Biochemical Pharmacology 80 (2010) 1580–1591

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Role of monoamine oxidase, nitric oxide synthase and regional brain monoamines in the antidepressant-like effects of methylene blue and selected structural analogues

Brian H. Harvey [a,*], Ingrid Duvenhage [a], Francois Viljoen [a], Nellie Scheepers [b], Sarel F. Malan [b,d], Gregers Wegener [c], Christiaan B. Brink [a], Jacobus P. Petzer [b]

[a] Division of Pharmacology, Unit for Drug Research and Development, School of Pharmacy, North-West University (Potchefstroom campus), Hofman street, Private Bag X6001, Potchefstroom 2520, South Africa
[b] Division of Pharmaceutical Chemistry, Unit for Drug Research and Development, School of Pharmacy, North-West University (Potchefstroom campus), Hofman street, Private Bag X6001, Potchefstroom 2520, South Africa
[c] Center for Psychiatric Research, University of Aarhus, Aarhus, Denmark
[d] School of Pharmacy, University of Western Cape, Cape Town, South Africa

ARTICLE INFO

Article history:
Received 20 May 2010
Accepted 29 July 2010

Keywords:
Dual action
Methylene blue
Structural analogues
Antidepressant
Monoamine oxidase
Nitric oxide
Serotonin
Norepinephrine
Dopamine

ABSTRACT

Dual action antidepressants have important therapeutic implications. Methylene blue (MB), a charged compound structurally related to tricyclic antidepressants, acts on both monoamine oxidase (MAO) and the nitric oxide (NO)-cGMP pathway, and has demonstrated antidepressant activity in rodents. We investigated the antidepressant properties of MB and selected structural analogues and whether their actions involve MAO, NO synthase (NOS) and regional brain monoamines.

Acute imipramine (IMI, 15 mg/kg), saline, MB, acriflavine (ACR), methylene green (MG), methylene violet (MV), thionine (THI) and tacrine (TAC) (1-60 mg/kg i.p.) were tested for antidepressant activity in the forced swim test (FST), as well as MAO-A/B inhibitory activity. Active antidepressant compounds were subsequently studied at their most effective dose during sub-chronic treatment, followed by behavioural sampling in the FST and assay of cortico-limbic monoamines and hippocampal nitrate (for NOS activity).

Only IMI, MB (15, 30, 60 mg/kg) and MG (7.5, 25, 40 mg/kg) reduced immobility in the acute FST. MB, MG and ACR were potent inhibitors of especially MAO-A. Following sub-chronic treatment, IMI (15 mg/kg) increased noradrenergic behaviour in the FST, while MB (15 mg/kg) and MG (15 mg/kg) enhanced serotonergic behaviour. MB and MG bolstered cortico-limbic serotonin (5HT) levels and to a lesser extent *l*-norepinephrine (*l*-NE), but did not significantly alter regional dopamine (DA) levels. MB, and to lesser degree MG, reduced hippocampal nitrate levels.

MB and MG present with structure-specific antidepressant-like effects following acute and sub-chronic treatment, possibly involving NOS and MAO-A inhibition and cortico-limbic 5HT and *l*-NE release. A role for MAO-B and DA appears minimal.

1. Introduction

Depression is a serious and burdensome psychiatric illness that is exacerbated by its recurrent nature. In the United States, one in six individuals will develop clinical depression during their life time [1,2]. Despite a broad range of antidepressants available to the clinician, a significant proportion of these patients will not respond to treatment, or will show only partial response [3]. Current antidepressants are primarily modulators of monoamine neurotransmission, whereas it is now increasingly being accepted that the biogenic amine hypothesis is insufficient to fully explain all the dimensions of this illness [4]. Indeed, neurotrophins, neurogenesis and the concepts of neuroplasticity has now taken centre stage in our understanding of depression and the mechanism of action of antidepressants [4,5].

The nitric oxide/cyclic guanosine 3′5′-monophosphate (NO/cGMP) cell signalling pathway has been proposed to play a role in the neurobiology and treatment of affective disorders [6]. Various studies have emphasised the role of this pathway in the neurobiology of depression [7,8,9] and in antidepressant response [10,11,12]. Originally these studies reported antidepressant-like effects following inhibition of NO synthase (NOS) or guanylyl cyclase [10,13], although a dual effect for NO donors and NOS inhibitors in rodent models of depression has been demonstrated

* Corresponding author. Tel.: +27 18 299 2238; fax: +27 18 299 2225.
E-mail address: Brian.Harvey@nwu.ac.za (B.H. Harvey).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.07.037

[14,15]. Since NO and cGMP plays a pivotal role in neuroplasticity [16], in intra- and inter-cellular communication [17,18], and regulation of neurotransmitter release [17,18], this pathway forms a functional bridge between the biogenic amine and neuroplasticity hypotheses. Although NOS inhibitors are both potent and cell-permeable, none are currently used in the treatment of any disorder, the foremost reasons for this being their low isoform-selectivity [19]. Moreover, the ubiquitous presence of multiple NOS isoenzymes [17,20] means that both beneficial and adverse central nervous system (CNS) responses related to NO inhibition can be expected [19,21].

Methylene blue (MB) has been used to treat neuropsychiatric illnesses from as early as 1899 [22]. The compound displays promising pre-clinical antidepressant and anxiolytic activity [23] and in fact has shown promise in clinical trials for affective disorders [24,25,26]. Although the tricyclic structure of MB bears a strong resemblance to the tricyclic antidepressants (TCA), it has other unique physico-chemical attributes [27], including an ionic charge. MB is also a noteworthy inhibitor of monoamine oxidase (MAO) [28,29], a known antidepressant target [30], while it is also widely recognised as a non-selective inhibitor of NOS and guanylate cyclase [21,31,32]. Despite the potential for adverse cardiovascular and CNS toxicity due to indiscriminate inhibition of the NOS-cGMP cascade, MB seems to be devoid of any significant side-effects related to this target [24]. Despite its earlier clinical promise, however, interest in MB faltered probably due to the introduction at that time of the highly successful serotonin reuptake inhibitors (SRI). Nevertheless, MB has attracted renewed interest recently as a novel treatment strategy for Alzheimer's disease [33], and to improve mood and cognitive deficits in bipolar disorder (see http://clinicaltrials.gov/show/NCT00214877).

Given the role of MAO and NO in depression and antidepressant action, a combined effect on these targets may jointly contribute to the psychotropic actions of MB. A recent review has emphasised the therapeutic benefit of multiple sites of action in a single molecule [34]. Considering the multi-factorial nature of depression, designing an antidepressant with multiple sites of action may offer distinct therapeutic advantages over the more traditional approach where only one neurobiological target is selected. The combined inhibitory actions of MB on MAO and the NO-cGMP system may therefore represent a more effective approach to treating disorders of mood. In order to investigate whether MB may represent a novel lead compound for the design of similar acting antidepressants, we studied the structure–activity relationship of MB and a number of its analogues with respect to antidepressant effects in the forced swim test (FST) after acute treatment, together with their inherent actions on MAO-A and -B. We also assessed antidepressant activity, as well as serotonergic vs catecholaminergic driven behaviours in the FST, after sub-chronic treatment, together with determination of hippocampal nitrate accumulation, a measure of NOS activity, and accumulation of dopamine (DA), serotonin (5HT), 5-hydroxyindole acetic acid (5-HIAA), *l*-norepinephrine (*l*-NE) and methoxy hydroxyl phenyl glycol (MHPG) in cortico-striato-limbic regions of the brain. The latter are important neurobiological substrates in the treatment of depression.

2. Materials and methods

2.1. Rationale

MB evokes an antidepressant-like effect in rodents over a distinct dosage range [23]. We confirmed the antidepressant-like activity of MB in the FST following acute administration to establish a dosage range under our conditions. Thereafter, we undertook a dose–response analysis for possible antidepressant-like activity of various tricyclic analogues of MB and compared this to MB and to imipramine (IMI), a tricyclic compound with known antidepressant activity (see Fig. 1). Four different doses of each compound were tested relative to control in the acute FST and with respect to locomotor behaviour. Since a closer reflection of antidepressant efficacy is only attainable after chronic treatment [35,36], our main objective was to study the antidepressant efficacy of MB, IMI and those MB analogues that have demonstrated efficacy in the acute FST, but now following a dosing protocol over 7 days. The most effective dose observed in the acute FST study for each compound was used in the latter study. In a separate analysis, all analogues, including IMI and MB, were evaluated with respect to inhibitory effects on MAO-A and -B. Diverse swimming behaviours were analysed following sub-chronic (not after acute) treatment in order to differentiate actions on serotonergic (increased swimming) and catecholaminergic (increased climbing) pathways [37]. In order to strengthen this association, regional brain levels of DA, 5HT, 5-HIAA, NE, and MHPG were analysed. Since the hippocampus is altered in depressed patients [4], and changes in NOS have been demonstrated in rat hippocampus following antidepressant treatment [11,38], the resulting effects of IMI, MB and those analogues tested in the FST after sub-chronic treatment, were assessed with respect to actions on hippocampal NOS activity *ex vivo*.

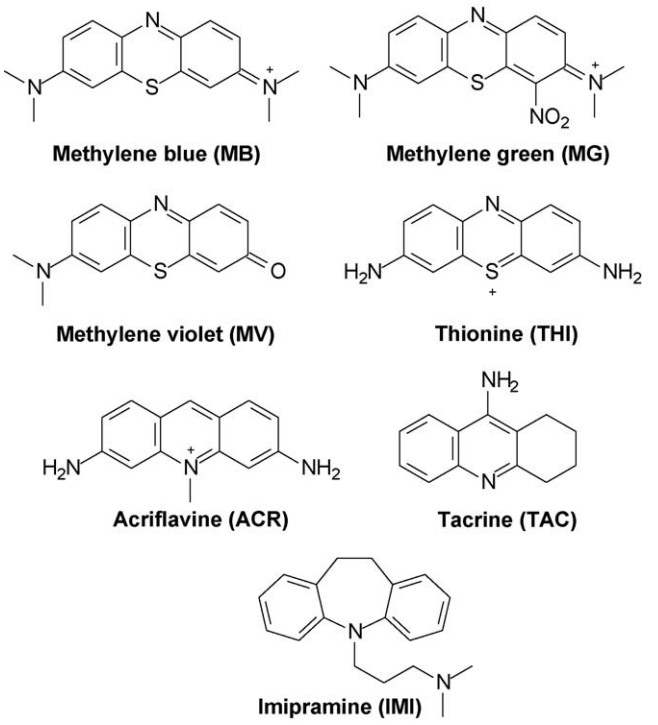

Fig. 1. Structures of imipramine (IMI) and methylene blue (MB) and related analogues of MB used in this study, including methylene green (MG), methylene violet (MV), thionine (THI), acriflavine (ACR) and tacrine (TAC).

