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A role for serotonin in the antidepressant activity of N^G-Nitro-L-arginine, in the rat forced swimming test

Valentina Gigliucci [a], Kathleen Niamh Buckley [a], John Nunan [b], Karen O'Shea [b], Andrew Harkin [a,b,*]

[a] Neuropsychopharmacology Research Group, School of Pharmacy & Pharmaceutical Sciences and Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland
[b] Department of Pharmacology & Therapeutics, National University of Ireland, Galway, Ireland

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ABSTRACT

The present study determined regional serotonin (5-HT) synthesis and metabolism changes associated with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NA) and the influence of 5-HT receptor blockade in the antidepressant-like actions of L-NA in the forced swimming test (FST). Regional effects of L-NA (5,10 and 20 mg/kg i.p.) on tryptophan hydroxylase (TPH) activity, the rate limiting enzyme for 5-HT synthesis, were determined by measuring accumulation of the transient intermediate 5-hydoxytryptophan (5-HTP) following in vivo administration of the amino acid decarboxylase inhibitor, NSD 1015 (100 mg/kg). L-NA (5–20 mg/kg) dose dependently increased 5-HTP accumulation, particularly in the amygdaloid cortex, following exposure to the FST. L-NA also provoked an increase in regional brain 5-HIAA concentrations and in the 5-HIAA:5-HT metabolism ratio. Co-treatment with NSD-1015 failed to consistently modify the antidepressant-like effects of L-NA in the FST. Sub-active doses of L-NA (1 mg/kg) and the 5-HT re-uptake inhibitor fluoxetine (2.5 mg/kg) acted synergistically to increase swimming in the test. Co-treatment with the non-selective 5-HT receptor antagonist metergoline (1, 2 and 4 mg/kg), attenuated the L-NA (20 mg/kg)-induced reduction in immobility and increase in swimming behaviours. Metergoline alone however provoked an increase in immobility and reduction in swimming behaviours in the test. A similar response was obtained following co-treatment with the preferential 5-HT_{2A} receptor antagonist ketanserin (5 mg/kg) and the 5-HT_{2C} receptor antagonist RO-430440 (5 mg/kg). Co-treatment with the 5-HT_{1A} receptor antagonist WAY 100635 (0.3 mg/kg) or the 5-HT_{1B} receptor antagonist GR 127935 (4 mg/kg) failed to influence the antidepressant-like activity of L-NA. Taken together these data provide further support for a role for 5-HT in the antidepressant-like properties of NOS inhibitors.

1. Introduction

To date a number of studies have demonstrated that inhibition of nitric oxide synthase (NOS) produces anxiolytic and antidepressant-like behavioural effects in a variety of animal paradigms (da Silva et al., 2000; Harkin et al., 1999, 2003, 2004; Jeffreys and Funder, 1996; Mutlu et al., 2009; Spiacci et al., 2008; Spolidório et al., 2007; Ulak et al., 2008; Yildiz et al., 2000a,b). Previously we reported that the NOS inhibitors, N^G-nitro-L-arginine (L-NA) and 7-nitroindazole (7-NI), dose dependently reduce immobility and increase swimming behaviour in the rat and mouse forced swimming test (FST), a test predictive of antidepressant activity (Harkin et al., 1999, 2003). These effects are overcome by administration of the NOS substrate, L-arginine, consistent with an involvement of NO in this behavioural response (Harkin et al., 1999; Jefferys and Funder, 1996; Joca and Guimarães, 2006; Volke et al., 2003; Yildiz et al., 2000a). However, it is presently unclear whether monoaminergic systems are involved in the antidepressant-like effects of NOS inhibitors.

The FST is the most widely employed screening test for antidepressants in rodents (Cryan et al., 2002a; Porsolt et al., 1978). When rodents are placed in a cylinder of water without an opportunity for escape, they typically display an immobile posture that is said to reflect a state of "behavioural despair". A variety of antidepressant drugs increase escape-oriented behaviour in the test. The test has undergone some adaptations since its introduction to improve its sensitivity and ability to discriminate between different classes of antidepressants. Lucki et al. (Detke et al., 1995; Lucki, 1997) adapted the original FST to score distinct active behaviours elicited by different classes of antidepressant drugs. Specifically, selective serotonin (5-HT) re-uptake inhibitors (SSRIs) and 5-HT receptor agonists provoke swimming behaviour (Cryan and Lucki, 2000; Detke et al., 1995; Page et al., 1999), whereas tricyclic antidepressants (TCAs) and selective noradrenaline and dopamine re-uptake inhibitors elicit climbing behaviour in the FST (Cryan et al., 2002b; Detke et al., 1995; Page et al., 1999; Reneric and Lucki, 1998). Thus the modified FST is employed as a behavioural tool to assess the role of monoamines in antidepressant action.

* Corresponding author. Neuropsychopharmacology Research Group, School of Pharmacy & Pharmaceutical Sciences and Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland. Tel.: +353 1 8962807; fax: +353 1 8962821.
E-mail address: aharkin@tcd.ie (A. Harkin).

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A more detailed analysis of the behavioural effects of NOS inhibitors in the FST offers clues to their potential mechanism of action which may involve monoamine neurotransmitters. The behavioural profile of NOS inhibitors in the adapted FST for rats parallels that obtained with the SSRI, fluoxetine. Moreover, depletion of endogenous 5-HT blocks the antidepressant-like activity associated with NOS inhibitors in the test (Harkin et al., 2003). From these observations it was proposed that NOS inhibitors may elicit their antidepressant-like activity in the FST through a 5-HT dependent mechanism. To further explore this mechanism we examined the ability of L-NA to influence the 5-HT synthetic pathway and 5-HT metabolism in the FST. Moreover, we examined if antagonists to 5-HT receptor subtypes could block the antidepressant-like activity of L-NA in the paradigm.

2. Materials and methods

2.1. Subjects and drug treatment

Male Sprague–Dawley rats (Harlan Olac, Bicester, UK) weighing approximately 250–300 g were used in this experiment. Rats were housed in groups of four and maintained on a 12 h:12 h light:dark cycle (lights on at 8 am) in a temperature controlled room (21–22 °C). Food and water were available *ad libitum*. L-NA (Sigma Aldrich Ireland), m-hydroxybenzylhydrazine dihydrochloride (NSD-1015, Sigma) metergoline (Sigma), fluoxetine hydrochloride (Eli Lilly and Co., USA), N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1′-biphenyl-4-carboxamide hydrochloride (GR 127935, Glaxo Wellcome, UK) and 4-fluoro-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-2-pyridinyl benzamide (WAY 100635 maleate, Wyeth, UK) were dissolved in saline and administered in an injection volume of 2 ml/kg. Ketanserin tartrate (Sigma) and benzofuran-2-carboxamidine (RO 430440, Hoffman-La Roche, Switzerland) were dissolved in 0.5% tween saline and administered in an injection volume of 1 ml/kg. All drugs were administered by the intraperitoneal (i.p.) route. Controls received injections of saline or tween saline in the same volume as the test groups. All experiments were in compliance with the European Community's Council directive, 1986 (86/609/EEC). All experiments were performed using independent groups of animals.

2.2. Forced swimming test

This test was performed using a modification of the method described by Detke et al. (1995). On the first day of the experiment, the rats were placed individually into a container 40 cm high and 18 cm in diameter containing 30 cm of water at 23 °C. The animals were left to swim in the water for 15 min before being removed, allowed to dry beside a heater and returned to their home cage. The animals received their first vehicle/drug injection 15 min after the first exposure to the FST. The second and third vehicle/drug injections were administered 5 h and 1 h prior to the second exposure to the FST exposure 24 h later. The drug administration protocol followed is standard for testing pharmacological agents in the rat FST (see Cryan and Lucki, 2000; Detke et al., 1995; Porsolt et al., 1978).

