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Corticolimbic changes in acetylcholine and cyclic guanosine monophosphate in the Flinders Sensitive Line rat: a genetic model of depression

Brand L, van Zyl J, Minnaar EL, Viljoen F, du Preez JL, Wegener G, Harvey BH. Corticolimbic changes in acetylcholine and cyclic guanosine monophosphate in the Flinders Sensitive Line rat: a genetic model of depression.

Objective: Depression is suggested to involve disturbances in cholinergic as well as glutamatergic pathways, particularly the *N*-methyl-D-aspartate receptor-mediated release of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP). The aim of this study was to determine whether the Flinders Sensitive Line (FSL) rat, a genetic model of depression, presents with corticolimbic changes in basal acetylcholine (ACh) levels and NO/cGMP signalling.
Methods: Basal levels of nitrogen oxides (NOx) and both basal and L-arginine-stimulated nitric oxide synthase (NOS) formation of L-citrulline were analysed in hippocampus and frontal cortex in FSL and control Flinders resistant line (FRL) rats by fluorometric and electrochemical high-performance liquid chromatography, respectively. In addition, ACh and cGMP levels were analysed by liquid chromatography tandem mass spectrometry and radioimmunoassay, respectively.
Results: Significantly elevated frontal cortical but reduced hippocampal ACh levels were observed in FSL versus FRL rats. Basal cGMP levels were significantly reduced in the frontal cortex, but not hippocampus, of FSL rats without changes in NOx and L-citrulline, suggesting that the reduction of cGMP follows through an NOS-independent mechanism.
Conclusions: These data confirm a bidirectional change in ACh in the frontal cortex and hippocampus of the FSL rat, as well as provide evidence for a frontal cortical ACh-cGMP interaction in the depressive-like behaviour of the FSL rat.

Linda Brand[1], Jurgens van Zyl[1,*,†], Estella L. Minnaar[1,*], Francois Viljoen[1], Jan L. du Preez[2], Gregers Wegener[3], Brian H. Harvey[1]

[1]Division of Pharmacology, Unit for Drug Research and Development, School of Pharmacy, North-West University, Potchefstroom, South Africa; [2]Analytical Technology Laboratory, Unit for Drug Research and Development, School of Pharmacy, North-West University, Potchefstroom, South Africa; and [3]Centre for Psychiatric Research, University of Aarhus, Denmark

*Both authors contributed equally to this manuscript.

†Present address: Department of Human Biology, Health Sciences, University of Cape Town, Cape Town, South Africa.

Keywords: cGMP; cholinergic; Flinders Sensitive Line rat; frontal cortex; hippocampus; major depression; nitric oxide

Linda Brand, Division of Pharmacology, Unit for Drug Research and Development, School of Pharmacy, North-West University, 2520 Potchefstroom, South Africa.
Tel: +27(18)2992233;
Fax: +27(18)2992225;
E-mail: linda.brand@nwu.ac.za

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Significant outcomes

- Frontal cortical ACh was elevated but reduced in the hippocampus of FSL versus FRL rats.
- Frontal cortical cGMP was reduced in FSL versus FRL rats, with no change in the hippocampus.
- Reduced frontal cortical cGMP occurred without altered NOS activity.

Limitations

This study was undertaken in stress-naïve animals, thus assessing basal levels of ACh and cGMP in FSL versus FRL rats. Since prior or on-going stress often precedes the psychopathology of depression in susceptible individuals, further studies in these animals under adverse conditions of stress may reveal a different neurochemical profile, one that more closely reflects the pathological condition.

Introduction

Major depressive disorder is a recurrent, stress-related heterogeneous neuropsychiatric disorder (1,2) that shows a significant genetic association (3–5). Furthermore, issues such as shortfalls in antidepressant efficacy (6), delayed onset of action and distressing side-effects (7) emphasise the need to identify new drug targets and new antidepressants. Although the monoamine hypothesis has heuristic value in our understanding of depression, it is less capable of explaining the complex dimensions of this illness (8). Instead, neurogenesis and the concepts of neuroplasticity have become central to our understanding of depression and the mechanisms of antidepressants (8,9).

The glutamate *N*-methyl-D-aspartate (NMDA) receptor and the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway play a pivotal role in neuroplasticity (10,11), while NO and cGMP are involved in intra- and intercellular communication as well as neurotransmitter release (12,13). Together, this suggests that the NO-cGMP signalling cascade is involved in both the neurobiology and treatment of mood disorders (14,15), although its exact role remains poorly defined.

