

α_2 -adrenoceptor binding in Flinders-sensitive line compared with Flinders-resistant line and Sprague-Dawley rats

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Objectives: Disturbances in the noradrenergic system, including alterations in the densities of α_2 -adrenoceptors, are posited to be involved in the pathophysiology of depression. In this study, we investigate the binding of α_2 -adrenoceptors in regions relevant to depression in an animal model of depression.

Methods: Using *in vitro* autoradiography techniques and the selective α_2 -ligand, [3 H]RX 821002, we investigated the density of α_2 -adrenoceptors in female Flinders-sensitive line (FSL) rats, a validated model of depression, and in two traditional control groups – female Flinders-resistant line (FRL) and Sprague-Dawley (SD) rats.

Results: The α_2 -adrenoceptor density was increased in most regions of the FSL rat brain when compared with SD rats (10% across regions). Moreover, the α_2 -adrenoceptor density was further increased in the FRL rats compared with both FSL (10% across regions) and SD rats (24% across regions).

Conclusions: The increase in α_2 -adrenoceptor binding in cortical regions in the FSL strain compared with the SD control strain is in accord with α_2 -adrenoceptor post-mortem binding data in suicide victims with untreated major depression. However, the differences in binding observed in the two control groups were unexpected and suggest the need for further studies in a larger cohort of animals of both sexes.

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Significant outcomes

- This study showed an increased α_2 -adrenoceptor binding across regions in the female Flinders-sensitive line (FSL) animals compared with control Sprague-Dawley (SD) rats.
- Increased binding in the FSL rats is in line with binding studies of brains from depressed suicide victims.
- Surprisingly, the density was further increased in Flinders-resistant line control rats when compared with both FSL and SD rats.

Limitations

- The animals used in this study were the sham controls of another study, and they were, therefore, handled daily for 10 days before the brains were removed.
- The sample size was small in some groups of animals, which might have masked significance in some regions.
- Female rats were studied because of the higher incidence rates of depression in females, making it difficult to compare data with previous studies carried out entirely on male animals.

Introduction

The World Health Organization expects that by 2030 depression will be the main burden of disease worldwide and the leading cause of ‘Years of life lived with disability’ (1). Today, ~350 million people globally are estimated to suffer from depression. Clinical symptoms can include anhedonia (i.e. inability to experience pleasure), feelings of guilt and low self-esteem, anxiety, disturbed sleep architecture and appetite, and suicidal ideation (2).

In the last 50 years, the monoamine hypothesis has dominated the understanding of the pathophysiology of depression, suggesting that a deficiency in monoamine transmission in the brain may lead to depression (3). Studies on the distribution of the monoaminergic receptors and transporters in the limbic, thalamic, and cortical neuronal circuits support that impairments in the serotonergic and noradrenergic systems are involved in the behavioural manifestations of depression (4).

Animal models simulating features of depressive disorders have been used in the study of the neurochemical mechanisms involved in depression. An example of a genetic model used to induce a partial depressive phenotype is the Flinders-sensitive line (FSL) rat model. Originally, the FSL rats and Flinders-resistant line (FRL) rats were selectively bred on a Sprague-Dawley (SD) background to differ in their sensitivity to an anticholinesterase compound (5). The FSL rats represent an animal model of depression with cholinergic supersensitivity (6,7), which is also a predisposing factor to depression in humans (8). Since the 1980s, the FRL has been used as a control strain for FSL in behavioural and biological studies as often as the background SD strain (9–11).

The FSL rat model meets the major criteria of high face, construct, and predictive validities for research on behavioural, neurochemical, and pathophysiological features of depression (7,10,12). In addition, the FSL rat exhibits certain characteristics analogous to depressed individuals – for instance, reduced appetite, disrupted sleep patterns, reduced activity, and abnormal immune function. Some antidepressant treatments can reverse the depressive behaviour of increased immobility in the forced swim test (7,13) and the suppression of neuronal plasticity in the FSL rats (14), reflecting the predictive validity for traditional antidepressant drug screening.