In the second exposure to the FST, rats were allowed to swim for a duration of 5 min, and their behaviour was videotaped from above using a CCD (Sanyo VCB 3372 high resolution monochrome) camera. The sequence of testing was randomised throughout the experiment so as to minimize any confounding effects of order of testing.

Behavioural scoring was performed using the manual event recording capability of the Ethovision, videotracking and behavioural analysis system (Noldus IT, The Netherlands). Keys (computer keyboard) were assigned to individual behaviours and used to record the duration of these behaviours in the test from video footage. We use event duration when scoring as opposed to a time sampling technique used by others (Cryan and Lucki, 2000; Detke et al., 1995). The main difference between these techniques is that time sampling provides an approximation of the behaviours scored. Behaviours scored included immobility, swimming and climbing. Immobility was defined as the animal floating in the water without struggling and making only those movements necessary to keep its head above the water. Swimming was scored as the animal making active swimming motions more than necessary to keep its head above water and involved moving horizontally or crossing the swim tank. Climbing behaviour consisted of the animal making active upward movements with forepaws in and out of the water directed against the walls of the swim chamber. These behaviours were scored in a mutually exclusive fashion throughout the trial. The rater of the behaviour was blind with respect to the drug treatment being scored.

2.3. Experimental plan

2.3.1. Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST

Rats were assigned to one of four groups: Group 1: Vehicle; Group 2: L-NA (5 mg/kg); Group 3: L-NA (10 mg/kg); Group 4: L-NA (20 mg/kg). Immediately prior to the last treatment of L-NA, the groups were subdivided into those receiving co-treatment with NSD-1015 or vehicle control. Animals receiving NSD-1015 were euthanized immediately following the FST on test day and regional brain biogenic amine concentrations were determined. A parallel experiment was carried out with identical NSD-1015 treatment groups for comparison purposes, where regional brain biogenic amine concentrations were determined in animals not subjected to the FST.

Both 5-HTP and L-DOPA usually occur as transient intermediates and are not normally detected in brain tissue, but administration of NSD-1015 prevents the decarboxylation step common to both compounds and thus allows them to accumulate *in vivo* (Carlsson et al., 1972; Conley et al., 2007; Evans et al., 2009). The selection of this dose and time was determined from data generated in our laboratory indicating that NSD-1015 (100 mg/kg, i.p.) produces optimal increases in 5-HTP and L-DOPA concentrations in rat brain 1 h after administration without altering behaviour and well-being of the animal (Nolan et al., 2000).

2.3.2. The effect of combining sub-active doses of L-NA and fluoxetine in the FST

Rats were assigned to one of four groups: Group 1: Vehicle Control; Group 2: L-NA (1 mg/kg) control; Group 3 fluoxetine (2.5 mg/kg) control; Group 4 L-NA (1 mg/kg) in combination with fluoxetine (2.5 mg/kg). The selection of doses was determined from previous dose response studies undertaken in our laboratory (Harkin et al., 2003).

2.3.3. Dose related effects of metergoline on the antidepressant-like activity of L-NA in the FST

Metergoline is a non-selective 5-HT receptor antagonist and was used at doses effective in blocking the *in vivo* effects induced by 5-HT receptor agonists in rats (Golozoubova et al., 2006; Mokler et al., 1983; Stachowicz et al., 2007). Rats were assigned to one of eight groups: Group 1: Vehicle; Group 2–4: Metergoline (1, 2 and 4 mg/kg); Group 5: L-NA (20 mg/kg); Group 6–8: L-NA in combination with metergoline (1, 2 and 4 mg/kg).

2.3.4. The effect of ketanserin, RO 430440, WAY 100635 or GR 127935 on the antidepressant-like activity of L-NA in the FST

In a separate series of experiments, the involvement of the 5-HT receptor subtypes in the antidepressant-like activity of L-NA in the FST was examined. Rats were co-treated with L-NA and ketanserin, a preferential 5-HT_{2A} receptor antagonist (Van Oekelen et al., 2003),

RO 430440, a selective 5-HT_{2C} receptor antagonist [RO 430440 has been shown to have a selectivity for 5-HT_{2C} (pKi = 7.1) over 5-HT_{2A} (pKi = 5.3) receptors (International application published under the Patent Cooperation Treaty, Benzofuryl derivatives and their use WO 97/42183; See also Cryan et al., 2000], WAY 100635, a 5-HT_{1A} receptor antagonist (Fletcher et al., 1996) or GR 127935 (a 5-$HT_{1B/D}$ receptor antagonist) (Skingle et al., 1996). In each experiment rats were assigned to one of 4 groups: Group 1: Vehicle; Group 2: ketanserin (5 mg/kg) or RO 430440 (5 mg/kg) or WAY 100635 (0.3 mg/kg) or GR 127935 (4 mg/kg); Group 3: L-NA (20 mg/kg); Group 4: L-NA in combination with each antagonist outlined in group 2. A single dose of each antagonist, obtained from the literature, was used. These doses have previously been shown to block 5-HT receptor mediated behaviours in a range of test paradigms in rats (Cryan et al., 2000; Hawkins et al., 2008; Martínez-Mota et al., 2002).

2.3.5. Locomotor activity

Animals were removed from their home cage and placed individually into activity monitor cages (32 cm × 20 cm × 18 cm; length × width × height) which were connected to AM1051 data logger (Benwick Electronics). Each activity monitor is equipped with 2 sets of horizontal infrared beams, positioned 3 cm and 15 cm above the base of the cage. Both sets of beams consist of a 12 beam × 7 beam matrix, forming a grid of 66 × 2.54 cm^2 cells within the cage. Activity is recorded as the number of times a beam changes from unbroken to broken. On the day of testing animals were placed in one of five test chambers and their activity was monitored over a 20 min period. The effects of metergoline, ketanserin, RO-430440 alone and in combination with L-NA were examined in groups of animals independent of those exposed to the FST. The doses of drugs and the treatment regime used were as described for the FST.

2.4. Determination of regional biogenic amine concentrations

Immediately following the FST, animals treated with NSD 1015 were euthanized by decapitation and the frontal cortex, striatum, hippocampus and amygdala were dissected on an ice cold plate for biogenic amine measurements as previously described (Harkin et al., 2001). 5-hydroxy tryptophan, 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA), L-dihydroxyphenylalanine (L-DOPA) dopamine and noradrenaline concentrations were measured by high performance liquid chromatography (HPLC) coupled with electrochemical detection as previously described (Harkin et al., 2003). Brain tissue was sonicated in 1 ml of mobile phase that was spiked with 20 ng/50 µl of N-methyl 5-HT (Sigma Aldrich Ireland) as an internal standard. The mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.1 mM EDTA (BDH Chemicals, UK), 1.4 mM octane-1-sulphonic acid (Sigma Aldrich) and 10% (v/v) methanol (Lab-Scan, Ireland) and was adjusted to pH 2.8 using 4 N NaOH (BDH Chemicals, UK). Homogenates were centrifuged at 15,000 × *g* in a Hettich Mikro/K refrigerated centrifuge for 15 min. A 20 µl sample of each supernatant was injected onto a reverse phase column (LI Chrosorb RP-18, 25 cm × 4 mm internal diameter, particle size 5 µm) for separation of the neurotransmitters (flow rate 1 ml/min). Neurotransmitter concentrations were quantified by electrochemical detection (Shimadzu) and chromatograms were generated using a Merck-Hitachi D-2000 integrator. Results are expressed as nanogram of neurotransmitter per gram fresh weight of brain tissue.