Cholinergic hyperfunction is suggested to occur in depression (16–18). Since anticholinergics are weak antidepressants (19,20), the exact role of acetylcholine (ACh) in the aetiology of depression remains unknown, although its involvement is possibly more supplementary to actions on other transmitters, such as glutamate and monoamines. Interestingly, a cGMP-ACh interaction has been suggested to have an important role in how the cholinergic system may interface with the neurobiology of depression and antidepressant action (19,20). Thus, cGMP is involved in crosstalk with cholinergic and other neurotransmitter systems (13,21), although the relevance of this interaction in depression remains obscure. For example, ACh release is reduced by NMDA receptor activation and modulated by NO (22,23), while ACh in turn depresses glutamate activity (24). By allowing inappropriate changes in monoamines, ACh and downstream messengers of the NMDA-NO pathway, an abnormality in glutamatergic pathways could directly and indirectly result in a mood disorder (25).

The Flinders Sensitive Line (FSL) rat is a genetic rodent model of depression that presents with extensive face and predictive validity for depression (26–28), including psychomotor retardation, anhaedonia following stress, loss of appetite/weight, sleep disturbances and anxiety (29,30) as well as increased responsiveness to environmental stressors (17,31,32). Neurochemically, the FSL rat displays a hypercholinergic response (29), with higher levels of hippocampal, striatal and hypothalamic muscarinic acetylcholine receptor (mAChR) noted in the adult rat (33), although with no difference observed with respect to mAChR density in the cortex of FSL versus Flinders resistant line (FRL) rats (33–35). Disturbances in serotonergic (36,37) and gamma amino butyrate (GABA) activity (35) as well as an exaggerated response of the NMDA-nitric oxide synthase (NOS) signalling cascade following stress (32) have also been reported in FSL rats. Consistent with the neuroplasticity hypothesis of depression, the FSL model also displays significant lower levels of the neurotrophic factors brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (38,39) and correspondingly a reduction in hippocampal volume and neuronal and synapse numbers (40).

The aims of the study are therefore to explore basal levels of ACh and NOS activity, as well as the role of NO and NO-ACh interactions, in the FSL rat relative to its control, the FRL rat. To this end, the study focuses on NO/cGMP and cholinergic signalling in two corticolimbic brain regions of importance in depression, viz. the hippocampus and frontal cortex.

Materials and methods

Animals

Approval of the study protocol was granted by the Animal Ethics Committee of the North-West University (Ethics approval number NWU0003207S2). All animals were treated according to the code of ethics in research as laid down by this Animal Ethics Committee. Breeding pairs of the FSL and FRL rats were originally gifted from Dr David Overstreet, University of North Carolina, USA. For this study, young, adult male rats, weighing 200 ± 20 g (Animal Centre of the North-West

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University, Potchefstroom campus), were reared and housed five rats per cage in identical cages at the Animal Research Centre, North-West University, under controlled conditions of temperatures (21 ± 1 °C), relative humidity (55 ± 5%), positive air pressure and a 12-h light-day cycle with free access to food and water. Animal Centre air was exchanged 16–18 times the volume (fresh uncirculated air) per hour, with air quality controlled with high-efficiency particulate air (HEPA) filters.

Neurochemical assays

Tissue dissection and storage. Animals were sacrificed by decapitation, after which the brain was swiftly removed and the hippocampus and frontal cortex dissected out on an ice-cooled stainless steel slab. The dissected tissue was individually placed in eppendorf tubes and immediately snap-frozen in liquid nitrogen to be stored at −86 °C until the day of analysis. Groups of 10 FSL and 10 FRL rats each were used in the NOS, cGMP as well as the ACh analyses.

Chemicals and apparatus. Chemicals were of analytical grade or higher and stored at specified conditions. All aqueous solutions were prepared using high-performance liquid chromatography (HPLC)-grade water, and volumetric glass apparatus was used throughout the analysis to make up the reagents and standards. For the nitrogen oxides (NOx) assay, all pipette tips, Eppendorf and HPLC vials were pre-rinsed with tris (hydroxymethyl)-aminomethan (TRIS) buffer for at least three times before use to remove trace amounts of nitrite that may offer interference at low standard concentrations (1–10 ng/ml).

Nitric oxide analysis.

NOx determination. Nitrogen oxides, viz. nitrate (NO_3^-) and nitrite (NO_2^-), the stable oxidative metabolites of NO, are extensively utilised as viable surrogate markers of NOS activity (41). Total neuronal nitrite and nitrate was measured with HPLC coupled to fluorescence detection. Analytical method validation met the general requirements of ISO 17025, 2005. The three-step method determines NOx (total nitrite and nitrate) and is based on the derivatisation of nitrite with the highly fluorescent compound 2,3-diaminonaphthalene (DAN) (42,43) and its subsequent assay by HPLC. After sample preparation, nitrates were converted to nitrite and the resultant nitrite then derivatised to allow detection by fluorescence following HPLC separation of DAN and 2,3-naphthotriazole (NAT). Fluorescence was detected at excitation and emission wavelengths of 363 and 425 nm, respectively, using a flow rate of 1 ml/min.