2.2. Drugs and chemicals

MB and selected MB analogues, viz. acriflavine (ACR), methylene green (MG), methylene violet (MV), thionine (THI) and tacrine (TAC), were purchased from Sigma–Aldrich (St. Louis, USA). The nitric oxide fluorometric assay kit was purchased from Biovision (California, USA). Kynuramine·2HBr and commercially available recombinant human MAO-A (5 mg/ml) and recombi-

nant human MAO-B (5 mg/ml) was obtained from Sigma–Aldrich (St. Louis, USA). All reference materials for the preparation of monoamine standards were purchased from Sigma–Aldrich, as were all other remaining chemicals and reagents for the HPLC analysis.

2.3. Animals

Male Sprague–Dawley rats (200–250 g) were used for both acute and sub-chronic studies. In all cases, rats were provided by the Animal Research Centre of the North-West University. Ethical approval for the study was granted by the Ethical Committee of the North-West University (NWU-0070-08-55), and all housing and experimental conditions were in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals. Rats were housed five rats per cage under controlled conditions of temperature (21 ± 0.5 °C), humidity ($50 \pm 5\%$), full spectrum cold white light (350–400 lx) over a 12 h light/dark (06:00–18:00 light), with free access to food and water.

For the initial screening test in the acute FST, animals were randomised ($n = 5$/group) to receive either saline, IMI, MB, or the MB analogues ACR, MG, MV, THI and TAC. For the more comprehensive sub-chronic treatment study, animals were randomised ($n = 10$/group) to receive either saline, IMI, MB plus the designated MB analogue/s found to be effective in the acute FST.

2.4. Drug treatment

Doses selected for MB (7.5–60 mg/kg i.p.) and IMI (15 mg/kg i.p.) were based on the literature [23,38,39]. Likewise, doses for MG (7.5–40 mg/kg i.p.; [40]), ACR (7.5–40 mg/kg i.p.; [41]), TAC (2.5–7.5 mg/kg i.p.; [42]), MV (3.75–10 mg/kg i.p.; [43]) and THI (1.0–5 mg/kg; [40]), were administered using the existing literature as a guideline, or as solubility allowed. All animals were weighed each morning, and their respective dosages calculated accordingly. Drug solutions, freshly prepared on the day of treatment, were dissolved in saline and administered in a volume of 0.5 ml i.p. However, MV and THI were dissolved in saline with a minimal amount of 5% glacial acetic acid, buffered with NaOH (pH 6), and administered in a volume of 0.2 ml i.p. The latter two vehicles were then also used as control, where required.

2.5. Locomotor activity

Locomotor activity was evaluated to ensure that changes in swim motivation are based on antidepressant response and not due to an indirect effect of the drug on locomotor activity. 20 min after the final injection, each rat was placed separately into an open field arena (1 m^2) marked with sixteen 25 cm × 25 cm blocks and their general locomotor behaviour recorded on video. The total number of line crossings expressed as the number of squares the rat entered during a 5 min time interval was scored as a measure of locomotor activity [12].

2.6. Forced swim test (FST)

2.6.1. Acute treatment study

The FST was performed as previously described [12,36,44]. On day 1 of the acute drug treatment challenge, the rats were allowed to habituate to their surroundings for a period of 30 min. Thereafter each animal was subjected to 15 min of pre-swimming in transparent Perspex cylinders (18 cm (d) × 40 cm (h)) containing 20 cm of clean water (25 °C) after which the animals were dried and returned to their home cages. Immediately thereafter, each rat received the first of three i.p. doses of the test drug, i.e. 24 h prior to the final swim test on day 2. The animals received their respective treatments at the same time each day (between 8:00 and 9:00 a.m.). On day 2, the animals received their second and final i.p. doses 6 h and again 1 h prior to the final 5 min test swim. After the last injection the rats were assessed in the open field arena to evaluate general locomotor activity (see Section 2.5.). The rats were then left in the room to habituate for a further 20 min before the final 5 min test swim commenced. During this period, immobility time was recorded digitally and later scored by three blinded investigators, two of whom were not involved in the study.

2.6.2. Sub-chronic treatment study

All animals received their i.p injections at the same time each morning for a period of 7 days (between 8:00 and 9:00 a.m.). On the penultimate day of treatment, i.e. 24 h prior to the final test swim, the rats were placed in the room to habituate for 60 min after which they were subjected to 15 min of pre-swimming, as described above. The rats were immediately dried and returned to their home cages where they received their final i.p. dose of test compound. On the final day of treatment, and after a 20 min habituation period in the test room, the rats were assessed in the open field arena to determine locomotor activity (Section 2.5). An hour later, the rats were reintroduced to the swimming cylinders for their final 5 min test swim in the FST apparatus. Apart from immobility time, all swimming and climbing behaviours, as described previously [12,37], were also recorded and scored. The rats were immediately dried after the test swim and returned to their home cages. Approximately 4 h after completion of the FST the rats were decapitated.

2.7. Recombinant human MAO-A and -B inhibition studies

All six compounds, including IMI, were evaluated for their ability to inhibit recombinant human MAO-A and MAO-B. This assay is based on the extent to which kynuramine is oxidized to 2-hydroxyquinoline by MAO, as described previously [45]. The formation of 4-hydroxyquinoline was measured fluorometrically at excitation and emission wavelengths of 310 and 400 nm, respectively. None of the test compounds fluoresced at these wavelengths or quenched the fluorescence of 4-hydroxyquinoline at the concentrations used for the inhibition studies (data not shown). In this way, the IC_{50} values (concentration of the inhibitor that produces 50% inhibition) for the inhibition of MAO-A and -B by each test compound were determined and categorised as very high (10–99 nM), high (100–999 nM), moderate (1–9 μM), low (10–99 μM) and very low (100–1000 μM) and referenced against moclobemide (selective MAO-A inhibitor), *l*-deprenyl (MAO-B inhibitor) and clorgyline (MAO-A and B inhibitor) [46].

Recombinant human MAO-A and -B (5 mg/ml) were pre-aliquoted and stored at −70 °C. All enzymatic reactions were carried out in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) containing MAO-A (0.0075 mg/ml) or MAO-B (0.015 mg/ml), plus various concentrations of the test inhibitor (0–3000 μM) and kynuramine (45 and 30 μM for MAO-A and -B, respectively). The final volume of the reactions were 500 μl and stock solutions of the test inhibitors were prepared in DMSO and added to reactions to yield a final concentration of 4% (v/v) DMSO. The reactions were incubated for 20 min at 37 °C and terminated with the addition of 200 μl NaOH (2 N). Distilled water (1200 μl) was added to each reaction before it was centrifuged for 10 min at 16,000 × *g*. The concentrations of the MAO generated 4-hydroxyquinoline in the reactions were determined by measuring the fluoroscence of the supernatant at excitation and emission wavelengths of 310 and 400 nm, respectively. Quantitative estimations of 4-hydroxyquinoline were made by means of a

linear calibration curve ranging from 0.188 to 6.25 μM. Each calibration standard was prepared to a final volume of 500 μl in potassium phosphate buffer (100 mM, pH 7.4) and contained 4% DMSO, 200 μl NaOH (2 N) and 1200 μl distilled water. The IC_{50} values were determined by plotting the initial rate of oxidation vs the logarithm of the inhibitor concentration to obtain a sigmoidal dose–response curve. These kinetic data were fitted to the one-site competition model incorporated into the Prism software package and the IC_{50} values subsequently determined in duplicate and expressed as mean ± standard deviation (SD).

2.8. Tissue dissection

Animals used in the acute FST study were sacrificed by decapitation. Animals subjected to the sub-chronic treatment study were sacrificed by decapitation, whereupon the total hippocampus, frontal cortex and striatum were rapidly dissected out on an ice-cooled dissection slab, weighed and snap frozen in liquid nitrogen (−198 °C) before being used in the monoamine analysis (Section 2.10). In addition, separate hippocampal tissue was also used for the nitrate assay, as described below (Section 2.9).

2.9. Nitric oxide fluorometric assay

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 [47]. On the day of the analysis, samples were weighed and allowed to thaw. The brain tissue (hippocampus) was then homogenized on ice with a Heidolph glass-teflon homogenizer and suspended in 10% phosphate buffered saline (PBS). The analysis of sample nitrate was conducted using a commercially available kit as per the manufacturer's protocol (Biovision, California, USA), and expressed in pmol/g wet weight of tissue (mean ± SEM).

2.10. Regional brain monoamine determination

Quantification of monoamines in the hippocampus, frontal cortex and striatum was performed by a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described [48]. Monoamine concentrations in the samples were determined by comparing the area under the peak of each monoamine in the sample to that of the monoamine standard (range 5–50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) were found in this particular range. Monoamine concentrations were expressed as ng/g wet weight of tissue (mean ± SEM).

2.11. Statistical analysis

In the acute FST studies, the effect of saline or vehicle (acid-buffered saline), and IMI in saline and IMI in vehicle, were analysed using a two-way analysis of variance (ANOVA) followed by a Bonferroni posttest, while the effect of the different analogues in either saline or vehicle respectively, as required (see Section 2.4.), were analysed together by a one-way ANOVA followed by the Dunnett's test (Graphpad software, version 5.0 for Windows®, San Diego, USA). For ease of viewing and interpretation, these data are presented separately for each drug. For the sub-chronic treatment studies, all behavioural and neurochemical data were subject to one-way ANOVA plus post hoc analysis using the Dunnett's or Tukey–Kramer tests, as indicated. In all cases, data are expressed as the mean ± standard error of the mean (SEM), with a p-value of <0.05 deemed statistically significant.