2.5. Statistical analysis of data

Data are expressed as group mean with standard errors and were analysed using a one factor or two factor analysis of variance (ANOVA). If any statistically significant change was found, post hoc comparisons were performed using a Dunnett's or Student Newman Keuls test respectively. Data were deemed significant when $P<0.05$.

3. Results

3.1. Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST

ANOVA of time spent immobile showed effects of L-NA [$F(3,63) = 14.84$, $P<0.001$] and a L-NA × NSD-1015 interaction [$F(3,63) = 3.17$, $P = 0.03$]. Post hoc comparisons revealed that L-NA (10 and 20 mg/kg) reduced immobility time when compared to vehicle treated controls ($P<0.01$; $P<0.05$ respectively). L-NA (5, 10 and 20 mg/kg) also reduced immobility time in NSD-1015 treated animals when compared to NSD-1015 treated controls ($P<0.01$). NSD-1015 did not influence immobility time or the L-NA-induced reduction in immobility time when compared to their non NSD-1015 treated counterparts (Fig. 1A).

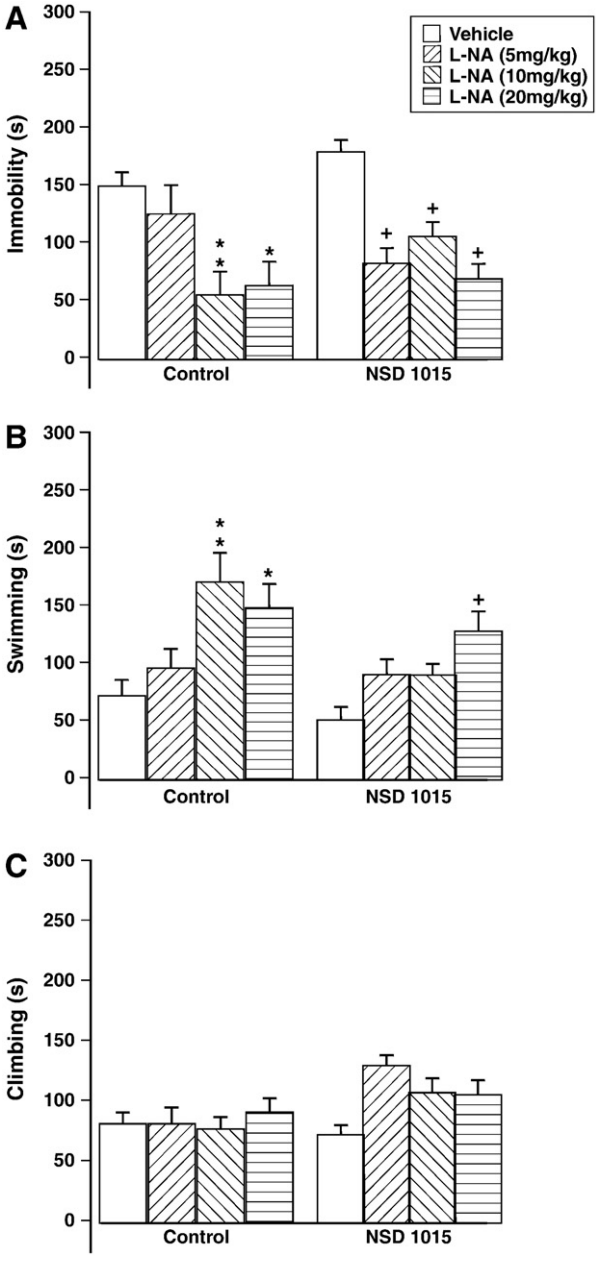

Fig. 1. Rats received L-NA (5, 10 and 20 mg/kg, i.p.) 24 and 5 h, and were co-treated with NSD-1015 (100 mg/kg, i.p.) 1 h, prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 7–10 animals. $^{*}P<0.05$; $^{**}P<0.01$ vs. Vehicle Control. $^{+}P<0.01$ vs. Vehicle + NSD-1015 group.

ANOVA of time spent swimming showed effects of L-NA [$F(3,63) = 9.73$, $P<0.001$] and NSD-1015 [$F(1,63) = 7.91$, $P = 0.007$]. Post hoc comparisons revealed that L-NA (10 and 20 mg/kg) increased swimming time when compared to vehicle treated controls ($P<0.01$; $P<0.05$ respectively). L-NA (20 mg/kg) also increased swimming time in NSD-1015 treated animals when compared to NSD-1015 treated controls ($P<0.01$). NSD-1015 did not influence swimming time when compared to vehicle treated controls. The L-NA (10 mg/kg)-induced increase in swimming time in NSD-1015 treated animals was reduced when compared to their non NSD-1015 treated counterparts ($P<0.01$), although co-treatment with NSD-1015 did not influence the response to L-NA (5 and 20 mg/kg) (Fig. 1B).

ANOVA of time spent climbing showed effects of NSD-1015 [$F(1,63) = 7.49$, $P = 0.008$] only. There were no effects of L-NA on the time spent climbing. Post hoc comparisons revealed that NSD-1015 did not influence climbing time when compared to vehicle treated controls (Fig. 1C).

3.2. L-NA increases regional brain 5-HTP accumulation and 5-HT metabolism following FST exposure

ANOVA of 5-HTP, 5-HT, 5-HIAA concentrations and the 5-HIAA:5-HT metabolism ratio failed to show effects of L-NA in the frontal cortex or striatum. A trend towards an increase in cortical 5-HTP concentrations following L-NA (20 mg/kg) administration was observed when compared to vehicle treated controls. However this failed to achieve significance [$F(3,33) = 2.55$, $P = 0.07$]. ANOVA of 5-HTP and 5-HT concentrations in the hippocampus failed to show effects of L-NA. Significant effects were observed for 5-HIAA [$F(3,36) = 4.5$, $P = 0.009$] and 5-HIAA:5-HT [$F(3,36) = 9.03$, $P<0.001$]. Post hoc comparisons revealed that 5-HIAA concentrations and 5-HIAA:5-HT ratios were increased in the L-NA (5, 10 and 20 mg/kg) treated groups when compared to vehicle treated controls (Table 1). ANOVA of 5-HTP concentrations in the amygdaloid cortex showed effects of L-NA [$F(3,34) = 8.63$, $P<0.001$]. Post hoc comparisons revealed a dose related increase in 5-HTP concentrations following L-NA(10 and 20 mg/kg) when compared to vehicle treated controls. Significant effects for 5-HIAA [$F(3,34) = 3.59$, $P = 0.023$] and 5-HIAA:5-HT [$F(3,34) = 6.39$, $P = 0.001$] were also observed in the amygdaloid cortex. Post hoc comparisons revealed that 5-HIAA concentrations and 5-HIAA:5-HT ratios were increased in the L-NA (5, 10 and 20 mg/kg) treated groups when compared to vehicle treated controls (Table 1). There were no effects of L-NA observed for L-DOPA, dopamine, its metabolites HVA and DOPAC or dopamine metabolism ratios in any of the brain regions tested with one exception. ANOVA of L-DOPA concentrations in the amygdaloid cortex showed effects of L-NA [$F(3,34) = 5.6$, $P = 0.003$]. Post hoc comparisons revealed a dose related increase ($P<0.05$, Dunnett's test) in L-DOPA concentrations following L-NA (10 and 20 mg/kg; 373 ± 18 and 408 ± 22 ng/g) when compared to vehicle treated controls (301 ± 10 ng/g).

