

**Structure-activity relationship of methylene blue and its analogues as
lead compounds for novel antidepressant development**

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Abstract

Antidepressants target serotonergic and/or catecholaminergic responses in an attempt at treating depression yet are at best 65% effective. New antidepressants, as well as new targets for antidepressant action, are thus urgently needed. The nitric oxide (NO)-cGMP cascade is implicated in the pathophysiology of depression. Methylene blue (MB) is a tricyclic compound that is structurally dissimilar to any known antidepressant, inhibits NO synthase (NOS) and guanylate cyclase and demonstrates significant antidepressive-like activity in rodents. The structural-activity relationship of MB and selected analogues will be studied as potential new lead antidepressant compounds using the acute and chronic forced swim test (FST), and compared to imipramine. Those analogues that demonstrate antidepressant-like activity will be studied with respect to their potential to inhibit monoamine oxidase (MAO) -A and -B *in vitro*, as well as modifying the NO-cGMP pathway in rat hippocampus following sub-chronic treatment.

MB analogues include methylene green (MG), methylene violet (MV), thionin acetate (TA), phenothiazine (PHE), tacrine (TAC) and acriflavine (ACR). MB and MB analogues were tested over a dosage range of 0.5-60 mg/kg in the acute FST study and compared to saline and imipramine. Swimming was analysed with respect to swimming and climbing behaviour to provide an indication of serotonergic and catecholaminergic properties. All the analogues, including imipramine, were tested for their inhibitory action on MAO-A (and B if required) by spectrofluorometric assay using human recombinant MAO. Those analogues with efficacy in the acute FST were tested in the chronic FST following 7 days treatment at the most effective dose identified in the acute FST protocol and compared to imipramine. Hippocampi were removed for analysis of nitrogen oxides, a surrogate marker of NOS activity.

IMI significantly reduced immobility in the acute FST, as did MB and MG, without effects on locomotor activity, thus indicating substantial antidepressant-like activity. ACR, TAC, MV, THI and PHE failed in this regard, while ACR and TAC significantly reduced locomotor activity. MB, MG and imipramine increased climbing behaviour in the acute FST, indicating catecholaminergic potentiation. MB ($IC_{50}=0.073 \mu\text{M}$), MG ($IC_{50}=0.169 \mu\text{M}$) and ACR ($IC_{50}=0.43 \mu\text{M}$) significantly inhibited MAO-A with moderate inhibition of MAO-B, while IMI and the other analogues were ineffective. In the chronic FST, MG was as effective as imipramine,

while MB was more effective than imipramine in reversing immobility, with limited locomotor effects. Interestingly, MB and MG increased swimming behaviour during chronic treatment, indicative of bolstering serotonergic neurotransmission, while imipramine again increased climbing behaviour. Neither imipramine, MG or MB had notable effects on hippocampal NOS. The antidepressant activity of MB and MG involves actions on MAO than on NOS, although MB is a more effective antidepressant than imipramine. Of the various analogues tested, only MG presents with antidepressant-like activity. Both MB and MG are charged entities that have unique structural characteristics, including a dimethylamine substituent at C-3 and C-7, which appears to be a requirement for antidepressant activity. These attributes provide important clues for novel antidepressant drug development.

Abstrak

Antidepressante teiken serotonergies en/of katesjolinergiese werking, ten einde major depressie te behandel, alhoewel dit net 65 % effektief is. Nuwe antidepressante, sowel as nuwe teikens vir antidepressante werking word dus dringend benodig. Die stikstofoksied (NO)-cGMP kaskade word met die patofisiologie van depressie geassosieer. Metileen blou (MB) is n trisikliese molekule wat struktureel verskillend is as enige ander bekende antidepressant, terwyl dit NO sintetase (NOS) sowel as guanylyl siklase inhibeer. MB demonstreer ook merkbare antidepressante aktiwiteit in knaagdiere. Die struktuur-aktiwiteit verhouding van MB en geselekteerde analoë sal studeer word as potensiële nuwe antidepressante molekules met behulp van die akute en kroniese forseerde swem toets (FST), terwyl dit met imipramine (IMI) vergelyk sal word. Die analoë wat die meeste antidepressiewe aktiwiteit betoon, se vermoë om monamien oksidase (MAO) –A en –B *in vitro* te inhibeer, sowel as modifiserende effekte op die NO-cGMP baan in die rot hippokampus na kroniese behandeling.

MB analoë sluit in; metileen groen (MG), metileen violet (MV), tionin asetaat (TA), fenotiasien (PHE), tacrine (TAC) en acriflavien (ACR). MB, sowel as MB analoë was getoets oor n dosis spektrum van 0.5-60 mg/kg in die akute FST studie en vergelyk met saline en imipramine. Swem en klim aksies was geanaliseer om aan te dui wat die serotonergiese of katesjolinergiese werking van die middels was. Al die analoë, insluitend imipramine, was getoets vir hul inhiberende vermoë op MAO-A (en B, indien nodig gevind) met behulp van n spektrofluorometriese bepaling met menslike rekombinante MAO. Die analoë met effektiwiteit in die akute FST, was ook getoets in die kroniese behandeling van 7 dae, met die mees effektiefste dosis wat in die akute studie geidentifiseer was en met imipramien vergelyk. Die hippokampus was dan verwyder vir analisering van stikstofoksiede, n surrogaat merker van NOS aktiwiteit.

IMI het merkbare vermindering in die immobiliteit getoon in die akute FST, so ook MB en MG, sonder enige effekte op die lokomotor aktiwiteite. Dus toon dit n merkbare antidepressiewe aktiwiteit. ACR, TAC, MV, THI en PHE en gefaal in hierdie opsig, terwyl ACR en TAC merbaar die lokomotor aktiwiteit verminder het. MB, MG en IMI het die klim gedrag verhoog tydens die akute FST, wat op katesjolinergiese werking dui. MB ($IC_{50}=0.073 \mu\text{m}$), MG ($IC_{50}=0.169 \mu\text{m}$) en ACR ($IC_{50}= 0.43 \mu\text{m}$) het merbaar MAO-A

geinhibeer met middelmatige inhibisie op MAO-B, terwyl IMI en die ander analoë oneffektief was. Tydens die kroniese FST behandeling, was daar gevind dat MG net so effektief soos IMI is, met min effekte op die lokomotor effekte. Beide MB en MG het die swem gedrag verhoog tydens kroniese behandeling, wat n aanduiding is van verhoogde serotonergiese neurotransmissie, terwyl imipramine die klim gedrag verhoog het. Nie IMI, MB of MG het enige merkbare effekte op die hippokampale NOS sisteem getoon nie. Die anitdepressante aktiwiteit van MB en MG sluit in effekte op MAO en NOS, alhoewel MB n meer effektiewe antidepressant is as IMI. Van al die analoë wat getoets was, het net MG antidepressiewe aktiwiteit vertoon. Beide MB en MG is gelaaide entiteite wat unieke strukturele karakteristieke het, insluitend a dimetilielamien substuent op C-3 en C-7. Dit blyk na n vereiste vir antidepressiewe aktiwiteit. Hierdie waarnemings verskaf waardevolle idees vir die ontwikkeling van n nuwe antidepressante middel.

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1.1 Problem statement

Depression is a serious and troublesome disorder that has severe effects on family life and personal relationships. Major depressive disorder is recurrent and a major cause of morbidity worldwide (Blazer, 2000). It is an affective disorder, characterized by disturbances in mood, sleep, motivation, appetite and energy (Leonard, 1997).

Effective antidepressant treatments have been available for many years and the considerable improvement of safety and tolerability of antidepressants that has come about after the introduction of selective reuptake inhibitors (SSRI's) has led to marked improvement in treatment outcome. However, a major problem with current treatment modalities is the delayed onset of action and that the accepted response rate to antidepressants is poor, in the order of 65%. It is now well accepted that antidepressant efficacy rests on increasing intrasynaptic levels of serotonin (5-HT) and/or noradrenalin (NA). This response occurs within hours after administration, but due to various presynaptic feedback mechanisms, as well as the dependence of these receptor-directed actions of the antidepressant on subcellular signalling processes, the eventual clinical response is invariably delayed for up to 4 to 6 weeks (Harvey, 1997; Popoli *et al.*, 2002; Harvey *et al.*, 2003). This discrepancy has prompted a shift to more diverse models to explain antidepressant action, to identify new antidepressant targets especially those located at a sub-cellular level, and to develop new antidepressants that will address the above-mentioned discrepancies. The nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway has not only been found to be implicated in the neurobiology of depression (Harvey, 1996), but its unique action that is vested in intra- and inter-cellular communication (Prast and Philippu, 2001; Bloom, 2001) provides an important putative target for novel antidepressant development.

Elevated levels of nitric oxide synthase (NOS) activity has been observed in depressed patients (Suzuki *et al.*, 2001), while pre-clinical studies have demonstrated the antidepressant effects of NOS inhibitors (Harkin *et al.*, 1999) as well as that typical antidepressants inhibit hippocampal NOS activity *in vivo* (Wegener *et al.*, 2003). Thus

strategies aimed at suppressing the NO-cGMP pathway may prove to be useful in treating depression. One such drug candidate is the tricyclic compound, methylene blue.

Methylene blue has been used to treat neuropsychiatric illnesses from as early as 1899 (Bodoni, 1899) and more recently it has been used in clinical trials for manic depressive patients with success (Naylor *et al.*, 1987). Methylene blue also displays promising pre-clinical activity as an antidepressant and anxiolytic agent (Eroglu and Caglayan, 1997). The mechanism of action of this compound remains unclear, but it has been the subject of speculation in recent years. It has been found to have noteworthy inhibitory actions on MAO (Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007) as well as the NOS-NO-cGMP pathway (Volke *et al.*, 1999). Recently it has been found that the compound may induce severe serotonin toxicity in conjunction with other serotonergic agents (Ramsay *et al.*, 2007; Ng *et al.*, 2008), which suggests that over and above the above-mentioned actions, the compound has powerful actions on the serotonergic system which may explain its psychotropic actions. Typical agents that selectively inhibit the NO-cGMP system have great potential for adverse effects, especially with respect to cardiovascular and central nervous system toxicity. In spite of this, methylene blue seems to be devoid of any significant side-effects (Kupfer *et al.*, 1994). Structurally, methylene blue is unique, unlike any known antidepressant compound. For one it is charged, which is highly unusual for a centrally acting drug. This study will investigate the structural-activity relationship of MB and a number of its analogues with respect to antidepressant effects, as well as effects on a known antidepressant target, MAO, and on hippocampal NOS activity. Dual actions on these two targets may constitute a potentially new group of lead antidepressant compounds.

1.2 Study aims

The main aims of this project are:

- To identify a series of chemical analogues that present with diverse structural characteristics that are similar to methylene blue, yet unique;
- To assess any dose-related antidepressant actions of methylene blue in the rat acute forced swim test (FST) using previously published guidelines (Eroglu and Caglayan, 1997),
- To confirm the FST set-up and to confirm that the antidepressant imipramine does indeed induce a robust antidepressant-like response in the acute FST, according to previously published guidelines (Harvey *et al.*, 2001),
- To perform a dose-response analysis on the methylene blue analogues in the acute FST to determine if any possess antidepressant-like activity, whether these

responses are dose dependent, and how they compare to a reference antidepressant, imipramine;

- To determine the effect of imipramine, methylene blue and its analogues on monoamine oxidase (MAO) activity;
- To determine whether methylene blue as well as any analogues active in the acute FST are also active in the FST following chronic treatment, and how they compare relative to chronic imipramine treatment; and
- To determine whether any of the active antidepressant compounds exert effects on the NO/cGMP pathway following chronic treatment.

1.3 Study layout

Six analogues of methylene blue have been chosen for this study. All six analogues including methylene blue will be tested in the acute FST study and compared to imipramine, using a dose-response analysis. To achieve this, male Sprague Dawley rats will be treated with five different doses of each compound and tested in the acute FST study, and compared to saline and imipramine. Thereafter, diverse aspects of swim behaviour will be studied in order to verify actions on serotonergic and catecholaminergic pathways (Cryan *et al.*, 2002), as well as immobility response in the FST.

All analogues will then be evaluated for their inhibitory effects on monoamine oxidase (MAO) A and B in an *in vitro* spectrofluorometric assay using human recombinant MAO. Thereafter, those analogues that have demonstrated antidepressant-like effects in the acute FST will be studied with respect to chronic antidepressant effects using a chronic treatment protocol. The rats will be treated for 7 days and tested in the FST relative to saline and imipramine. As above, behavioural analysis of swim activity as well as immobility in the FST will be analysed. Thereafter, the rats will be sacrificed and the hippocampi dissected out for analysis of NOS activity using a fluorometric assay kit.

2.1 Depression

Depression was first described as melancholia (black bile in Greek) by Hippocrates around 400 B.C. (Akiskal, 2000) It is a highly disabling illness that presents with significant long term morbidity that prevents the afflicted person from functioning normally. The National Institute of Mental Health estimates that approximately 1 in 18 people, or roughly 14, 8 million people, in the USA will develop depression at some point in their lives (Kessler *et al.*, 2005). Another study concluded that mental illnesses accounts for over 15% of the burden of disease in established market economies, such as the USA, which is more than the disease burden caused by all the cancers (Murray & Lopez, 1997; Kessler *et al.*, 2003). Major depressive episodes may occur only once in a person's life, but more often it recurs throughout a person's life (American Psychiatric Association, 1994). As with many illnesses, the earlier the treatment starts the more effective it is and the greater the likelihood that recurrence can be prevented.

Depression often co-exists with other illnesses which may precede depression by either directly or indirectly contributing to its development. Anxiety disorders such as post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder often accompany depression (Devane *et al.*, 2005) as does alcohol and any other substance abuse disorder (Conway *et al.*, 2006). Moreover, depression can also be co-morbid with other serious medical illnesses such as stroke, heart disease, diabetes, cancer and human immunodeficiency virus (HIV) (Harvey, 2008). Some studies suggests that where depression occurs in addition to another illness, the presenting symptoms tend to be more severe for both conditions, while the patients adapt with greater difficulty to their medical condition resulting in medical costs that are higher than those who do not have co-existing depression (Cassano & Fava, 2002). Increasing evidence suggests that treating the depression can also help improve the outcome of co-morbid illnesses (Katon & Ciechanowski, 2002). In South Africa it is particularly the depression-HIV co-morbidity that is a concern. According to the WHO (World Health Organization), the prevalence of HIV among South-African adults aged 15 – 49 years is a staggering 21.5 %, whilst HIV-positive individuals have nearly twice the likelihood of

presenting with a recent episode depression, compared to HIV-negative patients (Ciesla & Roberts, 2001). The key to effective antiretroviral therapy (ART) is adherence, while poor compliance to ART is associated with untreated depression (Carpenter *et al.*, 1998). In South-Africa, HIV/AIDS patients may be at greater risk for psychopathology due to stressful living conditions, including the high rate of unemployment, poverty as well as high crime rates and domestic violence (Moosa & Jeenah, 2007). These facts further emphasise the importance of research into depression in Southern-Africa, particularly with regard to effective management.

2.1.1 Symptomatology

Depressive disorder is characterized by a combination of symptoms that can interfere with a person's ability to work, sleep, study, eat and enjoy pleasurable activities. The severity, frequency and duration of symptoms will vary depending on the individual and his or her particular illness. The diagnosis of major depression is based on symptomatic criteria described by the Diagnostic and Statistical Manual of Mental disorders (American Psychiatric Association, 1994), as summarised in Table 2-1. The diagnosis of major depression can be made when five or more of the symptoms listed in Table 2-1 have been present for more than a two week period, and that a definitive period of depressed mood or clear loss of interest in activities can be identified (American Psychiatric Association, 1994).

Table 2-1: The diagnostic criteria of major depressive disorder

Depressed mood
Irritable mood
Diminished interest or pleasure in daily activities
Weight loss or weight gain
Insomnia or hypersomnia
Psychomotor retardation
Fatigue
Feelings of worthlessness and guilt
Lack of concentration or indecisiveness
Recurrent suicidal thoughts

2.1.2 Aetiology

There is no single known cause of depression. Rather, the origins of the illness are multifactorial, resulting from a combination of genetic, biochemical, environmental and psychological factors. Genetic predisposition, adverse early childhood experiences, the effect of psychosocial adversity as well as the biological and physiological effects of other physical diseases, all play a role in increasing vulnerability to developing depression. Consequently, the aetiology of depression cannot be attributed to a single biochemical imbalance (Shah, 2002). Genetic influences and the aggregation of certain mental disorders in families have been recognised for over 50 years (Shah, 2002). In some studies conducted in twins, for example, concordance is 60% for recurrent depression (Bertelsen *et al.*, 1977), while epidemiological studies suggests that roughly 40%-50% of the risk for developing depression is genetic (Sanders *et al.*, 1999; Fava & Kendler, 2000).

The heritability of depression is greater in women than in men (Kendler *et al.*, 2001) and approximately 40% of variance in both males and females can be accounted for by genetic factors (Goldberg, 2006). The discovery of a polymorphism in the serotonin transporter gene in depression has provided much needed hope in identifying the specific genetic pathways involved in depression, and how these may interact with adverse life events to predict the development of the illness (Ogilvie *et al.*, 1997; Shah, 2002; Caspi *et al.*, 2003; Goldberg, 2006).

Early life adversity is known to contribute to the expression or exacerbation of a variety of physical and psychological disorders (Kessler, 1997). Consequently, an important factor to consider is environmental influences, e.g. poor nurturing, maternal separation, child abuse, etc, and the effect that these may have on early childhood development and the risk for developing depression in adulthood (Parker & Hadzi-Pavlovic, 1984). Women are three times more likely to develop depression than men, subsequent to experiencing a stressful life event (Maciejewsky *et al.*, 2001). A possible explanation for the difference in prevalence of depression in gender can also be contributed to the hormone oestrogen, which may render women more susceptible to stress (Seeman, 1997), possibly by dysregulating the HPA (hypothalamic-pituitary adrenal) axis response to stress (Weiss *et al.*, 1999) or by modulating biogenic amine responses.

Depression can be regarded as being the result of a maladaptive response to stress, where individual vulnerability to stress plays a deciding role in determining the impact of stress on the body, and the later development of a mood disorder (Kendler *et al.*, 2001). Central to this is dysregulation of stress hormones and of the stress response (McEwen, 2004; De Kloet *et al.*, 2005). Following exposure to an aversive event, the body initiates a series of adaptive mechanisms that allow the individual to cope. This is a natural response and is referred to as allostasis (McEwen & Wingfield, 2003). Allostasis describes the biological and psychological processes involved in acquiring a learned adaptive response necessary for survival, and involves mounting an adequate response to stress and thereafter initiating a natural shut-off once the stressor has passed. However, an increased susceptibility to stress will prevent a natural beneficial response, eventually resulting in the stress response either not being “turned on”, or not being “turned off” or in fact being over-utilised. The latter condition is termed allostatic load, and defines a state of excessive “loading” that over time will lead to a maladaptive response, a lack of coping and eventually will conclude with the development of stress-related illnesses, such as depression (McEwen & Wingfield, 2003).

2.1.3 The neuroanatomy of depression

Several brain regions have been implicated in depression, although we still only have a rudimentary understanding of the neural circuitry and specific brain abnormalities underlying mood disorders (Nestler *et al.*, 2002). Indeed, human brain imaging studies have demonstrated changes in blood flow or related measures in several brain areas of depressed patients, although these can be broadly condensed to the hippocampus, prefrontal cortex and striatum (Drevets, 2001; Liotti & Mayberg, 2001; Nestler *et al.*, 2002).

The hippocampus plays a major role in the pathophysiology of major depressive disorder (MacQueen *et al.*, 2003) and mediates many of the cognitive aspects of depression, such as memory impairments, feelings of worthlessness, guilt, hopelessness, doom and suicide (Nestler *et al.*, 2002). Importantly, several clinical studies have reported a reduction in hippocampal volume in patients with major depressive disorder (Sheline, 2000; Steffens *et al.*, 2000; MacQueen *et al.*, 2003). While many of the studies which have reported a reduction in hippocampal volume in depression were conducted in older patients (Sheline, 2000; Steffens *et al.*, 2000), age is not predictive of hippocampal volume change, but rather the length of the illness as well as other variables associated with the illness (Sheline *et al.*, 1999). In fact, prolonged depression appears to be associated with more severe atrophy (Sapolsky, 2001), while MacQueen and colleagues (2003) have confirmed in young adults that reductions in the hippocampus increase as a function of multiple depressive episodes.

The striatum and amygdala are important in emotional memory, such that deficits here could result in anhedonia (decreased drive and lack of reward for pleasurable activities), anxiety and reduced motivation, all symptoms that predominate in many depressed patients (Nestler *et al.*, 2002). Structural and functional abnormalities of the amygdala have been reported in major depression (Thase *et al.*, 2002).

The prefrontal cortex serves as a mood regulator and is associated with a variety of learning and memory processes. We are able to identify different sub-categories of the prefrontal cortex. The ventromedial prefrontal cortex (VMPFC), for example, is responsible for mediating pain, aggression, sexual functioning and eating behaviours, whereas the lateral orbital prefrontal cortex (LOPFC) assesses risk and modulates affective states. The dorsolateral prefrontal cortex (DLPFC) maintains executive functioning, sustained attention and working memory processes (Swanson, 1987; Maletic *et al.*, 2007). Regional blood studies have found that patients with depression display hyperactivity in the VMPFC and LOPFC and hypoactivity in the DLPFC (Drevets, 1998), and might suggest that these abnormal activities may be responsible for the manifestations of some symptoms of depression (Maletic *et al.*, 2007). It is however, important to realise that these various brain regions operate in a series of highly interacting parallel circuits (Nestler *et al.*, 2002), thus enabling us to formulate a neural circuitry for depression.

2.1.4 Neurobiology/neuropathological hypotheses of depression

The neuropathological basis of depression remains poorly understood, despite many decades of research. The monoamine hypothesis remains the most well-known model, mainly because most if not all the clinically effective antidepressants in use today were

developed along the precepts of this model (Harvey, 1997; Harvey, 2008). However, the monoamine hypothesis has a number of flaws which has led to a modified monoamine hypotheses but also to a number of novel theories. These will be discussed below.

2.1.4.1 Monoamine hypothesis

The monoamine hypothesis (Schildkraut, 1965) has been the cornerstone of depression research for close on 50 years, beginning after the serendipitous discovery of monoamine oxidase inhibitors (iproniazid) and tricyclic antidepressants (imipramine) (Van Praag, 2001; Hindmarch, 2002). Upon realising that the aforementioned drugs all act to increase the synaptic levels of various monoamines, especially serotonin, noradrenalin and dopamine, this discovery led to the development of a whole spectrum of antidepressants, all with varying selectivity and specificity for one or more of these monoamines (Harvey, 1997).

This hypothesis suggests that depression is directly related to decreased monoaminergic neurotransmission in the brain, notably noradrenalin and serotonin (Randrup and Braestrup, 1997). This conclusion follows after drugs which have the ability to facilitate and enhance serotonergic and/or noradrenergic neurotransmission in the brain, were found to be useful as antidepressants (Hyman & Nestler, 1993). This theory was further augmented by observations that the tranquilizing and anti-hypertensive drug reserpine, which is known to deplete the brain of serotonin and other catecholamines, which can induce depression when administered over an extended period (Cooper *et al.*, 1996). The role of serotonin in mood modulation is also supported by evidence that depletion of tryptophan (the primary substrate for serotonin synthesis) can also engender a lowering in mood (Den Boer, 2006). An extension of the monoamine hypothesis, viz. the permissive theory (Coppin, 1967), states that low serotonin activity may permit the expression of the affective state, but that the type of mood disorder presented is determined by the level of norepinephrine. Thus, in the presence of reduced serotonin levels, low levels of norepinephrine will cause depression while high levels of norepinephrine will cause mania (Harvey 1997). Changes in sensitivity of norepinephrine or 5HT₂ receptors may also relate to the onset of depression (Lesch, 1992), and several authors have linked the onset of antidepressant activity to changes in serotonin and norepinephrine receptor density (Leonard, 1984; Blier & de Montigny, 1999).

While serotonergic and noradrenergic antidepressants have been the mainstay of treatment in recent years, several reports also suggest that increased dopamine neurotransmission in especially the nucleus accumbens is related to the mechanism of action of antidepressants (Wells, 2003). It has been proposed as early as the 1970's that dopamine may play a role in the pathophysiology of depression (D'Aquila *et al.*, 2000). Definitive dysfunctions of the

mesolimbic and mesocortical dopaminergic pathways are primarily implicated in the melancholic and cognitive features of depression (Naranjo *et al.*, 2001; Lehr *et al.*, 2002; Millan, 2004) and dopaminergic neurons are involved in the control of reward-related behaviour and incentive motivation which are impaired in depression (D'Aquila *et al.*, 2000).

Despite the success of the monoamine theory, it is unable to provide a comprehensive explanation of the mechanism of action of antidepressants, nor is it able to fully explain the pathophysiology of depression (Hindmarch, 2002). A major deficiency of this hypothesis is the fact that some drugs, for example cocaine, are effective inhibitors of catecholamine reuptake, and thus increase the availability of norepinephrine at central synapses, yet are not clinically effective antidepressants (Kosten *et al.*, 1994). While monoamine reuptake inhibitors, such as tricyclic antidepressants and monoamine oxidase inhibitors increase synaptic levels of norepinephrine and serotonin within hours of administration, they eventually bolster monoaminergic transmission after sustained use (Bymaster *et al.*, 2003; Leonard, 2003). Cocaine on the other hand is a monoamine releaser following acute treatment but depletes monoamines after chronic treatment, thus worsening mood. A second deficiency is that the above mentioned antidepressants all require treatment of at least 3-4 weeks before response can be expected, despite the fact that synaptic monoamine levels increase within hours of first administration (Harvey, 1997; Popoli *et al.*, 2002). This discrepancy indicates that the mechanisms underlying antidepressant response are not simply due to their immediate effects on synaptic monoamine receptors (Thase *et al.*, 2002), but that other more deep-seated sub-cellular events are involved that may explain this delayed time course before response (Harvey, 1997; Popoli *et al.*, 2002). This realisation has prompted new hypotheses in an attempt at understanding this phenomenon (see Section 2.1.5.1 below).

2.1.4.2 Dysfunction of the hypothalamic-pituitary adrenal axis

The endocrine hypothesis focuses on the fact that hormones have been found to play a critical role in the development and expression of a wide range of behaviours, including a contribution to the pathophysiology of a number of psychiatric disorders and the mechanism of action of psychotropic drugs, particularly in major depression (Checkley, 1996; Juruena *et al.*, 2004).

The hypothalamic-pituitary-adrenal axis (Figure 2-1) regulates the stress response along a sequence of events involving the hypothalamus, the anterior pituitary gland and the adrenal cortex (Hindmarch, 2002). Corticotropin-releasing hormone (CRH), a hormone released by CRH neurons of the endocrine hypothalamus, stimulates the anterior pituitary to secrete

adrenocorticotropin hormone (ACTH). ACTH in return acts on the cortex of the adrenal gland to stimulate secretion of the glucocorticoid hormone, cortisol (or corticosterone in rats) (Schimmer & Parker, 2001). The HPA (hypothalamic-pituitary adrenal) axis is regulated primarily by diurnal rhythms affecting basal steroidogenesis, through negative feedback regulation by adrenal corticosteroids, and finally through increases in steroidogenesis (Bao *et al.*, 2007). The negative feedback regulation occurs at multiple levels of the HPA axis and is responsible for maintaining physiological levels of circulating glucocorticoid levels in the appropriate ranges and according to a set circadian rhythm (De Kloet *et al.*, 2005). The HPA-axis is one of the central stress response centres of the body, and is responsible for the adequate handling of stressful stimuli as well as shutting down the stress response once the stressor has passed (McEwen, 2004). Indeed, a maladaptive stress response can override these normal negative feedback control mechanisms, leading to a marked increase in plasma levels of adrenocortical steroids (Schimmer & Parker, 2001). As a result, hypercortisolemia is a characteristic feature of depression (Gibbons & McHugh, 1962; Varghese & Sherwood Brown, 2001).