Among the symptoms of major depression are disruptions in attention, memory, and sleep, which are in part controlled by the central noradrenergic system. Post-mortem studies in the brains of suicide victims with major depressive disorder have confirmed increased α_2 -adrenergic receptor density in the

temporal and prefrontal cortex (15,16), hippocampus (17), and locus ceruleus (18). Moreover, De Paermentier et al. (16) found that antidepressant-treated suicide victims had significantly lower levels of α_2 -adrenoceptors in the cortex, hippocampus, caudate, and amygdala compared with controls, suggesting a normalising effect of antidepressants on the α_2 -adrenoceptors. Pre-clinically, Subhash et al. (19) also found a decrease in α_2 -adrenoceptors in cortical areas, but not in hippocampal areas, in the adult male rat brain after chronic treatment with tricyclic antidepressants. Conversely, other studies found no difference in α_2 -adrenoceptor binding in suicide victims compared with controls (20,21), and results remain inconclusive, probably due to the heterogeneities in depressive phenotypes, causes of death, and lack of complete knowledge about ante-mortem drug exposure(s).

The α_2 -adrenoceptors have a widespread distribution throughout the brain: they are localised pre-synaptically on noradrenergic nerve terminals and on the cell body of noradrenergic neurons of the pontine nuclei such as locus ceruleus, allowing them to modulate the release of noradrenaline via a negative-feedback mechanism (22). In the terminal projection areas, they are localised either pre-synaptically on afferent dopaminergic and serotonergic terminals or post-synaptically on intrinsic cell bodies (23,24). The vast majority of α_2 -adrenoceptors are located post-synaptic to noradrenaline terminals (25).

Aims of the study

This study aimed to investigate the densities of α_2 -adrenoceptors in different brain regions of the female FSL rats compared with its two traditional control groups – female FRL rats and SD rats. A single concentration binding study with the selective tritiated α_2 -adrenoceptor antagonist, [3 H]RX 821002, was used in an attempt to further investigate the noradrenergic system in the FSL rat model of depression.

Methods and Materials

Animals

This study was approved by the Danish Committee on Ethics in Animal Experimentation (2007/561-1378). Adult (12–14 weeks old at the start of the study) female FSL rats ($n = 8$) and FRL rats ($n = 6$) from breeding colonies maintained in the animal quarters of the Centre for Psychiatric Research, Aarhus University Hospital, were used along with female SD rats ($n = 5$) from Taconic farms located in Denmark. They were kept on a normal 12-h light/12-h dark cycle and had free access to food