Table 1
Effect of L-NA on regional brain 5-HTP accumulation following FST exposure.

Group	5-HTP Tissue concentration (ng/g wet weight)			
	Cortex	Striatum	Hippocampus	Amygdala
Vehicle	179 ± 10	364 ± 37	246 ± 12	344 ± 13
L-NA (5)	221 ± 21	431 ± 40	286 ± 18	403 ± 27
L-NA (10)	216 ± 11	398 ± 28	275 ± 10	411 ± 10*
L-NA (20)	223 ± 6	404 ± 49	280 ± 15	465 ± 16*
	5-HIAA Tissue concentration (ng/g wet weight)			
Vehicle	108 ± 6	439 ± 33	183 ± 16	172 ± 11
L-NA (5)	130 ± 6	496 ± 49	254 ± 16**	223 ± 14*
L-NA (10)	118 ± 8	458 ± 36	227 ± 9*	200 ± 8*
L-NA (20)	118 ± 5	442 ± 45	233 ± 14*	212 ± 12*
	5-HT Tissue concentration (ng/g wet weight)			
Vehicle	532 ± 28	753 ± 42	553 ± 39	748 ± 38
L-NA (5)	512 ± 18	751 ± 76	572 ± 17	773 ± 25
L-NA (10)	530 ± 20	707 ± 53	522 ± 19	738 ± 23
L-NA (20)	506 ± 22	685 ± 71	526 ± 29	752 ± 31
	5-HIAA:5-HT			
Vehicle	0.207 ± 0.017	0.584 ± 0.034	0.330 ± 0.014	0.229 ± 0.007
L-NA (5)	0.259 ± 0.020	0.671 ± 0.039	0.443 ± 0.022*	0.288 ± 0.015*
L-NA (10)	0.223 ± 0.011	0.653 ± 0.035	0.437 ± 0.013*	0.271 ± 0.008*
L-NA (20)	0.235 ± 0.009	0.656 ± 0.037	0.446 ± 0.023*	0.282 ± 0.011*

Dose related effects of L-NA on regional changes to 5-HTP, 5-HIAA, 5-HT and the 5-HIAA:5-HT ratio following FST exposure. L-NA (5, 10 and 20 mg/kg) was administered 24, 5 h and co-administered with NSD-1015 (100 mg/kg i.p.) 1 h prior to FST. Brain regions were dissected immediately after the FST. Data represents the mean and standard error of 8–10 animals per group and was analysed using a one factor ANOVA followed by a Dunnett's test. *$P<0.05$; **$P<0.01$ vs. Vehicle Control. 5-HTP: 5-hydroxytryptophan.

3.3. L-NA increases 5-HT metabolism in the striatum following NSD-1015 administration to rats

ANOVA of 5-HTP, 5-HT, 5-HIAA concentrations and the 5-HIAA:5-HT metabolism ratio failed to show effects in the frontal or amygdaloid cortex. ANOVA of 5-HTP, 5-HT and 5-HIAA concentrations in the striatum and hippocampus also did not show effects. Significant effects were observed for the 5-HIAA:5-HT ratio in the striatum [$F(3,25) = 3.01$, $P<0.05$] and there was a non significant trend in the hippocampus [$F(3,26) = 2.68$, $P<0.07$]. Post hoc comparisons revealed that 5-HT metabolism was increased in the striatum following L-NA (20 mg/kg) administration when compared to vehicle treated controls (Table 2). There were no effects of L-NA observed for L-DOPA, dopamine, its metabolites HVA and DOPAC or dopamine metabolism ratios in any of the brain regions tested.

Table 2
Effect of L-NA on regional brain 5-HTP, 5-HT and 5-HIAA concentrations following NSD 1015 administration to rats.

Group	5-HTP Tissue concentration (ng/g wet weight)			
	Cortex	Striatum	Hippocampus	Amygdala
Vehicle	158 ± 32	284 ± 59	246 ± 38	402 ± 54
L-NA (5)	233 ± 51	341 ± 30	282 ± 45	415 ± 48
L-NA (10)	160 ± 21	235 ± 37	255 ± 35	297 ± 17
L-NA (20)	184 ± 22	241 ± 27	276 ± 36	398 ± 32
	5-HIAA Tissue concentration (ng/g wet weight)			
Vehicle	109 ± 16	434 ± 84	202 ± 35	214 ± 23
L-NA (5)	160 ± 24	438 ± 28	258 ± 38	281 ± 44
L-NA (10)	138 ± 18	340 ± 36	236 ± 33	237 ± 39
L-NA (20)	157 ± 17	462 ± 27	298 ± 32	308 ± 24
	5-HT Tissue concentration (ng/g wet weight)			
Vehicle	463 ± 74	837 ± 145	452 ± 65	793 ± 90
L-NA (5)	553 ± 90	799 ± 33	529 ± 66	862 ± 80
L-NA (10)	446 ± 48	620 ± 70	463 ± 63	657 ± 65
L-NA (20)	502 ± 41	674 ± 37	519 ± 50	875 ± 63
	5-HIAA:5-HT			
Vehicle	0.245 ± 0.021	0.495 ± 0.033	0.431 ± 0.024	0.276 ± 0.017
L-NA (5)	0.296 ± 0.015	0.556 ± 0.046	0.482 ± 0.022	0.326 ± 0.042
L-NA (10)	0.322 ± 0.045	0.562 ± 0.052	0.528 ± 0.047	0.361 ± 0.038
L-NA (20)	0.318 ± 0.029	0.698 ± 0.055*	0.572 ± 0.042	0.359 ± 0.032

Dose related effects of L-NA on regional changes to 5-HTP, 5-HT and 5-HIAA concentrations in NSD-1015 treated animals. L-NA (5, 10 and 20 mg/kg) was administered 24, 5 h and co-administered with NSD-1015 (100 mg/kg i.p.) 1 h and 5 min prior to animal sacrifice when brain regions were dissected immediately. Data represents the mean and standard error of 7–8 animals per group and was analysed using a one factor ANOVA followed by a Dunnett's test. *$P<0.05$ vs. Vehicle Control. 5-HTP: 5-hydroxytryptophan; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindoleacetic acid.

3.4. L-NA augments the activity of fluoxetine in the FST

ANOVA of immobility time showed effects of fluoxetine only [$F(1,36)=4.30$, $P=0.045$]. Post hoc comparisons revealed no significant differences between the groups. Fluoxetine induced a small decrease in immobility time and a trend was observed for L-NA to potentiate this effect (Fig. 2A).

ANOVA of swimming time showed effects of fluoxetine [$F(1,36)=4.50$, $P=0.041$] and a L-NA × fluoxetine interaction approaching significance [$F(1,36)=3.36$, $P=0.075$]. Post hoc comparisons revealed that neither L-NA nor fluoxetine had an effect on swimming time. L-NA and fluoxetine together provoked an increase in swimming time when compared to vehicle treated controls, L-NA or fluoxetine treatments alone ($P<0.05$) (Fig. 2B).

ANOVA of climbing time showed no effects of treatment (Fig. 2C).