Standards and reagents. A 100 µg/ml stock solution containing 13.7 mg sodium nitrate ($NaNO_3$) and 15.0 mg sodium nitrite ($NaNO_2$), dissolved in 100 ml TRIS buffer, pH 7.6 was freshly prepared daily. A standard series of working concentrations in the range of 10–300 ng/ml was prepared from stock solutions by appropriate dilution before use. Standard regression analysis displayed significant positive linearity ($r^2 = 0.9988$). All standards were prepared in TRIS buffer with samples homogenised in the same buffer. All buffers were made up with HPLC-water and stored at −20 °C.

β-Nicotinamide adenine dinucleotidephosphate (β-NADPH) was freshly prepared daily to a final concentration of 0.22 µM, which was used in combination with an enzyme solution [176 mg D-glucose-6-phosphate monosodium salt (G-6-P) with 100 IU/0.05 mg glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (GD) in 26.04 ml of a 170 mM sodium phosphate buffer, pH 7.4] in order to cycle the $NADP^+$ to NADPH. The enzyme solution was stable for at least a month at −80 °C. Immediately before use, 50 µl of the enzyme mixture was added to freshly prepared nitrate reductase (NR) to yield a final NR concentration of 15 mU (42, 44). DAN was freshly prepared daily in 0.76 M HCl to a final concentration of 0.57 mM. The solution was protected from light and stored on ice but allowed to reach room temperature before addition to sample.

Chromatographic conditions. HPLC was performed using an Agilent 1100 series HPLC system, equipped with an isocratic pump, autosampler and a Shimadzu RF-551 fluorescence detector (excitation 363 nm and emission 425 nm). Chemstation Revision A.06.02 data acquisition and analysis software was used for calculating peak areas and sample concentrations. An Eclipse XDB-C18 column (4.6 × 150 mm, 5 µM; Agilent, Santa Clara, CA, USA) was used and protected by a SecurityGuard™ guard column (HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18–4.0 × 3.0 mm; Phenomenex, Torrance, CA, USA). The isocratic elution mobile phase comprised of 47.8% of 15 mM disodium orthophosphate buffer (pH = 7.5) and 52.2% HPLC-grade methanol (Merck, Darmstadt, Germany) adjusted with 85% orthophosphoric acid and delivered at a flow rate of 1 ml/min at a temperature of 26 °C.

NOx assay procedure.

Tissue extraction and preparation. On the day of analysis, samples were removed from −86 °C storage and allowed to thaw, and then it was weighed and immediately homogenised in approximately 750 µl

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of ice-cold TRIS buffer [1 mM ethylene glycol-bis (β-aminoethyl ether, N-N-N′ tetraacetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA) and 25 mM TRIS, pH = 7.4, adjusted with 10% HCl] with a Heidolph-Elektro KG glass/Teflon homogeniser (15 strokes at ±22 500 U/min) at ±2–6 °C. A sufficient amount of tissue homogenate was stored at −86 °C for routine determination of sample protein concentration by the Bradford method (45). The soluble fraction of the sample was obtained by centrifugation for 1 h at 5400 × g, 4 °C (46). Supernatant (200 µl) was directly transferred into an amber glass HPLC vial and kept on ice until use.

Conversion of nitrate to nitrite. Fifty microlitres of β-NADPH (1.3 µM; Sigma-Aldrich Chemicals, St Louis, MO, USA) was added to the sample/standard, immediately followed by a separate addition of 50 µl NR (15 mU; Sigma-Aldrich) in enzyme mixture, to avoid inhibition of NR by pre-incubation with NADPH (44). The nitrate in the brain sample/standard was optimally converted into nitrite by incubating the reaction mixture for 45 min at 20 °C (47).

Derivatisation of nitrite. The conversion reaction was terminated and a new reaction initiated with the addition of 50 µl DAN (Sigma-Aldrich) in HCl (44). This reaction is optimal at 24 °C. After 10 min, the pH was adjusted with 25 µl 1.71 M NaOH to stop the derivatisation reaction, stabilise the product (NAT) and allow fluorescence detection of NAT. A final centrifugation at 2800 × g for 5 min was necessary to pellet the remaining protein. HPLC fluorescence detection was used to perform separation of DAN and NAT using a runtime per sample of 9 min. NOx was expressed in micro molars. Protein was routinely assayed by the method of Bradford (45).

