arguelles et al., 2017), while selective α_{2C}-AR antagonists are anxiolytic (Sallinen et al. 1998a). This paradox reflects how non-selective α_2-AR agonists (e.g. dexmedetomidine) and selective α_{2C}-AR antagonists (ORM-10921) differently modify NA release. This may be due to differences in anatomical location and function of α_{2A} and α_{2C}-AR and how ligands differently compete with NA at these receptors (see Uys et al. 2017a for review). Agonists also tend to more bluntly shut down noradrenergic neurotransmission by inhibiting NA release whilst antagonists may work through block of postsynaptic α_2-AR as well as heteroreceptors.

The current study has some limitations that need to be considered. Assessing total tissue monoamine levels at a single time point can reflect altered rate of synthesis, rate of metabolism and rate of release. In vivo microdialysis studies could assist in determining which factors contribute to the observed tissue level changes. Also, the effects of three doses of ORM-10921 on hippocampal monoamine and metabolite levels were compared with those of a single dose of IMI and IDAZ. However, the primary unknown in this study was ORM-10921, with IMI and IDAZ used as reference controls at doses known to be active. Nevertheless, caveats such as single dose and differences in metabolism offer alternative explanations to the findings.

In conclusion, selective engagement of the α_{2C}-AR with ORM-10921 as opposed to non-selective binding to α_2-AR present with distinct, albeit different, hippocampal monoamine responses that are comparable to a known antidepressant, IMI. With α_{2C}-AR expressed at high levels in the hippocampus, the higher selectivity and receptor affinity of ORM-10921 compared to IDAZ at these receptors (Sallinen et al. 2013) may be the key driver for the differences in how the two compounds modulate monoamine levels and exert behavioural (antidepressant) effects. Indeed, selective α_{2C}-AR antagonists are more effective, while having no sedative effects (Uys et al. 2017b) as well as pose a lower propensity for cardiovascular and respiratory side effects. These preliminary findings suggest that selective α_{2C}-AR antagonists, ORM-10921 in particular, should be investigated as a pharmacological intervention in major depressive disorder in humans, as well as in animals presenting with anxiety and depressive-like symptoms.

Author contributions

BHH designed the study and the original protocol and prepared the manuscript. MMU contributed towards the study design, conducted all experiments and processed the data and contributed to the preparation of the manuscript. FPV contributed towards the bioanalysis of the brain samples. MS contributed towards the study design and preparation of the manuscript. QS and LM advised on article concept and writing.

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Declaration of Competing Interest

FPV, MMU, QS and LCRM have no conflicts of interest to declare. MS was an employee of Orion Pharma at the time this work was undertaken. Over the past three years, BHH has participated in advisory boards and received honoraria from Servier, and has received research funding from Servier, Lundbeck, HG&H Pharma, and Wildlife Pharma. BHH declares that, except for income from the primary employer and research funding from the above-mentioned organisations and agencies, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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