3. Results

3.1. Acute FST and locomotor studies

3.1.1. Imipramine, methylene blue (MB) and various MB analogues

Two-way ANOVA of control vs IMI in the FST, using saline and acid-buffered saline as co-variants, found no significant interaction with vehicle [$F(1,16) = 0.3$, $p = 0.59$], but a significant effect of IMI treatment vs saline ($p < 0.01$; Fig. 2) and vs acid-buffered saline ($p < 0.05$; Fig. 2). Similarly, two-way ANOVA of control vs IMI in the locomotor analysis, using saline and acid-buffered saline as co-variants, found no significant interaction with vehicle [$F(1,16) = 0.06$, $p = 0.81$], while no significant effect of IMI treatment vs saline or acid-buffered saline was evident in the post hoc analysis ($p > 0.05$, Fig. 2).

One-way ANOVA of the immobility data in the saline controlled studies describes a significant effect of treatment [$F(16,68) = 10.38$; $p < 0.0001$] as well as a significant effect on locomotor activity [$F(17,70) = 7.72$; $p < 0.0001$]. MB treatment induced a dose-dependent decrease in immobility, with a significant dose-related suppression of immobility at doses of 15 mg/kg ($p < 0.05$), 30 mg/kg ($p < 0.001$) and at 60 mg/kg ($p < 0.001$) compared to saline, whereas a lower dose of 7.5 mg/kg was ineffective (Fig. 2A). MB significantly decreased locomotor activity compared to saline at higher doses of 15 mg/kg ($p < 0.01$) and 60 mg/kg ($p < 0.001$; Fig. 2B), whilst it did not reach statistical significance at 30 mg/kg. MG was also markedly effective in the FST, inducing a significant decrease in immobility at doses of 7.5 mg/kg ($p < 0.001$), 15 mg/kg ($p < 0.001$), 25 mg/kg ($p < 0.001$) and 40 mg/kg ($p < 0.001$) compared to saline (Fig. 2E). MG also significantly decreased locomotor activity compared to saline-treated rats at doses of 7.5 mg/kg ($p < 0.05$; Fig. 2F), 25 mg/kg ($p < 0.001$; Fig. 2F) and 40 mg/kg ($p < 0.001$; Fig. 2F). ACR, however, was ineffective on immobility at all the doses tested (Fig. 2C), although it significantly decreased locomotor activity compared to saline at all doses tested, viz. 7.5 mg/kg ($p < 0.01$; Fig. 2D, 15 mg/kg ($p < 0.01$; Fig. 2D), 30 mg/kg ($p < 0.001$; Fig. 2D) and 40 mg/kg ($p < 0.001$; Fig. 2D). Similarly, TAC failed to induce a significant reduction in swim immobility in the FST (Fig. 2G), while it also significantly decreased locomotor activity at doses of 2.5 mg/kg ($p < 0.001$; Fig. 2H), 3.75 mg/kg ($p < 0.001$; Fig. 2H), 5 mg/kg ($p < 0.001$; Fig. 2H) and 7.5 mg/kg ($p < 0.001$; Fig. 2H).

One-way ANOVA of the immobility data in the acid-buffered saline controlled studies describes a significant effect of treatment [$F(8,36) = 2.57$; $p = 0.03$] as well as a significant effect on locomotor activity [$F(13,56) = 4.18$; $p < 0.0001$]. All doses of MV were ineffective in reducing immobility in the FST (Fig. 2I). However, MV evoked a trend for a general suppression of locomotor activity, although only attaining statistical significance vs vehicle control at a dose of 7.5 mg/kg ($p < 0.01$; Fig. 2J). Similarly, THI treatment was without affect on immobility time in the FST or on locomotor activity (Fig. 2K and L).

3.2. In vitro recombinant human MAO-A and MAO-B studies

Table 1 presents the IC_{50} values for MAO-A and MAO-B for the various compounds under study, and in comparison with the IC_{50} for known MAO-A and B inhibitors (according to Chimenti et al. [46]). In order to classify the various compounds under study according to their apparent potency to inhibit MAO-A or MAO-B, we have tabulated these data with respect to potency relative to MB, a known MAO inhibitor in this group (Table 2). According to this categorisation, MB is a very high potency inhibitor of MAO-A and a moderate inhibitor of MAO-B. Relative to MB, MG is a high potency inhibitor of MAO-A as well as a moderate inhibitor of MAO-B. On the other hand, IMI is a low to very low potency inhibitor of both MAO-A and -B. MV

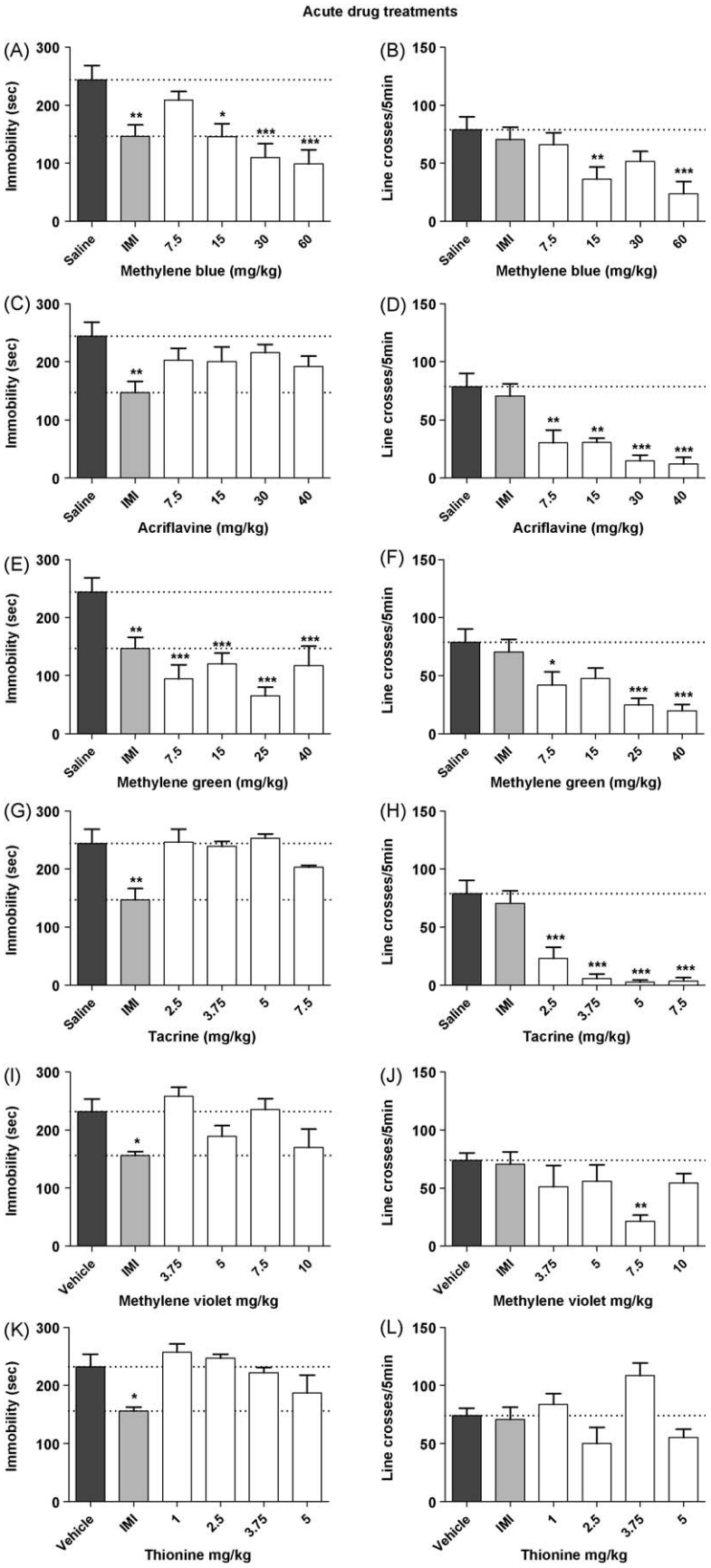

Fig. 2. FST: Effect of acute IMI (15 mg/kg; Bonferroni vs saline/vehicle) treatment and various doses of MB, ACR, MG, TAC, MV, and THI, as indicated, on the duration of immobility in the FST (A; C; E; G; I; K), and on locomotor activity in the open field test (B; D; F; H; J; L), compared to control (n = 5/group; Dunnett's test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1
The IC_{50} values for the inhibition of recombinant human MAO-A and -B by IMI, as well as by MB and its analogues.

Compound	MAO-A IC_{50} value[a] (μM)	MAO-B IC_{50} value[a] (μM)
Methylene blue	0.07 ± 0.17	4.37 ± 0.14
Methylene green	0.25 ± 0.11	5.5 ± 0.23
Imipramine	12500 ± 0.11	>10
Methylene violet	2.76 ± 0.42	4.20 ± 0.50
Acriflavine	0.43 ± 0.25	>10
Thionine	371.8 ± 0.36	2.66 ± 0.25
Tacrine[b]	424.5 ± 1.22	>1
Clorgyline[c]	0.004	61.35
l-Deprenyl[c]	67.25	0.02
Moclobemide[c]	361.38	None

[a] The IC_{50} values are expressed as the mean ± SD of duplicate determinations. Lower IC_{50} values suggest a more potent inhibitor. IC_{50} for clorgyline, *l*-deprenyl and moclobemide are provided for comparison.
[b] Tacrine could not be evaluated at higher concentrations since it fluoresces.
[c] Values obtained from literature [46].

can be regarded as a moderate inhibitor of MAO-A and a moderate inhibitor of MAO-B. ACR is a high potency inhibitor of MAO-A and a low-very low potency inhibitor of MAO-B. THI and TAC were found to be very low potency inhibitors of MAO-A. Interestingly, THI is a moderate potency MAO-B inhibitor with TAC a moderate to very low inhibitor of this enzyme.