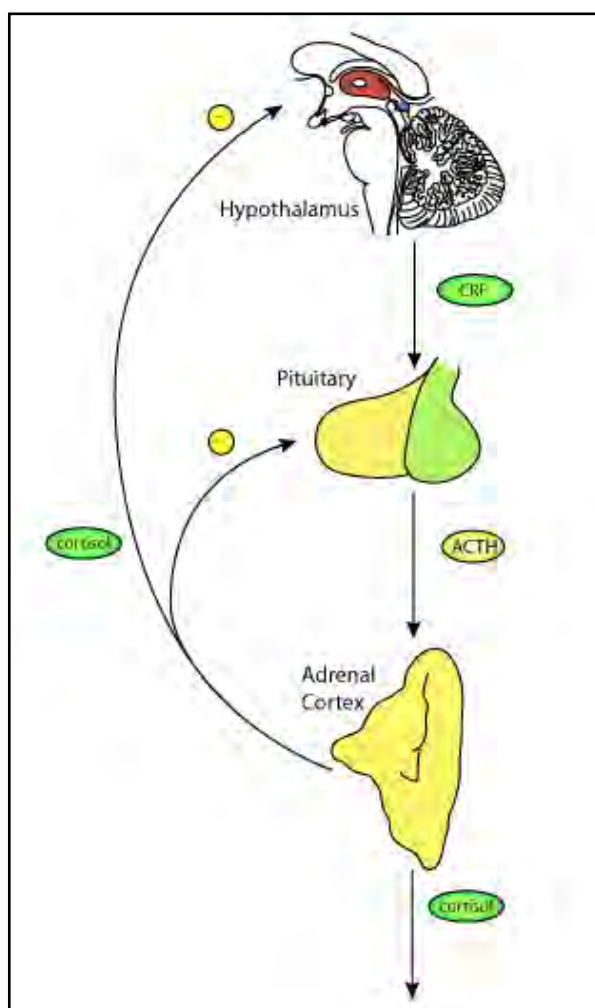


Figure 2-1: The hypothalamic-pituitary-adrenal (HPA) axis (Bingzheng *et al.*, 1990).

There are two types of corticoid receptor, namely glucocorticoid and mineralcorticoid, each with a distinct role in the brain. Glucocorticoid receptors play an important role in the pathophysiology of depression (Hindmarch, 2002). These receptors are necessary for the HPA feedback regulation when glucocorticoid levels are high in response to stress (De Kloet *et al.*, 1998; Spencer *et al.*, 1998; Juruena *et al.*, 2004). Furthermore, by virtue of negative feedback, cortisol acts to dampen down the stress-induced activation of the HPA axis and in so doing has an important role to shut down an over active stress response (Jacobson & Sapolsky, 1991; Plotsky *et al.*, 1998; Hindmarch, 2002). A link between depression and hypercortisolemia is not new (Gibbins & McHugh, 1962; Carpenter & Bunney, 1971). For example, high cortisol levels are typical of Cushing's syndrome, as are depressive symptoms, both of which are normalised following the effective reduction of hypercortisolemia (Sonino *et al.*, 1993). Signs of a hyperactive HPA axis are visible in many depressed patients (Nestler, 1998), including enhanced levels of cortisol in the urine and serum (Rubin *et al.*, 1995), non response to the normally suppressive actions of

dexamethasone (Rubin *et al.*, 1995), a blunted ACTH response after challenge with CRF (corticotropin releasing factor), as well as adrenal gland hyperplasia (Hindmarch, 2002). Interestingly, as in depression Cushing's syndrome patients also often present with hippocampal atrophy that is reversible upon successful lowering of circulating levels of cortisol (see Harvey *et al.*, 2003 for review).

It has also been suggested that an impaired cortisol negative feedback, or increased levels of CRF, accounts for the HPA overactivity and thus the possibility of a pathophysiological pattern of HPA dysfunction in depressed patients (Young *et al.*, 1991; Nemeroff, 1996; Hindmarch, 2002). Clinical antidepressants can exert their effects through indirect modulation of the glucocorticoid receptors via actions on serotonin (5-HT) (Cassano *et al.*, 2001; Weidenfeld *et al.*, 2002). Studies in depressed patients and animal models have also demonstrated that antidepressants increase glucocorticoid receptor expression resulting in reduced basal and stimulated HPA axis activity, which may in the end contribute to the therapeutic action of these drugs (Pariante & Miller, 2001; Juruena *et al.*, 2004). Focusing in on abnormalities of the HPA axis in depression holds great promise for identifying new targets for novel antidepressant development, including glucocorticoid and CRF receptor antagonists (e.g. mifepristone) or inhibitors of cortisol synthesis e.g. ketoconazole (Dinan, 2001; Hindmarch, 2002). Such approaches have been used in clinical studies with mixed results (Malison *et al.*, 1999; Wolkowitz *et al.*, 1999). It is thus clear that depression involves abnormalities of the HPA axis although further research is needed to confirm the safety and efficacy of treatment options aimed at correcting these abnormalities.

2.1.4.3 Impairment of neurotrophic mechanisms

The neurotrophin hypothesis is built on increasing evidence that implicates neurotrophic factors in the pathophysiology of depression (Sen *et al.*, 2008), particularly brain-derived neurotrophic factor (BDNF). BDNF is a small neuroprotective protein and a member of the neurotrophin family (Hofer *et al.*, 1990; Yulug *et al.*, 2009) that plays a critical role in the development and maintenance of the peripheral and central nervous systems, as well as being involved in neuronal survival and proliferation (Murer *et al.*, 2001). BDNF is found throughout the brain, with particular abundance in the hippocampus and cerebral cortex (Altar, 1999). As a result, BDNF has been identified as a target for antidepressant action (Cooke *et al.*, 2009). By modulating plasticity, inhibiting cell death cascades and increasing cell survival proteins responsible for neuronal proliferation and maintenance, BDNF plays a critical role in improving long-term potentiation and synaptic transmission, key elements in memory, cognition, mood as well as antidepressant response (Yulug *et al.*, 2009).

Prior and/or ongoing stress is an important precipitant of depression and has been found to reduce neurogenesis and neurotrophic factors in the brain (Duman, 2004; Sen *et al.*, 2008). Serum BDNF levels are reduced in depression and demonstrates decreased levels in the hippocampus and cortex (Dwivedi *et al.*, 2003). This is particularly important since the hippocampus plays a major role in the pathophysiology of depression, and is also susceptible to atrophic changes during chronic forms of the illness (MacQueen *et al.*, 2003; Sheline, 2000; Steffens *et al.*, 2000). Importantly, antidepressants promote neurogenesis and neurotrophic gene expression (Malberg *et al.*, 2000), while a crucial construct of the neurotrophin hypothesis is that reduced BDNF levels in depression normalise following antidepressant treatment (Sen *et al.*, 2008). Animal studies confirm that stress-induced reductions in BDNF are restored with antidepressant treatment (Suiciak *et al.*, 1997; Shirayama *et al.*, 2002; Sen *et al.*, 2008). Important to note is that these neuroplastic changes require chronic treatment, typically 3-4 weeks (Cooke *et al.*, 2009).

Further supporting evidence for this hypothesis is that infusion of BDNF into the brain of animals produces an antidepressant-like effect in the learned helplessness and forced swim test models of depression (Suiciak *et al.*, 1997; Shirayama *et al.*, 2002), while chronic antidepressant treatment in animals enhance the expression of BDNF with varying results depending on the duration of treatment, the brain regions examined, as well as the drug type used (DeFoubert *et al.*, 2004; Russo-Neustadt *et al.*, 2004; Calabrese *et al.*, 2007; Balu *et al.*, 2008; Cooke *et al.*, 2009). By bolstering BDNF, antidepressant treatment will therefore improve compromised cognition evident in depression, as well as improve connectivity in critical brain regions involved in mood and stress response, ultimately bringing about a reversal of reduced hippocampal volume together with improved cognitive performance as well as other symptoms of depression (Nibuya *et al.*, 1995; Duman *et al.*, 1998; Sen *et al.*, 2008; Yulug *et al.*, 2009). Polymorphism in the BDNF gene has also been associated with depression-like traits in some studies (Sen *et al.*, 2008), suggesting that low BDNF levels may precipitate depressive disorder and as such represent a possible genetic risk marker for developing depression (Yulug *et al.*, 2009).

2.1.4.4 Impairment of brain reward pathways

The exact mechanisms that underlie the symptoms of anhedonia in depression remains elusive, although it can be said that the reward system underscores many of the core symptoms of major depressive disorder, including anhedonic symptoms (Naranjo *et al.*, 2001). Animal studies have revealed that stress is linked to anhedonic behaviour and

dysfunctional reward-related neural circuitry in the brain (Bogdan & Pizzagalli, 2006). One study investigated the interplay between stress and anhedonia in humans, finding that patients reported a decreased ability to experience pleasure following a stressful period (Berenbaum & Connelly, 2003). There is thus clear evidence in support of a potential link between stress and anhedonia (Bogdan & Pizzagalli, 2006). Moreover, melancholia, a subtype of depression characterized by anhedonia, is often accompanied by hypercortisolemia (Gold & Chrousos, 1999). Thus stress might precipitate depression by inducing anhedonia (Bogdan & Pizzagalli, 2006).

The brain reward system consists of extensive neural pathways that mediate reward-orientated behaviour, such as pleasure and motivation for directing the subject's behaviour towards goals that are beneficial to them (Koob, 1996; Wise, 1996; Naranjo *et al.*, 2001). These rewards are biological or cognitive stimuli that produce and reinforce behaviours that will promote and/or sustain feelings of pleasure or positive emotional states (Schultz, 1998; Naranjo *et al.*, 2001). Animal models of depression have demonstrated that stress induces anhedonic-like behaviour (Anisman & Matheson, 2005) while protocols such as chronic mild stress (Willner, 2005), learned helplessness (Henn & Vollmayer, 2005), inescapable stress (Zacharko *et al.*, 1983) as well as early separation (Matthews & Robins, 2003) abrogate the animals' sensitivity towards reward (Bogdan & Pizzagalli, 2006). It is especially stressors affecting dopaminergic pathways associated with reward that impact on anhedonic behaviour (Zacharko *et al.*, 1983; Bogdan & Pizzagalli, 2006).

Dopamine is implicated in natural rewards such as sexual arousal and food intake (Melis and Argiolas, 1995; Salamone *et al.*, 1997). Dopamine releasing drugs will therefore promote reward behaviour while dopamine antagonists or dopamine depleters will reduce reward behaviours and/or inhibit response to rewards (Naranjo *et al.*, 2001). The mesocorticolimbic dopamine pathway plays a central role in the brain reward system, although the functional anatomy may vary depending on the type of reward. However, other neurotransmitters may also modulate the brain reward pathways in conjunction with dopamine, including serotonin (Rocha *et al.*, 1998) which is suggested to inhibit reward mechanisms (Naranjo *et al.*, 2001), as well as opioid systems (Wise, 1996).

2.1.4.5 Alterations in neurogenesis

Neurogenesis refers to the production of new neurons in the adult brain (Eriksson *et al.*, 1998), and can be altered by a variety of biological and environmental factors (Eriksson *et al.*, 1998). In the rat this is particularly noticeable in neurons of the dentate gyrus of the hippocampus and the olfactory bulb (Jacobs, 2002). Stress is an important controlling factor

in the process of neurogenesis (Gould *et al.*, 1998; Tanapat *et al.*, 1998; Jacobs, 2002) and, as highlighted earlier, is a causal factor in depression as well (Kendler *et al.*, 1999). Considering the latter point, it is not unexpected to find that adrenal steroids, which are released in response to stress, play an important role in neurogenesis. In fact adrenal steroids were the first neurochemical factor shown to affect neurogenesis in the dentate gyrus (Gould *et al.*, 1992), with increased plasma corticosterone levels decreasing neurogenesis (Cameron & Gould, 1994). It has also been reported that estrogen increases hippocampal neurogenesis in adult female rats (Tanapat *et al.*, 1999), while vitamin E deficiency has also been reported to increase neurogenesis in the dentate gyrus of rats (Ciaroni *et al.*, 1999). Serotonin, especially via the 5-HT_{1A} receptor, can significantly affect neurogenesis (Jacobs *et al.*, 1998) as it plays an important role in neuronal and synaptic plasticity in the central nervous system (Azmitia & Whitaker-Azmitia, 1997; Jacobs, 2002). These data are thus supportive of the conclusion that a stress-induced decrease in neurogenesis may be an important contributing factor in depression (Jacobs, 2002).

2.1.4.6 Glutamate hypothesis

Glutamate is one of the most widely distributed neurotransmitter systems in the mammalian brain (Monaghan *et al.*, 1989). Not only does it play an important role in its own right on brain function, it also exerts significant and important regulatory control over the release of other transmitters, such as dopamine, serotonin, acetylcholine (ACh) and noradrenalin (Flott & Seifert, 1991; Prast & Phillippu, 2001), which suggest a significant role in the regulation of mood. Especially important is its reciprocal relationship with γ -aminobutyric acid (GABA), and together these two transmitters exert pronounced effects on the levels of neuronal function and activation in the brain (Harvey *et al.*, 2003; Harvey, 2008). Clinical studies have demonstrated alterations in the serine/glycine ratio as well as glutamate in depressed patients (Altamura *et al.*, 1995), as well as changes in the NMDA receptor complex in various limbic brain regions in suicide victims. Importantly, chronic antidepressant treatment reduces serum levels of aspartate and glutamate (Maes *et al.*, 1998). Platelet glutamate receptor super-sensitivity is also evident in patients with depression. Glutamate has been found to play a major role in the regulation of hippocampal neurogenesis (Balu & Lucki, 2009), by increasing hippocampal cell proliferation (Gould *et al.*, 1994; Cameron *et al.*, 1995). The hippocampus and amygdala have high concentrations of N-methyl-D-aspartate (NMDA) receptors and it is likely that glutamate contributes to the progressive, deleterious neurocognitive effects of chronic stress, mania, and severe recurrent depression (Magarinos and McEwen, 1995; Maes *et al.*, 1998). Both stress and glucocorticoids have been found to increase glutamate concentrations in the hippocampal synapse and it is acknowledged as a prime mediator of glucocorticoid-induced neurotoxicity (Sapolsky *et al.*, 2000),

Antidepressants are known to modify glutamate N-methyl-D-aspartate (NMDA) receptors (Skolnick, 1999; Paul, 2001; Harvey *et al.*, 2003; Harvey *et al.*, 2002). This has prompted a shift towards glutamate as a basis to explaining the neurobiology of depression and of antidepressant action (Shiah and Yatham, 1998; Paul, 2001). Depression may therefore be driven by excessive activation of the glutamate NMDA receptor cascade (Harvey *et al.*, 2003; Harvey, 2008), so that treating depression may be addressed through the use of NMDA receptor antagonists. Indeed, this approach has realised some degree of success as an augmenting approach in resistant forms of depression (Zarate *et al.*, 2006).

Considering the above, attention has also begun to focus more on events subsequent to NMDA receptor activation. In this regard, glutamate mediated activation of subcellular calcium-dependent pathways, especially NO and its second messenger, cyclic guanosine monophosphate (cGMP) (Harvey, 1996; McLeod *et al.*, 2001; Paul, 2001), has met with interesting results (see Section 2.2). A number of studies (Finkel *et al.*, 1996; Suzuki *et al.*, 2001) have found evidence for elevated NOS activity in patients with depression, although this has not always been consistent, with depression also being associated with decreased NOS activity (Xing *et al.*, 2002). These opposing results have prompted intense investigation into the role of NO in stress related disorders, and especially in depression. This is also supported by pre-clinical studies which have not only demonstrated the antidepressant effects of NMDA antagonists (Skolnick, 1999), but also of NOS inhibitors (Harkin *et al.*, 1999) and guanylyl cyclase-cGMP inhibitors (Eroglu and Caglayan, 1997; Heiberg *et al.*, 2002). Importantly, NMDA antagonists (Rogoz *et al.*, 2002) and NOS inhibitors (Harkin *et al.*, 2004) exert synergistic antidepressant effects with classical antidepressant in the rat forced swim test. As alluded to earlier, a diverse array of known antidepressants, including electroconvulsive therapy, evoke adaptive changes to the glutamate NMDA receptor (Skolnick, 1999; Paul, 2001; Harvey *et al.*, 2003), while various antidepressants inhibit NOS activity over and above their known actions on monoamine uptake (Wegener *et al.*, 2003; Harvey *et al.*, 2006). Interestingly, bupropion which is an atypical antidepressant acting predominantly via dopaminergic mechanisms (Cooper *et al.*, 1980), also exerts its antidepressant effects via the NO-cGMP signalling pathway (Dhir *et al.*, 2007), while lithium too has been found to exert diverse effects on NO-cGMP signalling (Harvey *et al.*, 1990; Dehpour *et al.*, 1994; Harvey *et al.*, 1994).

The glutamate-NO cascade therefore offers many possible explanations for the neuropathology of depression. Nitric oxide is a neuromodulator and regulates the release of primary neurotransmitters such as noradrenalin, serotonin, dopamine and acetylcholine, excitatory and inhibitory amino acids as well as histamine in the CNS (Prast & Phillippu,

2001; Heiberg *et al.*, 2002). Wegener *et al* (2000) showed that endogenous NO exerts a negative control over 5-HT and dopamine levels in the rat hippocampus, while inhibitors of NO can decrease Ach release in certain brain areas (Prast & Phillippu, 1992; Prast *et al.*, 1995). NO inhibits synaptic reuptake of released glutamate into the neuron and as such will potentiate the actions of glutamate (Pogun *et al.*, 1994). These modulatory actions of NO highlight its potential role in the regulation of mood, anxiety, motor activity as well as disorders such as depression, bipolar disorder (Karatinos *et al.*, 1995), schizophrenia, anxiety- and stress disorders (Harvey, 1996).

Nitric oxide may also counter some of the neuropathological changes of depression through its neuroprotective actions by downregulating NMDA receptors via a negative feedback loop, or through its antioxidant actions. However, the opposite is also true. Under certain conditions of excessive glutamate release and/or excessive production of superoxide, NO reacts with superoxide to form the highly neurotoxic peroxynitrite anion (Dawson & Dawson, 1998). This mechanism, coupled with mitochondrial dysfunction and oxidative stress (Esplugues, 2002) is associated with many neurological disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Dawson & Dawson, 1998), and indeed in mood disorders as well (Harvey, 2008). These neurotoxic actions of NO are mediated by an increase in glutamate-mediated extracellular Ca^{2+} release (Dalkara *et al.*, 1998).

Depression is highly co-morbid with anxiety (Hudson & Pope, 1990), while it is known that antidepressants have anxiolytic effects (Nut *et al.*, 1999). Several findings indicate that NO plays an important role in anxiety-related disorders. NMDA antagonists (Dunn *et al.*, 1989), as well as NOS and guanlyl cyclase inhibitors have potent anxiolytic effects in animal models (Eroglu and Caglayan, 1997; Volke *et al.*, 1997). High concentrations of NOS are found in brain regions involved in the modulation of anxiety and defensive behaviour (Vincent and Kimura 1992), and exposure to stressful stimuli has been found to induce the activation of NO-producing neurons in those brain regions (Krukoff and Khalili 1997). This provides further importance of the inherent value of targeting the NO system in the treatment of depression as it will address the associated symptoms of anxiety as well. However, the Janus-faced qualities of NO mean that it may be both detrimental and beneficial in the neurobiology and aetiology of depression. In fact, both high and low levels of NO have been documented in depression, while antidepressants may either increase (Jopek *et al.*, 1999) or decrease (Wegener *et al.*, 2003; Harvey *et al.*, 2006) NOS activity. These attributes make the NO-cGMP pathway an attractive target for novel antidepressant development, although new and in-depth research is needed to establish exactly how NO may be utilised as a novel therapeutic target for treating depression. Furthermore, the use of NOS inhibitors as putative

antidepressants is also problematic due to the ubiquitous presence of NO in the brain and the periphery, and the relative lack of selectivity of these inhibitors for the neuronal isoform of NOS. This lack of selectivity for the NOS isoforms results in a high risk of potentially lethal side effects, particularly related to the cardiovascular system (Hobbs *et al.*, 1999; Ignarro *et al.*, 1999).

2.1.5 Drug treatment of depression

Current drug treatment of depression remains heavily dependent on the monoamine hypothesis. There are some exceptions, however, e.g. tianeptine and agomelatine that offer subtle nuances of novel thinking, and that are discussed briefly later. In spite of the large number of antidepressants available for the management of depression, there are clear shortcomings. Thus, approximately 30% of depressed patients do not fully respond to drug therapy while the remaining 70% do not achieve complete remission (Holtzheimer & Nemeroff, 2006; Fava & Davidson, 1996). Issues such as side effects and discontinuation syndrome are all receiving a lot of attention as factors that hamper patient compliance and that inevitably affect long-term outcome (Harvey *et al.*, 2003). Although there is some evidence for a more rapid onset of action for some antidepressants, such as escitalopram (Kasper *et al.*, 2006; Harvey, 2008), all available agents are disadvantaged by a slow onset of action despite that changes in synaptic monoamines occur within hours of administration. Therapeutic response thus requires 2-4 weeks of continuous administration (Zimmerman *et al.*, 2005), while maintenance treatment for a minimum of 9 months for the first episode is imperative (Harvey, 1997). A proposed hypothesis regarding the slow onset of action is based on the interactions of 5-HT in the forebrain (Mongeau *et al.*, 1997), especially important is the role of 5-HT_{1A} autoreceptors. When these 5-HT_{1A} receptors are stimulated, they inhibit 5-HT neuronal firing activity so that the release of serotonin is attenuated. Longer term exposure to high 5-HT levels results in the gradual down-regulation of these autoreceptors thus disinhibiting serotonin release. This delay in producing a sustained increase in serotonergic transmission is usually taken as the reason for the delayed onset of antidepressant action (Harvey, 1997), although increasing evidence now also supports a role for time-dependent changes in sub-cellular signalling as an important contributor to the slow onset of antidepressant action (Harvey, 1997; Harvey, 2008). One such signalling cascade is the subject of this investigation, viz. the NO-cGMP pathway. There is thus a dire need for the development of newer and target-specific antidepressant drugs that will address the above issues. However, in this section I will review the currently available antidepressants and their use in the treatment of depression.

2.1.5.1 Antidepressants targeting monoaminergic transmission

Monoaminergic pathways play a crucial role in the control of cognition, affect, endocrine secretion, chronobiotic rhythms, appetite and motor function, all of which is profoundly disrupted in depressive states (Millan, 2004). Moreover, all clinically available antidepressants increase corticolimbic monoamines. Despite this, however, their limited efficacy, delayed onset of action and undesirable side-effects prompt the search for new and improved agents (Millan, 2004).

Modern day antidepressants were discovered somewhat serendipitously in the 1950's. Isoniazid and its isopropyl derivative, iproniazid (Figure 2-2), were developed for the treatment of tuberculosis in 1951. It was later found that iproniazid had mood-elevating effects, a response that was later attributed to inhibition of the monoamine hydrolysing enzyme, monoamine oxidase (MAO) (Healy, 1997; Baldessarini, 2001). Iproniazid was to become the first clinically successful antidepressant (Healy, 1997; Baldessarini, 2001). Also in the 1950's, imipramine (Figure 2-2) was discovered out of a need to synthesize new antihistamines, sedatives, analgesics or antiparkinsonian drugs. However, it was found to be somewhat ineffective in psychotic patients, yet remarkably effective in depressed patients (Baldessarini, 2001). After establishing the basis of its efficacy to be due to inhibition of monoamine reuptake, imipramine became the archetypal tricyclic antidepressant and eventually spurred the so-called era of psychopharmacology as we know it. Imipramine is still used today as an effective antidepressant, despite the introduction of new compounds.

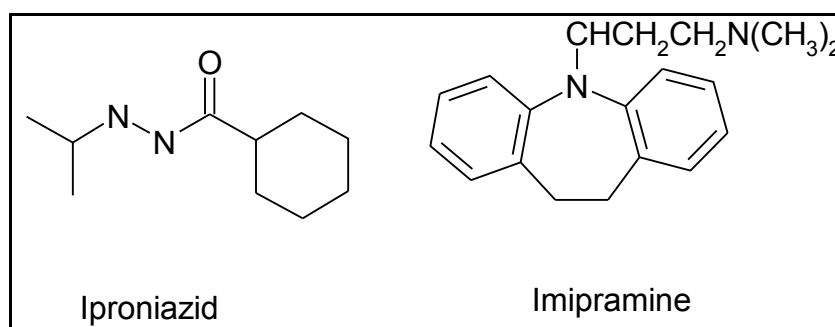


Figure 2-2: Chemical structures of iproniazid and imipramine

Currently used anti-depressant drugs either inhibit the uptake of 5-HT and/or norepinephrine, or block monoamine oxidase enzymes, leading to an immediate increase in synaptic levels of these monoamines. Bupropion, however, is unique in that it is a more potent inhibitor of dopamine reuptake (Baldessarini, 2001).

2.1.5.1.1 Monoamine oxidase inhibitors (MAOI)

Monoamine oxidase (MAO) inhibitors, or MAOI, were the first successful antidepressant drugs. MAO is a flavin-containing enzyme localized in the mitochondrial membranes of nerve terminals (Cesura & Pletscher, 1992; Baldessarini, 2001), and regulates the concentration of catecholamines and serotonin in the central nervous system (CNS) and peripheral tissues (Baldessarini, 2001). MAO is divided into MAO-type A and MAO-type B, while inhibition of these enzymes by MAOI will result in an increase in the bioavailability of monoamine neurotransmitters in the CNS or sympathetic nervous system. Serotonin and norepinephrine nerve terminals mainly contain MAO-A, such that selective inhibitors of MAO-A are usually more effective in the treatment of major depressive disorder than MAO-B inhibitors, such as L-deprenyl (Murphy *et al.*, 1987). Irreversible MAOI, such as iproniazid and phenelzine (figure 2-3), irreversibly block mitochondrial MAO resulting in a sustained elevation in monoamines which poses a risk for hypertensive crisis or serotonin syndrome (White & Simpson, 1981; Sternbach, 1991; Millan, 2004). However, reversible MAOIs, like moclobemide (Figure 2-3), are used more frequently for depression due to their lower potential to induce dangerous drug-drug and drug-food interactions (Leipzig & Mendelowitz, 1992; Hansten & Horn, 2000). Despite that these drugs attain rapid and maximum inhibition of MAO within days after administration; clinical effects are delayed for several weeks (Baldessarini, 2001). In recent years, the use of MAOI has declined significantly following the discovery of safer and more user-friendly alternatives to treating depression (see below).

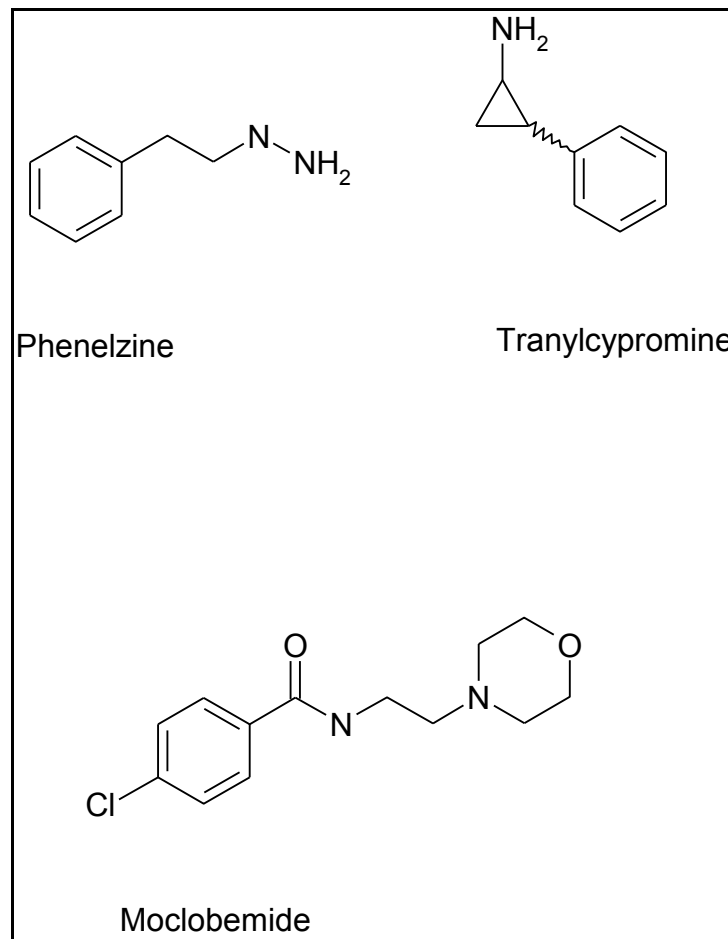


Figure 2-3: Chemical structures of some monoamine oxidase inhibitors

2.1.5.1.2 Tricyclic antidepressants (TCA)

The archetypal tricyclic antidepressants, imipramine and amitriptyline, have been used successfully as antidepressants since the 1960's. These drugs are referred to as "tricyclics" due to their 3-ring chemical structure (Figure 2-4). Imipramine is a dibenzazepine compound that differs from the phenothiazine antipsychotics by the replacement of the sulphur atom with an ethylene bridge to produce a seven-membered central ring (Baldessarini, 2001). Because of their close structural similarities, the TCA's share pharmacological and clinical properties.