NOS activity assessment. NOS stoichiometrically converts the amino acid L-citrulline to NO and L-citrulline. This enzyme reaction is used as an indication of NOS activity (48). In this study, basal and L-arginine-stimulated neuronal nitric oxide synthase (nNOS) activity (expressed in µM) was determined in the frontal cortex and hippocampus by using a customised and validated isocratic reversed-phase liquid chromatography method with amperometric electrochemical detection, based on a fluorescence detection method (49). The assay was carried out at an enzyme concentration of 30–50 µg protein, adequate to convert the added substrate to product, but prior to reaching steady state (50).

Standards and reagents. A 100 µg/ml amino acid containing stock solution (all obtained from Sigma-Aldrich) was freshly prepared daily by dissolving 1 mg L-citrulline, 1.21 mg L-arginine, 1 mg GABA and 1 mg glutamate in 10 ml borate buffer, pH 7.5. A standard series of working concentrations (0.1–5 µg/ml) L-citrulline was prepared from stock solutions by appropriate dilution before use. Standard regression analysis displayed a positive, significant linearity ($r^2 = 0.9970$). The enzyme mixture used for cycling β-NADPH and NR was prepared according to the same procedure as in the NOx assay. β-NADPH was freshly prepared daily to a final concentration of 0.2 mM. The NOx assay TRIS buffer was also replaced with a borate buffer to avoid the competition of the amino group (-NH_2) in TRIS with *O*-phthalaldehyde (OPA; Pierce, Rockford, IL, USA). The final concentration of each component in the reaction cocktail was β-NADPH, 27.36 µM; calmodulin (CaM) 13.7 µg/ml; flavin adenine dinucleotide disodium salt hydrate (FAD) 1 µM; riboflavin 5′-monophosphate sodium salt dihydrate (FMN) 1 µM; tetrahydrobiopterin (BH_4) 4 µM and L-arginine 18.3 µM.

Chromatographic conditions. HPLC was performed using an Agilent 1100 series HPLC system, equipped with an isocratic pump, autosampler and GBC LC 1260 electrochemical detector. Chemstation Revision A.06.02 data acquisition and analysis software was used for calculating peak areas and sample concentrations. The glassy carbon electrode was used at a potential of +0.600 V, range: 5 nA–500 pA, polarity: positive, filter: 64 point, filter (backside): 0.5 Hz. A Luna C18-2 column, 75 × 4.6 mm, 5 µm (Phenomenex) was used and protected by a SecurityGuard™ guard column (HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18–4.0 × 3.0 mm; Phenomenex). The mobile phase comprised of 0.1 M disodium orthophosphate, 0.13 mM ethylenediaminetetraacetic acid (EDTA disodiumsalt Na_2EDTA) and 28–35% methanol, pH ± 6.4 – adjusted with orthophosphoric acid (85%) – and was delivered at a flow rate of 0.8–1.5 ml/min at a temperature of 26 °C.

L-Citrulline assay procedure.

Tissue extraction and preparation. On the day of analysis, samples were weighed and homogenised in approximately 1 ml of ice-cold borate buffer (15 mM, pH 7.6 – adjusted with HCl, sodium tetraborate decahydrate and boric acid powder) with a Heidolph-Elektro KG glass/Teflon homogeniser (15 strokes at ± 22 500 U/min) at 2–6 °C. Samples were centrifuged for 1 h at 5 400 × g, 4 °C (46), and the supernatant decanted and separated from the tissue pellet. The standard amount of supernatant extraction

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for a particular brain part that contains approximately 30–50 μg protein (50) was calculated after spectrophotometric determination of protein concentration (45). Supernatant aliquots were transferred to an amber glass HPLC vial and kept on ice until use.