3.3. FST and locomotor studies vs IMI after sub-chronic treatment

According to the acute FST studies (Section 3.1.), only MB and MG could be deemed to be effective antidepressants. Based on the data presented in Fig. 2 for MB and on earlier studies [23], a dose of 15 mg/kg was selected for evaluating MB in the sub-chronic treatment study. Since no earlier studies have been performed on MG with limited rodent toxicology data, we selected a nominal mid-tier dose for MG, namely 15 mg/kg [40]. This dose is within the known dosage range of MB thus limiting the risk of unnecessary toxicity in the animal. The acute FST data also suggest that a dosage of 15 mg/kg for both MB and MG are equivalent with IMI so that any improved response in the FST over IMI after sub-chronic treatment would not be due to bias introduced by a higher dose.

Sub-chronic drug treatment evoked a marked effect on immobility in the FST [$F(3,36) = 15.42$; $p < 0.0001$], with post hoc analysis revealing a significant decrease in immobility in IMI vs saline-treated rats ($p < 0.01$; Fig. 3A). Both MB ($p < 0.001$; Fig. 3A) and MG ($p < 0.05$; Fig. 3A) significantly decreased immobility time vs saline-treated rats. Furthermore, MB evoked a greater decrease in immobility time compared to MG-treated rats ($p < 0.01$; Fig. 3A) and narrowly missed significance compared IMI-treated rats (Fig. 3A).

One-way ANOVA of the swimming data revealed a significant effect of treatment [$F(3,36) = 21.95$; $p < 0.0001$]. Post hoc analysis found no differences in swimming behaviour between saline and IMI-treated rats. MB significantly increased swimming behaviour compared to saline-treated rats ($p < 0.001$; Fig. 3B), vs IMI-treated rats ($p < 0.001$; Fig. 3B) and vs MG-treated rats ($p < 0.05$; Fig. 3B). MG also significantly increased swimming behaviour compared to saline-treated rats ($p < 0.01$; Fig. 3B) as well as vs IMI-treated rats ($p < 0.01$; Fig. 3B). One-way analysis of variance of the climbing data similarly revealed a significant effect of treatment [$F(3,36) = 11.80$; $p < 0.0001$]. Post hoc analysis of these data found that IMI significantly increased climbing behaviour compared to saline-treated rats ($p < 0.001$; Fig. 3C), vs MG-treated rats ($p < 0.001$; Fig. 3C) and vs MB treated rats ($p < 0.05$; Fig. 3C). Both MB and MG were ineffective in modifying climbing behaviour vs saline, although MB treatment did evoke a trend towards higher levels of climbing behaviours (Fig. 3C).

One-way ANOVA of the locomotor data revealed a significant effect of treatment [$F(3,36) = 6.81$; $p = 0.0009$]. Post hoc analysis indeed revealed there to be a significant decrease in locomotor activity in the IMI and MG-treated groups vs saline ($p < 0.05$ and $p < 0.001$, respectively; Fig. 3D), while MB treated rats revealed a slight but insignificant decrease in locomotor activity vs saline (Fig. 3D).

3.4. Regional brain monoamines following sub-chronic treatment

3.4.1. Frontal cortex

Drug treatment induced a significant effect on cortical levels of 5HT [$F(3,16) = 13.44$; $p = 0.0001$] and *l*-NE [$F(3,16) = 12.49$; $p = 0.0002$], and narrowly missed significance with respect to treatment effects on the levels of their associated metabolites, 5-HIAA [$F(3;16) = 2.91$; $p = 0.07$] and MHPG [$F(3,16) = 2.72$; $p = 0.08$]. Drug treatment had no effect on cortical DA levels [$F(3,16) = 0.51$, $p = 0.68$] (Fig. 4A–E). Post hoc analysis found that MB induced a significant increase in frontal cortical levels of both 5HT ($p < 0.001$; Fig. 4A) and *l*-NE ($p < 0.01$; Fig. 4C). MG induced a marginal but non-significant increase in 5HT (Fig. 4A), but a significant increase in *l*-NE levels ($p < 0.01$; Fig. 4C) in this brain region. Both MB ($p < 0.001$; Fig. 4A) and MG ($p < 0.05$; Fig. 4A) induced higher cortical 5HT levels than did IMI. Similarly, MB ($p < 0.01$; Fig. 4C) and MG ($p < 0.01$; Fig. 4C) induced significantly higher cortical *l*-NE levels than did IMI. Interestingly, IMI was without noteworthy effects compared to saline treatment, on any of the cortical monoamines analysed (Fig. 4A–E).

3.4.2. Hippocampus

Drug treatment induced a significant effect on hippocampal levels of 5HT [$F(3,16) = 18.90$; $p < 0.0001$] and *l*-NE [$F(3,16) = 5.19$; $p = 0.01$], as well as a significant effect on the levels of 5-HIAA [$F(3,16) = 4.93$; $p = 0.01$] but not on levels of MHPG [$F(3,16) = 1.28$; $p = 0.32$]. An effect of drug treatment narrowly missed significance with respect to hippocampal DA levels [$F(3,16) = 2.56$, $p = 0.09$] (Fig. 5A–E). Post hoc analysis found that MB induced a significant

Table 2
Relative MAO inhibition potency of the compounds under study.

Compound	MAO-A		MAO-B	
	[a]Potency relative to MB	Classification	[a]Potency relative to MB	Classification
Methylene blue	1.0	VH	1.0	M
Methylene green	3.6	H	1.3	M
Imipramine	178571.4	VL	2.3	L–VL
Methylene violet	39.4	M	1.0	M
Acriflavine	6.1	H	2.3	L–VL
Thionine	5311.4	VL	0.61	M
Tacrine	6064.3	VL	0.23	M–VL

IC_{50} values are converted to relative potency vs that of methylene blue (MB).
[a] Potency classification: VH, very high (10–99 nM); H, high (100–999 nM); M, moderate (1–9 μM); L, low (10–99 μM); VL, very low (100–1000 μM).

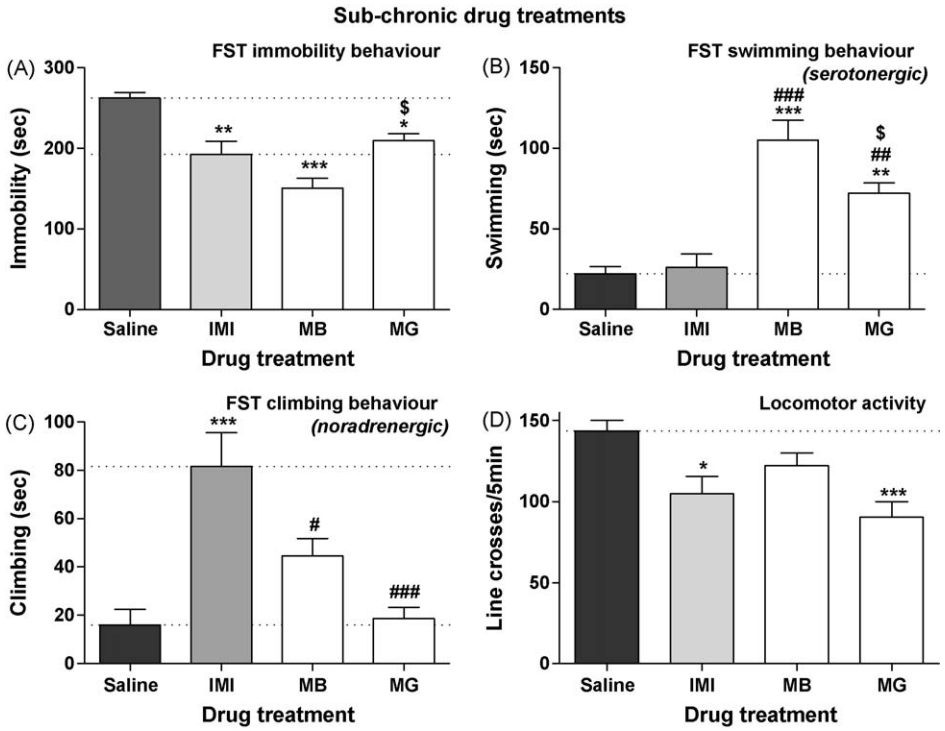

Fig. 3. FST: Effect of sub-chronic treatment with IMI (15 mg/kg), MB (15 mg/kg) and MG (15 mg/kg) as indicated, on (A) duration of immobility in the FST; (B) swimming (5HT) behaviour in the FST; (C) climbing (NE) behaviour in the FST; (D) locomotor activity in the open field test ($n = 10$/group; Tukey–Kramer test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs saline; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs IMI; $^{\$}p < 0.05$ vs MB.

increase in hippocampal levels of both 5HT ($p < 0.001$; Fig. 5A) and *l*-NE ($p < 0.01$; Fig. 5C), together with a significant lowering of 5-HIAA levels ($p < 0.01$; Fig. 5B). MG on the other hand only significantly elevated 5HT levels ($p < 0.05$; Fig. 5A), without affecting 5-HIAA (Fig. 5B). MG had no effect on *l*-NE levels in the hippocampus (Fig. 5C). Importantly, both MB ($p < 0.001$; Fig. 5A) and MG ($p < 0.01$; Fig. 5A) more significantly elevated hippocampal 5HT levels to a higher degree than that of IMI, while MB and MG were equally effective in raising 5HT levels in this brain region (Fig. 5A). IMI again was without noteworthy effects on 5HT and NE (Fig. 5A and C), but slightly lowered 5-HIAA (Fig. 5B) and DA (Fig. 5E) and slightly elevated MHPG levels (Fig. 5D). The latter changes, however, were not significant.

3.4.3. Striatum

Drug treatment induced a significant effect on striatal levels of 5HT [$F(3,16) = 33.28$; $p < 0.0001$] and *l*-NE [$F(3,16) = 4.13$; $p = 0.02$]. Similarly, a significant effect of treatment was observed on the levels of 5-HIAA [$F(3,16) = 5.80$; $p = 0.007$] and MHPG [$F(3,16) = 5.98$; $p = 0.006$]. Drug treatment had no effect on striatal DA levels [$F(3,16) = 2,11$, $p = 0.14$] (Fig. 6A–E). Post hoc analysis found that both MB ($p < 0.001$; Fig. 6A) and MG ($p < 0.05$; Fig. 6A) induced a significant increase in striatal levels of 5HT, although neither was able to alter striatal *l*-NE levels (Fig. 6C). Importantly, both MB ($p < 0.001$; Fig. 6A) and MG ($p < 0.001$; Fig. 6A) induced significantly higher 5HT levels compared to IMI. Furthermore, MB was more effective in raising 5HT than MG ($p < 0.05$; Fig. 6A). Interestingly, IMI evoked a significant reduction in striatal 5HT levels ($p < 0.05$; Fig. 6A), with a similar albeit insignificant response on striatal NE levels (Fig. 6C). IMI also induced a significant reduction in striatal 5-HIAA levels compared to saline ($p < 0.05$; Fig. 6B) and compared to MG ($p < 0.05$; Fig. 6B), while MB and MG were without effect (Fig. 6B). Both MB and MG elevated striatal levels of MHPG, although only the response to MG compared to saline ($p < 0.05$; Fig. 6D) and IMI ($p < 0.01$; Fig. 6D) was able to reach statistical significance.