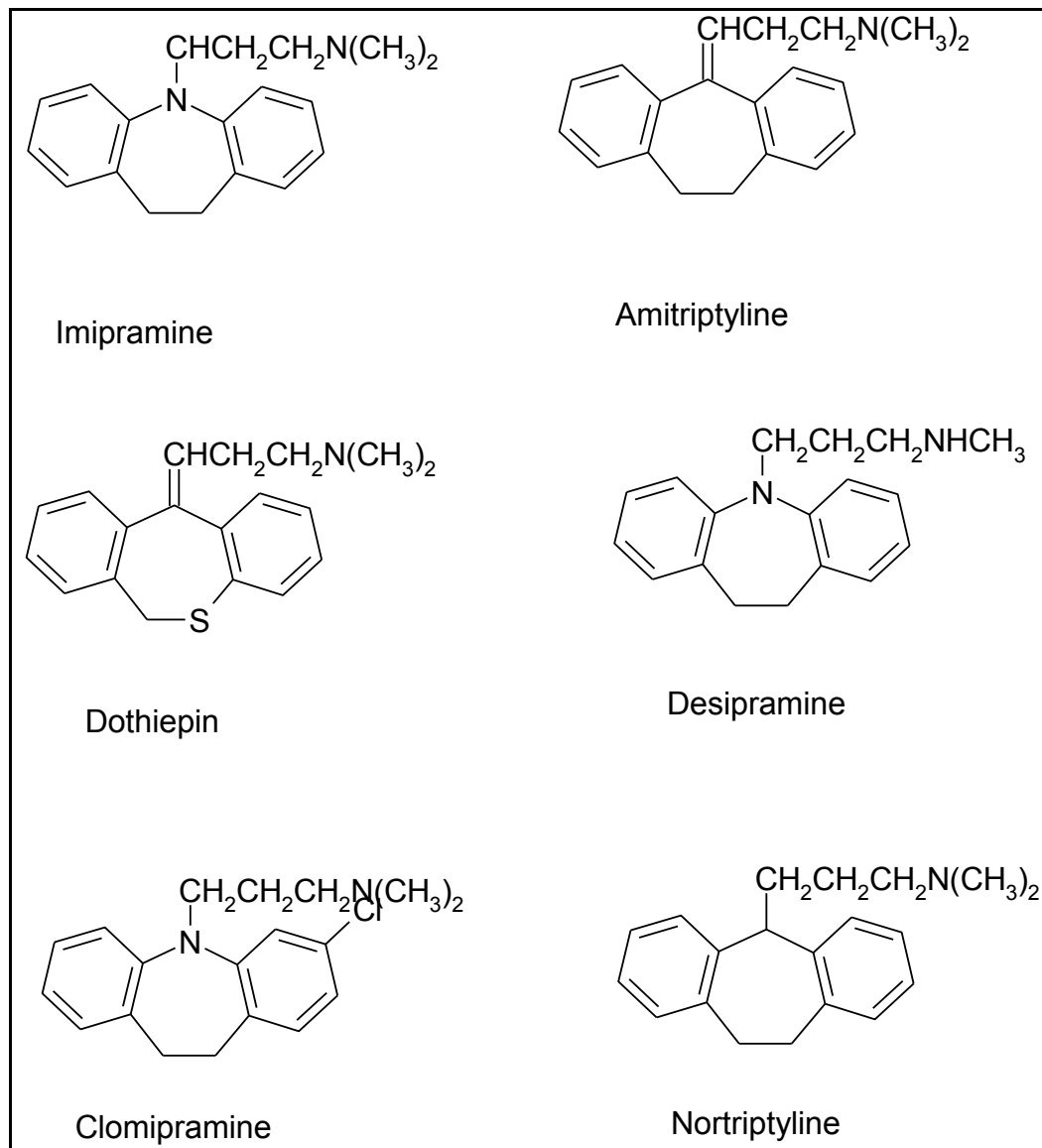


Figure 2-4: Chemical structures of some tricyclic antidepressants.

The action of the TCA's is based on their ability to inhibit the neuronal transport of norepinephrine and serotonin (Barker & Blakely, 1995). However, their selectivity for these two systems is relative. TCA's like desipramine and nortriptyline that have a secondary amine side chain are relatively selective inhibitors of norepinephrine transport, while those with a tertiary-amine, such as imipramine and clomipramine, more effectively inhibit the re-uptake of serotonin (Baldessarini, 2001). As a result of potent antimuscarinic effects, TCA's produce many autonomic side effects such as dry mouth, metallic taste, epigastric distress, constipation, dizziness and blurred vision. TCA's also have very prominent and dangerous interactions with central nervous depressants such as alcohol, as well as with MOA inhibitors, serotonergic agents and hepatic enzyme inducer or inhibitors (Baldessarini, 2001). In addition, TCA's induce diverse cardiovascular effects, including orthostatic hypotension,

sinus tachycardia and prolonged cardiac conduction times, which in turn can result into potential lethal arrhythmias, especially in overdose (Baldessarini, 2001).

Despite their widely recognised efficacy, in fact some meta-analyses suggest that TCA's are more effective than SSRI's in treating depression (Arrol *et al.*, 2005), they have largely been relegated to the status of second line treatment due to their side effect burden, particularly cardiovascular toxicity which poses a considerable risk for patients with unresolved suicidal ideation (Tihonen *et al.*, 2006). The main indications for TCA's today are for treatment resistant depression (Ananth, 1998), enuresis in children (Hazel, 1996) as well as attention deficit-hyperactivity disorder (ADHD) in patients who respond poorly to stimulants (Steingard *et al.*, 1995).

2.1.5.1.3 Selective serotonin reuptake inhibitors and norepinephrine reuptake inhibitors

Although the group of selective serotonin re-uptake inhibitors (SSRI's), including citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline and escitalopram (Figure 2-5), are not superior in efficacy to that of the older MAOI and TCA's, they have markedly improved safety and side effects profiles, making them far more tolerable for the patient (Harvey, 1997). In addition, their ease of use (once daily dosing) and improved patient compliance has led to their acceptance as the most commonly prescribed antidepressant drugs (Harvey, 1997; Baldessarini, 2001). Further appeal with these agents is that serotonin is now regarded as a neuromodulator that is involved to a lesser or greater degree in most psychiatric disturbances. Consequently, prescribing of an SSRI has escalated in disorders that may or may not be co-morbid with depression, such as obsessive compulsive disorder (OCD), anxiety disorders, bulimia and others. In fact today SSRI's are the agents of choice in the treatment of OCD as well as disorders of compulsivity, such as bulimia nervosa, Tourette' syndrome, trichotillomania and others (Geller *et al.*, 1998; Hoehn-Saric *et al.*, 2000). Subsequent to the development of the SSRI's, newer agents were developed to selectively inhibit the reuptake of norepinephrine (eg. reboxetine, nisoxetine) or both norepinephrine and serotonin (duloxetine, venlafaxine). The latter have been advocated to have a faster onset of action and to be more effective in resistance forms of depression due to their dual action on two key transmitters involved in depression (Harvey, 2008). Important to note is that unlike the TCA's that are all structurally similar, these drugs all have different drug structures with different pharmacological and pharmacokinetic properties (Figure 2-5; Harvey, 1997).

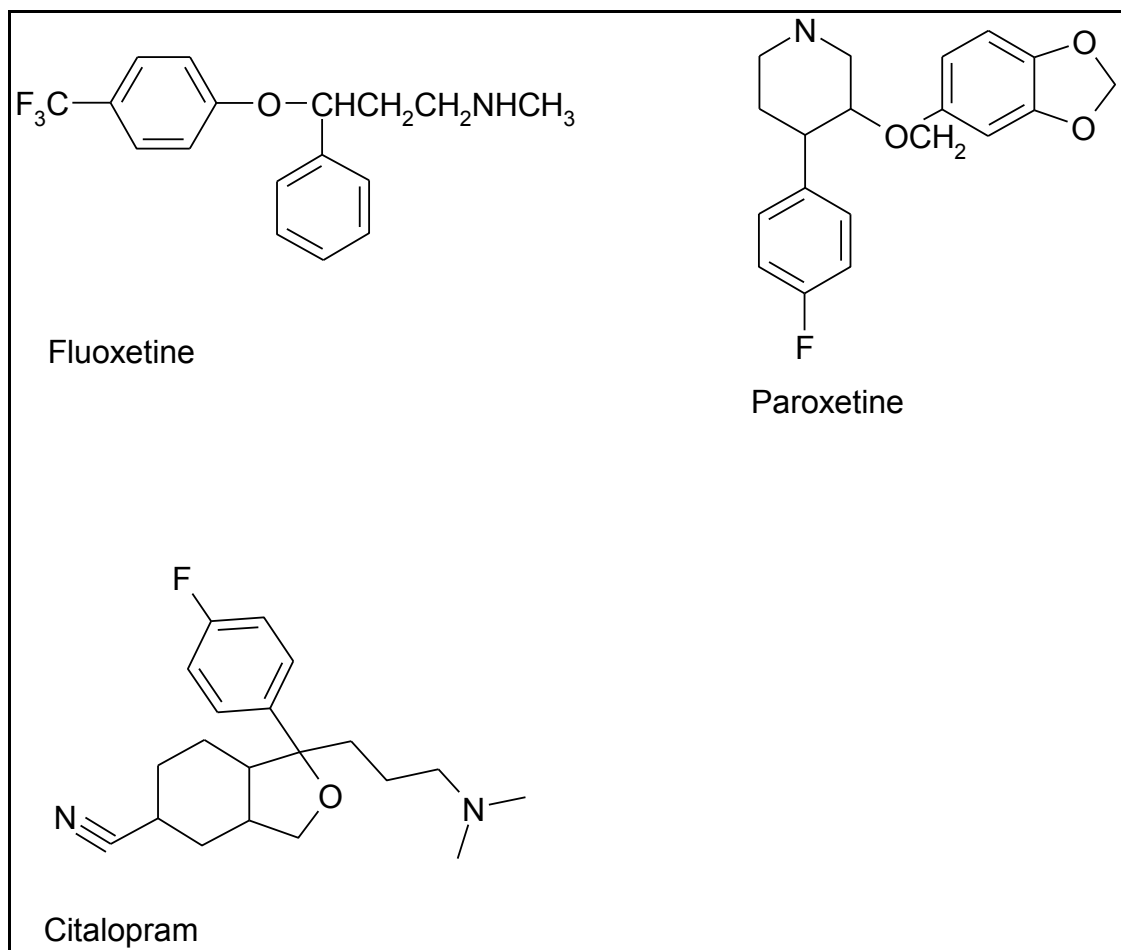


Figure 2-5: A representative sketch of some selective serotonin reuptake inhibitors

By blocking the neuronal transport of serotonin, SSRI's invoke a complex response that is directly or indirectly related to the non-specific activation of pre- and postsynaptic serotonin (5-HT) receptor types (Baldessarini, 2001) (Azmitia & Whitaker-Azmitia, 1995). Thus, 5-HT₃ receptor stimulation results in nausea and vomiting, while gastro-intestinal symptoms follow on from 5-HT₄ activation. Sleep disturbances, anxiety and jitteriness result from excessive activation of 5-HT_{2A/C} receptors, as well as the discontinuation syndrome upon abrupt stopping of the medication (Harvey *et al.*, 2003). Another aspect is the modulatory role of 5-HT on other transmitters such as dopamine, where excessive activation of 5-HT_{2A/C} receptors will lead to abrogation of dopaminergic function in the cortex, striatum and other areas resulting in motor side effects and sexual dysfunction (Harvey, 1997; Harvey *et al.*, 2003; Baldessarini, 2001). However, this cross-talk with cortico-striatal dopamine pathways has also been used to explain how SSRI's work in OCD (Harvey *et al.*, 2002). The sudden increase in 5-HT following inhibition of the reuptake transporter will activate 5-HT₁ autoreceptors that are responsible for maintaining synaptic 5-HT homeostasis. This negative feedback mechanism effectively shuts down further 5-HT release, and will “brake” the onset of action of the antidepressant (Azmitia & Whitaker-Azmitia, 1995; Baldessarini, 2001).

2.1.5.1.4 Other antidepressants

Between the diverse pharmacological actions of tricyclic antidepressants and the SSRI's that present with a single site of action, lies a newer group of antidepressants with no single feature in common that enables a convenient classification. This group are thus referred to as atypical antidepressants, and include bupropion, nefazadone and mirtazapine (Figure 2.6; Horst & Preskorn, 1998).

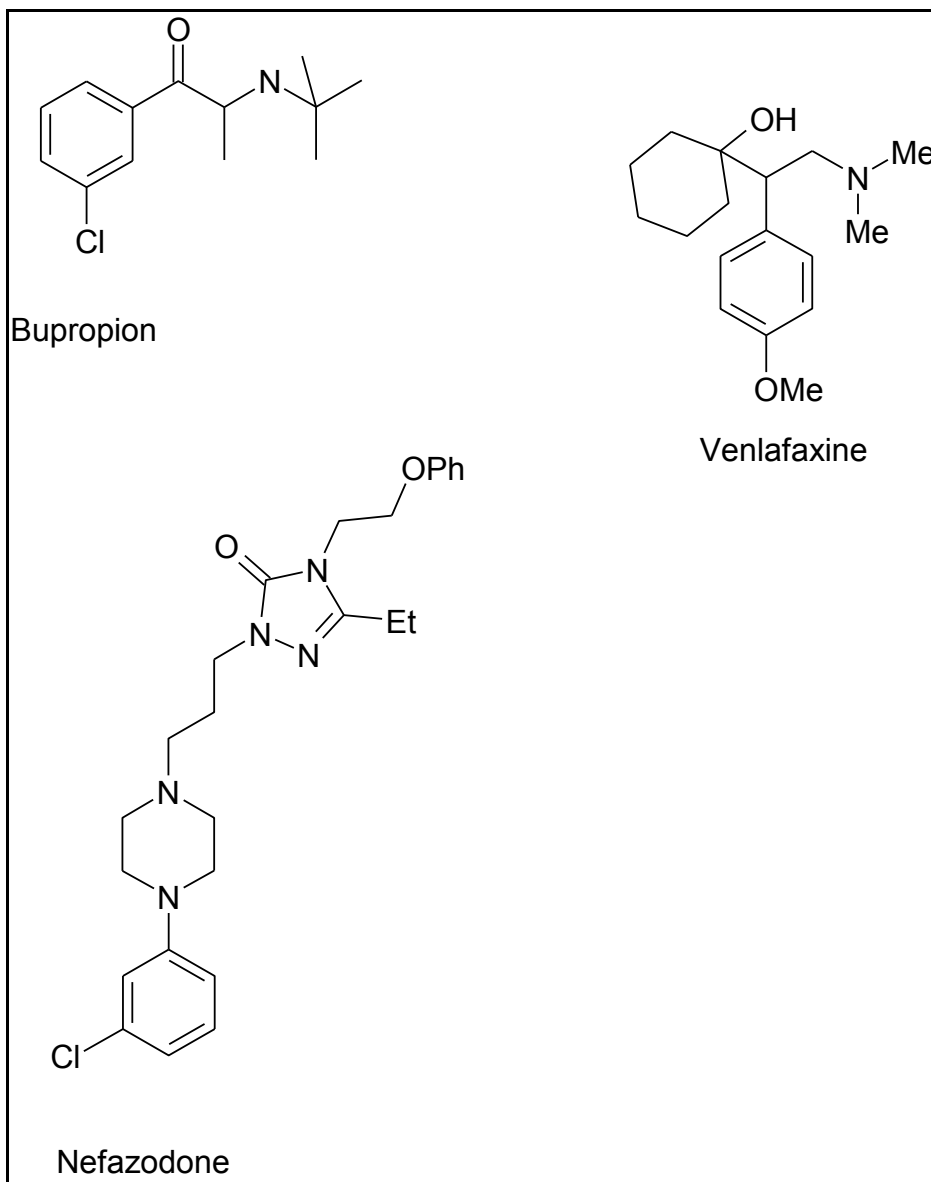


Figure 2-6: Chemical structures of some atypical antidepressants

As described earlier, dopamine plays an important role in mood. Typical antidepressants may indirectly enhance extracellular levels of dopamine by virtue of noradrenalin-dopamine cross-talk mechanisms. Thus, increasing noradrenalin through blocking of noradrenergic transporters or by acting on α_2 -adrenoreceptors and 5-HT_{2C} receptors will bolster dopamine

levels in mesolimbic and mesocortical dopaminergic pathways (Millan *et al.*, 2000). Bupropion is structurally and mechanistically different from all other types of antidepressants (Horst & Preskorn, 1998), being an inhibitor of noradrenalin and dopamine reuptake (Ascher *et al.*, 1995) with no influence on serotonin uptake (Horst & Preskorn, 1998). Although generally used as a second-line drug, it has found value in treating refractory depression or when side effects, especially sexual dysfunction, become intolerable with SSRI's (Jacobsen, 1996).

Nefazodone is a phenylpiperazine chemically related to trazodone (Horst & Preskorn, 1998). Its main mechanism of action is the blocking of 5-HT_{2A} receptors as well as presenting with weaker affinity for the serotonin reuptake transporter. It thus acts as an SSRI but with the added benefit of lesser side effects that follow from excessive 5-HT_{2A} receptor activation (Horst & Preskorn, 1998). Nefazodone presents with minimum cardiotoxicity or anticholinergic effects, and has less side-effects than trazodone, which is plagued by issues of sedation and symptoms related to alpha-1 blockade (Jacobsen, 1996). However, it was discontinued because of severe hepatotoxic effects (Stewart, 2002).

Venlafaxine is a bicyclic phenylethylamine compound (Horst & Preskorn, 1998) that inhibits the uptake of serotonin, noradrenalin but also dopamine to a lesser extent (Bolden-Watson & Richelson, 1993). Venlafaxine differs from tricyclic antidepressants as it has little or no activity on α - or β -adrenergic receptors, muscarinic cholinergic receptors as well as histaminergic receptors (Bolden-Watson & Richelson, 1993; Horst & Preskorn, 1998).

Evidence suggests that α_1 -adrenoreceptors play an important role in the actions of antidepressants. Blockade of α_1 -adrenoreceptors may mimic depressive states which is associated with α_1 -adrenoreceptor desensitisation (Stone *et al.*, 2003). Chronic antidepressant treatment gradually down-regulates α_1 -adrenoreceptors, an adaptive effect that is related to their delay to onset of action, and underpins the utility of antagonist properties at α_1 -adrenoreceptor sites for therapeutic effects (Millan *et al.*, 2000; Payne *et al.*, 2002; Millan, 2004). However, there are concerns for cardiovascular side-effects of drugs acting on these receptors. Mirtazapine potentiates both noradrenalin and serotonin neurotransmission at selected receptor subtypes, but its main mechanism of action is the antagonism of α_1 -adrenoreceptors and α_2 -adrenoreceptors (Den Boer, 1996). Yohimbine and idazoxan are α_2 -adrenergic receptor antagonists with some clinical evidence which suggest some antidepressant efficacy of these drugs although they are not classified or recognised as antidepressant drugs (Osman *et al.*, 1989; Cappiello *et al.*, 1995)

2.2 The nitric oxide/cGMP pathway

As has been described in Section 2.1.4.6, the glutamate-NO signalling cascade has become an important molecular target in our understanding of depression and antidepressant action. Nitric oxide is a free radical gas, long known as an air pollutant and a potentially toxic agent. However, it has recently been shown to be an endogenous cell-signalling molecule of great physiological importance (Moody *et al.*, 2001), particularly in the nervous system, while it acts as a mediator in the cardiovascular, renal, pulmonary, endocrine and immune systems as well (Cooper *et al.*, 1996). NO had been recognised as an important regulator of vascular and inflammatory mechanisms for more than a decade before its role in the central nervous system was appreciated (Garthwaite *et al.*, 1988), this following from the efforts of various laboratories to characterise brain nitric oxide synthase (NOS) (Snyder & Dawson, 1995; Bloom, 2001).

NO produced by endothelial cells is a primary determinant of vascular resting tone through basal release, but resulting in vasodilation when synthesized in response to shear stress and to a variety of vasodilating agents (Garland *et al.*, 1995). NO also plays an active role in inhibiting platelet aggregation and adhesion (Radomski, 1987). Impaired vascular NO production has been implicated in atherosclerosis, hypertension, cerebral and coronary vasospasm as well as ischemia-reperfusion injury (Krumenacker *et al.*, 2004). In the immune system, NO serves as an important effector of macrophage-induced cytotoxicity and its overproduction is an important mediator of inflammatory states (Moncada *et al.*, 1991; Nathan, 1992; Ignarro *et al.*, 1999; Moody *et al.*, 2001). In the brain, NO is involved in a host of phenomena including long-term potentiation, guanylyl cyclase activation, glutamate uptake and glutamate toxicity (Bloom, 2001), as well as mediating the release of a number of key transmitters, but most notably serotonin, noradrenaline, dopamine and acetylcholine (Prast and Phillipu, 2001).

2.2.1 NO biosynthesis

NO is produced from the semi-essential amino acid, L-arginine (Figure 2-7) via a family of NOS enzymes termed constitutive and inducible NOS (Moody *et al.*, 2001). Constitutive NOS includes endothelial NOS (eNOS) and neuronal NOS (nNOS) which are involved in the CNS and found in neuronal cells, while inducible NOS (iNOS) is involved in inflammatory or autoimmune processes and can be found in macrophages and hepatocytes (Oosthuizen *et al.*, 2005). NOS is a *flavin adenine dinucleotide* (FAD) and flavin mononucleotide (FMN) enzyme, requiring molecular O₂ as well as NADPH as coenzyme and tetrahydrobiopterin as cofactor in the reaction. The neuronal and endothelial enzyme is constitutively expressed

and requires the presence of Ca^{2+} and calmodulin for activity (Cooper *et al.*, 1996). iNOS, on the other hand, is calcium independent (Oosthuizen *et al.*, 2005).

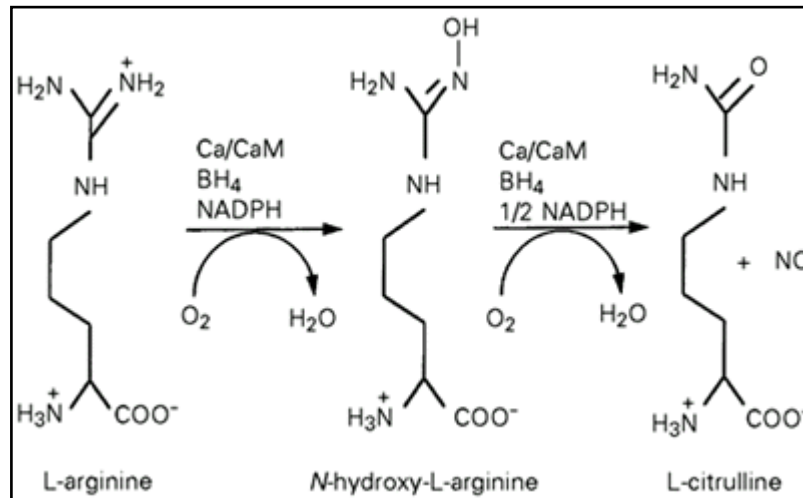


Figure 2-7: Biosynthesis of NO as depicted by Murad (1999)

NO is synthesized on demand, i.e. *de novo* (Esplugues, 2002) and diffuses from nerve terminals to act on enzymes and other elements in and adjacent to the cell of release. Thus, both neuronal and non-neuronal structures in close proximity of its release are influenced. This implies that NO acts as both a neurotransmitter and neuromodulator (see below and section 2.1.4.6) (Garthwaite & Boulton, 1995). Upon release, the primary action of this gaseous molecule is to bind to the iron containing heme of guanylyl cyclase to stimulate the enzyme and to increase the concentration of the second messenger molecule, cGMP (Moncada *et al.*, 1993). The rise in cGMP activates cGMP-dependent protein kinases (PKG) that catalyze the phosphorylation of substrate proteins that are responsible for its eventual physiological effects of the NO-cGMP cascade (Cooper *et al.*, 1996).

NO reacts with oxygen (O_2), and then undergoes spontaneous oxidation to form the inactive metabolites nitrite (NO_2^-) and nitrate (NO_3^-). It also reacts with $\text{O}_2^{\cdot -}$ to form the unstable neurotoxic intermediate peroxynitrate (ONOO^-) (Denninger & Marletta, 1999). ONOO^- acts on the respiratory chain (I-IV) complex to generate superoxide anions and hydrogen peroxide (H_2O_2) (Alderton *et al.*, 2001).

2.2.2 Regulation of nNOS

The most important regulator of nNOS activity is free cytosolic Ca^{2+} (Figure 2-8), which stimulates nNOS through interaction with calmodulin. As a result, any event linked to an

action potential that activates voltage-dependant Ca^{2+} channels will increase nNOS activity (Esplugues, 2002). However, in the central nervous system nNOS activation and subsequent NO synthesis is predominantly regulated by glutamate stimulated Ca^{2+} conductance via NMDA receptor-associated ion channels (Dawson *et al.*, 1992). NO then acts as a diffusible gaseous intercellular messenger to induce a cascade of important intracellular events (Deutsch *et al.*, 1997), foremost among these being activation of the soluble guanylyl cyclase-cGMP-protein kinase G (PKG) cascade, which acts as an important second messenger for glutamatergic function (Fedele *et al.*, 1999). Apart from effects on PKG, cGMP also targets various cyclic nucleotide phosphodiesterases (PDE's) and ion channels that together will illicit diverse effects on neuronal function (Figure 2-8; Harvey, 1996; Oosthuizen *et al.*, 2005).

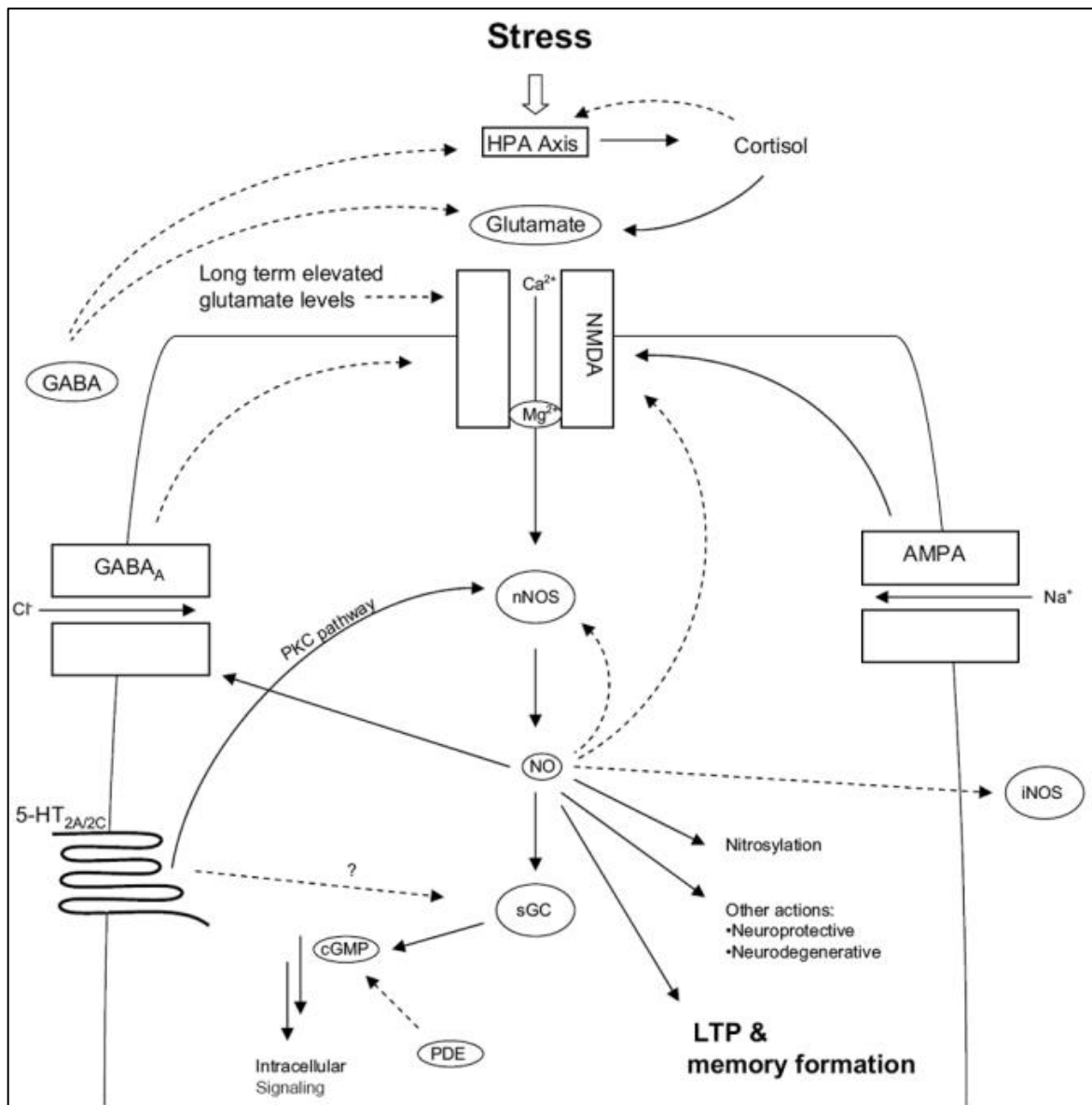


Figure 2-8: Activation of nNOS in the central nervous system as depicted by Oosthuizen *et al.*, (2005). Stress and altered HPA-axis activity provoke the release of glutamate and the opening of NMDA receptors leading to the calcium-dependent activation of NOS and NO release. Stress also induces the release of 5-HT, the latter acting on the 5-HT₂ receptor to further activate nNOS via protein kinase C (PKC). NO then acts as a modulator of further glutamate and GABA release, as well as promotes cellular plasticity, via the second messenger cGMP.