nNOS enzyme activation. nNOS represents the majority of constitutively expressed NOS in rat brain (51,52). To simulate the *in vitro* enzyme reaction, a reaction cocktail containing 15 μl BH_4 (100 μM), 5 μl FAD (100 μM) and FMN (100 μM), 50 μl β-NADPH (0.2 mM) and 12.5 μl CaM (400 μg/ml) was added to the brain supernatant aliquot after the reaction cocktail has been incubated for 2 min at 37 °C (53). Test samples were spiked with 50 μM L-arginine, while control samples received 134 μl of borate buffer. Immediately thereafter, 84 μl (25.2 mU) NR was dissolved in the cycling enzyme mixture (G-6-P and GD) containing glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (GD) and G-6-P in a phosphate buffer, pH 7.6, and potassium dihydrogen orthophosphate (KH_2PO_4). Sodium chloride ($NaCl_2$) was added to the vial, and the nNOS enzyme reaction initiated with 10 μl calcium chloride ($CaCl_2$; 130 mM). Following incubation for 10 min at 37 °C (53,54), the samples were removed from the oven, and the reaction was stopped by dilution with 50 μl ice-cold stop buffer [50 mM 4-(2-hydroxymethyl) piperazine-1-ethanesulfonic acid (HEPES), 5 mM EDTA, pH 5.5 – NaOH]. The pH of the sample was adjusted to pH 9 with the addition of 25 μl of potassium acetate (CH_3COOK, 10 M). The HPLC vials were centrifuged for 2.5 min at 2800 × g for removal of any remaining protein. Fifty microlitres of the resultant supernatant was pipetted into a HPLC-vial insert and placed into the HPLC autosampler after programming the software's injector program for pre-column *O*-phtaldialdehyde derivatisation and subsequent injection of 50 μl into the HPLC for separation of amino acids. L-Arginine-challenged NOS activity was calculated by subtracting induced L-citrulline sample concentrations from basal L-citrulline sample concentrations. The difference in L-citrulline concentrations represents the increase in L-arginine-activated NOS activity when the conversion reaction was initialised. nNOS activity is presented in the results as the difference between the basal and L-arginine-stimulated activity in each of the brain areas.

cGMP analysis. Since NOx determination is a surrogate marker of authentic NO, and L-citrulline for NOS activity, a third approach to measuring activity of the NOS pathway is to assay downstream signalling through accumulation of cGMP. A parallel relationship exists between NOS and NO-mediated accumulation of cGMP in the rat brain (55,56). The second messenger cGMP was therefore measured in the aforementioned brain areas using a direct competitive immunoassay (enzyme-linked immunosorbent assay) kit (Sigma-Aldrich) (57). Differences in sample basal cGMP concentration between FSL and FRL rats were determined according to the manufacturer instructions (Sigma-Aldrich Catalog number CG200), with the measured optical density expressed in pmol cGMP formed/mg protein. Protein was routinely assayed by the method of Bradford (45).

ACh analysis. At the day of analysis, brain tissue was weighed, using ±100 mg hippocampal and frontal cortical tissue for ACh analysis. A solution of 1 ml of 0.1 M $HClO_4$ and 4 μM physostigmine (Eserine®) was added to each vial of brain tissue. The vials were sonicated for 2 × 10 s after which it was centrifuged at 20 000 × g for 15 min (4 °C) in a Sigma 3K15 bench top centrifuge. The preparation of supernatant was performed on ice.

ACh was quantified by means of a liquid chromatography method coupled to electron spray ionisation tandem mass spectrometery (58). The chromatographic system consisted of an Agilent G1312A binary pump, a G1379B micro vacuum degasser and a thermostat autosampler fitted with a six port injection valve with a 100 μl loop capillary. The analytes ACh, choline and internal standard, neostigmine, were separated on a cation-exchange column (Hamilton PRP-X200; 150 × 4.1 mm internal diameter). The temperature of the column was maintained at 25 °C. Compounds were detected with an Agilent 6410 liquid chromatography/mass spectrometer (LC/MS/MS). The most abundant fragment ion was selected for each compound by performing a product ion scan. Multiple reaction monitoring (MRM) transitions of 146.2→87.1 for ACh, 104→60 for choline and 223.2→72.1 for neostigmine were chosen according to the most abundant fragment ion. Since ACh, choline and neostigmine are positively ionised in an environment with low pH, MRM was performed in positive electron spray ionisation mode. Compound analysis was optimised prior to sample analysis by direct infusion with a liquid chromatography flow rate of 0.3 ml/min. The collision energy voltage, fragmentation voltage and capillary voltage were adjusted to give the highest sensitivity with the injection program and set at voltages of 20, 80 and 5000, respectively. Nitrogen was used as nebuliser gas and desolvation gas. The gas temperature (°C), gas flow (l/min) and nebuliser pressure (psi) were set at 300, 10 and 45, respectively. The mobile phase consisted of 5 mM ammonium acetate and 100% acetonitrile. The solution was prepared by dissolving

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115.6 mg ammonium acetate in 300 ml of purified water (Milli Q). The pH was adjusted to 4.0 with glacial acetic acid and then 700 ml of acetonitrile was added and thoroughly mixed. The solution was filtered under vacuum through a 0.45 µm membrane filter. The HPLC system was purged with increased eluent flow before adjusting the flow to 0.3 µl/min for sample analysis with isocratic elution. The supernatant (250 µl) was added to 20 µl of 0.1 mg/ml neostigmine internal standard. An amount of 100 µl was added to an insert vial and 10 µl withdrawn from the vial and injected on the column. ACh was expressed as ng/mg tissue.