3.5. Hippocampal NOx accumulation following sub-chronic treatment

Fig. 7 provides the comparative effects of the three compounds studied in the sub-chronic study on hippocampal NOx (nitrite plus nitrate) accumulation, as determined by accumulation of nitrate. One-way ANOVA of the data found a significant effect of sub-chronic drug treatment on hippocampal nitrate accumulation ($F(3,16) = 3.89$; $p = 0.03$). Post hoc analysis (Dunnett's) found that while IMI treatment was without effect (Fig. 7), MB treatment significantly reduced hippocampal nitrate compared to saline-treated animals ($p < 0.05$; Fig. 7), while MG also demonstrated a definite trend towards lowered nitrate concentrations although failed to reach significance (Fig. 7).

4. Discussion

This paper has considered the proposition of a multi-mode of action antidepressant that targets the nitrergic and monoaminergic systems, based on the tricyclic compound MB and using IMI as reference antidepressant. IMI induced an antidepressant-like response in the acute FST without marked locomotor effects (Fig. 2) that could not be related to MAO-A/B inhibition (Table 1). Similarly, MB was antidepressant at doses between 15 and 60 mg/kg (Fig. 2A) without any confounding increase in locomotor activity (Fig. 2B). These results concur with earlier studies [23] describing MB's antidepressant-like effects between 15 and 30 mg/kg, but not at 60 mg/kg (possibly due to differences in study conditions). With the acute FST being sensitive to MAO inhibitors [44], earlier studies [28,29], as well as the current study, has confirmed MB to be a very high potency inhibitor of MAO-A and a moderate inhibitor of MAO-B (Tables 1 and 2). MG is also

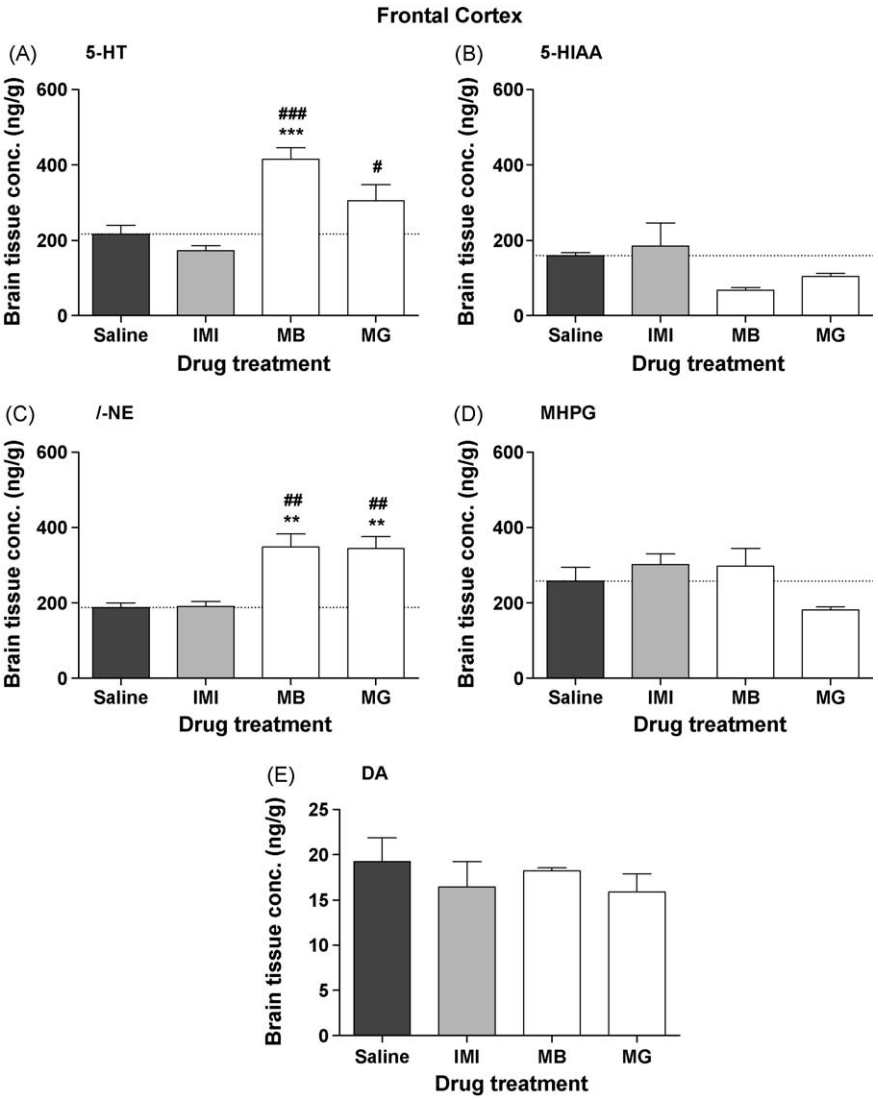

Fig. 4. Effect of sub-chronic treatment with IMI (15 mg/kg), MB (15 mg/kg) and MG (15 mg/kg) as indicated, on frontal cortical levels of (A) 5HT; (B) 5-HIAA; (C) *l*-NE; (D) MHPG and (E) DA (n = 5/group; Tukey–Kramer test). $^{**}p < 0.01$; $^{***}p < 0.001$ vs saline; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs IMI

antidepressant-like at doses of 7.5, 15, 25, and 40 mg/kg (Fig. 2E), despite a reduction in locomotor activity (Fig. 2F). We also found MG to be a high potency MAO-A inhibitor and a moderate MAO-B inhibitor (Tables 1 and 2) which very likely contributes towards its antidepressant-like effects.

Acute ACR, TAC, MV, and THI treatment failed to evoke a noteworthy antidepressant-like response in the acute FST (Fig. 2C, G, I, K). Since we noted that ACR displays high potency MAO-A inhibition, although with only low to very low MAO-B inhibitory activity (Tables 1 and 2), we were expectant of an antidepressant-like response. This was not the case. TAC and MV similarly are very low to moderate MAO inhibitors. However, ACR (7.5, 15, 30 and 40 mg/kg; Fig. 2D) as well as TAC (2.5, 3.75, 5, and 7.5 mg/kg; Fig. 2H) and MV (7.5 mg/kg; Fig. 2J) significantly suppressed locomotor activity. Although THI did not adversely affect locomotor activity (Fig. 2L) and also displayed very low to moderate MAO-A and MAO-B inhibition (Tables 1 and 2), the observed MAO inhibition may be insufficient for antidepressant effects. Although further dose-ranging studies may identify antidepressant-like properties without adverse locomotor effects, these preliminary data suggest a lack of antidepressant properties with these compounds. Nevertheless, ACR is an interesting compound as it did not suppress activity in the FST above baseline while both MG and ACR suppress locomotor activity to a similar degree, yet only MG remains effective in the FST. This difference may be due to MG's ability to inhibit both MAO-A and B while ACR is a low to very low potency MAO-B inhibitor (Table 2). This moderate potency inhibition of MAO-B, which is more DA selective [30], suggests a role for DA in MG action. However, as illustrated in the regional brain monoamine studies described below, DA may be less of an important player suggesting another possible mechanism, such as inhibition of NOS. Concluding, apart from IMI, MB and MG were the only effective antidepressant compounds in the acute FST, with MAO-A and possibly MAO-B inhibition evidently an important contributor to these effects. Upon subsequent testing after sub-chronic treatment, IMI again evoked a significant antidepressant-like response (Fig. 3A), as did equivalent doses of MB and MG (Fig. 3A). MB treatment proved more effective than MG and numerically superior than IMI, while MG evoked a similar response to that of IMI (Fig. 3A). Both IMI and MG significantly decreased locomotor activity, with MB engendering the same response, but not significantly (Fig. 3D), thus confirming the antidepressant effects of these compounds after sub-chronic treatment.

Although IMI is not a MAO inhibitor (Tables 1 and 2), it exerts a preferential action on monoaminergic responses by inhibiting *l*-NE