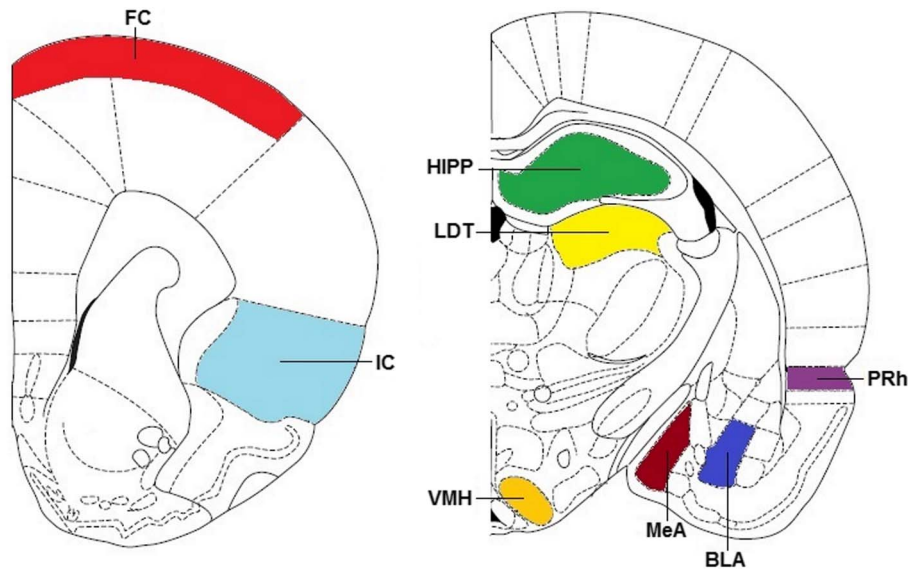


Fig. 1. A schematic presentation of the regions investigated as FC, IC, HIPP, LDT, VMH, MeA, BLA, and PRh. Adapted from Paxinos rat brain atlas at anterior–posterior: 12 (left) and 6.00 mm (right) (27). FC, frontal cortex; IC, insular cortex; HIPP, hippocampus; LDT, laterodorsal thalamic nucleus; VMH, ventromedial hypothalamic nucleus; MeA, medial amygdaloid nucleus; BLA, basolateral amygdaloid nucleus; PRh, perirhinal cortex.

and water. Rats were housed two per cage (average weight: 200–250 g). All the animals used in this study were from groups of animals that received sham treatment as part of another study about the effects of electroconvulsive shocks (26). The sham animals were handled once daily for 1–2 min for 10 consecutive days and had ear clips briefly attached but no current was passed.

Tissue preparation

Within 48 h of the last sham session, the rats were decapitated using a guillotine. The brains were rapidly removed and cut along the sagittal midline into two hemispheres after removing the cerebellum. The hemispheres were then flash-frozen using isopentane cooled to -40°C with dry ice and stored at -80°C until further processing. Each hemisphere was cut into 20- μm -thick coronal sections using a cryostat (Microm HM 500 OM Cryostat, Microm, Walldorf, Germany) at -20°C and thaw-mounted on Polysine adhesion microscope slides (Thermo Scientific, Braunschweig, Germany). The slides were stored at -80°C until the binding studies were performed.

Autoradiography

Slices at the approximate levels of the frontal cortex (FC, anterior–posterior: 12.24–11.76 mm), the insular cortex (IC, anterior–posterior: 12.24–11.76 mm), the perirhinal cortex (PRh, anterior–posterior: 6.00–

5.76 mm), the basolateral amygdaloid nucleus (BLA, anterior–posterior: 6.00–5.76 mm), the medial amygdaloid nuclei (MeA, anterior–posterior: 6.00–5.76 mm), the ventromedial hypothalamic nucleus (VMH, anterior–posterior: 6.00–5.76 mm), the laterodorsal thalamic nucleus (LDT, anterior–posterior: 6.00–5.76 mm), and the dorsal hippocampus (HIPP, anterior–posterior: 6.00–5.76 mm) were selected according to the stereotaxic co-ordinates of the rat brain atlas (27) (Fig. 1). For each region, two adjacent slides were used to measure total binding and a third was used to measure non-specific binding.

In vitro autoradiography was carried out according to the method of Booze et al. (28) with some modifications. In brief, the slides were thawed at room temperature (22°C) for 5 min and pre-incubated in baths containing 25 mM GLYGLY (Sigma-Aldrich, St. Louis, MO, USA) buffer (pH 7.6) for 5 min. The incubations with 2 nM [^3H]RX 821002 (Specific activity: 40–70 Ci/mmol; PerkinElmer, Skovlunde, Denmark) were carried out at 22°C in the same buffer for 90 min. Non-specific binding was determined by the addition of 10 μM unlabelled phentolamine (Sigma-Aldrich). After incubation, the slides were washed twice for 2 min in fresh buffer (4°C), rinsed quickly in distilled water (4°C) to remove buffer salts, dried under a steam of cold air, and stored overnight for further drying under vacuum. On day 2, the slides were placed in standard film cassettes (BAS-Cassette 2025; Fujifilm, Tokyo, Japan) along with Tritium standards (American Radiolabeled Chemicals, St. Louis, MO, USA) and