2.2.3 NO signalling

Soluble guanylyl cyclase (sGC) is the enzyme considered to be the major physiological target for neuronal NO, while subsequent synthesis and release of cGMP mediates a large number of the physiological actions of NO (Esplugues, 2002), these include effects on ion channels, phosphodiesterases and transporters. Importantly, NO has been linked to the

release of other neurotransmitters, including increasing release of acetylcholine (Gustafsson *et al.*, 1990), increasing noradrenalin (Li & Rand, 1989), increasing dopamine (Hanbauer *et al.*, 1992), decreasing glutamate (Sorkin, 1993) and increasing γ -aminobutyric acid (GABA) (Kuriyama & Ohkuma, 1995). Thus the effects of NO will to a lesser or greater degree be inseparable from many of the physiological effects of these transmitters, especially in the brain. Direct S-nitrosylation of receptors, activation of cGMP-dependent protein phosphorylation cascades, regulation of neuronal energy dynamics and a modulating effect on transporters, are examples of these interactions (Choi *et al.*, 2000; Pieper *et al.*, 2000; Kiss & Vizi, 2001).

2.2.4 NO in the central nervous system

Not only will NO be involved in maintaining vascular tone in the cerebral tissue, but through its involvement as a down-stream messenger of the NMDA receptor, it will serve as an important physiological regulator of neurotransmission, neuromodulation, cell survival and cell death mechanisms (Prast and Philippu, 2001). Although NO is critical under normal NMDA-directed activity for functions such as cognition, conditions where a sustained and/or excessive activation of NMDA receptors is evident (Colasanti *et al.*, 2000) will result in high levels of Ca^{2+} influx into the postsynaptic neuron resulting in an overproduction of NO and eventual expression of its cytotoxic qualities (Esplugues, 2002). This is typically the scenario during ischemia-reperfusion injury and glutamate-related neurodegenerative illnesses such Alzheimer's disease and Huntington's disease (Colasanti *et al.*, 2000).

Neuronal NOS can be located either pre- or post-synaptically and is implicated in neurotoxicity, synaptic plasticity and modulation of behavioural pathways such as learning, anxiety, mood and expression of pain (Esplugues, 2002). Endothelial NOS is mainly involved in the regulation of vascular function, although it is not unreasonable to assume that NO released from the vascular endothelium may influence nearby neuronal tissue as well (Qu *et al.*, 2001). Inducible NOS is implicated in the non-specific immune response in the brain, and is usually associated with inflammatory conditions of the brain (Murphy, 2000). Despite their distinct physiological functions, all three isozymes may play definite roles in neuropsychiatric disorders. For example, it has already been mentioned earlier that depression is an inflammatory condition (Harvey, 2008), while cardiovascular disorders are also recognised as being co-morbid with depression (Harvey, 2008), thus highlighting the possible co-involvement of iNOS, eNOS and of course nNOS, in a psychiatric illness such as anxiety and depression. In fact, stress which is so integral to the neurobiology of depression and other stress-related conditions is associated with activation of nNOS and iNOS, although the two

isoforms may occupy different roles at different stages of the illness (Harvey *et al.*, 2004; 2005; Oosthuizen *et al.*, 2005).

NO is involved in two forms of synaptic plasticity; long-term potentiation (LTP) and long-term depression (LTD). LTP is a synaptic correlate of learning and memory and is most pronounced in the higher brain centres involved in cognitive functions, particularly the cerebral cortex and hippocampus (Bohme *et al.*, 1991; Haley *et al.*, 1992; Esplugues, 2002). With regards the NO system, guanylate cyclase activation seems to be a primary initiator of NO-mediated events controlling the induction of LTP (Bohme *et al.*, 1991; Haley *et al.*, 1992; Esplugues, 2002). LTP is a property of many central excitatory synapses characterized by a prolonged enhancement of synaptic transmission or an activity-dependent increase in synaptic strength, lasting from hours or even weeks. LTP induction involves glutamate acting on amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA) in concert with NMDA receptors, to activate a series of Ca^{2+} /calmodulin-dependent events. LTD, on the other hand, is a reduction in synaptic strength characterized by a long lasting depression of parallel fibre synapses due to repeated excitation. LTD appears to be a result of diminished sensitization of postsynaptic AMPA receptors mediated by protein kinase C, as well as PKG from the NO-cyclic GMP signalling pathway (Shibuki & Okada, 1991; Daniel *et al.*, 1993). This would suggest that the glutamate-NO-cGMP pathway will have important actions on memory and other cognitive processes. Since these are well recognised deficits in depression, it opens up the NO-cGMP cascade as a novel approach to not only treating depression, but also disorders of cognition (Brink *et al.*, 2008).

2.2.5 Pharmacological manipulation of the NO-cGMP cascade

Various compounds have been synthesised in an attempt to modulate the NO-cGMP system, including so-called selective inhibitors of the various NOS isoenzymes. Typical agents include L-nitromethyl arginine, a non-specific inhibitor of the constitutive NOS isoforms (i.e. nNOS and eNOS), the selective nNOS inhibitors N-nitro-arginine and 7-nitroindazole, and the selective iNOS inhibitor aminoguanidine (Oosthuizen *et al.*, 2005). Non-selective and selective inhibitors of sGC include methylene blue and 1-(2-trifluoromethylphenyl)-imidazole (TRIM), respectively (Mayer *et al.*, 1993; Thippeswamy, 2001). Agents that bolster the NO-cGMP pathway include releasers of NO such as amyl nitrate, nitroglycerine and molsidomine, and drugs that increase cGMP levels by inhibiting cGMP-phosphodiesterase such as sildenafil and tadalafil (Corbin, 2000). To date, only the nitrates and the PDE5 inhibitors have found clinical application, this being for angina pectoris and erectile

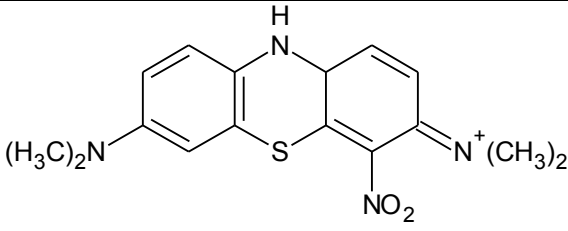
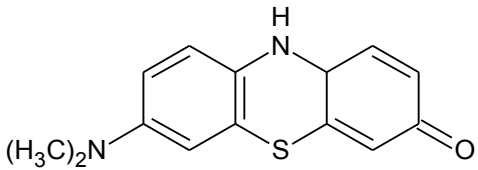
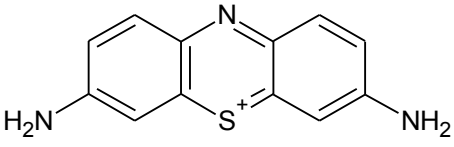
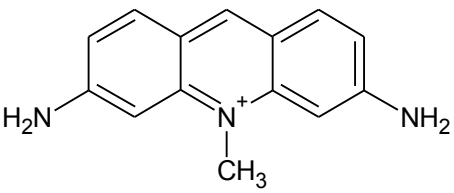
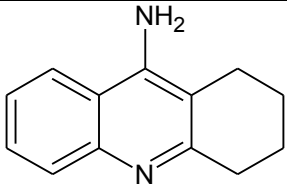
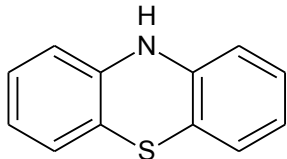
dysfunction, respectively (Ghofrani *et al.*, 2006). However, the use of NOS inhibitors have been much more problematic due to the ubiquitous presence of NO in brain and the periphery, the relative lack of selectivity of these inhibitors and the associated risk of potentially lethal cardiovascular and other adverse effects (Hobbs *et al.*, 1999; Ignarro *et al.*, 1999). It is thus pertinent that new molecules for targeting the NO-system be developed for possible therapeutic application in neurological and psychiatric disorders.

One molecule of particular interest is methylene blue (MB), a compound known to have multiple modulatory actions on the NO-cGMP system, and the subject of this investigation. Due to its long history of clinical use for various clinical conditions, including that of the central nervous system, as well as its relative safety, MB may represent a valuable lead compound for novel drug discovery that directly exploits the NO-cGMP system.

2.3 Methylene blue (MB)

MB was first synthesised in 1876 by Heinrich Caro as a non-toxic cotton dye. Indeed, MB is well known for its staining properties in biology (Wainwright *et al.*, 2002) as well as its indication in redox reactions (Wainwright *et al.*, 2002). It is the latter characteristic that prompted its most well known clinical application, namely as an antidote for the emergency treatment of methemoglobinemia (Cawein *et al.*, 1964). MB has been widely used for medicinal purposes since the late 1800's (Bodoni, 1899), although its clinical value has remained largely unexplored. However, this is set to change as the compound is now registered for 11 clinical trials in the USA in 2008 (Oz *et al.*, 2009). When considering its potential as a psychotropic, MB displays promising pre-clinical activity as an antidepressant and anxiolytic (Eroglu and Caglayan, 1997), while the compound has also demonstrated clinical utility in the treatment of bipolar disorder and schizophrenia (see below). Its mode of action remains unclear, although recent studies have found it to have noteworthy inhibitory actions on MAO (Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007) as well as the NOS-NO-cGMP pathway (Eroglu and Caglayan, 1997), the details of which are discussed below. Since both these targets present with functional relevance for antidepressant action, the principle aim of this study is to investigate MB and its structural congeners as putative lead compounds in the search for a new class of antidepressant. The following section will review the chemistry and psychobiology of MB.

Table 2-2: Methylene blue analogues which have been chosen for this study

 <p>Methylene green</p>	 <p>Methylene violet</p>
 <p>Thionin acetate</p>	 <p>Acriflavine</p>
 <p>Tacrine</p>	 <p>Phenothiazine</p>

2.3.1 Methylene blue: Physical chemistry

MB is a solid green powder at room temperature and yields a blue solution when dissolved in water. The characteristic deep blue colour, which is due to its oxidised state (shown on the left in Figure 2-9), is the stable form of MB and demonstrates an absorption spectrum of 609 to 668 nm (Ramsay *et al.*, 2007). Methylene colourless is the more unstable reduced form of MB known as leucomethylene blue, which does not absorb in the visible spectrum (shown on the right in Figure 2-9; Oz *et al.*, 2009). These two forms of the dye exist as a redox couple in equilibrium, so that together they form a reversible oxidation-reduction system or electron donor-acceptor couple (Oz *et al.*, 2009). Reducing agents such as NADPH can convert MB to leucomethylene blue which is then oxidised by O₂. Each reaction cycle, catalyzed by the MB-enzyme ensemble, can lead to the consumption of NADPH and O₂ and to the production of reactive oxygen species, predominantly H₂O₂ (Buchholz *et al.*, 2008; Oz *et al.*, 2009).

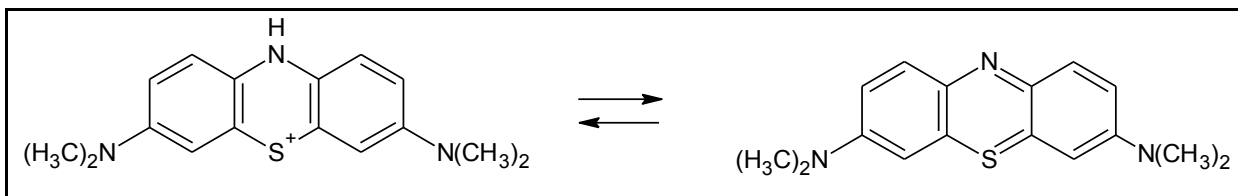


Figure 2-9: The electron donor-acceptor couple typified by methylene blue and its reduced state; leucomethylene blue

Despite it being a charged molecule, MB does pass the blood-brain barrier (Peter *et al.*, 2000). Indeed, patients receiving MB (after parathyroidectomy) in conjunction with any SSRI or other serotonergic drugs, may develop prominent neurological signs and symptoms (Sweet & Standiford, 2007), suggesting that MB can cause severe neurological toxicity through a bolstering of central serotonergic mechanisms (Ramsay *et al.*, 2007; Stanford *et al.*, 2009). Isobolic potential curves encompassing the MB molecule indicates that charges on the nitrogen and sulphur atoms are not localized and are almost equally distributed on the surface of the molecule (Oz *et al.*, 2009) as seen in Figure 2-10. Methylene blue's passage into the brain, despite ionization may be facilitated by the dispersed charge distribution around the molecule, which may facilitate its passage through the membranes (Wagner *et al.*, 1998; Oz *et al.*, 2009).

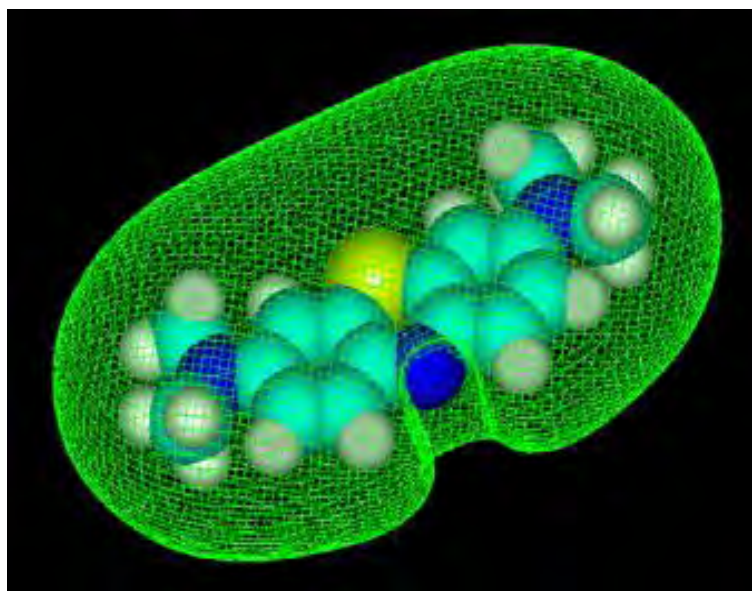


Figure 2-10: The isopotential surface surrounding the methylene blue structure in 3D space, as described by Oz *et al.*, 2009.

Other possibilities which may influence the membrane penetration of MB are the differences in ionization and lipophilicity between MB and leucomethylene blue, its reduced form. Since

lipophilic compounds easily cross the blood-brain barrier (Lorke *et al.*, 2008) and since there are significant differences in the biological activities of methylene blue and leucomethylene blue, it can be assumed that the more lipophilic form of the compound would be biologically more active (Oz *et al.*, 2009).

2.3.2 Methylene blue in clinical medicine

2.3.2.1 Methemoglobinemia

Methemoglobinemia is a condition of elevated methemoglobin in the blood, which does not bind to oxygen and in turn can result in hypoxia and cyanosis (Wright *et al.*, 1999). The iron of the haemoglobin molecule is oxidised from the ferrous (Fe^{2+}) state to the ferric state (Fe^{3+}), resulting in the inability to transport oxygen. MB is effective in the treatment of methemoglobinemia by assisting in the reduction of the ferric iron back to the ferrous state (Wright *et al.*, 1999).

2.3.2.2 Encephalopathy

Ifosfamide is an alkylating agent used in the treatment of germ cell testicular cancer, paediatric and adult sarcomas, carcinomas of the cervix, the lung and various lymphomas (Alici-Evicimen & Breitbart, 2007), and is a common component of high-dose chemotherapy regimens with bone marrow or stem cell rescue (Chabner *et al.*, 2001). However, the drug may cause severe neurological toxicity, including encephalopathy, which has been ascribed to mitochondrial toxicity induced by the metabolites of ifosfamide. By acting as an electron acceptor, MB can substitute for the flavoprotein deficiency and thus treat ifosfamide-induced encephalopathy (Küpfer *et al.*, 1994; Aeschlimann *et al.*, 1996). The prophylactic treatment for encephalopathy with MB, however, resides in another mechanism; the oxidation of excessive NADH formed during ifosfamide metabolism. MB allows for the re-oxidation of NADH to allow hepatic glucose production to return to normal and to correct intracellular redox balance (Küpfer *et al.*, 1996). MB is a potential inhibitor of drug oxidation by interfering with the microsomal electron transport chain (Küpfer *et al.*, 1996; Aeschlimann *et al.*, 1996). The drug inhibits the systemic and mitochondrial activation of 2-chloroethylamine (CIEA) and as such inhibits multiple amine oxidases *in vitro*, thus justifying its use as an antidote treatment (Küpfer *et al.*, 1996; Aeschlimann *et al.*, 1996). It reverses chloroacetaldehyde (CIAA) toxicity *in vivo*, but also inhibits multiple extra hepatic amine oxidases, which makes it effective for prophylactic treatment to prevent ifosfamide encephalopathy (Aeschlimann *et al.*, 1996).

Disturbances in the glutamate-NMDA pathway are well recognised to be the pathological basis for various encephalopathies (Harvey, 2008), while various authors have considered depression to have strong similarities with a metabolic (Dager *et al.*, 2004; Kruse *et al.*, 2006) or hepatic encephalopathy (Harvey, 2008). Moreover, this includes the involvement of NMDA evoked sub-cellular signalling mechanisms as well, especially NO and cGMP (see Harvey, 2008 for review). Wolin *et al.* (1990) have demonstrated that MB blocks the activation of guanylate cyclase in preparations that generate NO, while more recently Volke and colleagues (1999) have demonstrated its ability to inhibit NOS *in vivo* in rat brain. However, the exact mechanism of inhibition remains uncertain, although it is believed to involve the ability of MB to modify electron shuttling between various cofactors within the NOS and guanylyl cyclase enzyme complexes (Wolin *et al.*, 1990). NOS and the activation of guanylate cyclase by hydrogen peroxide are known to be inhibited by superoxide anion (Wolin *et al.*, 1990), while leucomethylene blue (reduced MB) inactivates NOS extracellularly through generation of superoxide anion during the auto-oxidation of MB *in vivo* (Wolin *et al.*, 1990; McCord *et al.*, 1970). On the other hand, Küpfer *et al.* (1996) reports that MB is also a potent inhibitor of superoxide formation, acting as an alternative electron acceptor for tissue oxidases, thus leading to the production of hydrogen peroxide instead of superoxide. This contradiction may be explained by MB acting to inhibit superoxide generation, while leucomethylene does the opposite.

2.3.2.3 Psychotic disorders

Pre-treatment with MB has anti-psychotic-like effects when tested in an animal model of schizophrenia (Klamer *et al.*, 2004), while it also reduces phencyclidine (PCP)-induced hyperlocomotion (Bujas-Bobanovic *et al.*, 2000), another putative marker of antipsychotic efficacy. However, PCP-induced hyperlocomotion is also potentiated by NOS inhibitors (Bujas-Bobanovic *et al.*, 2000). A possible explanation may be that MB acts independent of NO-cGMP, such as via the inhibition of cytochrome oxidase (Callaway *et al.*, 2002). The PCP analogue MK-801, which binds with high-affinity and selectivity to the NMDA receptor, is known to evoke hyperactivity, stereotypic behaviours and episodic explosive jumping (popping) behaviour in rodents (Deutsch *et al.*, 1993) which has been suggested to represent a useful animal model of schizophrenia. These popping behaviours are markedly reduced by MB (Deutsch *et al.*, 1996), thus suggesting that NOS inhibitors or inhibitors of NO function do indeed have antipsychotic activity. In support of this, early studies by Deutsch *et al.* (1997) have shown that MB has therapeutic value as an adjuvant in the treatment of schizophrenia,

as well as an adjuvant in manic-depressive illness (Narsapur *et al.*, 1983; Naylor *et al.*, 1984; Naylor *et al.*, 1986).

Whether the above actions of MB directly involve the NO-cGMP pathway are of considerable interest. Certainly we know that MB can modulate iron-containing enzymes (Kelner *et al.*, 1988), while stoichiometrical amounts of iron are present in both sGC and NOS (Gerzer *et al.*, 1981). Thus MB will antagonise NO stimulation of cGMP production by binding to the heme moiety of sGC (Mayer *et al.*, 1993). MB also shares a property noted with some antipsychotics and that is to sequester calmodulin, and affect that will directly abrogate constitutive NOS activity (Dawson *et al.*, 1992). This close relation with antipsychotic drugs has importance since a number of widely used anti-psychotic drugs inhibit nNOS *in vitro* (Hu *et al.*, 1994), while inhibition of striatal NOS activity *in vivo* has been described for haloperidol in rats (Harvey & Nel, 2003). Lubeluzole, a NO-dependent sGC inhibitor (Lesage *et al.*, 1994), is a neuroprotective agent used after acute stroke (Lesage *et al.*, 1994). Experiments on neuronal cultures have shown that lubeluzole inhibits anoxia and glutamate-induced NO-related neurotoxicity and inhibits neurotoxicity induced by NO donors (Lesage *et al.*, 1996). Since neurodegeneration forms an important component of schizophrenia, this emphasises the importance of exploring NOS inhibitors and drugs that interfere with the NO-cGMP cascade as novel treatments for the management of this disorder.

2.3.2.4 Mood disorders

Pre-clinical studies have reported on the potent anxiolytic and anti-depressant-like effects of MB (Eroglu *et al.*, 1997). As alluded to previously, MB has occasionally been used as an adjuvant in manic-depressive illness (Narsapur *et al.* 1983; Naylor *et al.*, 1984; Naylor *et al.*, 1986). Interestingly, lithium salts which are the drug of choice for the treatment of manic depression, also display noteworthy effects on the NOS-cGMP system (Harvey *et al.*, 1990; 1994), while MB is a useful addition to lithium in the long-term treatment of manic-depressive psychosis (Naylor *et al.*, 1986) and may also be of therapeutic value in depressive psychosis (Naylor *et al.*, 1981). Naylor *et al.* (1987) also found that as little as 15 mg/day MB effectively treats severely depressed patients. When considering the biogenic amine hypothesis of depression (see Section 2.1.4.1), it is of note that local perfusion by retro-microdialysis, as well as systemic administration, of MB increases the extra cellular levels of 5-HT in the hippocampus (Wegener *et al.*, 2000) as well as increasing the efflux of 5-HT and dopamine (Volke *et al.*, 1997). Moreover, MB powerfully inhibits MAO (Aeschlimann *et al.*, 1996). Since increasing synaptic levels of monoamines such as 5HT play an integral role in the efficacy of

clinically effective antidepressants, the above findings provide a robust rationale for how MB may exert its mood altering effects, and that further development of similar acting agents may represent a novel class of antidepressant compounds.

2.3.2.5 Disorders of cognition

Another important consideration is the role of NO and cGMP in memory (Chapman *et al.*, 1992). Indeed, recent pre-clinical evidence has demonstrated the beneficial effects of PDE5 inhibitors such as sildenafil on cognition, as well as having plausible antidepressant-like effects (Prickaerts *et al.*, 2002; Brink *et al.*, 2008). Cognitive disturbances form a central feature of Alzheimer's disease (Wells, 2003) as well as schizophrenia and depression (Green, 1996; Wells, 2003). Drugs targeting impaired mitochondrial respiration may improve memory retention deficits found in some neurodegenerative diseases (Callaway *et al.*, 2002). Impaired mitochondrial respiration is associated with deficits in learning and memory in various diseases, particularly Alzheimer's disease (Bowling *et al.*, 1995). Cytochrome c oxidase, the mitochondrial enzyme that catalyses the utilisation of oxygen for the electron transport chain during cellular respiration, is known to decline in Alzheimer's disease (Kish *et al.*, 1992), while its inhibition causes significant deficits in memory and learning (Wada *et al.*, 1996). MB increases mitochondrial respiration *in vitro* and hence has the potential to increase oxygen consumption and to compensate for decreased cytochrome c oxidase activity (Martinez *et al.*, 1978). Indeed, 1 mg/kg MB was reported to completely restore impaired memory retention induced by sodium azide, an inhibitor of cytochrome c oxidase (Martinez *et al.*, 1978). MB acts as an electron shuttle to oxygen that bypasses cytochrome c oxidase in the electron transport chain in mitochondria (Visarius *et al.*, 1997). This suggests that it compensates for impaired mitochondrial respiration and in so doing improves spatial memory retention (Callaway *et al.*, 2002). *In vitro* studies also indicate that high doses of MB inhibit superoxide generation (Salaris *et al.*, 1991) and also prevent aggregation of Alzheimer's disease-like tau protein (Wischik *et al.*, 1996). These observations have special relevance not only for Alzheimer's disease but also for depression, which not only is associated with deficits in various cognitive parameters (Austin *et al.*, 2001), but also shows evidence of degenerative phenomena in brain regions involved in memory and learning.

2.3.2.6 Side effects of methylene blue

The side effects of MB in the clinical setting have been well documented by Narsapur *et al.* (1983) in a study where 300 mg/day MB was administered to 19 manic-depressive patients. Several patients experienced nausea with occasional vomiting that appeared shortly after each dose, although this could often be prevented by taking the medication after a meal with a cold drink. MB may also irritate the bladder and urethra, with dysuria and increased

frequency of micturation (Narsapur *et al.*, 1983). A major drawback is the wide spread dissatisfaction among patients using MB regarding the blue colouration of the urine and often of the patient's underwear. In another study, doses of 15 mg/day MB were found to be associated with very few complications with regards to toxicity and side effects, while it still appeared to be a potent antidepressant (Naylor *et al.*, 1987). Together, these studies would suggest that MB is relatively safe for human ingestion. However, it was recently found that the compound may induce severe serotonin toxicity in conjunction with any SSRI (Ramsay *et al.*, 2007; Ng *et al.*, 2008). These and other side effects, although benign, will ultimately pose a problem for some patients and hence negatively influence compliance.

2.3.3 Structural analogues of methylene blue and their biological activity

2.3.3.1 Phenothiazine

Phenothiazine (Figure 2-11) was derived from MB as a synthetic dye (Wainwright *et al.*, 2002), but is known today as the parent molecule from which the typical class of antipsychotics were developed. The compound therefore has powerful antipsychotic and antihistaminergic properties (Baldessarini & Tarazi., 2001). However, there is a close relationship between the structure of the phenothiazine ring and its biological activity. It has a three-ring structure in which two benzene rings are linked by a sulphur atom and a nitrogen atom. Substitution of an electron-withdrawing group at position 2 increases anti-psychotic efficacy of phenothiazines and other tricyclic congeners (Baldessarini & Tarazi, 2001).

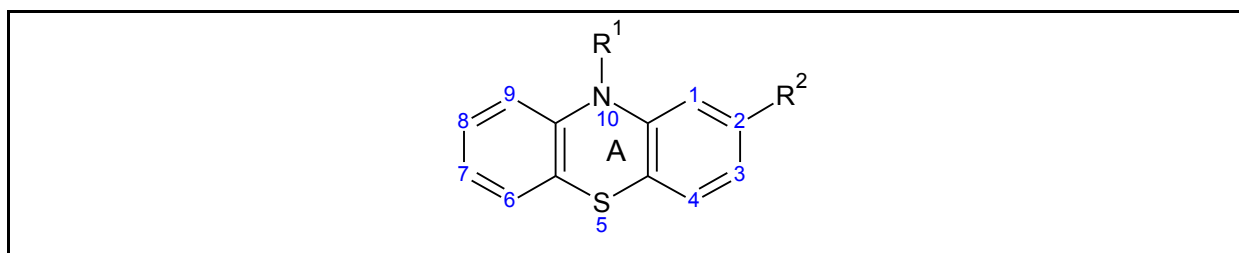


Figure 2-11: The chemical structure of phenothiazine

Favourable Van Der Waal's interactions between the side chain amino group of phenothiazine and the 2-substituent on ring A promote a conformation mimicking dopamine (Feinberg *et al.*, 1975). The nature of the substituent at position 10 also influences pharmacological activity and is necessary for the tranquilising potential, which increases with mounting electronegative substitution on position 2. Substitution on position 1, 3 and 4 causes a decrease in calming activity (Baldessarini & Tarazi, 2001).