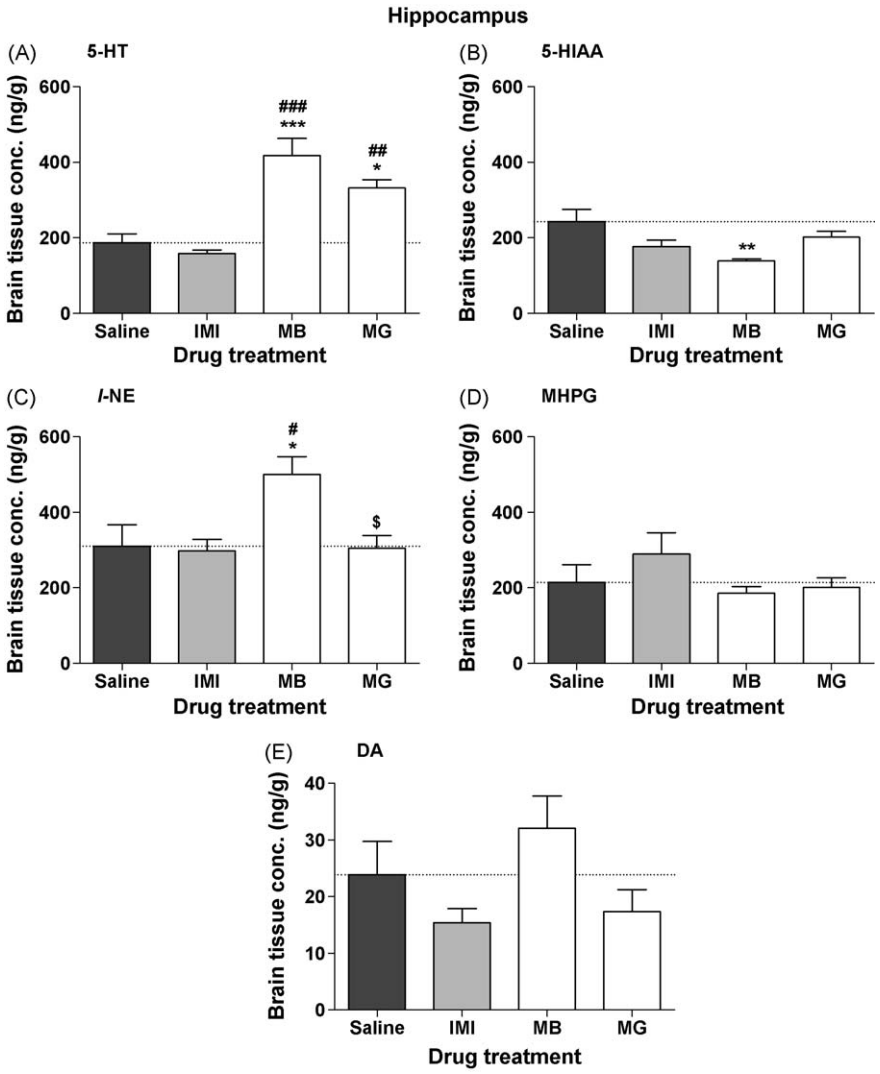

Fig. 5. Effect of sub-chronic treatment with IMI (15 mg/kg), MB (15 mg/kg) and MG (15 mg/kg) as indicated, on hippocampal levels of (A) 5HT; (B) 5-HIAA; (C) *l*-NE; (D) MHPG and (E) DA (n = 5/group; Tukey–Kramer test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs saline; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs IMI; $^{\$}p < 0.05$ vs MB

and 5HT reuptake [49]. IMI significantly increased *l*-NE (climbing) related behaviour (Fig. 3C) but with little effect on 5HT (swimming) behaviours (Fig. 3B). The latter observation is unusual considering its dual noradrenergic and serotonergic activity [49], although has been observed before [12]. This observation may be due to IMI being metabolised to desipramine, a predominantly noradrenergic antidepressant [49]. However, as will be described later, IMI failed to increase *l*-NE in either of the brain regions studied, actually lowering it in the striatum (Figs. 4–6). It is generally accepted that *l*-NE and 5HT systems interact at various levels [30,50] which make it difficult to accurately define and separate noradrenergic vs serotonergic behaviours under all conditions. This blurring of the lines emphasises the value of combined bio-behavioural (i.e. monoamines, MAO, FST) analysis, as utilised in this study. These data thus suggest the presence of, although arguably not exclusively, a noradrenergic basis for the efficacy of sub-chronic IMI treatment in the FST.

MB, however, significantly increased serotonergic mediated behaviours while only slightly (albeit insignificantly) increasing noradrenergic behaviours (Fig. 3B and C). Similarly, MG also selectively increased serotonergic related behaviours (Fig. 3B), without affecting noradrenergic behavior (Fig. 3C), thus affirming the serotonergic nature of both MB and its psychoactive analogue, MG. Interestingly, in an earlier pilot study, we found that IMI, MB and MG selectively increase noradrenergic- over serotonergic-mediated behaviour following acute treatment [51], thus emphasising the discrepancy in behavioural response to acute and sub-chronic drug treatment. Importantly, sub-chronic MB treatment was superior to both IMI and MG with respect to its ability to increase serotonergic behaviours (Fig. 3B) while on the other hand IMI was decidedly more effective on noradrenergic signalling than MB or MG (Fig. 3C). Since antidepressant efficacy requires long-term and not acute treatment, the sub-chronic FST data more accurately reflects the clinically relevant actions of these compounds on synaptic monoamine levels. Indeed, these findings are in support of clinical evidence for 5HT syndrome following the administration of MB with other serotonergic agents [28,29,52].

Recent studies have emphasised that multiple target drugs [34], or that combining more than one antidepressant with different modes of action, may offer distinct therapeutic advantages in treating depression. MAO is a recognised target for antidepressant action [30,49], while various studies have emphasised the importance of NOS inhibition in antidepressant response [10,11,38]. We have confirmed the MAO-A and -B inhibitory actions of MB and MG (Tables 1 and 2). MB is also an inhibitor of the NO-cGMP system [21,32], although no studies to date have evaluated MG in this regard. Although not significant, our study did indicate a numerical advantage in the FST response after sub-

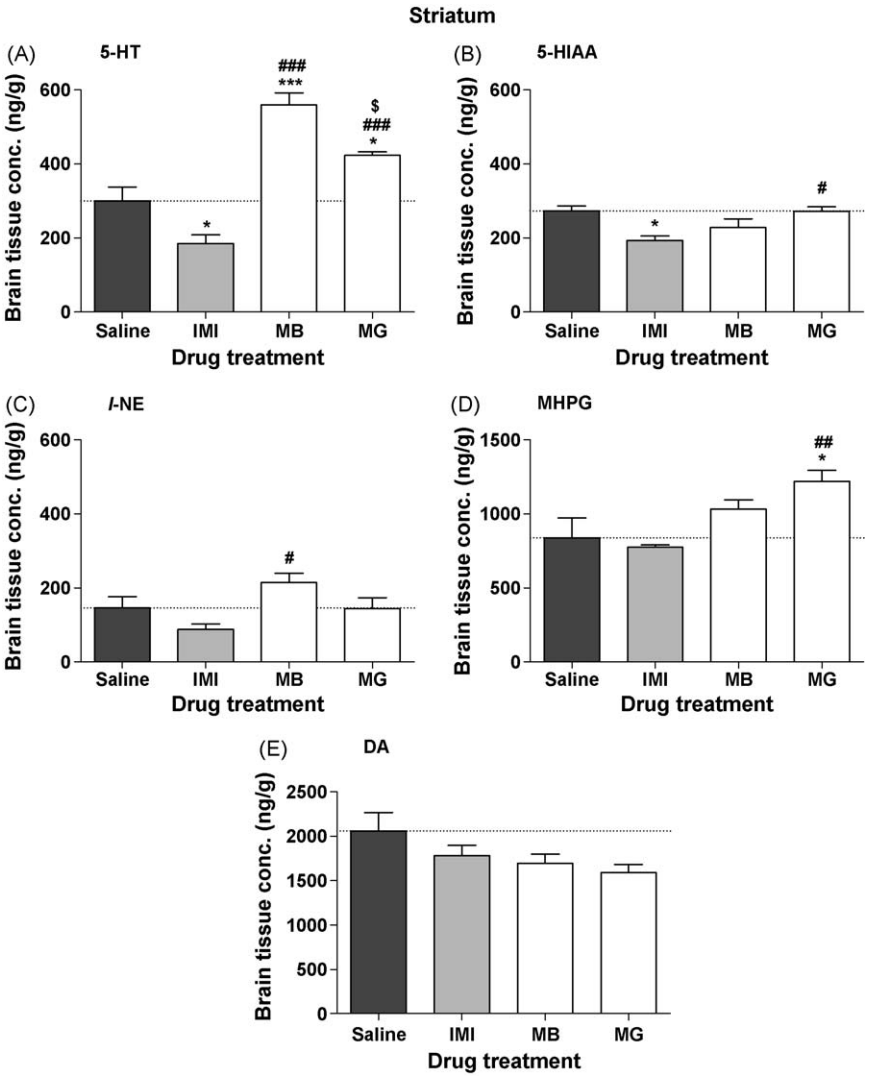

Fig. 6. Effect of sub-chronic treatment with IMI (15 mg/kg), MB (15 mg/kg) and MG (15 mg/kg) as indicated, on striatal levels of (A) 5HT; (B) 5-HIAA; (C) *l*-NE; (D) MHPG and (E) DA ($n = 5$/group; Tukey–Kramer test). $^{*}p < 0.05$; $^{***}p < 0.001$ vs saline; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs IMI; $^{\$}p < 0.05$ vs MB

chronic treatment in favour of MB compared to IMI (Fig. 3A). Since available antidepressants have distinct shortfalls in efficacy [3], the combination of MAO and NOS inhibition may represent a more effective approach to treating disorders of mood, as well as representing a novel approach to improving antidepressant drug design. Animal studies have confirmed the benefit in adding a NOS inhibitor to traditional antidepressant treatment [53]. Indeed, the improved antidepressant response of MB over MG (Fig. 3A) may reside in the ability of MB to more powerfully inhibit MAO-A but also NOS. Accordingly, sub-chronic MB treatment significantly reduced hippocampal nitrate levels, with MG less effective but producing a similar trend ($p = 0.07$, Student's *t*-test plus Bonferroni; Fig. 7). IMI however failed to alter hippocampal nitrate concentrations, contrary to an earlier study where chronic IMI treatment significantly reduced hippocampal NOS activity [38]. A variable NOS response to IMI is, however, not unusual [54,55], and various factors can influence its ability to inhibit NOS in vivo. Thus, for example, NOS involvement may only become prominent following exposure of the animal to a stressor [9] so that NOS inhibition by an antidepressant may only be evident in a pathological model [11]. Treatment-induced adaptive changes in the NMDA receptor complex [38,54] may also explain the lack of effect. Also dose and duration of IMI treatment may contribute, with a more pronounced response more likely following a longer treatment period, e.g., 15 mg/kg × 1 week i.p. (current study) vs 15 mg/kg × 3 weeks i.p. [38].