Statistical analysis. All data were analysed with Statistica® and graphically presented with Graphpad Prism® (Statistica Data Analysis Software System, version 8; Statsoft Inc., 2007; Graphpad software, version 5.0 for Windows®, San Diego, CA, USA). All data were non-parametrically analysed in view of the small sample sizes and possible non-normal distribution of the data. The Mann-Whitney U test was used, as will be indicated under the Results section. The representation of data was expressed as means ± standard error of the mean, and statistical significance defined as $p < 0.05$ in all instances.

Results

Nitric oxide analysis in frontal cortex and hippocampus

NOx determination. Mann-Whitney U test analysis showed no significant differences in endogenous NOx concentrations between the FRL control rats and the FSL rats in either the frontal cortex (23.63 ± 3.43 vs. 20.71 ± 1.60, $z = -0.04$, $p = 0.97$) or the hippocampus (23.20 ± 1.73 vs. 27.33 ± 3.99, $z = 0.94$, $p = 0.34$).

nNOS activity. Mann-Whitney U test analysis showed no significant differences in L-arginine-activated NOS activity between FRL and FSL rats in either the frontal cortex (0.74 ± 0.86 vs. 1.60 ± 0.83, $z = 0.64$, $p = 0.52$) or the hippocampus (3.19 ± 1.78 vs. 4.42 ± 1.95, $z = 0.79$, $p = 0.43$).

cGMP analysis in frontal cortex and hippocampus

Mann-Whitney U test analysis showed a significant difference in endogenous cGMP concentrations in the frontal cortex of FSL versus FRL control rats, with a significant decrease in cGMP concentrations noted in FSL relative to FRL rats (14.05 ± 0.44 vs. 15.57 ± 0.85, $z = -2.61$, $p = 0.009$; Fig. 1a). However, there were no significant differences in hippocampal cGMP concentrations in FSL versus FRL rats (17.80 ± 1.05 vs. 18.56 ± 1.57, $z = -0.94$, $p = 0.34$; Fig. 1b).

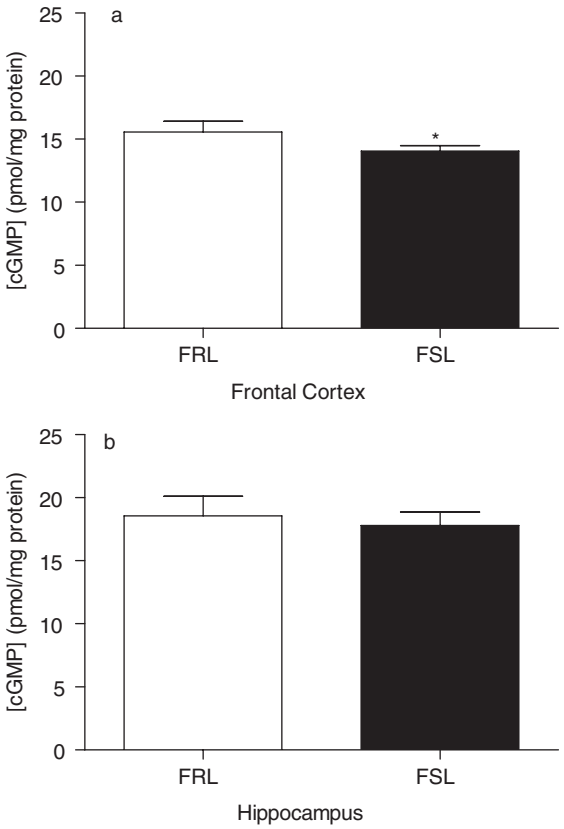

Fig. 1. cGMP concentrations in pmol/mg protein in the frontal cortex (a, $p = 0.009$) and hippocampus (b, $p = 0.34$) of FSL and FRL rats. Data were analysed by the Mann-Whitney U test ($n = 10$, mean ± standard error of the mean).

ACh levels in frontal cortex and hippocampus

The Mann-Whitney U test revealed significant differences between FSL and FRL control rats with respect to endogenous ACh levels in the frontal cortex and hippocampus. ACh levels in the frontal cortex were significantly elevated in FSL rats (0.63 ± 0.02 vs. 0.47 ± 0.04, $z = 2.83$, $p = 0.005$; Fig. 2a) but significantly lower in the hippocampus (0.64 ± 0.03 vs. 1.21 ± 0.06, $z = -3.74$, $p = 0.0002$; Fig. 2b) compared to their FRL controls.