3.5. Effects of metergoline on the antidepressant-like activity of L-NA in the FST

ANOVA of immobility time showed effects of metergoline [$F(3,70)=6.63$, $P<0.001$] and L-NA [$F(1,70)=31.95$ $P<0.001$]. Post hoc comparisons revealed that metergoline (4 mg/kg) increased immobility time when compared to vehicle treated controls ($P<0.01$). Metergoline (4 mg/kg) also increased immobility time in L-NA treated animals when compared to L-NA treated controls ($P<0.05$). L-NA reduced immobility time when compared to vehicle treated controls ($P<0.05$) (Fig. 3A).

ANOVA of swimming time showed effects of metergoline [$F(3,70)=7.33$, $P<0.001$] and L-NA [$F(1,70)=43.27$ $P<0.001$]. Post hoc comparisons revealed that metergoline (1, 2 and 4 mg/kg) decreased swimming time when compared to vehicle treated controls ($P<0.05$). Metergoline (4 mg/kg) also decreased swimming time in L-NA treated animals when compared to L-NA treated controls ($P<0.05$). L-NA increased swimming time when compared to vehicle treated controls ($P<0.05$) (Fig. 3B).

ANOVA of climbing time showed an interaction between metergoline and L-NA [$F(3,70)=2.79$, $P=0.047$]. Post hoc comparisons

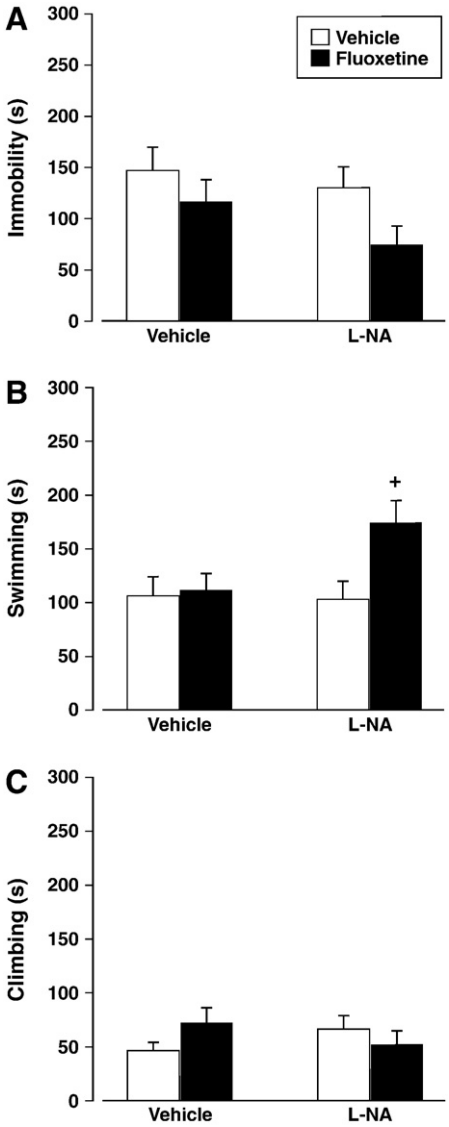

Fig. 2. Rats received L-NA (1 mg/kg, i.p.) and fluoxetine (2.5 mg/kg, i.p.) alone and in combination 24, 5 and 1 h prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 10 animals. $^{+}P<0.05$ vs. vehicle control, L-NA and fluoxetine alone treated groups.

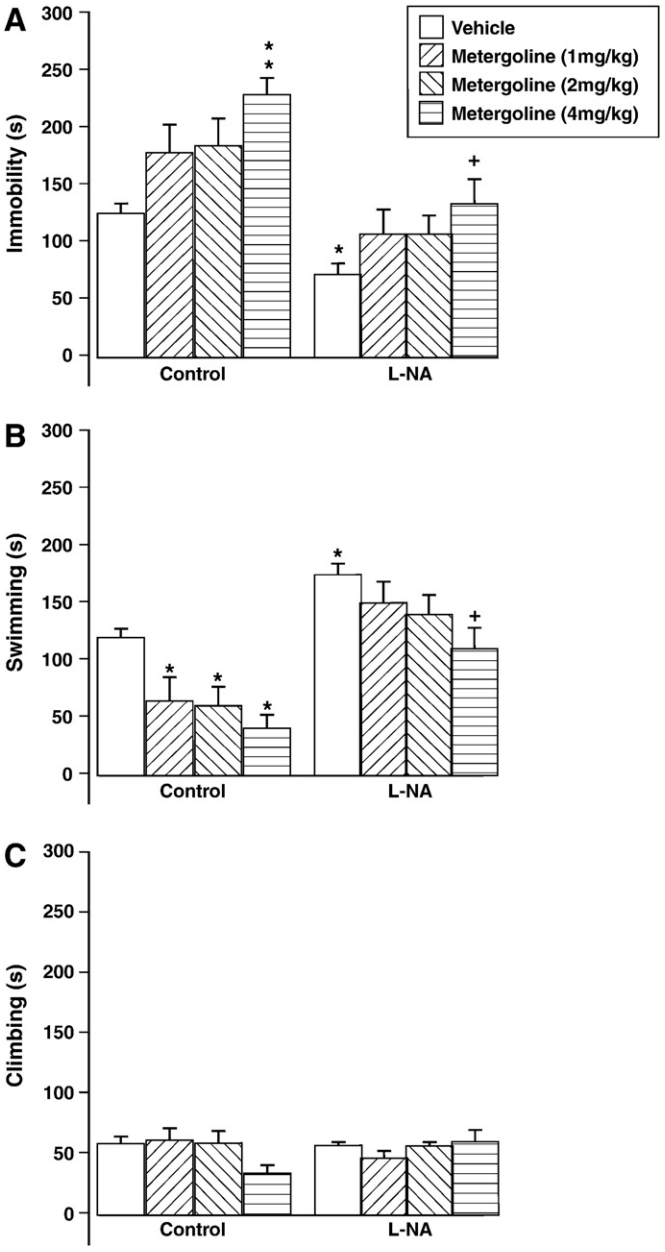

Fig. 3. Rats received L-NA (20 mg/kg, i.p.) and metergoline (1, 2 and 4 mg/kg, i.p.) alone and in combination 24, 5 and 1 h prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 9–10 animals. $^{*}P<0.05$; $^{**}P<0.01$ vs. Vehicle Control; $^{+}P<0.05$ vs. Vehicle + L-NA.

revealed no significant differences between the treatment groups (Fig. 3C).

3.6. Treatment with ketanserin and RO 430440 provoke a similar response to metergoline in the FST attenuated by co-treatment with L-NA

ANOVA of immobility time showed effects of ketanserin [$F(1,36) = 9.77$, $P = 0.004$] and L-NA [$F(1,36) = 40.21$, $P < 0.001$]. Post hoc comparisons revealed that ketanserin increased immobility time when compared to vehicle treated controls ($P < 0.01$). There was a reduction in immobility time following L-NA treatment when compared to vehicle treated controls ($P < 0.01$) (Fig. 4A).

ANOVA of swimming time showed effects of ketanserin [$F(1,36) = 13.46$, $P < 0.001$] and L-NA [$F(1,36) = 30.31$, $P < 0.001$]. Post hoc comparisons revealed that ketanserin decreased swimming time when compared to vehicle treated controls ($P < 0.01$). Ketanserin reduced swimming time in L-NA treated animals when compared to L-NA treated controls ($P < 0.05$). There was an increase in swimming time following L-NA treatment when compared to vehicle treated controls ($P < 0.01$) (Fig. 4B).

ANOVA of climbing time showed effects of L-NA [$F(1,36) = 6.23$, $P = 0.017$]. Post hoc comparisons revealed no differences between the treatment groups (Fig. 4C).