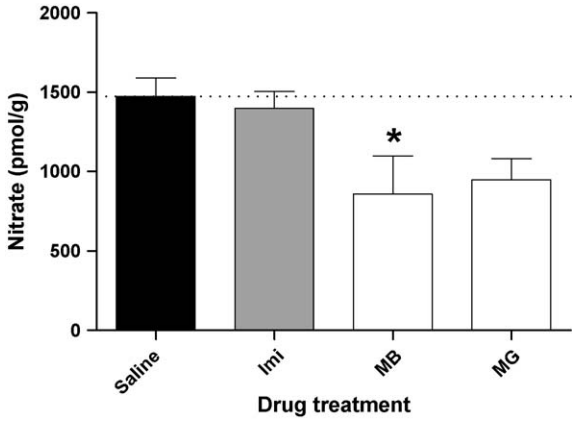

Fig. 7. Effect of sub-chronic treatment with saline, IMI (15 mg/kg), MB (15 mg/kg) and MG (15 mg/kg), as indicated, on nitrate levels in the rat hippocampus, expressed as mean ± SEM, $^{*}p < 0.05$ vs saline ($n = 5$/group; Dunnett's test).

Mood disorders involve the hippocampus, prefrontal cortex and striatum which operate within highly interacting parallel circuits

[56,57,58]. Mood related changes in the hippocampus mediate many of the cognitive aspects of depression, while the striatum mediates symptoms related to anhedonia, anxiety and reduced motivation [58]. The prefrontal cortex exerts "top-down" regulation of the above regions, and is also associated with various learning and memory processes [58]. The serotonergic system is intimately linked to stress and anxiety responses [59], while antidepressant action involves actions on hippocampal $5HT_{1A}$ and cortical $5HT_{2A}$ receptors [30,50]. *l*-NE on the other hand acts as an arousal/alerting system in many brain regions [60], while stress in various guises elevates *l*-NE in the prefrontal cortex and hippocampus [61,62] and underlies the symptoms and pathology of various anxiety disorders [63]. The mesolimbic dopamine system, especially the ventral striatum, is associated with reward and plays a role in anhedonia, reduced motivation, and decreased energy level in individuals with depression [64]. This system is also responsive to aversive events [48], possibly representing a positive, coping mechanism by increasing an individual's motivation to cope actively with a stressor [64].

Given the ability of MB and MG to inhibit MAO-A and -B (Tables 1 and 2), an effect on the levels of 5HT and *l*-NE as well as DA (via MAO-B inhibition) can be expected. In the current study, sub-chronic MB treatment significantly increased frontal cortical, hippocampal and striatal 5HT levels (Figs. 4A, 5A, 6A). MB also significantly lowered hippocampal 5-HIAA levels (Fig. 5B). MG, however, induced a trend towards elevated 5HT levels in the frontal cortex (Fig. 4A), but significantly elevated 5HT in the hippocampus (Fig. 5A) and striatum (Fig. 6A). Both compounds significantly increased cortical *l*-NE levels (Fig. 4C), although only MB significantly elevated hippocampal *l*-NE levels (Fig. 5C), with neither compound significantly affecting striatal *l*-NE (Fig. 6C). These data support earlier findings that MB increases hippocampal release of 5HT but also DA [65], although this was via acute local or systemic administration. Considering that MB and MG are significant MAO-B inhibitors, and that NOS inhibitors, including MB, increase hippocampal DA release [17,65], it is of interest that neither MB nor MG altered DA levels in any of the brain regions studied, but especially the striatum where DA plays a dominant role in depression (Figs. 4E–6E). However, a more specific analysis of the ventral striatum may have revealed a different profile. It is possible that an effect on DA may only become evident under more aversive conditions, or that sub-chronic treatment may activate a negative feedback mechanism that will attenuate transmitter synthesis [66,67]. That MB failed to increase noradrenergic-mediated behaviour in the FST (Fig. 3C) is somewhat at odds with its ability to increase *l*-NE. However, since *l*-NE and 5HT interact at various levels of cortico-limbic circuits [30] it cannot be considered definitive that *l*-NE has a limited role in the MB response. Indeed, when we re-analysed these groups (Fig. 3C) with the Student's *t*-test plus Bonferroni correction (instead of the Tukey–Kramer test), we found MB significantly increased climbing behaviour ($p = .036$). Together our data support a prominent action on especially 5HT but also *l*-NE signalling for MB and MG in cortico-limbic circuits, although with a limited involvement of DA. Not only is this brain circuit critically involved in mood regulation, but also both signalling systems are important neurobiological substrates involved in antidepressant action, as evinced by the accompanying sub-chronic FST data (Fig. 3).

Despite its marked antidepressant effects noted clinically and in the current study (Figs. 2 and 3) as well as its relationship with elevated noradrenergic vs serotonergic behaviours (Fig. 3B and C), sub-chronic IMI treatment failed to induce any noteworthy changes in cortical or hippocampal monoamines (Figs. 4 and 5). Furthermore, opposite to the elevations in 5HT induced by MB and MG in the striatum, IMI evoked a significant reduction in both 5HT and 5-HIAA levels in this brain region (Fig. 6A and B). This observation is unexpected, but may be explained by the sub-chronic treatment regime used, where chronic antidepressant treatments are known to reduce neurotransmitter turnover [66,67]. However, the process of neuronal adaptation to IMI and MAO inhibitors may also exert seemingly different effects on terminal 5HT autoreceptors [68], resulting in opposing effects in synaptic levels of 5HT. Ultimately both will evoke tonic activation of postsynaptic $5HT_{1A}$ receptors responsible for antidepressant action [69].

From a structural chemistry perspective, MB and MG are unique in possessing a permanent charge and the dimethylamine functional group (Fig. 1). That an effective antidepressant may bare an ionic charge is unique yet attractive, especially considering the increasing evidence for altered redox states in mood disorders [70–72]. Indeed, MB's planar structure, redox chemistry and ionic charge may allow it to interact with novel targets involved in the neuropathology of depression, including guanylyl cyclase, NOS and various mitochondrial reactions [27]. This study has provided conclusive evidence of the antidepressant activity of MB and MG following acute and sub-chronic treatment, most likely involving increases in 5HT and *l*-NE via MAO-A inhibition, while a contributory role via NOS inhibition is also apparent. Although MAO-B inhibition may be evident, this is not adequate to involve DA in the action of these compounds. Further structure–activity studies are needed to extend these findings and to confirm the value of MB and its congeners as multi-mode of action antidepressants that target the nitrergic and monoaminergic systems.

Conflicts of interest

Brian Harvey has participated as a speaker and/or advisory board member, and has received honoraria, from Bristol-Myers Squibb, Merck-Schering Plough, Pfizer and Servier, and has received research funding from Lundbeck. No conflicts of interest exist with these companies, the above-mentioned funding agencies, and the work presented herein.

Acknowledgements

The authors would like to acknowledge the South African National Research Foundation (NRF) for a post-graduate study bursary (ID), as well as the Danish Medical Research Council (GW) and the Hede Nielsen Foundation, Denmark (GW), for financial support. We would also like to thank Cor Bester, Petri Bronkhorst and Antoinette Fick for assistance with the breeding and welfare of the animals.

References

[1] Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 2005;62:593–602.
[2] Kessler RC, Chiu WT, Demler O, Walters EE. Prevalence, severity, and comorbidity of twelve-month DSM-IV disorders in the National Comorbidity Survey Replication (NCS-R). Arch Gen Psychiatry 2005;62:617–27.
[3] Sartorius N, Baghai TC, Baldwin DS, Barrett B, Brand U, Fleischhacker W, et al. Antidepressant medications and other treatments of depressive disorders: a CINP Task Force report based on a review of evidence. Int J Neuropsychopharmacol 2007;10(Suppl. 1):S1–207.
[4] Krishnan V, Nestler EJ. The molecular neurobiology of depression. Nature 2008;455:894–902.
[5] Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff K, Gray A, et al. Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. Biol Psychiatry 2003;53:707–42.
[6] Harvey BH. Affective disorders and nitric oxide: a role in pathways to relapse refractoriness. Hum Psychopharmacol 1996;11:309–19.
[7] Bernstein HG, Stanarius A, Baumann B, Henning H, Krell D, Danos P, et al. Nitric oxide synthase-containing neurons in the human hypothalamus: reduced number of immunoreactive cells in the paraventricular nucleus of depressive patients and schizophrenics. Neuroscience 1998;83:867–75.
[8] Suzuki E, Yagi G, Nakaki T, Kanba S, Asai M. Elevated plasma nitrate levels in depressive states. J Affec Dis 2001;63:221–4.
[9] Wegener G, Harvey BH, Bonefeld B, Müller HK, Volke V, Overstreet DH, et al. Increased stress-evoked nitric oxide signalling in the Flinders sensitive line