Phenothiazines can be divided into three groups on the basis of the substitution on position 10. Compounds with aliphatic side chains include chlorpromazine and triflupromazine and are relatively low in antipsychotic potency but not in clinical efficacy. Compounds with a piperidine ring in the side chain include thioridazine and mesoridazine, and have a somewhat lower risk of extra pyramidal effects, possibly due to increased central anti-muscarinic activity (Baldessarini & Tarazi, 2001). Potent antipsychotic compounds have a piperazine side chain. These derivatives have relatively weak anti-cholinergic activity and this carry a greater risk for inducing extra pyramidal side effects, although have a lower tendency to produce sedation or autonomic side effects such as hypotension (Baldessarini & Tarazi, 2001). Phenothiazine derivatives with sedating and tranquilising effects have three carbon atoms interposed between position 10 of the central ring and the first amino nitrogen atom of the side chain at this position. Anti-histaminic phenothiazines, or strongly anti-cholinergic phenothiazines, have only two carbon atoms separating the amino group from position 10 of the central ring. Metabolic *N*-dealkylation of the side chain or increasing the size of amino *N*-alkyl substituents reduces antidopaminergic and antipsychotic activity (Baldessarini & Tarazi, 2001).

2.3.3.2 Tacrine

Tacrine, or 9-amino-1,2,3,4-tetrahydroacridine (Figure 2-12), is the analogue of the acridine structure and the first drug in this family to be marketed for Alzheimer's disease (Kozikowski *et al.*, 1992). The rationale for the use of tacrine in Alzheimer's disease is related to its ability to block acetylcholine esterase and to elevate acetylcholine levels thereby compensating for the central cholinergic deficiency associated with this disease (Fang *et al.*, 2008). Structure activity relationship studies of the tacrine nucleus shows that a substituent on position 6 exerts more favourable effects with respect to inhibitory effects on acetylcholine esterase activity than substituents in position 7 (Recanatini *et al.*, 2000). Steinberg *et al* (1975) concludes that the planar ring system of tacrine interacts with acetylcholinesterase and it is thought that the cyclohexyl ring is important for blocking the catalytic site of the enzyme (Proctor *et al.*, 2000).

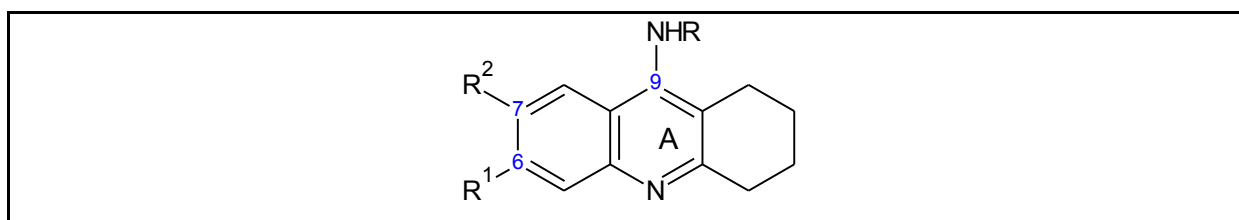


Figure 2-12: The chemical structure of 9-amino-1,2,3,4-tetrahydroacridine (Recanatini *et al.*, 2000).

Tacrine administration however was found to cause extreme hepatotoxicity in Alzheimer's disease patients (Watkins *et al.*, 1994), forcing its eventual withdrawal from clinical use. However, soon afterwards so-called NO-donor-tacrine hybrids were synthesized to be hepatoprotective anti-Alzheimer drugs (Figure 2-13; Fang *et al.*, 2008). NO increases blood flow in the brain and relieves inflammatory reactions in the brain, both of which are therapeutically significant in the treatment of Alzheimer's disease. The tacrine hybrids, synthesized by Fang and colleagues (2008), are attached to NO-donating nitrate- and diazeniumdiolate (NONOate) moieties connected via an alkylenediamine-type spacer. The rationale for their development was that the synergistic action of the NO donor, the tacrine-like heterocycle and the alkylenediamine spacer should yield potent but less toxic drug candidates for improved and safer treatment of Alzheimer's disease. The alkylenediamine side chain is introduced at the 9-position of the tacrine-like heterocycle. The spacer is also beneficial for inhibiting the periphery anion site (PAS) of AChE (Rosini *et al.* 2005), which is thought to relate to the neurotoxic cascade of Alzheimer's disease through AChE-induced amyloid- β aggregation (Bartolini *et al.*, 2003).

Little to no evidence exists for any clinical applications for other MB analogues, although there has been some evidence of studies on the antifilarial properties of methylene violet, but it was proven to be unsuccessful (Hawking *et al.*, 1952). Methylene green was also used in the same study, but was also found not to possess any antifilarial properties (Hawking *et al.*, 1952). However, methylene green at high doses was found to be an effective antimalarial agent *in vivo* against certain malaria species (Luond *et al.*, 1998).

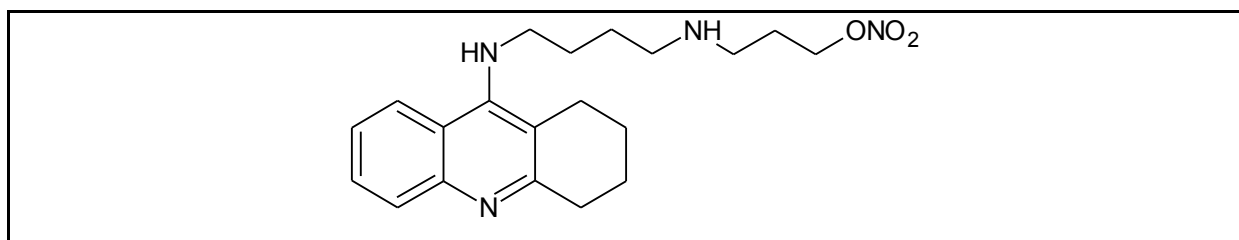


Figure 2-13: The chemical structure of a NO-donating-tacrine hybrid (Fang *et al.*, 2008).

This chapter presents and discusses the experimental methods employed in the current study, including experimental layout, animal models, materials, drugs and dosages as well as assay procedures.

The treatment of animals and behavioural testing were conducted in the Centre for Laboratory Animals at the North-West University, Potchefstroom Campus after the approval by the Ethics Committee of the North-West University (approval number: NWU-0070-08-55) and according to the internationally accepted ethics guidelines.

3.1 Study layout

In brief, this study may be divided into two experimental sections:

- **Experimental procedure 1:** This will be an acute treatment study aimed at confirming the antidepressant action of methylene blue in the rat forced swim test (FST), and to determine if the selected analogues of methylene blue also possess antidepressant-like activity. Furthermore the effect of methylene blue and its analogues will be investigated with respect to monoamine oxidase (MAO) inhibitory activity.
- **Experimental procedure 2:** This will be a chronic treatment study aimed at determining if methylene blue as well as the analogues which exhibited antidepressant-like action in the acute studies, also possesses antidepressant-like activity in the chronic treatment. The effects of the test compounds on the NO/cGMP pathway will also be investigated.

3.2 The rat forced swim test

The rat forced swim test was developed by Porsolt and colleagues (1978). This antidepressant screening model is based on the observation that when rats are forced to swim in a restricted space from which there is no possibility of an escape, they eventually cease to struggle, surrendering themselves to the experimental conditions (i.e. they show

despair or helplessness; Kulkarni & Dhir, 2007). This state is akin to some of the characteristic symptoms of depression (Porsolt *et al.*, 1978; American Psychiatric Association, 1994). This condition is then used to evaluate whether a putative antidepressant drug is able to reverse this state by promoting struggling and swimming (escape driven) behaviour. The widespread use of this model is largely due to its ease of use, reliability across laboratories and ability to detect a broad spectrum of antidepressant drugs (Borsini & Meli, 1988).

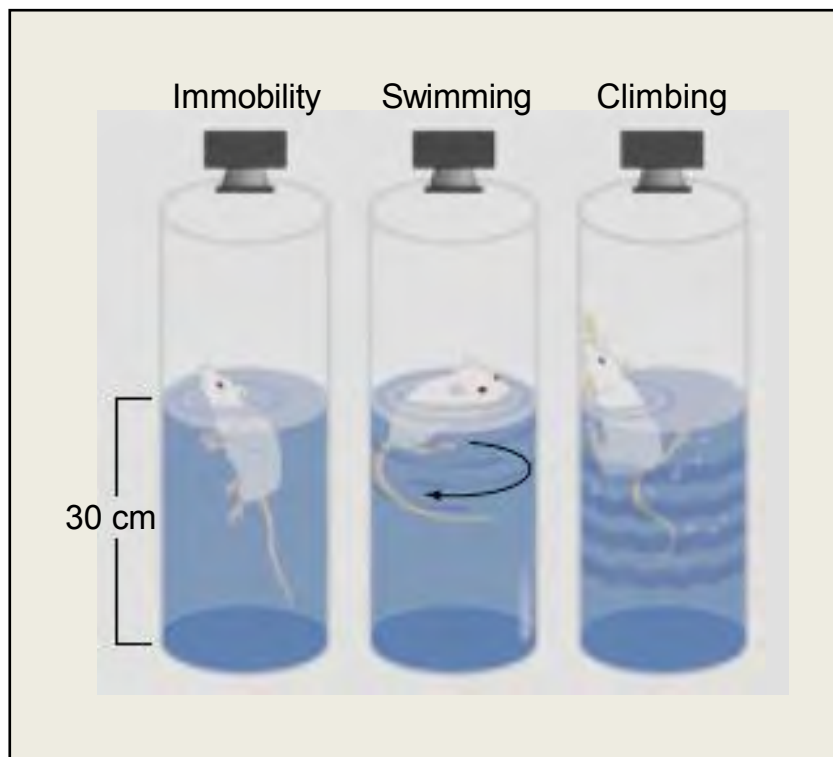


Figure 3-1: The behavioural parameters measured in the modified FST (Cryan *et al.*, 2002).

3.2.1 Scoring technique in the FST

The traditionally described FST has one major drawback however, and that is its inability to effectively detect the antidepressant activity of serotonin reuptake inhibitors (SRI's) (Detke *et al.*, 1995), which are the most widely prescribed group of antidepressant drugs in use today. Some procedural modifications, however, have been made in an effort to enhance the sensitivity of the traditional FST in the rat to be SRI-responsive (Lucki, 1997). These

behaviours, and how they have improved the capabilities of the FST, are described below and depicted in Figure 3-1 (Cryan et al., 2002).

- Immobility is defined when no additional activity is observed other than that required to keep the rat's head above the water.
- Climbing behaviour (also known as struggling) is defined as upward directed movements of the forepaws along the side of the cylinder.
- Swimming behaviour is defined as swimming movements (usually horizontal) throughout each cylinder.
- Antidepressants that primarily potentiate serotonin mediated neurotransmission increase swimming behaviour while those with primary actions on catecholaminergic neurotransmission increase climbing behaviour (Cryan *et al.*, 2002).

The above mentioned behavioural components are now used to distinguish between newer antidepressant agents and also to provide more definite information on the type (selectivity) of the antidepressant being tested. The above-mentioned behavioural components were measured in terms of the amount of time (in seconds) in which these specific behavioural component was observed for a total period of 5 minutes, with the combined time of all three components adding up to 300 seconds.

3.3 Animals and materials used

3.3.1 Animals

Male Sprague-Dawley rats weighing between 200-250 g were used for the acute FST studies while rats used in the chronic FST study weighed \pm 200 g on the final day of testing. 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 (no: NWU-0070-08-55). Suffering and discomfort to animals were minimised and the number of animals per group was the minimum needed for meaningful statistical evaluation of the data. The rats were housed in cages (5 rats per cage) with a width of 28 cm, a length of 44.5 cm and a height of 12.5 cm. The conditions in the animal centre were controlled at 21 ± 0.5 °C and $50\pm 5\%$ humidity. Full spectrum cold white light, with a light

intensity of 350-400 lux was provided over a 12 hour light-12 hour dark cycle (06:00-18:00 light). A positive air pressure was maintained in the laboratory with air filtration 99.7% effective for a particle size of 2 μm and 99.9% for a particle size of 5 μm . All animals had free access to food and water and were acclimatized to the laboratory conditions before the experiments.

For the acute FST study, animals were randomised into 9 groups comprising 5 animals each, and destined to receive acute treatment with saline, imipramine, methylene blue, methylene green, acriflavine, tacrine, methylene violet, thionin acetate and phenothiazine. For the sub-chronic treatment study, animals were randomised into 4 groups comprising 10 animals each, and destined to receive either saline, imipramine, methylene blue plus any of the methylene blue analogues that presented with antidepressant-like effects in the FST in the acute study described above.

3.3.2 Drug treatment

Methylene blue, methylene violet, methylene green, tacrine, acriflavine, phenothiazine and thionin acetate were all purchased from Sigma-Aldrich (St. Louis, USA). All animals were weighed each morning, and their respective dosages calculated accordingly and drug solutions were prepared fresh on the day of treatment.

In the acute FST treatment protocol, methylene blue, methylene green, acriflavine and tacrine were dissolved in a 0.9 % saline solution and administered i.p. at a maximum volume of administration of 0.5 ml. Methylene violet, thionin acetate as well as phenothiazine were administered in a volume of 0.2 ml i.p. and were dissolved in a 0.9 % saline solution containing a minimal amount of 5 % glacial acetic acid which were buffered with NaOH (pH = 6). Imipramine was dissolved in 0.9 % saline solution and administered i.p. at a maximum volume of administration of 0.5 ml.

In the chronic treatment protocol, all compounds were administered in a volume of 0.5 ml i.p., with all four compounds, i.e. saline, imipramine, methylene blue and selected analogues, dissolved in 0.9 % saline solution.

3.3.3 Other chemicals

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 recombinant human MAO-B enzyme (5 mg/ml) were obtained from Sigma-Aldrich (St. Louis, USA). All other reagents were analytical grade.

3.3.4 Instruments

Fluorescence spectrophotometry was conducted using a Varian Cary Eclipse fluorescence spectrophotometer for the monoamine oxidase assays. A Sony Digital Video Camera Recorder (model: DCR-TRV330E) was used to monitor animal behaviour. The Porsolt FST apparatus and the open field apparatus were manufactured “in-house” at NWU.

3.3.5 Selection of drugs and dosages for the acute FST study

Methylene blue analogues were chosen according to their structural similarities. Imipramine was used as a positive control mainly because of its known activity in the FST, while its chemical structure is similar to that of methylene blue and its analogues, i.e. all are tricyclic compounds.

The doses of the compounds were determined according to previous studies or equivalent to the accepted human dose. Some of these compounds have not been widely researched and data on their bioavailability and metabolism are not available yet. Solubility of these drugs also posed a significant problem. Consequently, in order to improve safety and tolerability in the animals, five different dosages of each compound were selected and administered intraperitoneally. These doses are below the reported LD₅₀ level of each compound.

Imipramine was administered intraperitoneally at a dose of 15 mg/kg in both FST studies, (Harvey *et al.*, 2002; 2006; Kulkarni and Dhir, 2007; Brink *et al.*, 2008) and was used as a reference antidepressant (positive control).

The intraperitoneal dose of methylene blue was used according to the work of Eroglu and Caglayan (1997). The dosage used was administered in a solution at doses of 3.75, 7.5, 15, 30 and 60 mg/kg.

Methylene green has been used successfully in doses ranging between 50 to 100 mg/kg i.p. in rats (Sewell & Hawking, 1950), and in his study was administered intraperitoneally in doses of 3.75, 7.5, 15, 25 and 40 mg/kg. Studies on the bioavailability and metabolism of this drug are not available as it has not been researched widely.

Acriflavine has previously been administered at a dose of 50 mg/kg i.p. in rats (Steinman & Leonara, 1971). The drug was administered intraperitoneally in doses of 3.75, 7.5, 15, 30 and 40 mg/kg.

Tacrine was administered in the same dosage range as a study done by Cousins *et al* (1997). It was administered intraperitoneally in doses of 1, 2.5, 3.75, 5, and 7.5 mg/kg.

Methylene violet has been safely used in rats at doses of 10 mg/kg intraperitoneally (Hawking *et al.*, 1952), and in the present study was administered intraperitoneally in doses of 2.5, 3.75, 5, 7.5 and 10 mg/kg.

Thionin acetate has been used safely in rats at a dose of 100 mg/kg (Sewell & Hawking, 1950). In the present study, the drug was administered intraperitoneally in doses of 0.5, 1, 2.5, 3.75 and 5 mg/kg.

Phenothiazine has previously been administered intraperitoneally in doses ranging between 10 to 20 mg/kg (Daniel *et al.*, 1998), and in this study was administered intraperitoneally in doses of 0.5, 1, 2.5, 3.75 and 5 mg/kg.

Normal saline was used as a solvent and control.

3.4 Experimental protocols

3.4.1 Experimental procedure 1:

3.4.1.1 Acute FST study

The rat FST is a validated and well-described screening test for the detection of antidepressant activity of known and unknown compounds. The traditional FST follows an acute treatment protocol where the animal receives a series of injections over a period of 24hrs (Porsolt *et al.*, 1978), although a chronic treatment protocol has also been developed (Detke & Lucki, 1995; Detke *et al.*, 1997). For the purpose of our study, we have selected the aforementioned acute study design, the reasons being not only its ease of use and accuracy in screening for novel antidepressant-like activity, but also because many of the drugs to be tested in this project do not have well defined dosage regimes or established toxicology. Consequently an acute treatment regimen would limit immediate toxicity to the animal. To further limit discomfort to the animals, we initiated a dose response analysis for each drug starting at very low doses and moving gradually up, and also to more accurately identify a likely therapeutic dose for rats for later application in the chronic treatment study.

The experimental protocol for the acute study was followed as described previously by Eroglu & Caglayan (1997). Firstly we wanted to demonstrate that a state of behavioural despair in rats induced by forced swimming can be reversed by acute treatment with methylene blue, and relate said antidepressant efficacy to the positive control, imipramine.

Secondly we wanted to perform a dose response study in order to establish a minimum effective dose for methylene blue in the FST with minimum toxicity to the animal, starting at a dose of 3.75 mg/kg (according to Eroglu and Caglayan, 1997). This would form the basis for deciding the approximate dosage range for the methylene blue analogues. Thereafter we wanted to determine if the methylene blue analogues have antidepressant-like activity in the acute FST, the minimum dosage being initiated in the same range as that of methylene blue, but their ceiling dose being dependent on presenting toxicity, assessed by initial pilot studies with each compound and visual evaluation, and issues of solubility. The methylene blue analogues were divided into two groups based on solubility complications:

Test compounds A (Figure 4-2): Methylene blue was designated test compound 1 (TC1), methylene green was designated test compound 2 (TC2), acriflavine was designated test compound 3 (TC3) and tacrine was designated test compound (TC4).

Test compounds B (Figure 4-3): Methylene violet was designated test compound 5 (TC5), thionin acetate was designated test compound 6 (TC6) and phenothiazine was designated test compound 7 (TC7).

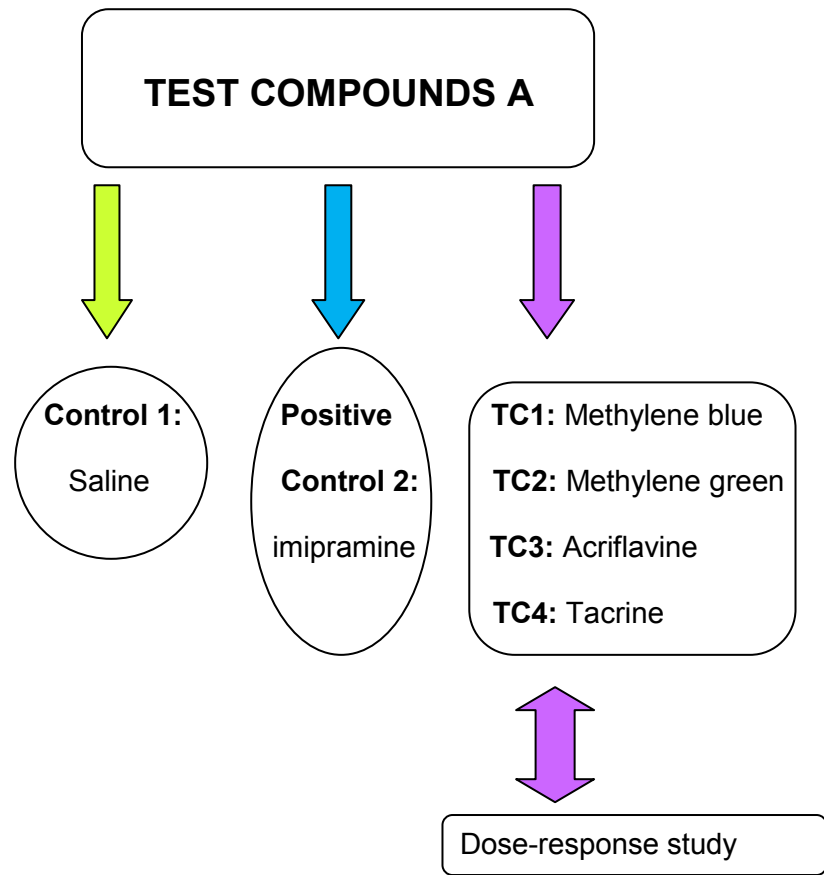


Figure 3-2: Schematic illustration of the treatment protocol of the rats with test compounds A.

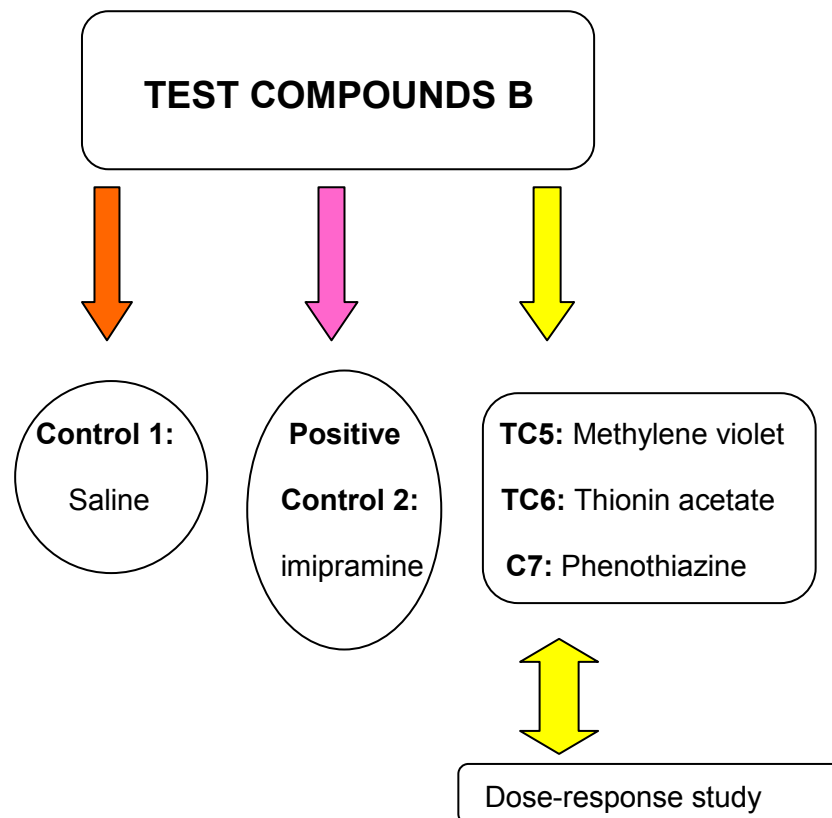


Figure 3-3: Schematic illustration of the treatment protocol of rats with test compounds B.

Five groups of rats were used per compound (n=5). On day one of the challenge, the rats were allowed to habituate to their surroundings for a period of 30 minutes. Thereafter each animal was subjected to 15 minutes of pre-swimming in transparent Perspex cylinders (diameter 18 cm, height 40 cm) 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 their first intra-peritoneal (i.p.) injection of the test drug, being 24 hours prior to the final swim test. The animals received their respective treatments at the same time each day (between 8:00 and 9:00 am) for the 24 hour treatment period. On day 2, 6 hours and again 1 hour prior to the final 5 minute test swim the rats received their second and third i.p. injection of the drug. After the last injection and following a 20 minute habituation period in the room, the rats were assessed in the open field arena to evaluate general locomotor activity (see Section 1.4.1.2 below). The rats were then left in the room to habituate for a further 20 minutes before the final 5 minute test swim commenced. For the final 5 minute swim test, rats were placed in the same cylinders as above containing 20 cm of clean water at 25 °C for

5 minutes. During this period, all swimming behaviours, as outlined in Section 1.2.1, were digitally recorded for later evaluation by the investigators. In the present study, the performance of each rat in the FST was video-taped using a digital camera and scored individually by 3 blinded investigators, 2 of whom were not involved in the study.

1.4.1.2. Assessment of locomotor activity in the open field arena

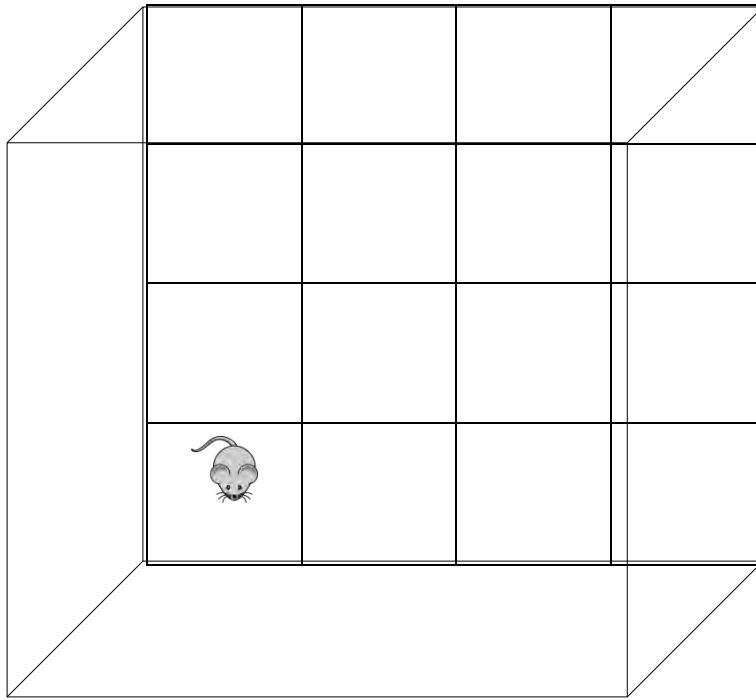


Figure 3-4: Illustration of the open field arena, which comprises 16 identical squares inside a square box in which each rat is placed separately and studied for 5 minutes.

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. Some 20 minutes after the final 5 minute swim in the FST procedure, each rat was placed separately into the open field arena (Figure 4-4) and studied for 5 minutes. The open field arena comprises of 16 identical squares drawn on the base of the box as seen in Figure 4-4. The animal's activity in the arena was digitally recorded and its levels of general locomotor activity was determined by counting line crossings over a given time period. Thus line

crossings were expressed as the number of squares the rat entered during a 5 minute time interval (Walsh & Cummings, 1976; Podhorna & Brown, 2002).

3.4.1.2 Recombinant human MAO-A and –B inhibition studies

All seven compounds including imipramine were evaluated as inhibitors of recombinant human MAO-A. Any compounds that expressed antidepressant-like activity in the FST were also tested for recombinant human MAO-B inhibition. In this test, the IC₅₀ values (concentration of the inhibitor that produces 50% inhibition) for the inhibition of MAO by each test compound were determined.

The compounds were evaluated as potential inhibitors of recombinant human MAO-A and MAO-B using a patented procedure (Novaroli *et al.*, 2005). The enzyme activity measurements were based on the extent to which kynuramine is oxidized to 2-hydroxyquinoline by the MAO isoforms (Novaroli *et al.*, 2005). The formation of 4-hydroxyquinoline was measured fluorometrically at excitation and emission wavelengths of 310 and 400 nm respectively. None of the test inhibitors fluoresced at these wavelengths or quenched the fluorescence of 4-hydroxyquinoline at the concentrations used for the inhibition studies.

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), various concentrations of the test inhibitor (0-3000 µM) and kynuramine. The final concentrations of kynuramine which served as a substrate in the reactions were 45 µM 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 minutes 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 10 minutes at 16,000 g. The concentrations of the MAO generated 4-hydroxyquinoline in the reactions, were determined by measuring the fluorescence of the supernatant at 400 nm (Novaroli *et al.*, 2005). Quantitative estimations of 4-hydroxyquinoline were made by means of a linear calibration curve ranging from 0.188-6.25 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 were added to each standard. The IC₅₀ values were determined by plotting the initial rate of oxidation versus the logarithm of the inhibitor concentration to obtain a sigmoidal dose-

response curve. This kinetic data were fitted to the one site competition model incorporated into the Prism software package and the IC₅₀ values were determined in duplicate and expressed as mean ± SEM.