ANOVA of immobility time showed effects of RO-430440 [$F(1,36) = 9.49$, $P = 0.004$] and L-NA [$F(1,36) = 61.95$, $P < 0.001$]. Post hoc comparisons revealed that RO-430440 increased immobility time when compared to vehicle treated controls ($P < 0.01$). There was a reduction in immobility time following L-NA treatment when compared to vehicle treated controls ($P < 0.01$). RO-430440 failed to influence the L-NA-induced reduction in immobility time when compared to vehicle + L-NA treated controls (Fig. 5A).

ANOVA of swimming time showed effects of RO-430440 [$F(1,36) = 11.62$, $P = 0.002$] and L-NA [$F(1,36) = 53.14$, $P < 0.001$]. Post hoc comparisons revealed that RO-430440 decreased swimming time when compared to vehicle treated controls ($P < 0.01$). There was an increase in swimming time following L-NA treatment when compared to vehicle treated controls ($P < 0.01$). RO-430440 failed to influence the L-NA-induced increase in swimming time when compared to vehicle + L-NA treated controls (Fig. 5B).

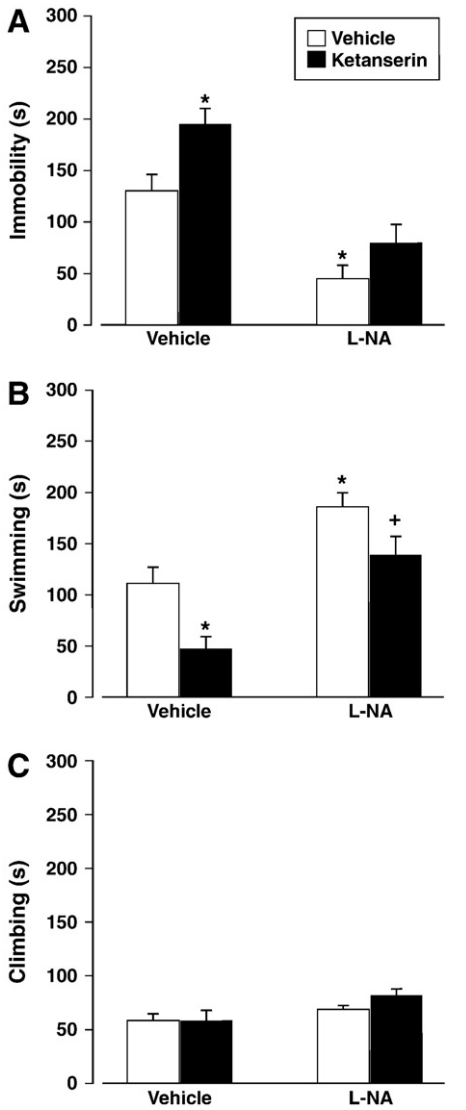

Fig. 4. Rats received L-NA (20 mg/kg, i.p.) and ketanserin (5 mg/kg, i.p.) alone and in combination 24, 5 and 1 h prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 10 animals. *$P < 0.01$ vs. Vehicle Control; $^{+}P < 0.05$ vs. Vehicle + L-NA.

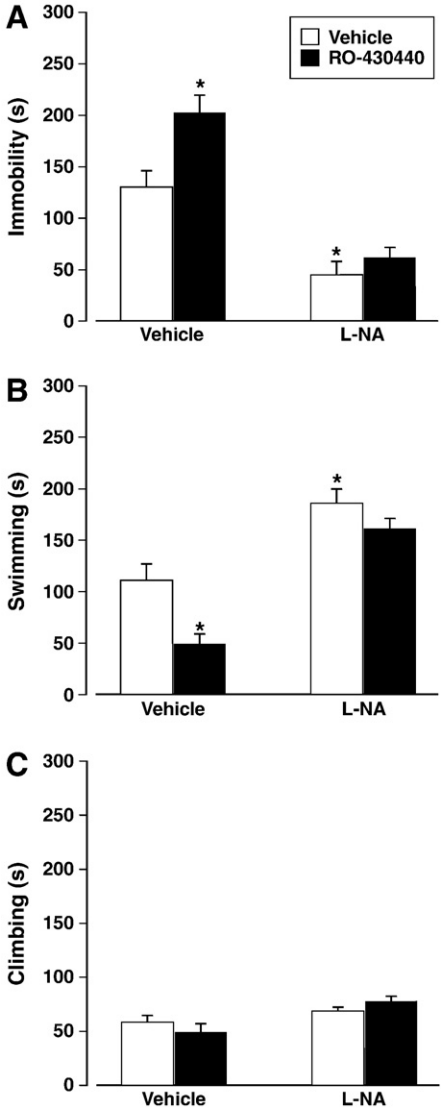

Fig. 5. Rats received L-NA (20 mg/kg, i.p.) and RO-430440 (5 mg/kg, i.p.) alone and in combination 24, 5 and 1 h prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 10 animals. *$P < 0.01$ vs. Vehicle Control.

ANOVA of climbing time showed effects of L-NA [$F(1,36)=10.70$, $P=0.003$]. Post hoc comparisons revealed no differences between the treatment groups (Fig. 5C).

3.7. Co-treatment with WAY 100635 or GR 127935 do not influence the antidepressant-like activity of L-NA in the FST

ANOVA of immobility time showed effects of L-NA [$F(1,36)=43.45$, $P<0.001$]. Post hoc comparisons revealed that L-NA reduced immobility time when compared to vehicle treated controls ($P<0.01$). WAY 100635 failed to influence immobility time or the L-NA-induced reduction in immobility time when compared to L-NA treated counterparts (Fig. 6A).

ANOVA of swimming time showed effects of L-NA [$F(1,36)=30.99$, $P<0.001$]. Post hoc comparisons revealed that L-NA increased swimming time when compared to vehicle treated controls ($P<0.01$). WAY 100635 failed to influence swimming time or the L-NA-induced increase in swimming time (Fig. 6B).

ANOVA of climbing time showed no effects of treatments (Fig. 6C).

For GR 127935, ANOVA of immobility time showed effects of L-NA [$F(1,34)=41.14$, $P<0.001$]. Post hoc comparisons revealed that L-NA reduced immobility time when compared to vehicle treated controls ($P<0.01$). GR 127935 failed to influence immobility time or the L-NA-induced reduction in immobility time when compared to L-NA treated counterparts. Mean immobility times for each treatment group were as follows: Vehicle Control: 167 ± 18; GR 127935 Control: 169 ± 17; Vehicle L-NA: 47 ± 17*; GR 127935 + L-NA: 60 ± 19. *$P<0.05$ vs. Vehicle Control.