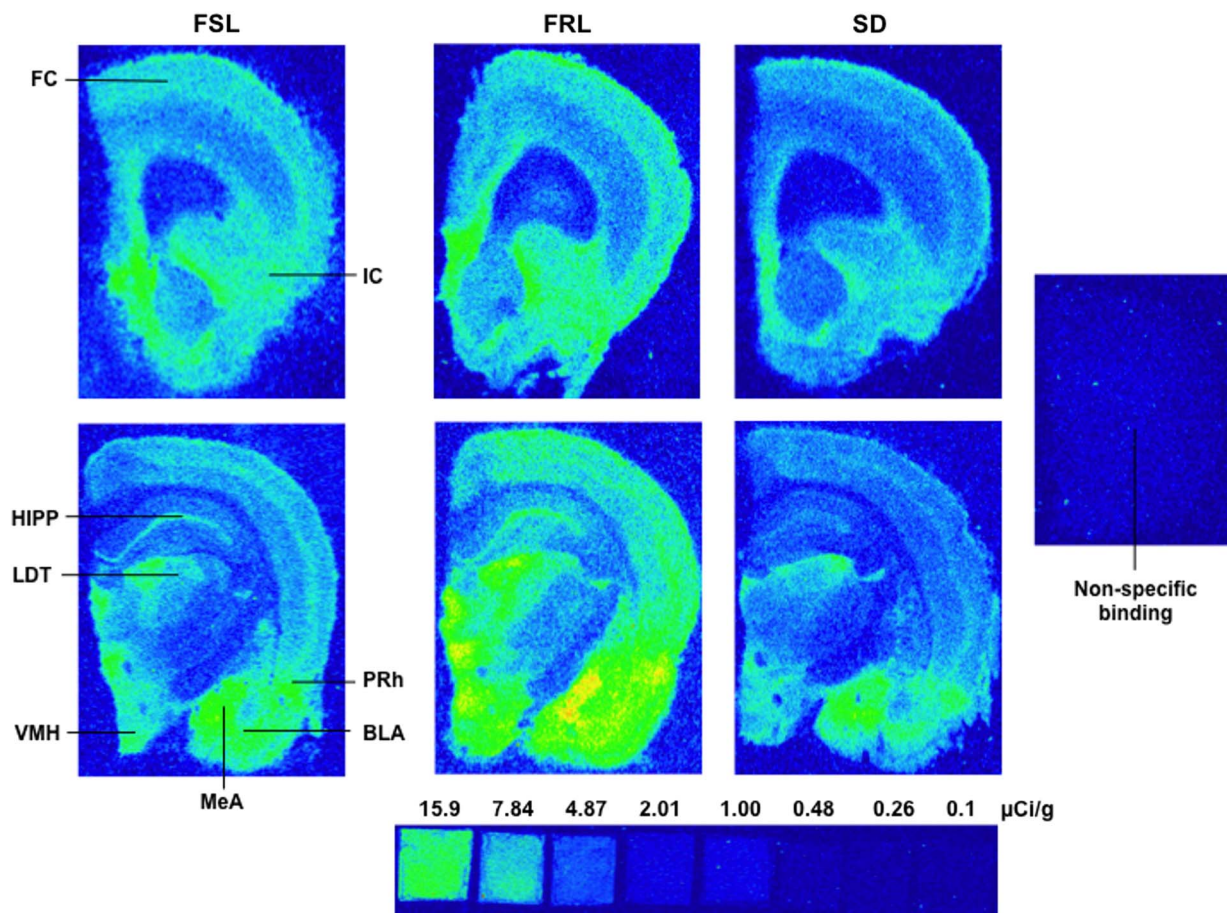


Fig. 2. A set of representative autoradiograms illustrating the regional distribution of α_2 -adrenoceptor binding or non-specific binding obtained at different coronal sections corresponding to the anterior–posterior: 12.24–11.76 (upper) and 6.00–5.76 mm (lower) (27) from FSL, FRL, and SD groups. FSL, Flinders-sensitive line; FRL, Flinders-resistant line; SD, Sprague-Dawley; FC, frontal cortex; IC, insular cortex; HIPP, hippocampus; LDT, laterodorsal thalamic nucleus; VMH, ventromedial hypothalamic nucleus; MeA, medial amygdaloid nucleus; BLA, basolateral amygdaloid nucleus; PRh, perirhinal cortex.

exposed to blank tritium storage phosphor screens (BAS-IP TR2025 Fuji Imaging Plate; Fujifilm Corporation, Tokyo, Japan) for 6 days at room temperature. On day 7, the screens were removed from the cassettes and scanned immediately using a Fujifilm BAS-5000 Phosphoimager (Fujifilm, Tokyo, Japan).