3.4.2 Experimental procedure 2:

3.4.2.1 Chronic treatment studies

In this experiment we wanted to determine whether sub-chronic treatment with methylene blue, and any of its analogues that had demonstrated antidepressant-like actions in the acute FST test described earlier, displays any antidepressant-like activity after a 7 day treatment regime and evokes changes in hippocampal nitrate accumulation (a surrogate marker of nitric oxide) after 7 days treatment. As before, saline was used as a control and imipramine (15 mg/kg) was used as a positive control.

Two groups of five rats each were assigned to each compound, one for the test compound and one for saline. All animals received their i.p injections at the same time each morning for a period of 7 days. On the penultimate day of treatment, the rats were placed in the room to habituate for 60 minutes after which they were subjected to 15 minutes of pre-swimming in 18 cm of clean water (25 °C). This occurred 24 hours prior to the final test swim. The rats were dried down immediately afterwards. On the final day of treatment the rats were assessed in the open field arena and their locomotor activity was measured after a 20 minute habituation period in the room. Each rat was placed separately in the open field arena for 10 minutes while their movements were digitally recorded. Line crossings expressed in the number of squares entered per 10 minutes was used to define locomotor activity. Rats were then allowed to habituate for 60 minutes to their surroundings. Thereafter, the rats were reintroduced to the swimming cylinders for their final 5 minute swim in 18 cm of clean water (25 °C). Again their behaviour was recorded using a digital camera. The rats were immediately dried down afterwards. When the FST was completed, on day 7, the rats were decapitated, their brains removed and the hippocampus rapidly dissected on ice and snap-frozen with liquid nitrogen (-198 °C). The brain tissue was then stored at -86 °C for the subsequent nitric oxide fluorometric assay.

3.4.2.2 Nitric oxide fluorometric assay

Nitric oxide has a biological half life of minutes (Moncada & Higgs, 1993) making its determination in tissue samples a complicated analysis that requires expensive equipment. NO is rapidly converted by oxygen to nitrite (NO_2^-) and nitrate (NO_3^-) (Titheradge, 1998). The total concentration of nitrite and nitrate in biological samples can therefore be used as an indirect yet quantitative measure of NO production (Titheradge, 1998).

In the current study, we have used a commercially available NO fluorometric analysis to determine nitrate levels in hippocampal extracts. Firstly, sample nitrate is converted to nitrite by nitrate reductase (NR). Thereafter, nitrite reacts with a fluorescent probe known as 2,3-diaminonaphthalene (DAN) which, when combined with the addition of sodium hydroxide (NaOH) to enhance the fluorescent yield, provides a read-out of the fluorescent intensity that is proportional to the total nitrate in the sample. By extrapolation, the latter reading also provides an expression of nitric oxide production in the sample.

3.4.2.2.1 Preparation of brain tissue homogenate

On the day of the analysis, samples were weighed and allowed to thaw. The tissues were then homogenized on ice with a Heidolph glass-teflon homogenizer and suspended in a 10% phosphate buffered saline (PBS). Brain tissue from 5 animals per treatment was used in this assay.

3.4.2.2.2 Assay procedure

A standard curve was first constructed to validate this assay method in our laboratory. Two calibration curves were employed: one with nitrate reductase (nitrate + NR) and one without (nitrate –NR). The nitrate standard (10 mM) was reconstituted with assay buffer to generate a 50 μM working solution and added in duplicate to five wells to generate 0, 200, 400, 600 and 800 pmol/well nitrate. Thereafter 5 μl “enzyme cofactor” was added to each well and 5 μl nitrate reductase was added to the first replicate set (nitrate + NR). 5 μl assay buffer was then added to the second replicate set (nitrate – NR). The plate was allowed to incubate for 2 hours at room temperature to allow the conversion of nitrate to nitrite. On completion of this incubation period, 5 μl “Enhancer” was added and the plate was incubated for another 30 minutes to quench interfering compounds. Thereafter 5 μl DAN reagent was added to each well and the plate incubated for 10 minutes during which time the nitrite reacted with the fluorescent reagent. 5 μl of NaOH was then added to each well and allowed to incubate for 10 minutes to enhance the fluorescent yield. All incubations occurred at room temperature.

The fluorescence of each well was measured at excitation and emission wavelengths of 360 and 450 nm, respectively. The standard curve was plotted using fluorescence intensity vs. nitrate concentration from which the value for the sample was determined by extrapolation.

To determine nitrate concentration in the tissue homogenates, 10 μ l of tissue homogenate was added to each well followed by 65 μ l of the assay buffer. The same assay procedure was then followed as described above. The nitrate concentration in each sample was determined by the following formula:

$$C = \frac{Sa}{Sv}$$

where Sa is the amount of samples as read from the standard curve line (in pmole), and Sv is the volume of the sample added to the well (in μ l), multiplied by the dilution factor, where dilution is the sample dilution done prior to addition of the sample to the well .

This chapter will present all experimental results obtained. This study is divided into two experimental procedures:

- **Acute study (experimental procedure 1):** The acute treatment study aims to confirm the antidepressant action of methylene blue in the rat forced swim test (FST), and to determine if the selected analogues of methylene blue also possess antidepressant-like activity. Furthermore the effect of the methylene blue and its analogues will be investigated with respect to monoamine oxidase (MAO) inhibitory activity. The following methylene blue analogues were tested: methylene green, acriflavine, tacrine, methylene violet, thionin acetate and phenothiazine.
- **Chronic study (experimental procedure 2):** The chronic treatment study aims to determine if methylene blue, as well as any analogues that have demonstrated antidepressant-like action in the acute treatment studies (procedure 1), also have antidepressant-like activity in a chronic treatment protocol. In addition, this experiment also determined the effects of the test compounds on the NO/cGMP pathway in the rat brain. The rationale being that acute treatments with an antidepressant are not antidepressant in humans (Leonard, 2003), despite producing a positive response in the acute FST in rodents. Thus, any antidepressant-like effects described in the acute FST studies could amount to a false positive.

4.1 The effect of acute treatment with methylene blue and its analogues in the rat forced swim test (FST)

The aim of this study was to confirm the antidepressant efficacy of methylene blue in the FST, as previously reported by Eroglu and Caglayan (1997), and thereafter to determine whether any of its structural analogues present with similar biological activity. Secondly, we wanted to determine the dosage at which methylene blue, and any of its analogues, is effective as an antidepressant using a dose-response study design. Finally, in order to allow a more specific interpretation of the nature of the swimming activity observed in the FST that may relate to noradrenergic or serotonergic driven mechanisms, swimming behaviour was

further analysed by a process of behavioural sampling, as described by Cryan *et al* (2002), whereby duration of immobility, climbing and swimming behaviours were specifically measured. In all instances, the tricyclic antidepressant imipramine (IMI; 15 mg/kg) was used as the positive control. Data were analysed by means of one-way analysis of variance (ANOVA) across all groups, and were subsequently subjected to the Dunnett's post test. Data were expressed as the mean \pm SEM with statistical significance defined at the 95% ($p < 0.05$) level. Descriptive statistics are provided in accompanying tables.

4.1.1 Effect of acute methylene blue treatment in the FST

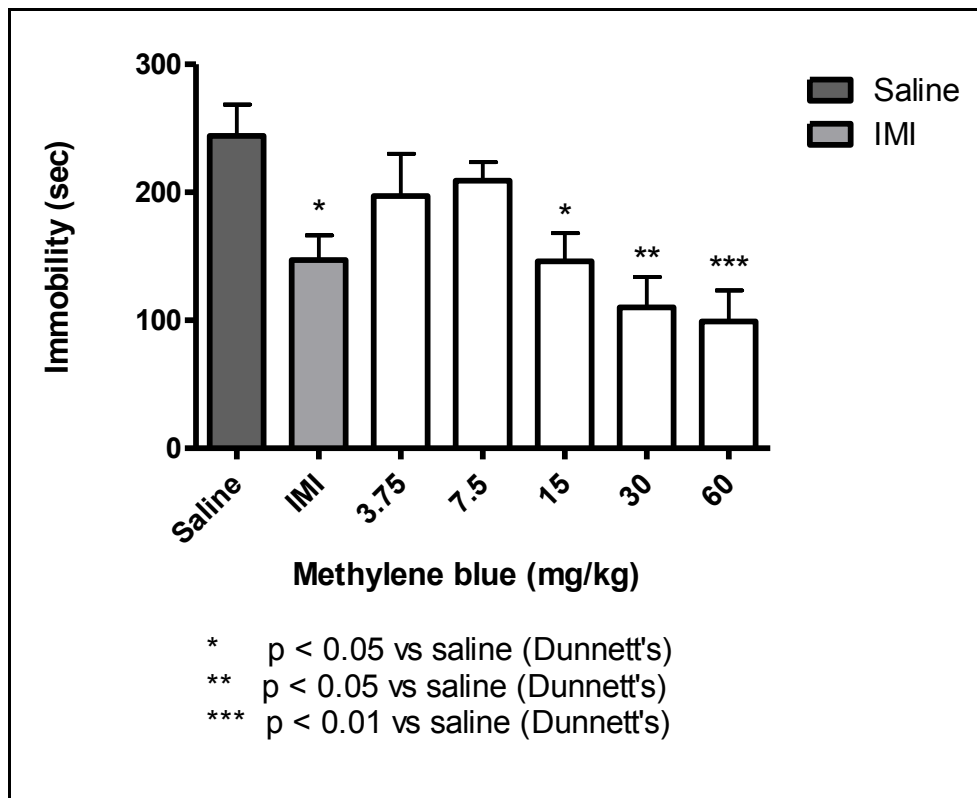


Figure 4-1: Effect of various doses of methylene blue, as indicated, on the duration of immobility in the FST, compared to saline-treated animals ($n = 5/\text{group}$).

One way analysis of variance of the data revealed a significant effect of treatment [$F(6;28)=5.116;p=0.0012$]. Post-hoc analysis of these data (Table 4-1) revealed a significant decrease in immobility time for imipramine (positive control) versus saline treatment, as

shown in Figure 4-1. Imipramine decreased immobility from 244 ± 24.57 sec to $147 \pm 19.27^*$ sec (* $p < 0.05$). Methylene blue demonstrated a dose-dependent decrease in immobility, with a significant response at doses of 15 mg/kg (from 244 ± 24.57 sec to $146 \pm 22.1^*$ sec, * $p < 0.05$), 30 mg/kg (from 244 ± 24.57 sec to $110 \pm 23.77^{**}$ sec, ** $p < 0.01$) and at 60 mg/kg (from 244 ± 24.57 sec to $99 \pm 24.46^{***}$ sec, *** $p < 0.001$), as compared to saline. Doses of 3.75 and 7.5 mg/kg were ineffective.

Table 4-1: The effect of drug treatment, as indicated, on immobility of rats in the FST, where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.01$ vs saline-treated animals (n=5) (MB=methylene blue).

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	244 ± 24.57 sec
Imipramine	$147 \pm 19.27^*$ sec
MB 3.75 mg/kg	197 ± 33.11 sec
MB 7.5 mg/kg	209 ± 14.61 sec
MB 15 mg/kg	$146 \pm 22.1^*$ sec
MB 30 mg/kg	$110 \pm 23.77^{**}$ sec
MB 60 m/kg	$99 \pm 24.46^{***}$ sec

Locomotor activity (Figure 4-2) was routinely evaluated to ensure that changes in swim motivation were based only on antidepressant-like response and not due to an indirect effect of the drug on locomotor activity.

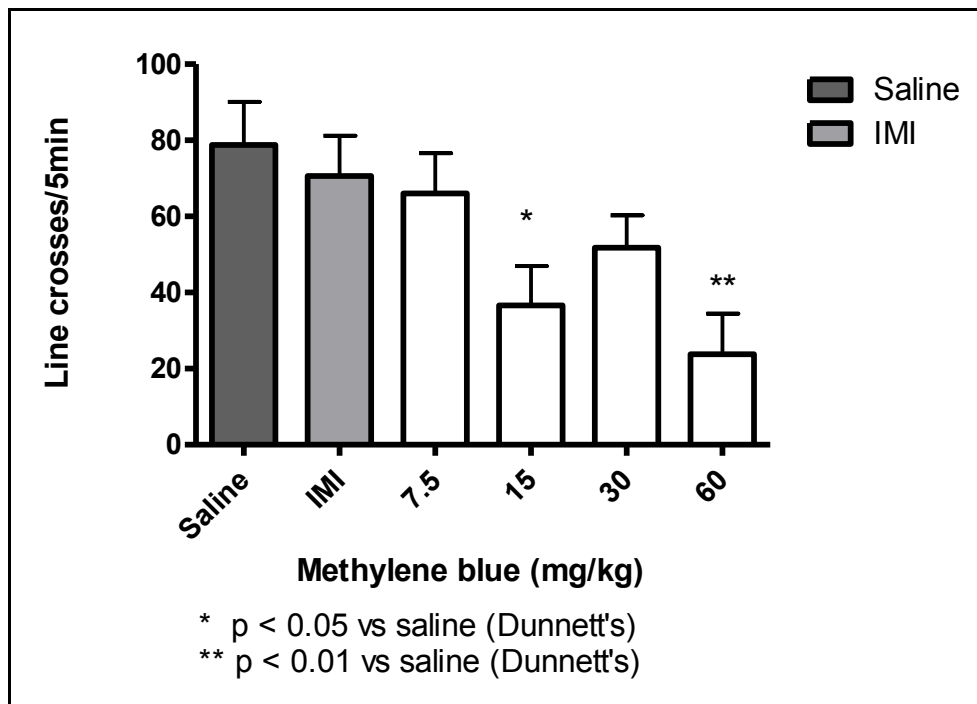


Figure 4-2: The locomotor activity of drug treatment, as indicated, as measured in the open field test, where * p < 0.05 and ** p < 0.01 vs saline-treated animals (n=5).

One way analysis of variance of the data revealed a significant effect of treatment [F(6;28)=4.189;p=0.0075]. Post-hoc analysis of these data (Table 4-2) revealed no significant decrease in the locomotor activity between saline and imipramine (Figure 4-2). As depicted in Figure 4-2, methylene blue significantly decreased locomotor activity compared to saline at a dose of 15 mg/kg and 60 mg/kg, with 30 mg/kg showing a trend towards significance.

Table 4-2: The effect of drug treatment, as indicated, on locomotor activity in the open field test, where *p<0.05 and **p < 0.01 vs saline treated animals (n=5) (MB=methylene blue).

Treatment group	Mean \pm SEM, line crosses
Saline	78.80 \pm 11.31
Imipramine	70.6 \pm 10.63
MB 3.75 mg/kg	66 \pm 10.59
MB 7.5 mg/kg	66 \pm 10.59
MB 15 mg/kg	36.6 \pm 10.39*
MB 30 mg/kg	51.75 \pm 8.56
MB 60 m/kg	23.8 \pm 10.63**

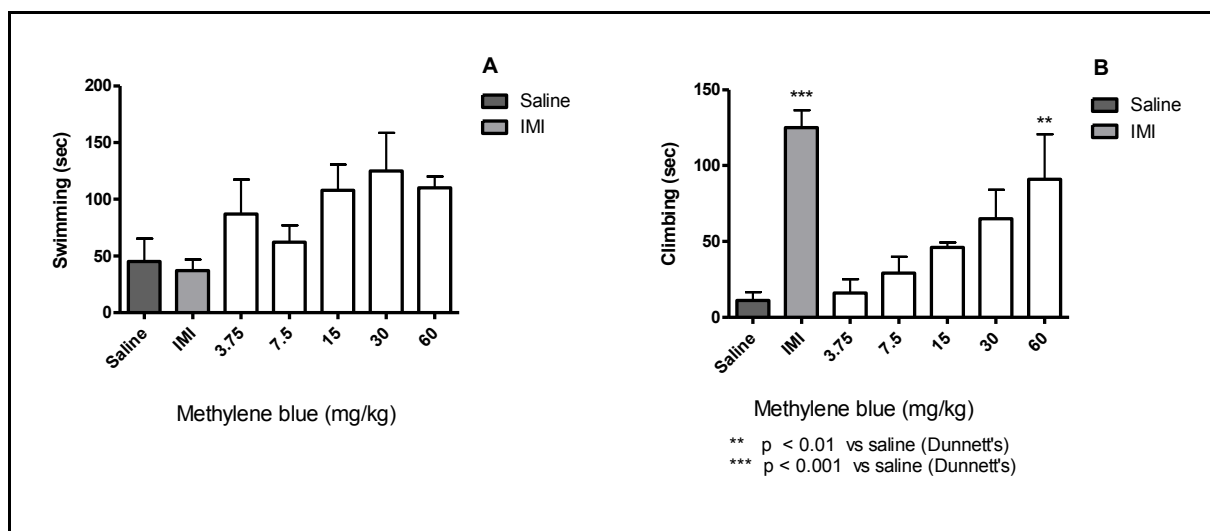


Figure 4-3: Effect of various doses of methylene blue, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

Known antidepressants that primarily potentiate serotonin mediated neurotransmission increase swimming behaviour in the FST, while those with primary actions through catecholaminergic neurotransmission increase climbing behaviour (Cryan *et al*, 2002; section 4.2). One way analysis of variance of the swimming data narrowly missed a significant effect of treatment [$F(6;28)=2.445;p=0.05$]. Nevertheless, post-hoc analysis of the swim data (Table 4-3) obtained in this analysis reveals that there are no significant differences in swimming

behaviour of rats after treatment with imipramine or methylene blue at any dose compared to saline treated animals (Figure 4-3, A). One way analysis of variance of the climbing data revealed a significant effect of treatment [$F(6;28)=7.534;p=0.0075$], while post-hoc analysis of these data (Table 4-3) showed that imipramine significantly increased climbing behaviour compared to saline treated rats (from 11 ± 5.57 to $125 \pm 11.62^{***}$, $^{***}p < 0.001$; Figure 4-3, B). Methylene blue demonstrated a dose-dependent increase in climbing behaviour, eventually becoming significant versus saline treatment at a dose of 60 mg/kg (from 11 ± 5.57 to $91 \pm 29.68^*$, $^{**}p < 0.01$; Figure 4-3, B).

Table 4-3: The effect of drug treatment, as indicated, on the swimming behaviour of rats (n=5). (MB=methylene blue)

Treatment group	Mean \pm SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	37 ± 9.7
MB 3.75 mg/kg	87 ± 10.48
MB 7.5 mg/kg	62 ± 14.97
MB 15 mg/kg	108 ± 22.56
MB 30 mg/kg	125 ± 33.5
MB 60 m/kg	110 ± 10.12

Table 4-4: The effect of drug treatment on climbing behaviour of rats in the FST, where $^{**}p < 0.01$ and $^{***}p < 0.001$ (n=5).

Treatment group	Mean \pm SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	$125 \pm 11.62^{***}$
MB 3.75 mg/kg	16 ± 9.138
MB 7.5 mg/kg	29 ± 10.89
MB 15 mg/kg	46 ± 3.321
MB 30 mg/kg	65 ± 19.17
MB 60 m/kg	$91 \pm 29.68^{**}$

4.1.2 Effect of acute methylene green treatment in the FST

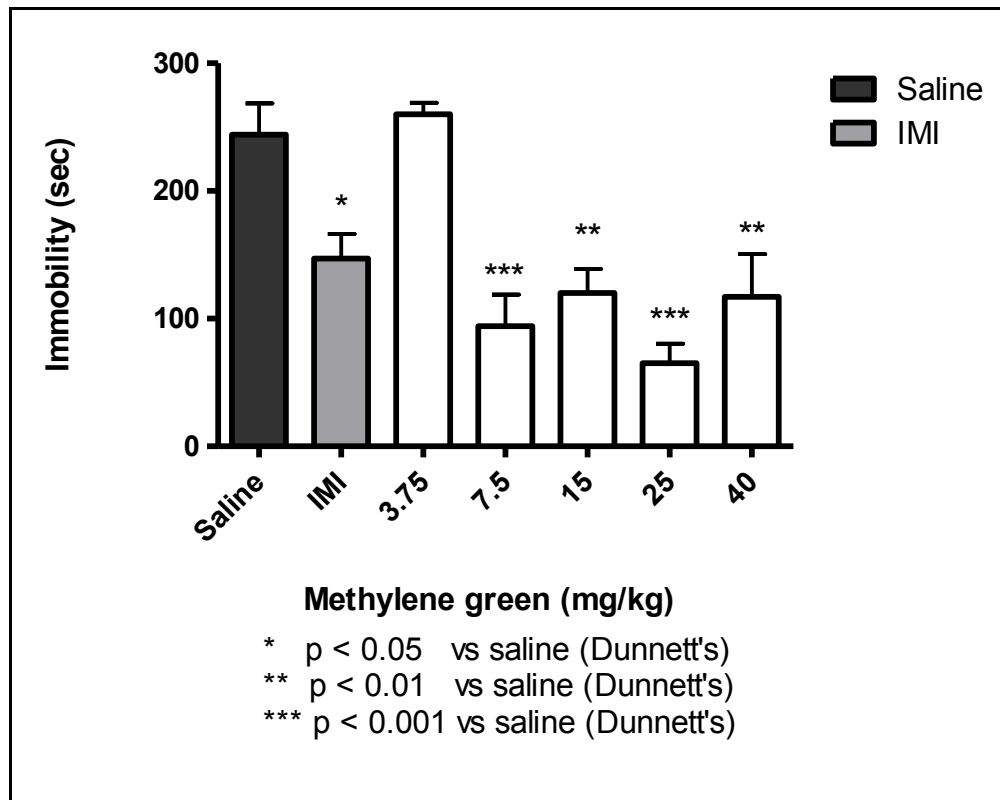


Figure 4-4: Effect of various doses of methylene green, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the data revealed a significant effect of treatment [F(6;28)=10.26;p=0.0001]. Post-hoc analysis of the data (Table 4-5) revealed a significant decrease in immobility time in imipramine treated animals compared to saline treated rats (244 ± 24.57 to 147 ± 19.27*; *p < 0.05; Figure 4-4). Methylene green also significantly decreased immobility times at doses of 7.5 mg/kg (from 244 ± 24.57 to 94 ± 24.87***, ***p < 0.001), 15 mg/kg (from 244 ± 24.57 to 120 ± 18.91**, ** p < 0.05), 25 mg/kg (from 244 ± 24.57 to 65 ± 15.41***, *** p < 0.001) and 40 mg/kg (from 244 ± 24.57 to 117 ± 33.71**, ** p < 0.01) as compared to saline, although a dose of 3.75 mg/kg was ineffective.

Table 4-5: The effect of drug treatment, as indicated, on immobility of rats in the FST, where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (n=5). (MG=methylene green)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	244 \pm 24.57
Imipramine	147 \pm 19.27*
MG 3.75 mg/kg	260 \pm 9.27
MG 7.5 mg/kg	94 \pm 24.87***
MG 15 mg/kg	120 \pm 18.91**
MG 25 mg/kg	65 \pm 15.41***
MG 40 m/kg	117 \pm 33.71**

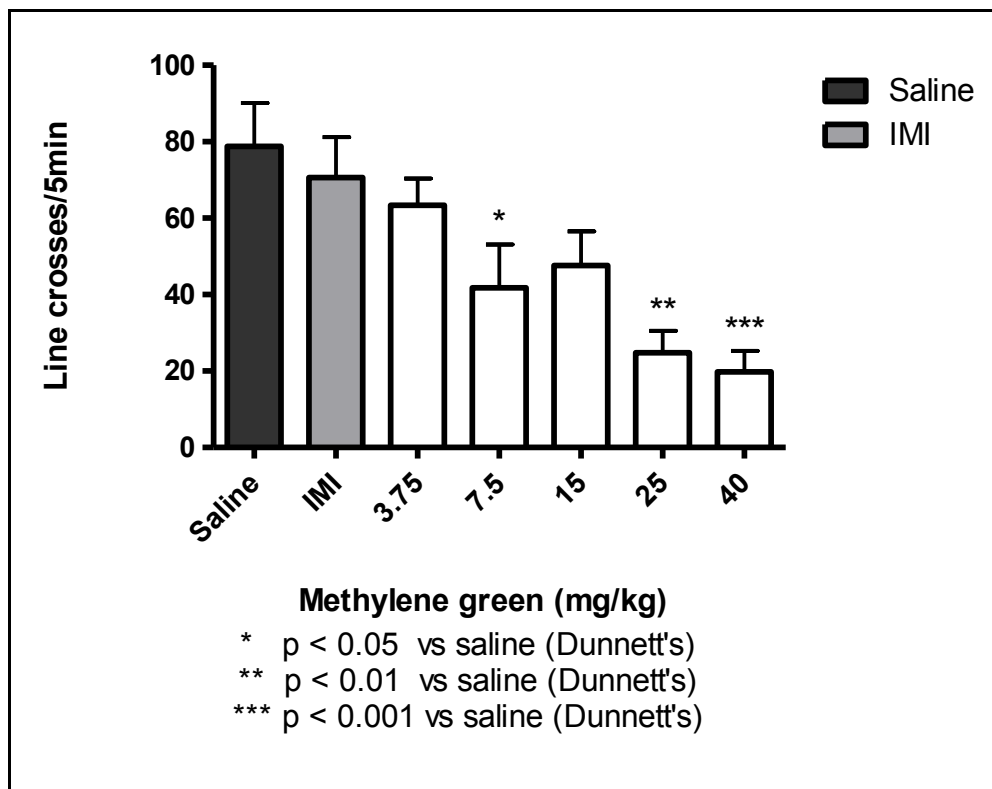


Figure 4-5: The effect of drug treatment, as indicated, on locomotor activity as measured in the open field test, where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs saline-treated animals (n=5).

Variance of the locomotor data revealed a significant effect of treatment [F(6;28)=5.889;p=0.0005]. Post-hoc analysis of these data (Table 4-6) revealed that there was no significant decrease in the locomotor activity in imipramine treated compared to saline treated rats, as depicted in Figure 4-5. Although without effect on locomotor behaviour at a dose of 3.75mg/kg, methylene green decreased locomotor activity significantly compared to saline treated rats at doses of 7.5 mg/kg (from 78.80 ± 11.31 to 41.8 ± 11.35*, *p < 0.05) 25 mg/kg (from 78.80 ± 11.31 to 24.8 ± 5.70**, **p < 0.01) and 40 mg/kg (from 78.80 ± 11.31 to 19.75 ± 5.513***, ***p < 0.001), as shown in Figure 4-5.

Table 4-6: The effect drug treatment, as indicated, on locomotor activity in the open field test, where *p<0.05, **p < 0.01 and ***p < 0.001 (n=?). (MG=methylene green)

Treatment group	Mean ± SEM, line crosses
Saline	78.80 ± 11.31
Imipramine	70.6 ± 10.63
MG 3.75 mg/kg	63.4 ± 6.99
MG 7.5 mg//kg	41.8 ± 11.35*
MG 15 mg/kg	47.6 ± 8.91
MG 25 mg/kg	24.8 ± 5.70**
MG 40 m/kg	19.75 ± 5.513***

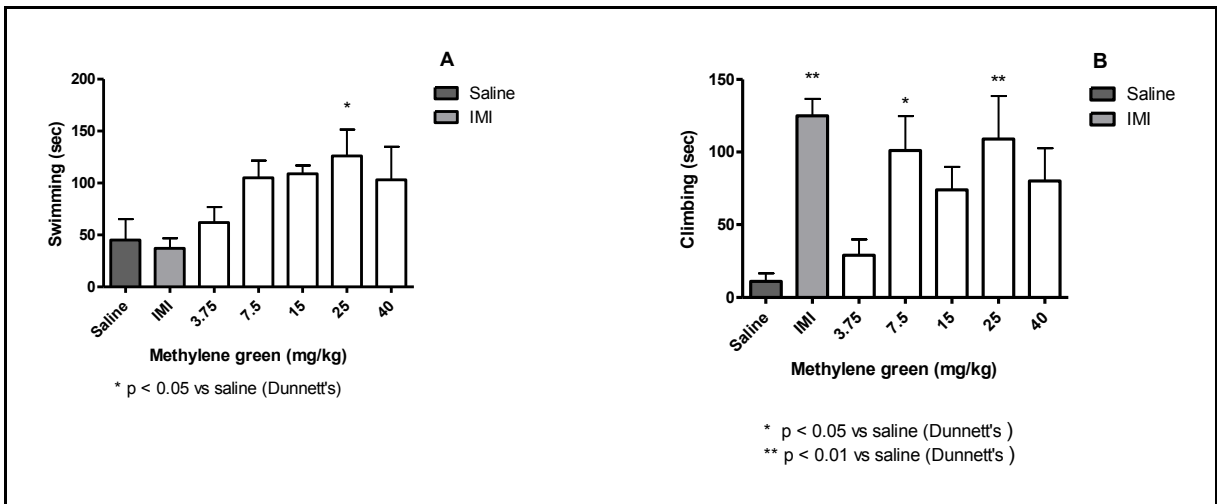


Figure 4-6: Effect of various doses of methylene green, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

When considering the effects of methylene green treatment on swimming behaviour in the FST, we find that one way analysis of variance of the data revealed a significant effect of treatment [F(6;28)=3.128;p=0.0179]. Post-hoc analysis of the data (Table 4-7), however, only revealed a significant increase in the swimming behaviour at a dose of 25 mg/kg (from 45 ± 20.49 to $126 \pm 25.66^*$, *p < 0.05; Table 4-7) with no significant differences in the swimming behaviour after treatment with either imipramine or methylene green at the other doses tested (Figure 4-6, A). Regards climbing behaviour, one way analysis of variance of the data revealed a significant effect of treatment [F(6;28)=4.956;p=0.0074]. Post-hoc analysis of the data (Table 4-8) indicated as described earlier, that imipramine significantly increased climbing behaviour compared to saline treated rats (from 11 ± 5.57 to $125 \pm 11.62^{***}$, ***p < 0.001; Figure 4-6, B). Similarly, methylene green treatment significantly increased climbing behaviour at doses of 7.5 mg/kg (from 11 ± 5.57 to $101 \pm 23.79^*$, *p < 0.05; Table 4-8) and 25 mg/kg (from 11 ± 5.57 to $109 \pm 29.56^*$, *p < 0.05; Table 4-8). An increase was also noted at doses of 15 mg/kg and 40 mg/kg, but did not reach significance (Table 4-8).