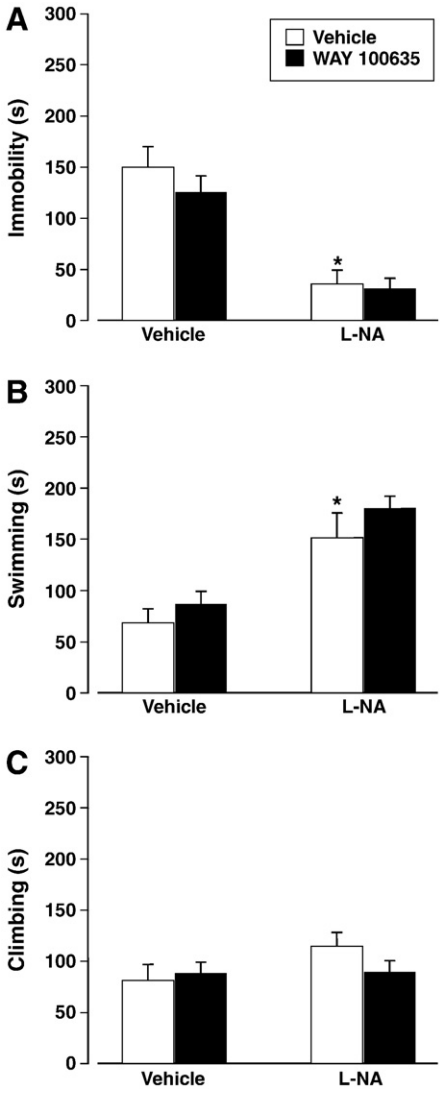

Fig. 6. Rats received L-NA (20 mg/kg, i.p.) and WAY 100635 (0.3 mg/kg, i.p.) alone and in combination 24, 5 and 1 h prior to test. Immobility (A), Swimming (B) and Climbing (C) data in seconds (s) are expressed as mean and standard error of 10 animals.*$P<0.01$ vs. Vehicle Control.

ANOVA of swimming time showed effects of L-NA [$F(1,34)=58.56$, $P<0.001$]. Post hoc comparisons revealed that L-NA increased swimming time when compared to vehicle treated controls ($P<0.01$). GR 127935 failed to influence swimming time or the L-NA-induced increase in swimming time. Mean swimming times for each treatment group were as follows: Vehicle Control: 65 ± 13; GR 127935 Control: 51 ± 8; Vehicle L-NA: 181 ± 14*; GR 127935 + L-NA: 152 ± 20. *$P<0.05$ vs. Vehicle Control.

ANOVA of climbing time showed no effects of treatments.

3.8. The effects of metergoline, ketanserin and RO-43-0440 and L-NA alone or in combination on locomotor activity

Placement of the animals in the activity chambers resulted initially in exploratory activity and a gradual acclimatization to the novel surroundings over the 20 min test period. ANOVA of activity following ketanserin or L-NA administration alone or in combination showed effects of ketanserin [$F(1,16)=4.47$, $P=0.05$], L-NA [$F(1,16)=14.11$, $P=0.002$] and a ketanserin × L-NA interaction [$F(1,16)=5.43$, $P=0.033$]. Post hoc comparisons revealed that ketanserin (2285 ± 311) and L-NA (1790 ± 252) reduced activity when compared to vehicle treated controls (3622 ± 381) ($P<0.05$). Ketanserin failed to influence the L-NA related reduction in activity (1855 ± 240) when compared to L-NA treatment alone. There were 5 animals per treatment group. The numbers in parentheses represent mean activity counts over the 20 min observation period with standard error of the mean. Metergoline or RO-43-0440 did not affect activity over the trial period when compared to vehicle treated controls. Neither metergoline nor RO-430440 influenced the response to L-NA (data not shown).

4. Discussion

L-NA produced a reduction in immobility together with an increase in active swimming behaviour without any significant change in climbing consistent with previous reports of its antidepressant-like activity in the FST (Harkin et al., 2003). This behavioural profile is similar to that elicited by SSRIs or serotonergic agonists (Cryan and Lucki, 2000; Detke et al., 1995; Page et al., 1999). In the present investigation, co-treatment with NSD-1015 did not influence the antidepressant-like activity of L-NA in the FST. With or without NSD-1015, L-NA produced a dose-dependent reduction in immobility that occurs as a result of a selective increase in swimming behaviour. NSD-1015 inhibits the 5-HT biosynthetic pathway, although the acute treatment regime of NSD-1015 employed was not sufficient to provoke a depletion of endogenous 5-HT. In this regard, we have previously confirmed that depletion of 5-HT, via treatment with the tryptophan hydroxylase inhibitor p-chlorophenylalanine (pCPA), attenuates the antidepressant-like properties of NOS inhibitors in the FST (Harkin et al., 2003).

By determination of the accumulation of 5-HTP following NSD-1015 administration, the results of the present study indicate that tryptophan hydroxylase is activated in response to L-NA administration. L-NA provoked an increase in 5-HTP concentrations in the amygdaloid cortex suggesting that NOS inhibitors may act in a region specific manner. Moreover, the effects obtained were dose dependent and evident in FST exposed animals only. Inactivation of brain tryptophan hydroxylase by NO via nitrosylation has been previously described (Kuhn and Arthur, 1996, 1997) and such a mechanism may account for the increase in tryptophan hydroxylase activity observed in the current study. Raised glutamate and nitrergic neuronal

transmission in response to exposure to a stressful stimulus such as the FST (Joca et al., 2007; Kirby et al., 2007; Shirakawa et al., 2004) may increase NO-dependent nitrosylation of TPH leading to its inactivation. L-NA, in turn, may reverse this process by limiting the availability of NO, reducing the inactivation of tryptophan hydroxylase. Further experiments however are required to verify this mechanism linking stress-induced NO to the regulation of tryptophan hydroxlase activity.

L-NA also provoked a regional increase in 5-HIAA concentrations and 5-HT metabolism indicative of a possible enhancement of 5-HT release, re-uptake and/or metabolism to 5-HIAA. These effects were also largely dependent on exposure to the FST. Consistent with this are reports that NOS inhibitors increase extracellular levels of 5-HT in the rat brain after local or systemic administration (Kiss, 2000; Smith and Whitton, 2000; Segieth et al., 2001; Wegener et al., 2000). Inhibition by NO of 5-HT uptake into rat brain synaptosomes (Asano et al., 1997) and the human 5-HT transporter (Bryan-Lluka et al., 2004) has been previously described. More recently accounts of a physical interaction between the 5-HT transporter and neuronal NOS that underlies a reciprocal modulation of their activity provides further evidence for the regulation of 5-HT by NO (Chanrion et al., 2007; Garthwaite, 2007). NO may also inhibit monoamine oxidase activity (Muriel and Perez-Rojas, 2003) although the mechanism by which NO inhibits monoamine oxidase remains unknown at present. There are therefore a number of mechanisms by which NOS inhibitors may influence 5-HT metabolism. Overall, as 5-HT function is implicated in stress related disorders such as depression, it is tempting to speculate that chemically reactive species like NO influence conditions where 5-HT deficits have been identified. The current study provides evidence in support of enhanced 5-HT synthesis and increased 5-HT metabolism indicative of overall 5-HT activation which may account for the antidepressant-like properties of NOS inhibitors.

We have previously reported that NOS inhibitors can augment the behavioural effects of SSRIs and the tricyclic antidepressant imipramine in the mouse FST, but not antidepressants affecting noradrenergic transmission (Harkin et al., 2004). In the current study we extend this in the rat FST where a combination of sub-active doses of fluoxetine and L-NA produce an antidepressant response in the test. The ability of L-NA to increase the activity of fluoxetine is consistent with the qualitative similarities between the antidepressant-like activity of NOS inhibitors and SSRIs and the dependency of these effects on endogenous 5-HT (Harkin et al., 2003; Page et al., 1999). Coupled to the aforementioned evidence of enhanced 5-HT synthesis and metabolism, it is not unreasonable to suggest that NOS inhibitors may produce their antidepressant augmenting properties by modulating the availability and release of 5-HT. In this regard, it is also of interest to note that some antidepressants can inhibit the activity of NOS. For example, the SSRI paroxetine inhibits NOS activity at concentrations comparable to those achieved in clinical therapy (Finkel et al., 1996; Wegener et al., 2003). Such studies have raised the possibility that NOS inhibition may be a clinically relevant feature of at least some antidepressants.