Autoradiography analyses

The imaging plates were analysed using the software ImageGauge 4.0 provided with the imager. Photo-stimulated luminescence values per mm^2 obtained after background subtraction were calibrated using the Tritium microscales with concentrations ranging from 0.1 to 15.9 $\mu\text{Ci/g}$ (American Radiolabeled Chemicals, Saint Louis, MO, USA). As mentioned above, the total binding was measured in manually drawn regions of interest encompassing the PRh, layer II-III of the FC, the IC, the BLA, the MeA, the VMH, the LDT, and a

HIPP region covering the CA1-CA3 fields (Fig. 1). Non-specific binding for each rat was measured on the slides incubated with both tracer and blocker: circular regions were drawn on each brain slice, as the non-specific binding was homogeneous and an average for each region was made for each rat. These averages were subtracted from the average total binding measurements for corresponding rats to find the specific binding levels. Some slides were excluded due to tissue damage during the cryostat sectioning.

Statistical analyses

Initially, the groups were confirmed to be normally distributed using the Normal Q–Q Plot and the Shapiro–Wilks test and to have equal variance using the Bartlett’s test in statistical R software version 3.1.0 (29). Using GraphPad Prism (GraphPad software Inc, version 5.0), one-way analysis of variance (ANOVA) was performed in each region for comparing control and ‘depressed’

Table 1. Regional values of [3 H]RX 821002 densities

ROIs	FSL	N	FRL	N	SD	N
HIPP	7.28 \pm 1.29	(8)	8.20 \pm 0.61	(6)	6.49 \pm 0.80	(4)
LDT	17.64 \pm 1.24	(8)	22.69 \pm 0.91 ^{a,c}	(6)	16.33 \pm 0.74	(4)
VMH	18.03 \pm 0.98	(8)	22.12 \pm 1.43	(4)	17.45 \pm 1.64	(4)
MeA	28.79 \pm 1.26	(8)	33.81 \pm 0.92 ^{a,c}	(4)	28.54 \pm 0.80	(4)
BLA	25.04 \pm 1.41	(8)	28.09 \pm 0.96	(6)	23.41 \pm 2.97	(4)
PRh	25.96 \pm 0.51 ^b	(6)	29.18 \pm 0.91 ^{a,c}	(6)	22.24 \pm 0.84	(4)
FC	12.67 \pm 0.44 ^b	(8)	12.15 \pm 0.22 ^c	(6)	10.49 \pm 0.26	(5)
IC	17.35 \pm 0.61 ^b	(7)	17.20 \pm 0.24	(6)	15.28 \pm 0.49	(5)

BLA, basolateral amygdaloid nucleus; FC, frontal cortex; FRL, Flinders-resistant line; FSL, Flinders-sensitive line; HIPP, hippocampus; IC, insular cortex; LDT, laterodorsal thalamic nucleus; MeA, medial amygdaloid nucleus; N, number of measurements; PRh, perirhinal cortex; ROIs, regions of interest; SD, Sprague-Dawley; VMH, ventromedial hypothalamic nucleus.

Data are represented as mean [μ Ci/g] \pm standard error of the mean.

^a $p < 0.05$ between FSL and FRL; ^b $p < 0.05$ between FSL and SD; ^c $p < 0.05$ between FRL and SD.

groups followed by a Bonferroni multiple comparison test between all groups. Differences were considered significant if the probability of error was $< 5\%$.

Results

Photostimulated luminescence signals attributed to [3 H]RX 821002 binding were observed in different areas of the rat brain, and representative images at both coronal sections are shown in Fig. 2. The quantitative results, calculated from the measured light densities and the linear calibration curve created from the tritium microscale, are expressed as mean \pm SEM and are presented in Table 1.

Our *a priori* hypothesis was that the two control groups (FRL and SD) had equal receptor densities, and therefore could be merged into one group. However, both visual observation of the raw data (Fig. 2) and *post-hoc* analysis with Bonferroni correction revealed significant differences in four out of eight brain regions between the FRL and SD rats. Careful inspection of the raw autoradiograms (Fig. 2) also suggested that the low number of measurements in both the FRL and the SD groups may have obscured significant differences in the remaining regions including MeA, BLA, and IC. As this suggested unexpected differences between the two 'control' groups, we decided to run the analysis treating the FRL as a second experimental group.