Table 4-7: The effect of drug treatment, as indicated, on swimming behaviour of rats in the FST, where * p < 0.05 (n=5). (MG=methylene green)

Treatment group	Mean ± SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	37 ± 9.7
MG 3.75 mg/kg	62 ± 14.97
MG 7.5 mg/kg	105 ± 16.66
MG 15 mg/kg	109 ± 8.12
MG 25 mg/kg	126 ± 25.66*
MG 40 m/kg	103 ± 31.96

Table 4-8: The effect of drug treatment as indicated, on climbing behaviour of rats in the FST, where * p < 0.05 and **p < 0.01 (n=5). (MG=methylene green)

Treatment group	Mean ± SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	125 ± 11.62**
MG 3.75 mg/kg	29 ± 10.89
MG 7.5 mg/kg	101 ± 23.79*
MG 15 mg/kg	74 ± 15.84
MG 25 mg/kg	109 ± 29.56*
MG 40 m/kg	80 ± 22.64

4.1.3 Effect of acute acriflavine treatment in the FST

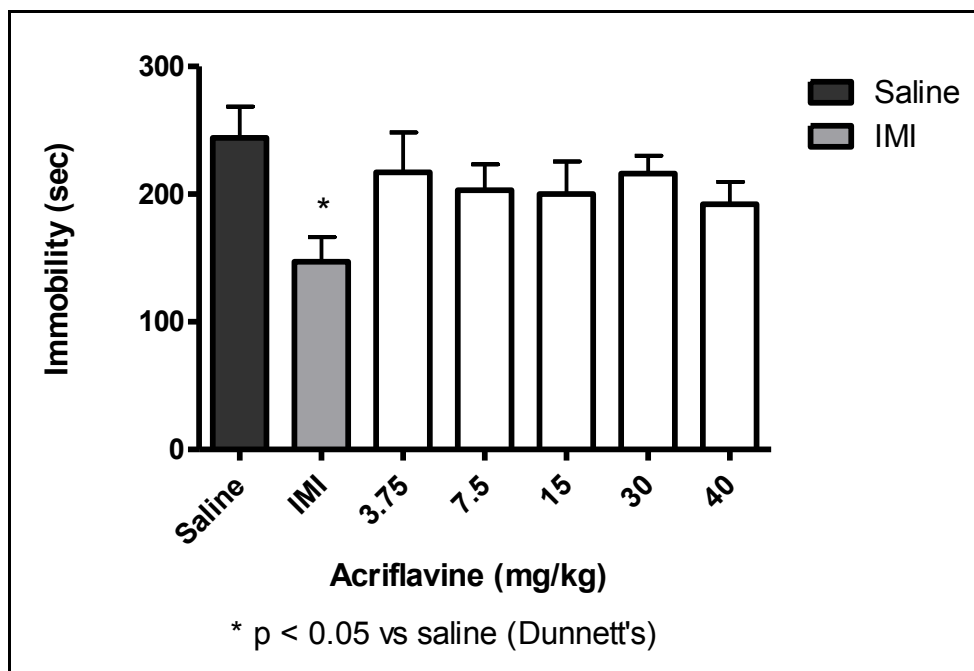


Figure 4-7: Effect of various doses of acriflavine, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the data did not reveal a significant effect of treatment [F(6;28)=1.749;p=0.1465], although post-hoc analysis (Table 4-9) of the data revealed a significant decrease in immobility time of imipramine versus saline treated rats (from 244 ± 24.57 to 147 ± 19.27*, *p < 0.05; Figure 4-7). However, acriflavine was without effect on immobility at any of the doses tested (Figure 4-7 and Table 4-9).

Table 4-9: The effect of drug treatment, as indicated, on immobility of rats in the FST, where * p < 0.05 (n=5).

(ACR=acriflavine)

Treatment group	Mean ± SEM, immobility time (sec)
Saline	244 ± 24.57
Imipramine	147 ± 19.27*
ACR 3.75 mg/kg	217 ± 31.41
ACR 7.5 mg//kg	203 ± 20.35
ACR 15 mg/kg	200 ± 25.64
ACR 30 mg/kg	216 ± 14.09
ACR 40 m/kg	192 ± 17.65

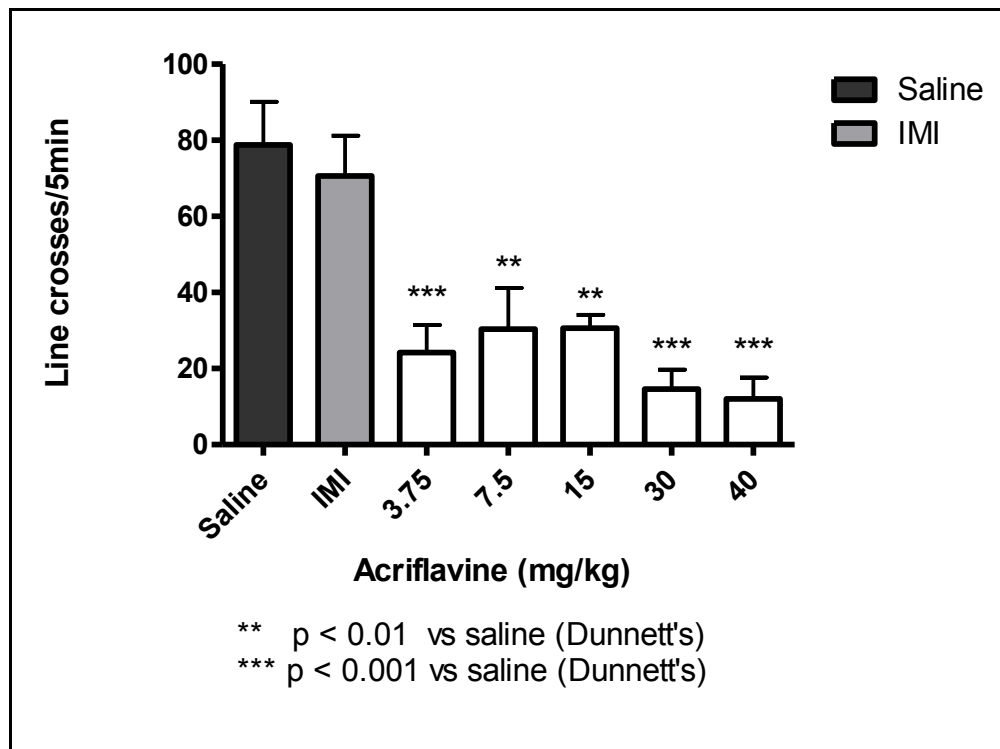


Figure 4-8: The effect of drug treatment, as indicated, on locomotor activity as measured in the open field test, where ** p < 0.01, *** p < 0.001 vs saline-treated animals (n=5).

One way analysis of variance of the locomotor data revealed a significant effect of treatment [F(6;28)=10.31;p=0.0001]. Post-hoc analysis of these data (Table 4-10) revealed that imipramine did not affect locomotor activity compared to saline treated rats (Figure 4-8). However, as depicted in Figure 4-8, acriflavine treatment significantly decreased locomotor activity compared to saline treatment at all doses tested, viz. 3.75 mg/kg (from 78.80 ± 11.31 to 24.2 ± 7.23***, ***p < 0.001), 7.5 mg/kg (from 78.80 ± 11.31 to 30.4 ± 10.84**, **p < 0.01), 15 mg/kg (from 78.80 ± 11.31 to 30.6 ± 3.53**, **p < 0.01), 30 mg/kg (from 78.80 ± 11.31 to 14.6 ± 12***, ***p < 0.001) and 40 mg/kg (from 78.80 ± 11.31 to 12 ± 5.61***, ***p < 0.001).

Table 4-10: The effect of drug treatment, as indicated, on locomotor activity in the open field test, where **p < 0.01 and ***p < 0.001 (n=5). (ACR=acriflavine)

Treatment group	Mean ± SEM, line crosses
Saline	78.80 ± 11.31
Imipramine	70.6 ± 10.63
ACR 3.75 mg/kg	24.2 ± 7.23***
ACR 7.5 mg/kg	30.4 ± 10.84**
ACR 15 mg/kg	30.6 ± 3.53**
ACR 30 mg/kg	14.6 ± 12***
ACR 40 m/kg	12 ± 5.61***

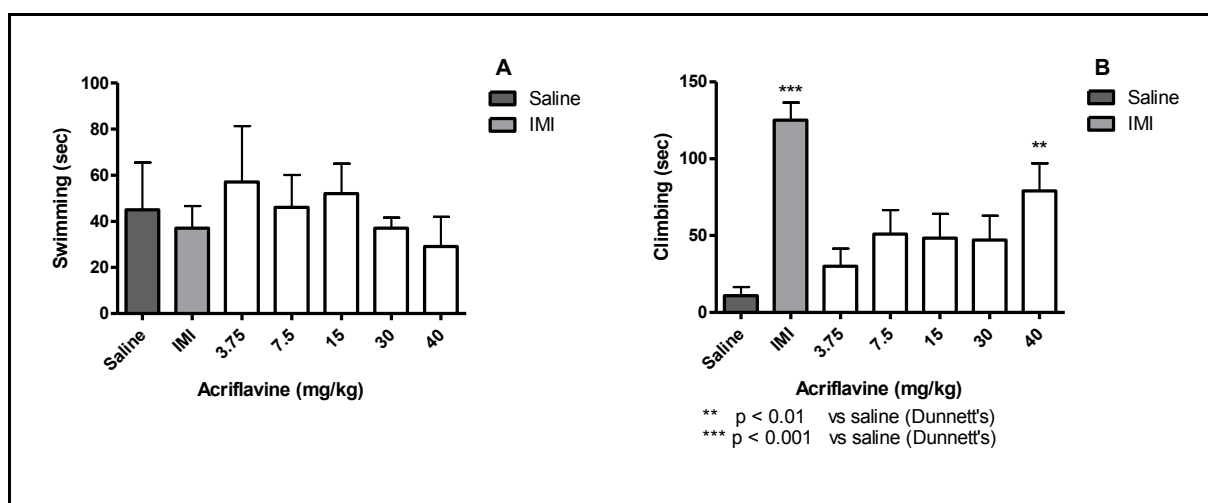


Figure 4-9: Effect of various doses of acriflavine, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the swimming data did not reveal a significant effect of treatment [F(6;28)=0.3916;p=0.8781], with post-hoc analysis of the data (Table 4-11) finding no significant difference in the swimming behaviour of rats after treatment either imipramine or acriflavine at any of the doses tested (Figure 4-9, A). However, one way analysis of variance of the climbing data revealed a significant effect of treatment [F(6;28)=6.954;p=0.0001]. Post-hoc analysis of the latter data indicate that both imipramine (from 11 ± 5.57 to 125 ± 11.62***, ***p < 0.001; Figure 4-9, B) and high dose (40mg/kg) acriflavine (from 11 ± 5.57 to 79 ± 17.92*, *p < 0.05; Figure 4-9, B) significantly increased climbing behaviour compared to saline treated rats.

Table 4-11: The effect of drug treatment, as indicated on swimming behaviour of rats in the FST (n=5). (ACR=acriflavine)

Treatment group	Mean ± SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	37 ± 9.7
ACR 3.75 mg/kg	57 ± 24.27
ACR 7.5 mg//kg	46 ± 14.09
ACR 15 mg/kg	53 ± 13
ACR 30 mg/kg	37 ± 4.64
ACR 40 m/kg	29 ± 12.98

Table 4-12: The effect of drug treatment, as indicated on climbing behaviour of rats in the FST, where * p < 0.05 and ***p < 0.001 (n=5).

Treatment group	Mean ± SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	125 ± 11.62***
ACR 3.75 mg/kg	30 ± 11.51
ACR 7.5 mg//kg	51 ± 15.6
ACR 15 mg/kg	48.4 ± 15.84
ACR 25 mg/kg	47 ± 15.94
ACR 40 m/kg	79 ± 17.92**

4.1.4 Effect of acute tacrine treatment in the FST

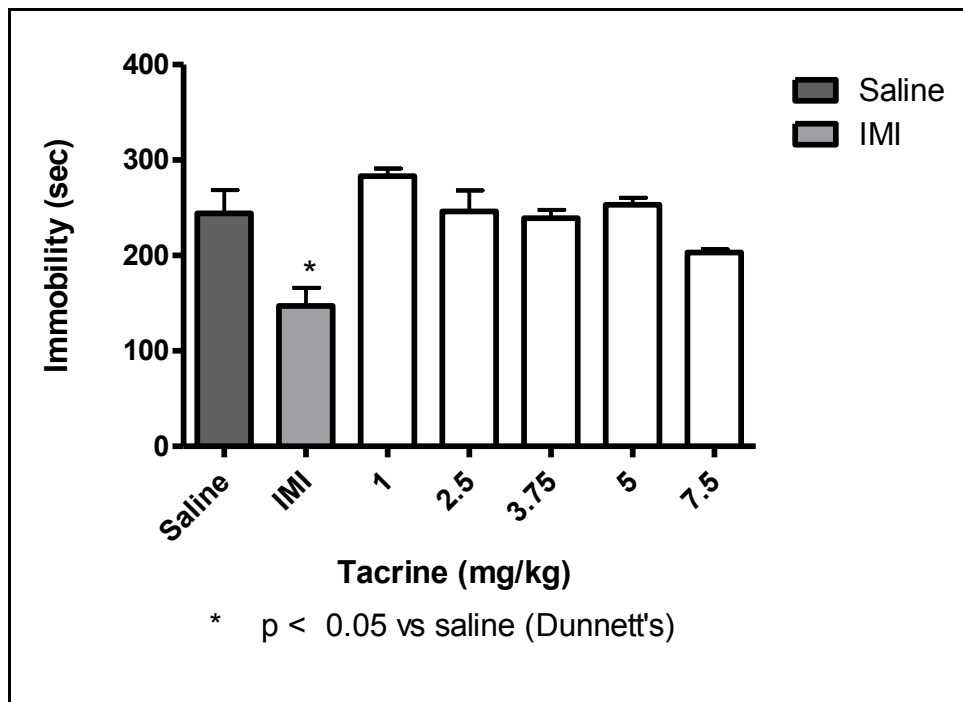


Figure 4-10: Effect of various doses of tacrine, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the immobility data revealed a significant effect of treatment [F(6;28)=7.945;p<0,0001]. Post-hoc analysis of the data (Table 4-13) revealed a significant decrease in immobility in imipramine versus saline treated rats (decreasing immobility from 244 ± 24.57 to 147 ± 19.27*, *p < 0.05; Figure 4-10). However, tacrine had no noticeable effect on immobility score at any dose tested.

Table 4-13: The effect of drug treatment, as indicated, on immobility of rats in the FST, where * p < 0.05 (n=5).

(TAC=tacrine).

Treatment group	Mean ± SEM, immobility time (sec)
Saline	244 ± 24.57
Imipramine	147 ± 19.27*
TAC 1 mg/kg	283 ± 8.12
TAC 2.5 mg//kg	246 ± 22.33
TAC 3.75 mg/kg	239 ± 8.86
TAC 5 mg/kg	253 ± 7.52

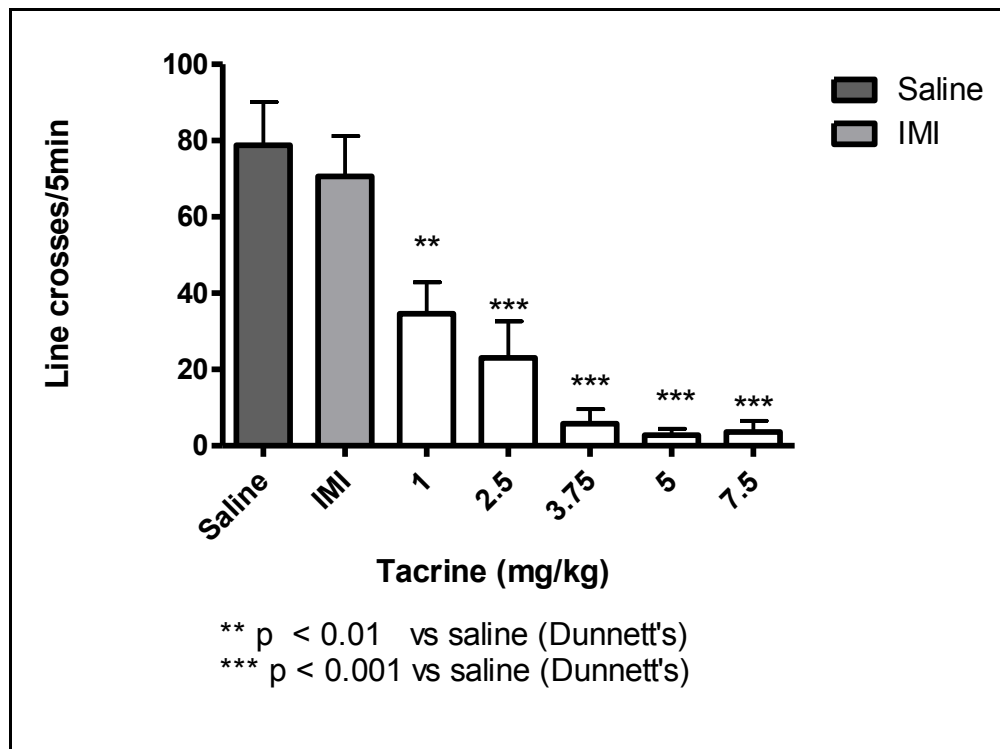


Figure 4-11: The effect of drug treatment, as indicated, on locomotor activity, as measured in the open field test, where** p < 0.01, *** p < 0.001 vs saline-treated animals (n=5).

One way analysis of variance of the locomotor data revealed a significant effect of treatment [F(6;28)=16.64;p<0.0001]. Post-hoc analysis of these data (Table 4-14) revealed that imipramine did not affect locomotor activity in any way relative to saline treated animals. As depicted in Figure 4-11, tacrine treatment dose-dependently decreased locomotor activity significantly compared to saline treatment at doses of 1 mg/kg (from 78.80 ± 11.31 to 34.6 ± 8.29**, **p < 0.01), 2.5 mg/kg (from 78.80 ± 11.31 to 23 ± 9.63***, ***p < 0.001), 3.75 mg/kg (from 78.80 ± 11.31 to 5.8 ± 3.84***, ***p < 0.001), 5 mg/kg (from 78.80 ± 11.31 to 2.8 ± 1.66***, ***p < 0.001) and 7.5 mg/kg (from 78.80 ± 11.31 to 3.6 ± 2.91***, ***p < 0.001), and as showed in Table 4-14.

Table 4-14: The effect of drug treatment, as indicated, on locomotor activity in the open field test, where **p < 0.01 and ***p < 0.001 (n=5). (TAC=tacrine)

Treatment group	Mean \pm SEM, line crosses
Saline	78.80 \pm 11.31
Imipramine	70.6 \pm 10.63
TAC 1 mg/kg	34.6 \pm 8.29**
TAC 2.5 mg/kg	23 \pm 9.63***
TAC 3.75 mg/kg	5.8 \pm 3.84***
TAC 5 mg/kg	2.8 \pm 1.66***
TAC 7 m/kg	3.6 \pm 2.91***

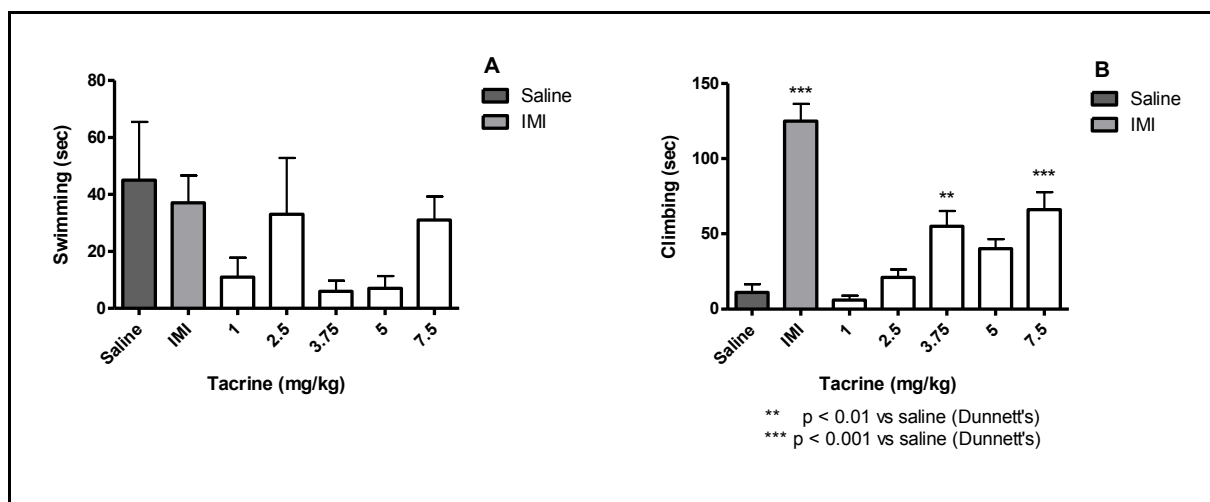


Figure 4-12: Effect of various doses of tacrine, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the swimming data did not reveal a significant effect of treatment [F(6;28)=1.686;p=0.1613]. Likewise post-hoc analysis of these data (Table 4-15) revealed no significant effect on swimming behaviour of rats after treatment with imipramine or tacrine at any dose (Figure 4-12, A). However, various doses of tacrine did indicate a strong trend to reduce swim activity, although these changes did not reach statistical significance versus saline, viz. 1 mg/kg, 3.75 mg/kg and 5 mg/kg. One way analysis of

variance of the climbing data revealed a significant effect of treatment [F(6;28)=24.35;p=0.0001]. Post-hoc analysis of these data (Table 4-16) indicated a significant increase in climbing behaviour in imipramine treated animals compared to saline treated animals (from 11 ± 5.57 to 125 ± 11.62***, ***p < 0.001; Figure 4-12, B). However, tacrine also significantly increased climbing behaviour at doses of 3.75 mg/kg (from 11 ± 5.57 to 55 ± 10.37**, **p < 0.01) and 7.5 mg/kg (from 11 ± 5.57 to 66 ± 11.66***, ***p < 0.001).

Table 4-15: The effect of drug treatment, as indicated, on swimming behaviour of rats in the FST (n=5). (TAC=tacrine)

Treatment group	Mean ± SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	37 ± 9.7
TAC 1 mg/kg	11 ± 6.78
TAC 2.5 mg/kg	33 ± 19.79
TAC 3.75 mg/kg	6 ± 3.67
TAC 5 mg/kg	7 ± 4.36
TAC 7 m/kg	31 ± 8.28

Table 4-16: The effect of drug treatment, as indicated, on climbing behaviour of rats in the FST, where **p < 0.01 and ***p < 0.001 (n=5). (TAC=tacrine)

Treatment group	Mean ± SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	125 ± 11.62***
TAC 1 mg/kg	6 ± 2.92
TAC 2.5 mg/kg	21 ± 5.34
TAC 3.75 mg/kg	55 ± 10.37**
TAC 5 mg/kg	40 ± 6.519
TAC 7 m/kg	66 ± 11.66***

4.1.5 Effect of acute methylene violet treatment in the FST

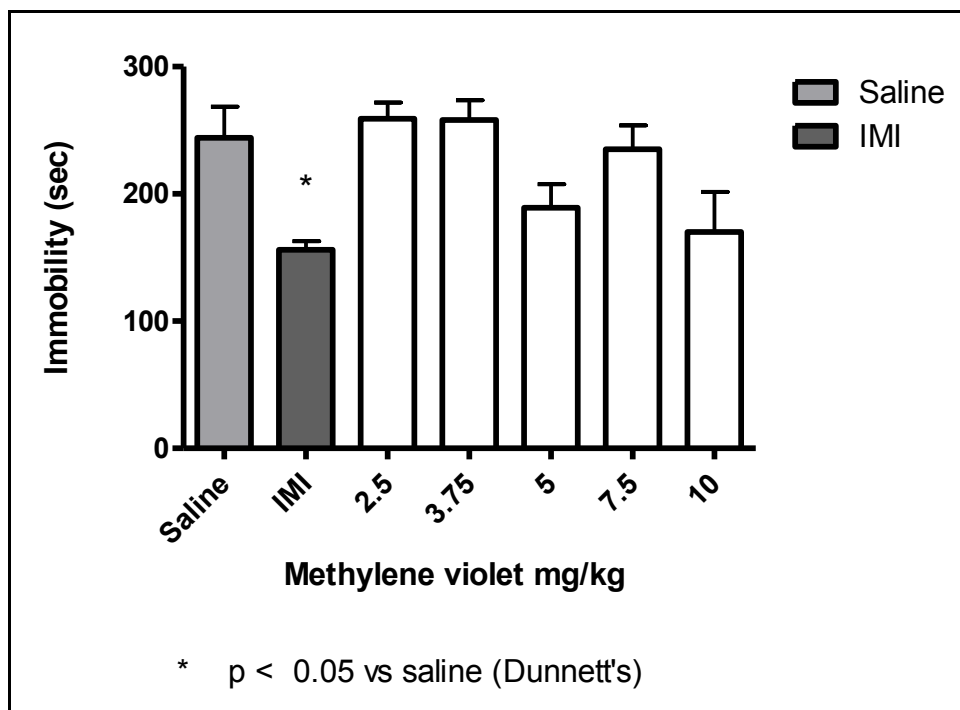


Figure 4-13: Effect of various doses of methylene violet, as indicated, on the duration of immobility in the FST, compared to saline-treated animals ($n = 5/\text{group}$).

One way analysis of variance of the immobility data revealed a significant effect of treatment [$F(6;28)=4.709;p=0.002$]. Post-hoc analysis of these data (Table 4-17) revealed a significant decrease in immobility time for imipramine compared to saline treated rats (from 244 ± 24.57 to $156 \pm 6.96^*$, $*p < 0.05$; Figure 4-13). In the methylene violet treated rats, a small albeit insignificant decrease in immobility time was obtained at doses of 5 mg/kg and 10 mg/kg, with all other doses being ineffective as well.

Table 4-17: The effect of drug treatment, as indicated, on

immobility of rats in the FST, where * $p < 0.05$ (n=5).