Discussion

The present study has investigated basal activity levels of the NO-cGMP pathway in FSL rats compared to their FRL counterparts (control), specifically with respect to NOS activity and the accumulation of NOx and cGMP. Furthermore, we have also studied corticohippocampal ACh accumulation in these animals. The most important observations from this study is that although NOS activity remains unaltered

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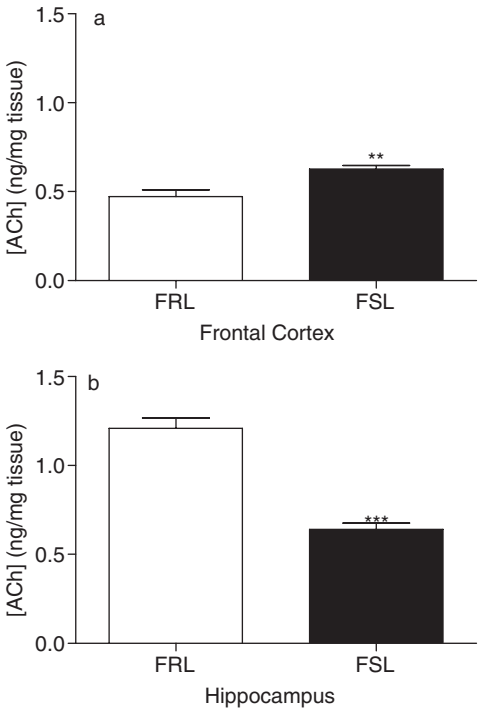

Fig. 2. Endogenous ACh levels in ng/mg tissue in the frontal cortex (a, $^{**}p = 0.005$) and hippocampus (b, $^{***}p < 0.001$) of FSL and FRL rats. Data were analysed using the Mann-Whitney U test ($n = 10$, mean ± standard error of the mean).

in stress-naïve FSL rats, these animals present with increased ACh and reduced cGMP levels in the frontal cortex. Moreover, there is an opposing reduction in ACh in the hippocampus but without any changes in cGMP. Importantly, reduced levels of frontal cortical cGMP occurred without concomitant changes in NOS activity or NOx accumulation.

Recent studies have begun to highlight the involvement of ACh in depression (18,59,60). The FSL rat model presents with an increased behavioural sensitivity to cholinergic agonists (61,62), increased ACh synthesis in the cortex and an increased concentration of mAChR in striatal and hippocampal brain areas (34,63). Since increased cholinergic activity has been proposed to underlie the development of depression (16–18,60), hypercholinergia has been proposed to mediate the depressive-like phenotype of these animals (29). The current study has confirmed this attribute, although we have now shown that while hypercholinergia may be evident in certain brain regions of FSL rats, such as the frontal cortex, a relative hypocholinergia is evident in the hippocampus.

Functionally, the hippocampus is implicated in spatial and contextual memory, while the frontal cortex mediates regulation of stress-related neuroendocrine function (64,65) and the interplay between emotions and memory formation (66). A lack of or inappropriate crosstalk between these areas forms the basis of the corticolimbic model of depression (67,68). Indeed, depression is associated with decreased activation of cortical regions and increased activation of limbic regions as a result of imbalances in connectivity in this circuit (69). With abundant expression of mAChR in the cerebral cortex and hippocampus (70), cholinergic input from the basal forebrain complex (71,72) is able to influence cortical arousal, consciousness, memory and learning (73). Various forms of stressful experience promote ACh release in the hippocampus and frontal cortex (74,75), while cortical-hippocampal dysfunction is implicated in aversive behaviour and cognitive disturbance following stress and re-experience in rats (76,77). These opposing changes in corticolimbic ACh levels in the FSL rat may underlie its stress-sensitive and depressogenic phenotype.

The importance of the NO/cGMP pathway in the pathology and treatment of depression and other stress-related illnesses is becoming more evident (78,79). Moreover, there is pre-clinical evidence for the interplay between ACh and NO-cGMP signalling in antidepressant action (19,20). Except for one paper from our laboratory (20), no other studies have investigated NO-ACh interactions in a genetic animal model of depression. NO and cGMP are among the principle messengers of the glutamatergic system (10,80), while cGMP is also an important messenger for the cholinergic system where it is involved in crosstalk between cholinergic and other neurotransmitter-mediated pathways (21). Indeed, the actions of well-known psychotropic compounds, such as phosphodiesterase type 5 (PDE-5) inhibitors (19,20) and lithium salts (81,82), have shown an interaction between the NO-cGMP and cholinergic systems.