One-way ANOVA was performed to determine the significant differences between the three rat groups for each region (Factor: strain). In the LDT, significant differences were found between the FSL and FRL rats (95% CI: -9.12 to -0.97 , $p < 0.05$) and between the FRL and SD rats (95% CI: 1.49 to 11.23 , $p < 0.01$). Similarly, in the MeA, significant

differences were found between the FSL and FRL rats (95% CI: -6.86 to -3.18 , $p < 0.0001$) and between the FRL and SD rats (95% CI: 3.15 to 7.40 , $p < 0.0001$). In the PRh, a significant difference was found between all rat groups, FSL versus FRL (95% CI: -4.43 to -2.01 , $p < 0.0001$), FSL versus SD (95% CI: 2.37 to 5.07 , $p < 0.0001$), and FRL versus SD (95% CI: 5.59 to 8.29 , $p < 0.0001$), whereas in the FC a significant difference was found between the FSL and SD rats (95% CI: 0.77 to 3.59 , $p < 0.01$) and the FRL and SD rats (95% CI: 0.17 to 3.16 , $p < 0.05$). In the IC, a significant difference was also found between the FSL and SD rats (95% CI: 0.15 to 3.99 , $p < 0.05$).

When the FRL group was compared with the FSL group, it was found that the FRL group had a higher density of α_2 -adrenoceptors in most of the brain regions of interest (10% across regions), and significant differences were found in three out of eight regions. Comparing the FSL animals with the SD group, a greater mean α_2 -adrenoceptor binding was found in most of the regions (10% across regions), and this reached statistical significance in three out of eight regions. An increased binding of α_2 -adrenoceptors was found in all the regions of the FRL brains compared with the SD group (24% across regions), and significant differences were found in four out of eight regions.

Discussion

The main finding in this study is the demonstration of neurobiological differences between the female FSL, FRL, and SD rats with respect to α_2 -adrenoceptors. The FSL rats have been found to be a valid model of some symptoms present in depression (10). We found significantly increased α_2 -adrenoceptor binding in the cortical regions of the FSL rats compared with control SD rats. This is consistent with our initial hypothesis based on studies in suicide victims with untreated major depression (16,17,30) and also with the suggested therapeutic action of antidepressants being partly ascribed to the downregulation of cortical α_2 -adrenoceptors in rat brain (19). The lack of significant differences in some of the limbic regions, despite a small trend (Table 1), may be the result of the modest sample size of this study.

Hippocampus, amygdala, thalamus, hypothalamus, PRh, IC, and FC have all been implicated to varying degrees in the aetiology of depression. Neuroimaging studies of patients with major depression have identified neurophysiological abnormalities in all these brain areas (4,31). We, therefore, examined α_2 -adrenoceptor binding in these cortical and sublimbic regions, which play a crucial role in depression as seen from a therapeutic standpoint (31).

Table 1 shows the regional distribution of receptor densities and demonstrates high variability across regions but consistency across animals. In all three strains, the highest densities of α_2 -adrenoceptors among the regions investigated were found in the two amygdaloid areas and in the PRh. The PRh, a subdivision of the medial part of the temporal lobe, plays an essential role in visual perception as well as in memory formation (32), and in humans decreased expression of glutamatergic receptors has been previously found in the PRh of patients with major depressive disorder (33). The PRh has strong reciprocal connections to higher cortical sensory areas and other memory-related structures, including the amygdala and the hippocampal formation, which are mostly involved in the regulation of emotions, anxiety, and memory processes (34). Hippocampus and amygdala are the major central structures that control the hypothalamus–pituitary–adrenal axis, and damage or abnormal activity in either of these areas, such as altered noradrenergic receptor function, could start a vicious cycle predisposing an individual to an episode of depression (35,36).