(FSL) rat: a genetic animal model of depression. Int J Neuropsychopharmacol 2010;13:461–73.
[10] Harkin AJ, Bruce KH, Craft B, Paul IA. Nitric oxide synthase inhibitors have antidepressant-like properties in mice. Acute treatments are active in the forced swim test. Eur J Pharmacol 1999;372:207–13.
[11] Wegener G, Volke V, Harvey BH, Rosenberg R. Local, but not systemic, administration of serotonergic antidepressants decreases hippocampal nitric oxide synthase activity. Brain Res 2003;959:128–34.
[12] Liebenberg N, Harvey BH, Brand L, Brink CB. Antidepressant-like properties of phosphodiesterase 5 inhibitors and cholinergic dependency in a genetic rat model of depression. Behav Pharmacol 2010;21:540–7.
[13] Heiberg IL, Wegener G, Rosenberg R. Reduction of cGMP and nitric oxide has antidepressant-like effects in the forced swimming test in rats. Behav Brain Res 2002;134:479–84.
[14] da Silva GD, Matteussi AS, dos Santos AR, Calixto JB, Rodrigues AL. Evidence for dual effects of nitric oxide in the forced swimming test and in the tail suspension test in mice. Neuroreport 2000;11:3699–702.
[15] Inan SY, Yalcin I, Aksu F. Dual effects of nitric oxide in the mouse forced swimming test: possible contribution of nitric oxide-mediated serotonin release and potassium channel modulation. Pharmacol Biochem Behav 2004;77:457–64.
[16] Kleppisch T, Feil R. cGMP signalling in the mammalian brain: role in synaptic plasticity and behaviour. Handb Exp Pharmacol 2009;191:549–79.
[17] Prast H, Philippu A. Nitric oxide as modulator of neuronal function. Prog Neurobiol 2001;64:51–68.
[18] Feil R, Kleppisch T. NO/cGMP-dependent modulation of synaptic transmission. Handb Exp Pharmacol 2008;184:529–60.
[19] Paige JS, Jaffrey SR. Pharmacologic manipulation of nitric oxide signaling: targeting NOS dimerization and protein–protein interactions. Curr Top Med Chem 2007;7:97–114.
[20] Garthwaite J, Boulton CL. Nitric oxide signaling in the central nervous system. Annu Rev Physiol 1995;57:683–706.
[21] Moore PK, Handy RL. Selective inhibitors of neuronal nitric oxide synthase–is no NOS really good NOS for the nervous system? Trends Pharmacol Sci 1997;18:204–11.
[22] Bodini P. Le bleu de methylene comme calmant chez les alienes. Semaine Med 1899;7:56.
[23] Eroglu L, Caglayan B. Anxiolytic and antidepressant properties of methylene blue in animal models. Pharmacol Res 1997;36:381–5.
[24] Narsapur SL, Naylor GJ. Methylene blue. A possible treatment for manic depressive psychosis. J Affect Disord 1983 May 1;5(2):155–61.
[25] Naylor GJ, Smith AH, Connelly P. A controlled trial of methylene blue in severe depressive illness. Biol Psychiatry 1987;22:657–9.
[26] Naylor GJ, Smith AH, Connelly P. Methylene blue in mania. Biol Psychiatry 1988;24:941–2.
[27] Oz M, Lorke DE, Hasan M, Petroianu GA. Cellular and molecular actions of methylene blue in the nervous system. Med Res Rev 2010, doi:10.1002/med.20177 [Epub ahead of print]
[28] Oxenkrug GF, Sablin SO, Requintina PJ. Effect of methylene blue and related redox dyes on monoamine oxidase activity; rat pineal content of N-acetylserotonin, melatonin, and related indoles; and righting reflex in melatonin-primed frogs. Ann NY Acad Sci 2007;1122:245–52.
[29] Ramsay RR, Dunford C, Gillman PK. Methylene blue and serotonin toxicity: inhibition of monoamine oxidase A (MAO A) confirms a theoretical prediction. Br J Pharmacol 2007;152:946–51.
[30] Leonard BE. Fundamentals of psychopharmacology, 3rd ed., Chichester UK: John Wiley and Sons; 2003.
[31] Luo D, Das S, Vincent SR. Effects of methylene blue and LY83583 on neuronal nitric oxide synthase and NADPH-diaphorase. Eur J Pharmacol 1995;290:247–51.
[32] Volke V, Wegener G, Vasar E, Rosenberg R. Methylene blue inhibits hippocampal nitric oxide synthase activity in vivo. Brain Res 1999;826:303–5.
[33] Oz M, Lorke DE, Petroianu GA. Methylene blue and Alzheimer's disease. Biochem Pharmacol 2009;78:927–32.
[34] Millan MJ. Dual- and triple-acting agents for treating core and co-morbid symptoms of major depression: novel concepts, new drugs. Neurotherapeutics 2009;6:53–77.
[35] Detke MJ, Johnson J, Lucki I. Acute and chronic antidepressant drug treatment in the rat forced swimming test model of depression. Exp Clin Psychopharmacol 1997;5:107–12.
[36] Porsolt RD, Brossard G, Hautbois C, Roux S. Models of affective illness: Forced Swimming and Tail Suspension Tests in Rodents. In: Enna SJ, Williams M, Barret JF, Ferkany JW, Kenakin T, Porsolt RD, editors. Current protocols in pharmacology. New York: John Wiley & Sons Inc.; 2000. p. 581–9.
[37] Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci 2002;23:238–45.
[38] Harvey BH, Retief R, Korff A, Wegener G. Increased hippocampal nitric oxide synthase activity and stress responsiveness after imipramine discontinuation: Role of $5HT_{2A/C}$-receptors. Metab Brain Dis 2006;21:211–20.
[39] Harvey BH, Jonker LP, Brand L, Heenop M, Stein DJ. NMDA receptor involvement in imipramine withdrawal-associated effects on swim stress. GABA levels and NMDA receptor binding in rat hippocampus. Life Sci 2002;71:43–54.
[40] Sewell P, Hawking F. Chemotherapy of experimental filariasis. Br J Pharmacol Chemother 1950 Jun;5(2):239–60.
[41] Steinman RR, Leonara J. Relationship of fluid transport through the dentin to the incidence of dental caries. J Dent Res 1971;50:1536–43.
[42] Cousins MS, Carriero DL, Salamone JD. Tremulous jaw movements induced by the acetylcholinesterase inhibitor tacrine: effects of antiparkinsonian drugs. Eur J Pharmacol 1997 Mar 19;322(2–3):137–45.
[43] Hawking F, Ormerod WE, Thurston JP, Webber WA. The antifilarial action and toxicity of methylene violet and of other compounds of the phenosafranine series. Br J Pharmacol Chemother 1952 Sep;7(3):494–508.
[44] Porsolt RD, Anton G, Blavet N, Jalfre M. Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol 1978;47:379–91.
[45] Novaroli L, Reist M, Favre E, Carotti A, Catto M, Carrupt PA. Human recombinant monoamine oxidase B as reliable and efficient enzyme source for inhibitor screening. Bioorg Med Chem 2005;13:6212–7.
[46] Chimenti F, Secci D, Bolasco A, Chimenti P, Bizzarri B, Granese A, et al. Synthesis, molecular modeling, and selective inhibitory activity against human monoamine oxidases of 3-carboxamido-7-substituted coumarins. J Med Chem 2009;52:1935–42.
[47] Schmidit HHHW, Kelm M. Determination of nitrite and nitrate by the Griess reaction. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. Chichester, England, UK: John Wiley and Sons; 1996. p. 491–8.
[48] Harvey BH, Brand L, Jeeva Z, Stein DJ. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. Physiol Behav 2006;87:881–90.
[49] Baldessarini RJ. Drug therapy of depression and anxiety disorders. In: Brunton LL, Lazo JS, parker LK, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 11th ed., McGraw-Hill Publishers; 2006. p. 429–59.
[50] Harvey BH. The neurobiology and pharmacology of depression. S Afr Med J 1997;87:540–50.
[51] Harvey BH, Duvenhage I, Petzer JP, Malan SF, Wegener G. Monoamine oxidase-A inhibition by methylene blue and selected analogues and antidepressant effects in the rat forced swim test.In: XXVII CINP Congress; Int J Neuropsychopharmacol 2010 (13 Supplement S1: 1-268, poster P-03-018).
[52] Ng BK, Cameron AJ, Liang R, Rahman H. Serotonin syndrome following methylene blue infusion during parathyroidectomy: a case report and literature review. Can J Anaesth 2008;55:36–41.
[53] Harkin A, Connor TJ, Burns MP, Kelly JP. Nitric oxide synthase inhibitors augment the effects of serotonin re-uptake inhibitors in the forced swimming test. Eur Neuropsychopharmacol 2004;14:274–81.
[54] Jopek R, Kata M, Nowak G. The activity of rat brain nitric oxide synthase following chronic antidepressant treatment. Acta Pol Pharm 1999;56:307–10.
[55] Suzuki E, Nakaki T, Kanba S, Shintani F, Miyaoka H. Long-term imipramine treatment increases nitrate levels in the rat hypothalamus. Cell Mol Neurobiol 2003;23:953–62.
[56] Drevets WC. Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. Curr Opin Neurobiol 2001;11:240–9.
[57] Liotti M, Mayberg HS. The role of functional neuroimaging in the neuropsychology of depression. J Clin Exp Neuropsychol 2001;23:121–36.
[58] Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. Neuron 2002;34:13–25.
[59] Graeff FG, Guimarães FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. Pharmacol Biochem Behav 1996;54:129–41.
[60] Koob GF. Corticotropin-releasing factor, norepinephrine and stress. Biol Psychiatry 1999;46:1176–80.
[61] Rosario LA, Abercrombie ED. Individual differences in behavioural reactivity: correlation with stress-induced nor-epinephrine efflux in the hippocampus of Sprague–Dawley rats. Brain Res Bull 1999;48:595–602.
[62] Finlay JM, Zigmond MJ, Abercrombie ED. Increased dopamine and norepinephrine release in medial pre-frontal cortex induced by acute and chronic stress: effects of diazepam. Neuroscience 1995;64:619–28.
[63] Sullivan GM, Coplan JD, Kent JM, Gorman JM. The noradrenergic system in pathological anxiety: a focus on panic with relevance to generalized anxiety and phobias. Biol Psychiatry 1999;46:1205–18.
[64] Nestler EJ, Carlezon Jr WA. The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 2006;59:1151–9.
[65] Wegener G, Volke V, Rosenberg R. Endogenous nitric oxide decreases hippocampal levels of serotonin and dopamine in vivo. Br J Pharmacol 2000;130:575–80.
[66] Karoum F, Korpi ER, Linnoila M, Chuang LW, Wyatt RJ. Reduced metabolism and turnover rates of rat brain dopamine, norepinephrine and serotonin by chronic desipramine and zimelidine treatments. Eur J Pharmacol 1984;100:137–44.
[67] Karolewicz B, Paul IA, Antkiewicz-Michaluk L. Effect of NOS inhibitor on forced swim test and neurotransmitters turnover in the mouse brain. Pol J Pharmacol 2001;53:587–96.
[68] Blier P, Chaput Y, de Montigny C. Long-term 5-HT reuptake blockade, but not monoamine oxidase inhibition, decreases the function of terminal 5-HT autoreceptors: an electrophysiological study in the rat brain. Naunyn Schmied Arch Pharmacol 1988;337:246–54.
[69] Haddjeri N, Blier P, de Montigney C. Long-term antidepressant treatments result in a tonic activation of forebrain 5HT1A receptors. J Neurosci 1998;18:10150–6.
[70] Harvey BH. Is major depressive disorder a metabolic encephalopathy? Hum Psychopharmacol 2008;23:371–84.
[71] Ng F, Berk M, Dean O, Bush AI. Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. Int J Neuropsychopharmacol 2008;11:851–76.
[72] Mokoena ML, Harvey BH, Oliver DW, Brink CB. Ozone modulates the effects of imipramine on immobility in the forced swim test, and nonspecific parameters of hippocampal oxidative stress in the rat. Metab Brain Dis 2010;25:125–33.