Data from the current study, however, have failed to indicate any differences in the corticolimbic accumulation of NOx and L-citrulline used as an index of NOS activity in FSL rats versus FRL controls. However, this response in stress-naïve animals may not be so unexpected. Significant activation of the NMDA-NOS signalling cascade occurs in the hippocampus of FSL but not FRL rats following exposure to a sub-chronic stressor. However, this does not occur in the basal state (32). It would thus appear that under ambient (basal) conditions as evinced in this study, activity of the NO cascade appears to be unchanged in the frontal cortex and hippocampus in FSL rats. However, the presence of an environmental stressor sets in place a hyper-responsive NO cascade that may be a susceptibility marker for developing depression in stress-sensitive individuals (32). This is not unlike that seen in depression where a significant gene-environment interaction is evident (83).

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Of particular note in this study is that despite an absence of any change with respect to NOx accumulation or NOS activity in FSL rats in either of the two brain areas studied, we observed a significant decrease in frontal cortical cGMP in FSL rats, with no change in the hippocampus. This is particularly interesting in view of the importance of cGMP signalling in cortical function, especially in relating decreased levels of cGMP to deficits in cognition and its role in depressive symptoms (15,84). In this regard, lithium salts for example increase cortical cGMP levels in rats which may have relevance for its mood stabilising actions (14,81,85). The decrease in frontal cortex cGMP observed here not only suggests neuroanatomical differences between FSL and FRL rats but also implies differences in cortical function, such as cognition and goal-directed behaviour, which all contribute to the symptoms of depression (86).

The significant increase in frontal cortical ACh in FSL rats, together with a significant reduction in cGMP in this same brain region, raises the interesting caveat that the latter observation, and the well-known depressogenic phenotype of the FSL rat (30), may be related to increased ACh-mediated suppression of cortical cGMP. In fact, earlier reports have documented a decrease in cGMP levels in the cortex after ACh administration (87,88). This interaction is quite plausible since the antidepressant properties of PDE-5 inhibitors such as sildenafil are disinhibited following concurrent antimuscarinic receptor blockade (19,20). Although the exact mechanism responsible for such an ACh-cGMP interaction remains speculative, recent studies have established that the cholinergic-cGMP interaction regulating the antidepressant response of sildenafil involves the activation of protein kinase G and subsequent enhancement of serotonergic neurotransmission (89). Reduced cGMP and elevated ACh tone in the frontal cortex of these animals may in fact be related to the increased stress sensitivity and pro-depressive phenotype of these animals.

Contrary to that in the frontal cortex, we noted a significant reduction in hippocampal ACh levels in FSL versus FRL rats, which is in line with earlier studies describing an upregulation of hippocampal mAChR in FSL rats (33). Interestingly, we did not observe any simultaneous change in hippocampal cGMP levels. This suggests that while an ACh-cGMP interaction in the frontal cortex may be causally linked to depression and antidepressant response, this may not immediately apply to the hippocampus. However, this observation can possibly be ascribed to the stress-naïve state of the animals used in this study which may preclude an immediate involvement of the hippocampus (32). In fact, the role of hippocampal NO-cGMP signalling in depression and antidepressant response is robust (77,90,91), although our data would suggest less dependence on cholinergic involvement.

NO is an anterograde messenger in cholinergic neurons (22), and various NO donors have been found to enhance ACh release (92–94), so that reduced frontal cortical NO-cGMP signalling as noted here may be responsible for the observed increase in ACh levels. However, we were unable to show any associated changes in NOx accumulation or NOS activity in the frontal cortex of FSL rats, suggesting that increased ACh may originate from a NOS-independent mechanism of cGMP synthesis. For example, recent studies with the selective cGMP-PDE-5 inhibitor, sildenafil (which will increase cGMP), have emphasised its ability to augment cholinergic signalling (19,95,96). This prompts further investigation into the regulation of cGMP levels by various cGMP-specific PDEs (97,98) and which might further elucidate the mechanisms underlying ACh-NO-cGMP signalling in the FSL rat.

In conclusion, our data confirm that while corticolimbic NO-related changes are absent in stress-naïve FSL rats, these animals present with reduced frontal cortical (but not hippocampal) cGMP levels. This study has also confirmed and expanded on the hypercholinergic status of the FSL rat model of depression. Changes in ACh are regionally specific, with elevated ACh levels evident in the frontal cortex and reduced levels in the hippocampus. Increased synthesis of ACh and reduced cGMP levels may be causally related, especially in the frontal cortex, and confirm that frontal cortical cGMP-ACh interactions are an important consideration in the neurobiology and treatment of depression.

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