The LDT, which was investigated in this study, provides massive projections to the FC in the rat brain and plays a role in learning and memory (37). The information flux in the thalamo-cortical pathway has shown to be reduced in a stressed model of depression, suggesting a decreased control of cortical activity by the thalamus, which can be reflected by cognitive deficits (38). Moreover, patients with major depressive disorder have an elevated number of neurons in the limbic thalamus relative to non-depressed controls (39). Collectively, the impairments in the firing pattern between the LDT and the FC and the increased number of α_2 -bearing neurons in the thalamus may contribute to the increased adrenoceptor density found in our laterodorsal thalamic region in the FSL and FRL rats.

Alternatively, and this possibility exists in all our examined regions, a sustained functional decrease in noradrenaline release, most likely due to increased number or over-activity of α_2 inhibitory autoreceptors localised on the neuronal cell bodies of locus coeruleus, would lead to compensatory upregulation of the postsynaptic α_2 -receptors in the projections areas. To address this hypothesis, it would have been necessary to perform autoradiography at the level of the brainstem and pontine nuclei housing the noradrenergic cell bodies. Unfortunately, the original study required the two halves of the brain to be processed separately for different analyses, and the brainstem areas were insufficiently preserved, especially medially, to allow for adequate sectioning and labelling.

Other neurochemical differences between the FSL rat model of depression and its two controls have been

described in the literature. Overstreet et al. (40) found FSL rats to have higher concentrations of both the muscarinic acetylcholine receptor and endogenous acetylcholine than their controls, FRL rats, in specific brain regions, explaining their increased sensitivity to anticholinergic drugs. Differences in the serotonergic systems have also been found; Hasegawa et al. (41) described a reduction in serotonin synthesis in the FSL rats compared with both FRL and SD rats, an effect ameliorated by chronic citalopram treatment (42). Moreover, the glutamate receptors have been found to be reduced in the FSL rats compared with both the controls (43). Overall, the neurochemical phenotype of the FSL rats is distinct from its two controls, displaying a pattern mimicking what has been found in brains of depressed patients.

Our use of two control groups proved to be a strength in detecting important findings. The higher binding found in FRL rats compared with SD rats was surprising, and only little is known about the differences between FRL and SD rats. Although the FRL strain is an inbred line, originally derived from the outbred SD rats, they are, in the literature, used interchangeably with SD rats as controls for FSL rats. The present results suggest that such substitution should be done with care. Moreover, the concept of SD rats as a uniform entity is questionable, due to the many different lines bred by commercial suppliers today, where variable husbandry practices may lead to variable early-life experiences of which we are not aware.

The fact that female rats were investigated in this study is important for balancing studies in male and female models, and it is also a strength because higher depression rates are observed in women than in men. However, differences between male and female Flinders rats have not been thoroughly investigated to account for validity of the FSL/FRL females as a proper model system for affective disorders.

The condition under which the study was conducted may have exacerbated the differences between the two control strains: studies suggest that the FRL animals express the most differences from the FSL during stressful situations (9,44). The animals whose brains were used in this autoradiography study were the control (sham) animals of a larger study regarding the effects of electroconvulsive shocks (26). As such, all groups were handled for 10 days, a situation that could produce mild stress in more sensitive rat strains such as the FRL and FSL rats, and may have possibly played a role in the upregulation of α_2 -adrenoceptors in this study.

The present study should be replicated in a larger group of animals of both sexes, bred and raised together and tested at multiple time points before final conclusions for or against the validity of FRL animals as controls can be reached.

In conclusion, our findings of higher α_2 -adrenoceptor density, especially in cortical regions, in the FSL animals compared with control SD rats is consistent with human post-mortem studies and further validate the implication of the noradrenergic system in the pathophysiology of depression and support the FSL rat as a model of depressive/anxiety type disorders with construct validity. Furthermore, our preliminary findings in a small sample of female animals suggest that it is advisable to always include SD rats as an extra control for the FSL rat model when conducting neurochemical studies.

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Conflicts of Interest

G.W. is Editor-in-Chief in *Acta Neuropsychiatrica*. However, G.W. actively withdrew from any decisions related to the review of the present manuscript.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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