In a separate series of experiments, the involvement of the 5-HT receptor subtypes in the antidepressant-like activity of L-NA in the FST was studied. To this end, rats were co-treated in turn with the non-selective 5-HT receptor antagonist metergoline, the preferential 5-HT_{2A} receptor antagonist ketanserin, the selective 5-HT_{2C} receptor antagonist RO-430440, the selective 5-HT_{1A} receptor antagonist WAY 100635, the 5-$HT_{1B/D}$ receptor antagonist GR 127935 and L-NA. Here, involvement of 5-HT receptor subtypes in the antidepressant-like effect of L-NA is supported by the demonstration that metergoline and ketanserin attenuate the antidepressant-like effect of L-NA and L-NA attenuates the increase in immobility and reduction in escape oriented behaviours induced by these 5-HT receptor antagonists in the FST.

Co-treatment with metergoline dose dependently attenuated L-NA-induced antidepressant-like activity in the FST. Metergoline alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant-like action of the drug in the test. Co-treatment with L-NA reduced these behavioural effects. In rats, metergoline can influence components of behaviour including a suppression of rearing, sniffing and locomotion at doses of 2 mg/kg and higher (Halford and Blundell, 1996; Mokler et al., 1983; Wilson et al., 1998). Thus the question arises, if the ability of higher doses of metergoline to reduce locomotor and other general behaviours might confound any conclusions regarding a reduction in the antidepressant-like activity of L-NA. In the current investigation we did not observe any effect of metergoline (4 mg/kg) on locomotor activity. We have previously reported that active doses of L-NA in the FST cannot be attributed to any psychomotor stimulant action (Harkin et al., 2003), yet L-NA attenuates the ability of metergoline to increase immobility in the FST. Taken together with the ability of metergoline to reverse the antidepressant-like actions of L-NA, these findings are consistent with the participation of 5-HT receptors in the antidepressant-like activity of L-NA in the FST.

Co-treatment with ketanserin produced a qualitatively similar response to that obtained with metergoline. Ketanserin alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant-like action and these behavioural effects were reduced by co-treatment with L-NA. Moreover, ketanserin attenuated L-NA-induced swimming activity in the FST. In the current investigation, in contrast to metergoline, ketanserin reduced locomotor activity. Co-administration with L-NA failed to influence this response suggesting that the ability of L-NA to attenuate the actions of ketanserin in the FST are related to an inhibition of the depressive phenotype independent of locomotor activity. Other investigators have not reported effects of ketanserin alone in the FST but this may be related to the lower doses used and differences in the manner in which the FST was carried out and scored (Martínez-Mota et al., 2002; Savegnago et al., 2007). The dose of ketanserin in the present study was selected based on the observed lack of interaction of lower doses of ketanserin with L-NA in pilot experiments undertaken in the laboratory. Similar doses have been employed by others where ketanserin (1–5 mg/kg) showed no effect on behavioural measures in the open field but was sufficient to block 5-HT agonist-induced behaviours (Hawkins et al., 2008).

Activation of 5-HT_{2C} receptors has previously been reported to provoke antidepressant-like activity in the FST. Moreover the effects of the SSRI fluoxetine may be blocked by pre-treatment with the 5-HT_{2C} receptor antagonist SB 206533 (Cryan and Lucki, 2000). It was therefore of interest in the current study to determine if blockade of 5-HT_{2C} receptors might influence the antidepressant-like response to L-NA. Co-treatment with RO 430440 produced a similar response to that obtained with ketanserin although RO 430440 did not significantly attenuate L-NA-induced antidepressant-like activity in the FST. Like ketanserin, Ro 430440 alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant-like action and these behavioural effects were attenuated by co-treatment with L-NA. In contrast to ketanserin, RO 430440 did not affect locomotor activity in the current study. The dose of RO 430440 was selected based on our observations that pre-treatment with RO 430440 at doses not less than 2.5 mg/kg can block fenfluramine-induced hypophagia and hypothermia which have previously been reported to be mediated via the stimulation of 5-HT_{2C} receptors (Cryan et al., 2000; Gibson et al., 1993).

The apparent differences between responses obtained with metergoline, ketanserin and RO 430440 suggest that the 5-HT_{2A} receptor is the predominant receptor subtype involved in the attenuation of the antidepressant-like activity of L-NA and that 5-$HT_{2A/2C}$ receptors play a role in promoting pro-depressant-like behaviours in the FST which may be attenuated by co-treatment with L-NA. To clarify the role of 5-HT_2 receptors further, treatment with the selective 5-HT_{1A} and 5-$HT_{1B/1D}$ receptor antagonists, WAY 100635 and GR 127935 respectively, failed to

influence behaviours in the FST either alone or in combination with L-NA. WAY 100635 is a full antagonist at both pre and post-synaptic 5-HT_{1A} receptors and doses of 0.1 mg/kg or lower are required to block both the pre and post-synaptic effects of 8-OH-DPAT (Critchley et al., 1994; Forster et al., 1995; Moser and Sanger, 1999). Both agents have previously been reported to lack activity in the FST (De Vry et al., 2004; Tatarczyńska et al., 2004) although co-treatment with WAY 100635 (0.3 mg/kg) can attenuate the effects of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT in the FST (DeVry et al., 2004; Moser and Sanger 1999). Moreover WAY 100635 (0.3 mg/kg) has previously been reported to partially attenuate the antidepressant activity of the SSRI fluoxetine in the rat FST. Whilst higher doses have been reported to demonstrate a potential role of 5-HT_{1A} receptors in the FST, these experiments have used alternative strains (Martinez-Mota, 2002), female rats (Estrada-Camarena et al., 2006a) and/or rats which have been ovariectomized (Estrada-Camarena et al., 2006b) and direct comparisons are not possible with the present study.

Similarly, pre-treatment with the 5-HT_{1B} antagonist GR-127935 (at doses up to 4 mg/kg) attenuates the behavioural effects of 5-HT_{1B} receptor agonists in various rat behavioural paradigms (de Boer and Koolhaas, 2005; Chaouloff et al., 1999; Tomkins and O'Neill, 2000) but could not influence the antidepressant-like effect of L-NA in the present experiment. Given the evidence to implicate 5-HT in the antidepressant-like activity of L-NA, it is perhaps surprising that inhibition of pre-synaptic autoreceptors by WAY 100635 or GR-127935 did not facilitate the effects of L-NA in the FST, even if post-synaptic 5-HT_{1A} receptors are not involved. This outcome suggests that L-NA may modulate synaptic 5-HT availability by mechanisms independent of pre-synaptic autoreceptors. Support for a role of 5-HT_{1A} and/or 5-HT_{1B} receptors in the antidepressant-like effects of L-NA in the FST is currently lacking, although additional studies with selective compounds might be worthwhile.

In conclusion the current data further demonstrate the antidepressant-like potential of the NOS inhibitor L-NA, and also suggest that the behavioural effects have a 5-HT related mechanism of action due to the additive effects of low doses of L-NA and fluoxetine and the influence of L-NA in swimming but not climbing behaviour. Interpretation of the results obtained with the non-selective and 5-HT_{2A} receptor antagonists must be approached with caution since the antagonists caused effects by themselves that were still apparent following co-administration with L-NA. The antidepressant-like effects of L-NA however were not modified by RO-430440 or WAY 100635 suggesting that 5-HT_{2C} and 5-HT_{1A} receptors are not involved. The fact that WAY 100635 can block both pre and post-synaptic receptors may contribute to the lack of effects observed with this compound.

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