(MV=methylene violet)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	244 \pm 24.57
Imipramine	156 \pm 6.96*
MV 2.5 mg/kg	259 \pm 12.79
MV 3.75 mg/kg	258 \pm 15.7
MV 5 mg/kg	189 \pm 88.67
MV 7.5 mg/kg	235 \pm 18.91
MV 10 m/kg	170 \pm 31.74

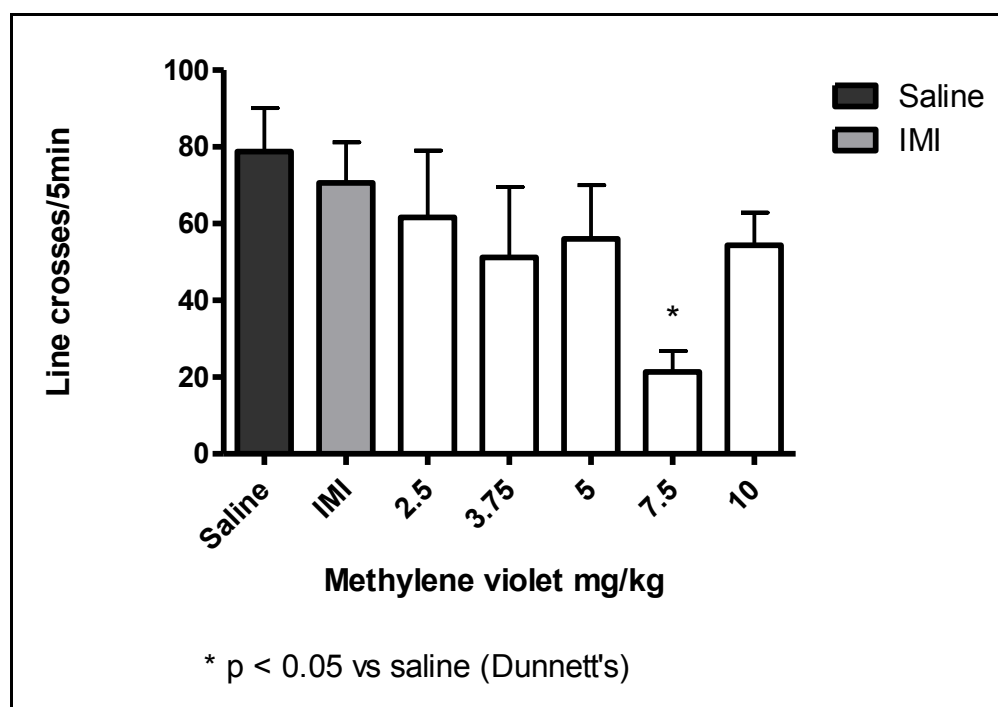


Figure 4-14: The effect of drug treatment, as indicated, on locomotor activity as measured in the open field test (n=5).

One way analysis of variance of the locomotor data did not reveal a significant effect of treatment [$F(6;28)=1.967;p=0.1045$]. However, although post-hoc analysis of the open field data (Table 4-18) revealed that imipramine did not affect locomotor activity in any way

relative to saline treated animals, a significant decrease on locomotor activity versus saline was observed in animals treated with methylene violet at a dose of 7.5 mg/kg (from 78.80 ± 11.31 to $21.4 \pm 5.409^*$, * $p < 0.05$; Figure 4-14).

Table 4-18: The effect of drug treatment, as indicated, on locomotor activity in the open field test, where * $p < 0.05$ vs saline treated animals (n=5).

(MV=methylene violet)

Treatment group	Mean \pm SEM, line crosses
Saline	78.80 ± 11.31
Imipramine	70.6 ± 10.63
MV 2.5 mg/kg	61.6 ± 17.42
MV 3.75 mg//kg	51.2 ± 18.36
MV 5 mg/kg	56 ± 14
MV 7.5 mg/kg	$21.4 \pm 5.409^*$
MV 10 m/kg	54.4 ± 8.442

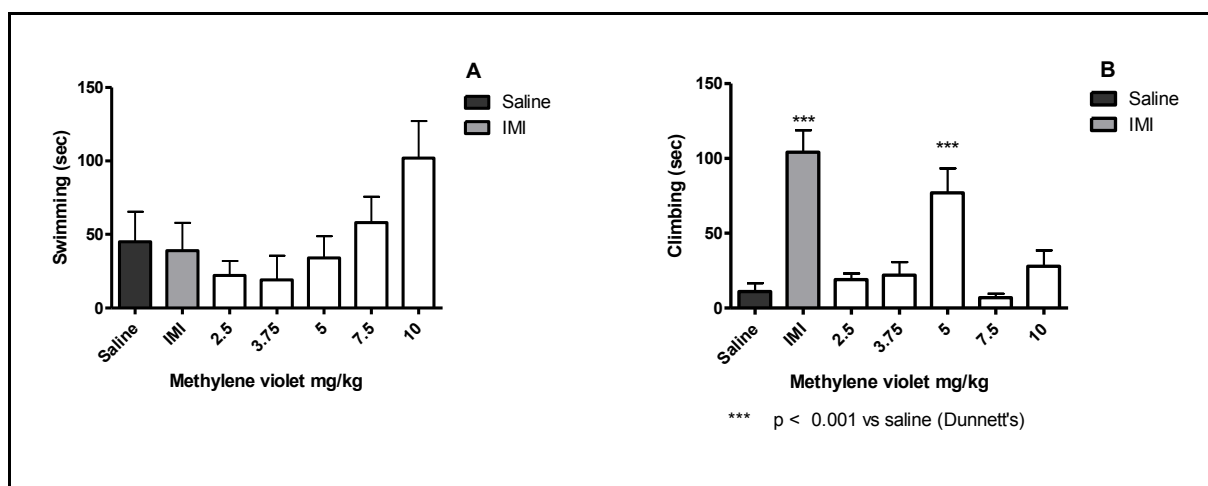


Figure 4-15: Effect of various doses of methylene violet, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the swimming data did not reveal a significant effect of treatment [$F(6;28)=2.405;p=0.0532$], with subsequent post-hoc analysis (Table 4-19) revealing no significant difference in swimming behaviour of rats after treatment with either imipramine or methylene violet as compared to saline (Figure 4-15, A). Nevertheless,

methylene violet did evoke a gradual increase in swimming behaviour across the dosage range (Figure 4-15, A). One way analysis of variance of the climbing data revealed a significant effect of treatment [$F(6;28)=13.30;p=0.0001$]. Subsequent post-hoc analysis (Table 4-20) found that imipramine significantly increased climbing behaviour compared to saline treated rats (from 11 ± 5.57 to $104 \pm 14.78^{***}$, $^{***}p < 0.001$; Figure 4-15, B). Methylene violet, on the other hand, had no effect on climbing except at a dose of 5 mg/kg, where climbing behaviour was significantly increased (from 11 ± 5.57 to $77 \pm 16.32^{***}$, $^{***}p < 0.001$; Figure 4-15,B).

Table 4-19: The effect of drug treatment, as indicated, on swimming behaviour of rats in the FST (n=5). (MV=methylene violet)

Treatment group	Mean \pm SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	39 ± 18.8
MV 2.5 mg/kg	22 ± 10.07
MV 3.75 mg/kg	19 ± 16.61
MV 5 mg/kg	34 ± 14.78
MV 7.5 mg/kg	58 ± 17.72
MV 10 m/kg	102 ± 25.18

Table 4-20: The effect of methylene violet, saline and imipramine on climbing behaviour of rats in the FST, where $^{***}p < 0.001$.

Treatment group	Mean \pm SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	$104 \pm 14.78^{***}$
MV 2.5 mg/kg	19 ± 4
MV 3.75 mg/kg	22 ± 8.75
MV 5 mg/kg	$77 \pm 16.32^{***}$
MV 7.5 mg/kg	7 ± 2.55
MV 10 m/kg	28 ± 10.56

4.1.6 Effect of acute thionin acetate treatment in the FST

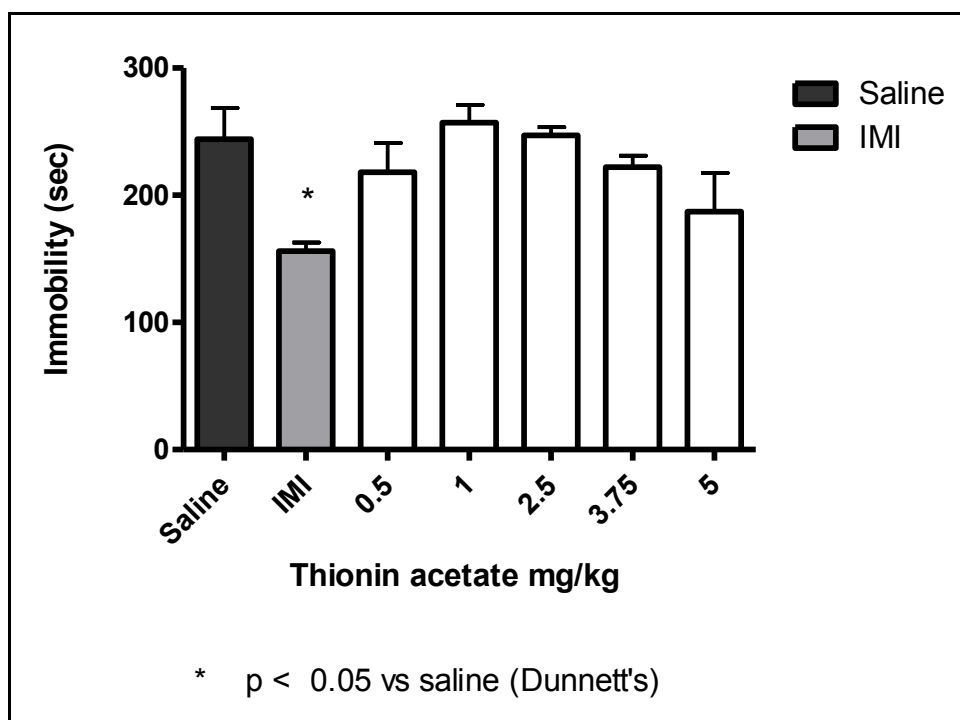


Figure 4-16: Effect of various doses of thionin acetate, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the immobility data revealed a significant effect of treatment [F(6;28)=3.745;p=0.0073]. Subsequent pos-hoc analysis of the data (Table 4-21) revealed as before that imipramine induced a significant decrease in immobility versus saline treated animals (from 244 ± 24.57 to 156 ± 6.96*, *p < 0.05; Figure 4-16). Thionin acetate, however, did not show any significant effect on immobility time at any of the doses tested (Figure 4-16; Table 4-21).

Table 4-21: The effect of drug treatment, as indicated, on immobility of rats in the FST, where * $p < 0.05$ ($n=5$).

(TA=thionin acetate)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	244 \pm 24.57
Imipramine	156 \pm 6.96*
TA 0.5 mg/kg	218 \pm 23.05
TA 1 mg/kg	257 \pm 14.2
TA 2.5 mg/kg	247 \pm 6.63
TA 3.75 mg/kg	222 \pm 9.03
TA 5 mg/kg	187 \pm 30.6

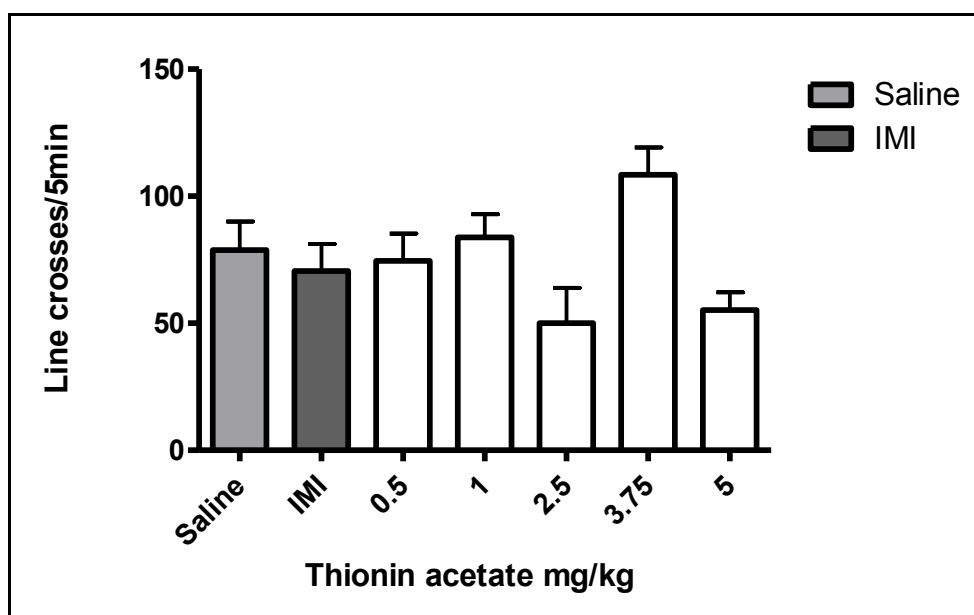


Figure 4-17: The effect of drug treatment, as indicated, on locomotor activity as measured in the open field test, where * $p < 0.05$ vs saline-treated animals ($n=5$).

One way analysis of variance of the locomotor data revealed a significant effect of treatment [$F(6;28)=3.280;p=0.0143$], although post-hoc analysis of the open field data (Table 4-22) revealed that neither drug had any marked effect on locomotor activity, compared to saline treated animals (Figure 4-17; Table 4-22).

Table 4-22: The effect of drug treatment, as indicated, on locomotor activity in the open field test (n=5). (TA=thionin acetate)

Treatment group	Mean \pm SEM, line crosses
Saline	78.80 \pm 11.31
Imipramine	70.6 \pm 10.63
TA 0.5 mg/kg	74.6 \pm 10.65
TA 1 mg/kg	83.8 \pm 9.1
TA 2.5 mg/kg	50 \pm 13.99
TA 3.75 mg/kg	108.4 \pm 10.79
TA 5 mg/kg	55.2 \pm 6.93

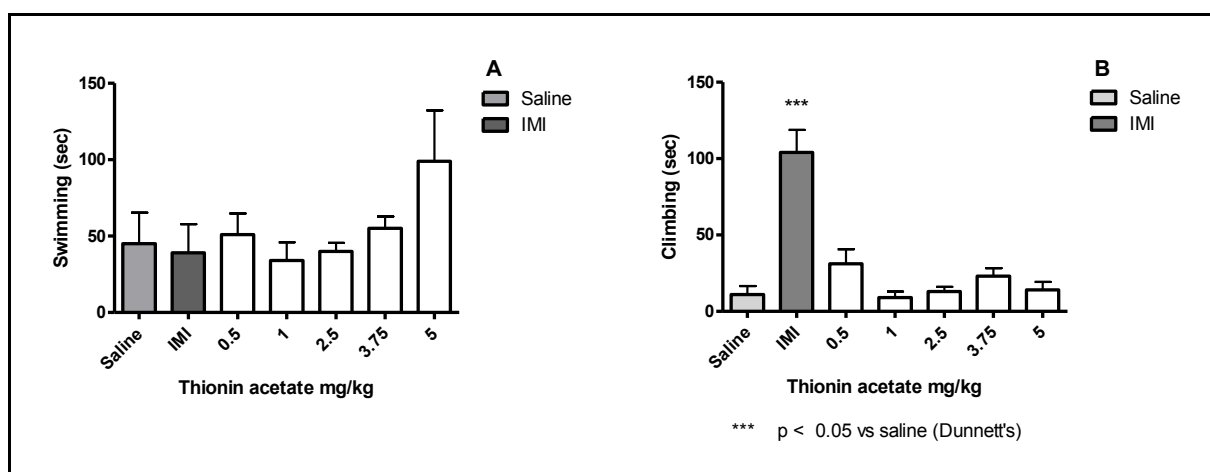


Figure 4-18: Effect of various doses of thionin acetate, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the swimming data did not reveal a significant effect of treatment [F(6;28)=1.473;p=0.2236], with subsequent post-hoc analysis (Table 4-23) revealing that neither imipramine, nor any of the tested doses of thionin acetate, affected swimming activity (Figure 4-18,A; Table 4-23). Thionin acetate marginally increased swimming behaviour at a dose of 5mg/kg, but this was not significant. One way analysis of variance of the climbing data revealed a significant effect of treatment [F(6;28)=18.95;p=0.0001]. Here post-hoc analysis (Table 4-24) found that, as before, imipramine significantly increased climbing behaviour compared to saline treated rats (from 11 \pm 5.57 to 104 \pm 14.78***, ***p < 0.001; Figure 4-18,B), while thionin acetate, on the other hand, did not evoke any noteworthy effects on climbing behaviour (Figure 4-18,B; Table 4-24).

Table 4-23: The effect of drug treatment, as indicated, on swimming behaviour of rats in the FST (n=5). (TA=thionin acetate)

Treatment group	Mean ± SEM, swimming (sec)
Saline	45 ± 20.49
Imipramine	39 ± 18.8
TA 0.5 mg/kg	51 ± 13.82
TA 1 mg/kg	34 ± 11.98
TA 2.5 mg/kg	40 ± 5.7
TA 3.75 mg/kg	55 ± 7.91
TA 5 mg/kg	99 ± 33.14

Table 4-24: The effect of drug treatment, as indicated on climbing behaviour of rats in the FST, where ***p < 0.001 (n=5).

Treatment group	Mean ± SEM, climbing (sec)
Saline	11 ± 5.57
Imipramine	104 ± 14.78***
TA 0.5 mg/kg	31 ± 9.67
TA 1 mg/kg	9 ± 4
TA 2.5 mg/kg	13 ± 3
TA 3.75 mg/kg	23 ± 5.15
TA 5 mg/kg	14 ± 5.34

4.1.7 Effect of acute phenothiazine treatment in the FST

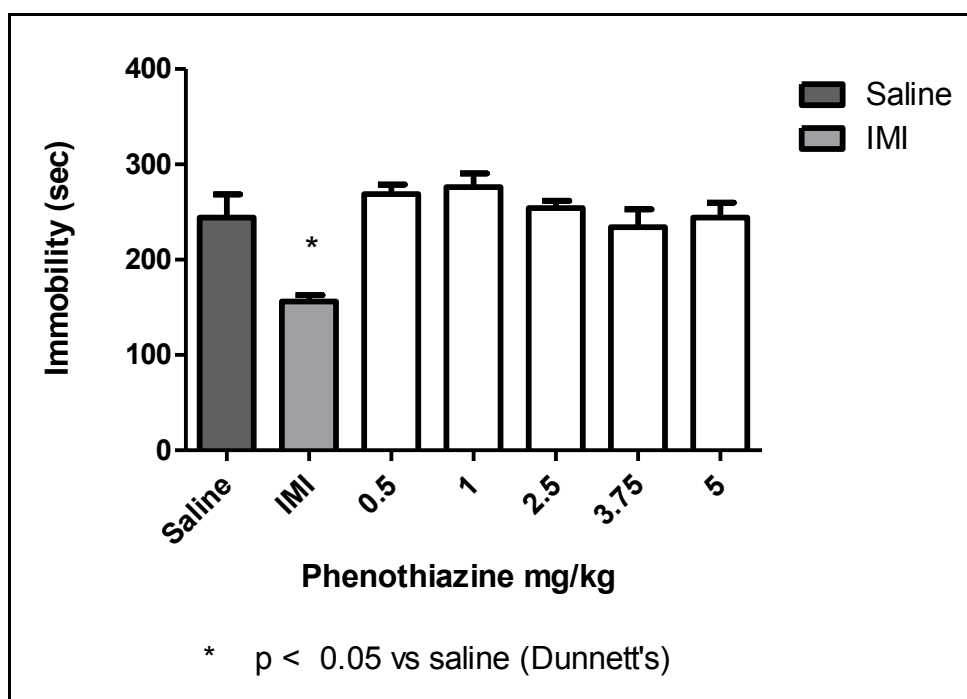


Figure 4-19: Effect of various doses of phenothiazine, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the immobility data revealed a significant effect of treatment [F(6;28)=6.756;p=0.0002]. Post-hoc analysis (Table 4-25) revealed, as before, that imipramine significantly decreased immobility in the FST compared to saline treated rats (from 244 ± 24.57 to $147 \pm 19.27^*$, $*p < 0.05$; Figure 5-19). There was no significant decrease in the immobility time of rats treated with phenothiazine at any dose when compared to saline (Figure 4-19; Table 4-25).

Table 4-25: The effect of drug treatment, as indicated, on immobility of rats in the FST, where $* p < 0.05$, vs saline-treated animals (n=5). (PHE=phenothiazine)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	244 ± 24.57
Imipramine	$156 \pm 6.96^*$
PHE 0.5 mg/kg	268.8 ± 9.98
PHE1 mg//kg	276 ± 14.53
PHE 2.5 mg/kg	254 ± 7.81
PHE 3.75 mg/kg	234 ± 18.93
PHE 5 mg/kg	244 ± 15.76

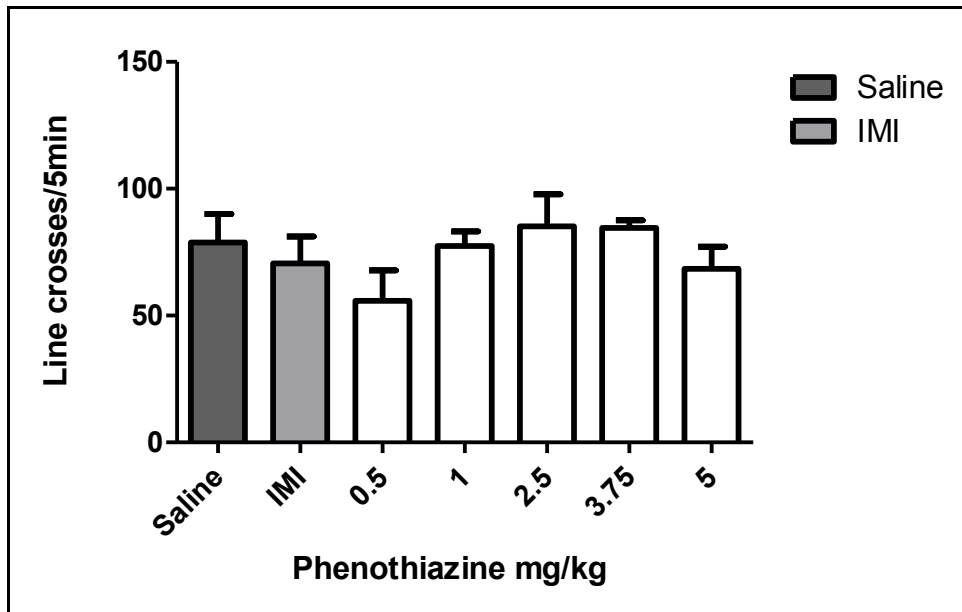


Figure 4-20: The effect of drug treatment, as indicated, on locomotor activity as measured in the open field test (n=5).

One way analysis of variance of the locomotor data did not reveal a significant effect of treatment [$F(6;28)=1.135;p=0.3679$]. Post-hoc analysis of these data (Table 4-26) reveals that neither imipramine, nor any dose tested for phenothiazine, had a marked effect on locomotor performance, compared to saline treated animals (Figure 4-20; Table 4-26).

Table 4-26: The effect of drug treatment, as indicated, on locomotor activity in the open field test (n=5). (PHE=phenothiazine)

Treatment group	Mean ± SEM, line crosses
Saline	78.80 ± 11.31
Imipramine	70.6 ± 10.63
PHE 0.5 mg/kg	74.6 ± 10.65
PHE 1 mg/kg	83.8 ± 9.1
PHE 2.5 mg/kg	50 ± 13.99
PHE 3.75 mg/kg	108.4 ± 10.79
PHE 5 mg/kg	55.2 ± 6.93

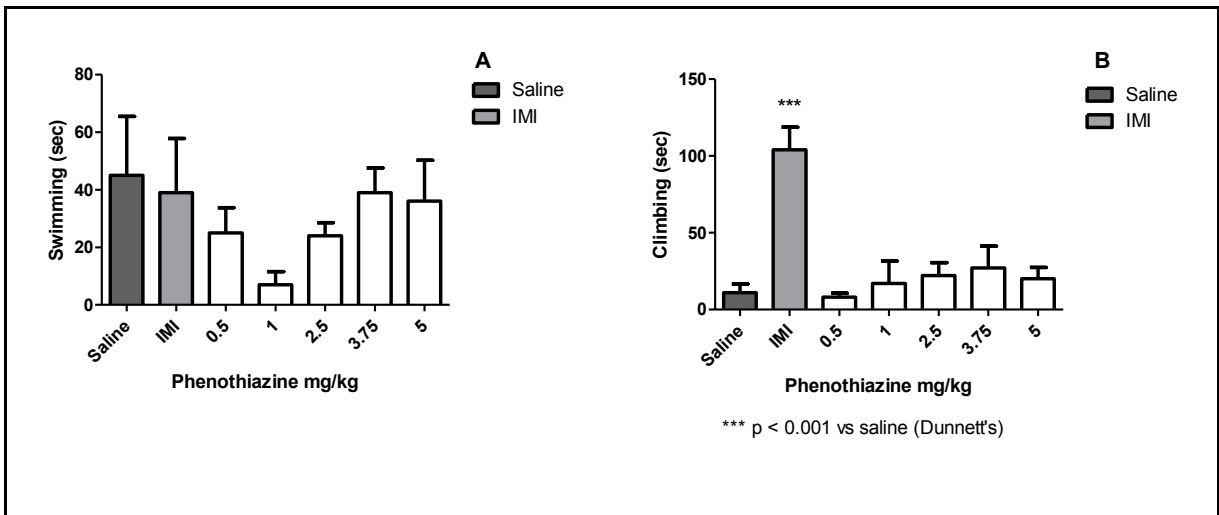


Figure 4-21: Effect of various doses of phenothiazine, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 5/group).

One way analysis of variance of the swimming data did not reveal a significant effect of treatment [$F(6;28)=1.006;p=0.44111$], and concurred by the post-hoc data analysis (Table 4-27) that neither imipramine nor phenothiazine have significant effects on swimming behaviour compared to saline treated rats (Figure 4-21,A). Phenothiazine at a dose of 1 mg/kg tended to decrease swimming behaviour but not significantly so (Figure 4-21, A; Table 4-27). One way analysis of variance of the climbing data revealed a significant effect of treatment [$F(6;28)=9.680;p=0.0001$]. Here post hoc analysis (Table 4-28) found that imipramine significantly increased climbing behaviour compared to saline treated animals (from 11 ± 5.57 to $104 \pm 14.78^{***}$, $^{***}p < 0.001$; Figure 4-21,B), although phenothiazine did not increase climbing behaviour at any of the doses tested (Figure 4-21,B; Table 4-28).

Table 4-27: The effect of drug treatment, as indicated, on swimming behaviour of rats in the FST (n=5). (PHE=phenothiazine)

Treatment group	Mean \pm SEM, swimming (sec)
Saline	45 \pm 20.49
Imipramine	39 \pm 18.8
PHE 0.5 mg/kg	25 \pm 8.8
PHE1 mg//kg	7 \pm 4.64
PHE 2.5 mg/kg	24 \pm 4.58
PHE 3.75 mg/kg	39 \pm 8.57
PHE 5 mg/kg	36 \pm 14.27

Table 4-28: The effect of drug treatment, as indicated on climbing behaviour of rats in the FST (n=5). (PHE=phenothiazine)

Treatment group	Mean \pm SEM, climbing (sec)
Saline	11 \pm 5.57
Imipramine	104 \pm 14.78***
PHE 0.5 mg/kg	8 \pm 2.5
PHE1 mg//kg	17 \pm 14.54
PHE 2.5 mg/kg	22 \pm 8.6
PHE 3.75 mg/kg	27 \pm 14.37
PHE 5 mg/kg	20 \pm 7.42

4.1.8 Summary and conclusions of the acute FST studies

The acute FST confirmed the antidepressant efficacy of imipramine whilst most importantly it also confirmed the efficacy of methylene blue, in agreement with an earlier acute study conducted by Eroglu & Caglayan (1997). The decrease in locomotor activity observed in methylene blue does not refute the decrease in immobility observed in the FST which is also in agreement with the aforementioned study. Of all the analogues that have been tested, methylene green was the only analogue to display significant antidepressant-like activity. The decrease in immobility time by methylene green was not altered by the decrease in locomotor activity at the same dose and thus reflects true antidepressant-like activity. Acriflavine and tacrine display no significant decrease in immobility time, but a significant decrease in locomotor activity. Methylene violet did not decrease immobility time significantly and only slightly decreased locomotor activity. Thionin acetate as well as phenothiazine did not alter immobility time significantly and did not have any significant effects on the locomotor activity. It can thus be concluded that only methylene blue and methylene green were successful in demonstrating an antidepressant-like effect in the acute FST study, and hence these two analogues will be carried forward to the chronic treatment FST studies (Section 4.2). Importantly, behavioural sampling in the FST indicated that imipramine, but also

methylene blue and methylene green, more specifically augment noradrenergic (climbing) behaviours, possibly explaining their antidepressant-like activity in the acute FST.

4.2 Chronic treatment study

In the clinic, antidepressants are not active over acute periods. In order to prevent the possibility of a false positive response and thus to confirm a likelihood that the analogues that were tested positively in the acute FST could indeed be antidepressant in humans if so tested, the aim of this study was to determine if methylene blue, as well as any of the successfully tested analogues in the acute studies, also demonstrate antidepressant-like activity in a chronic treatment study. Again, imipramine was used as a positive control. In all cases, drugs were administered once daily for 7 days at the doses shown to be effective in the acute FST. From the acute FST data described above, the following drugs were selected for the chronic FST study: Saline, imipramine (positive control), methylene blue and methylene green. Based on the data presented in the acute FST study for methylene blue and on earlier studies (Eroglu and Caglayan, 1997), 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 methylene green, namely 15 mg/kg (Sewell and Hawking, 1950). 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 15mg/kg for both methylene blue and methylene green are equivalent with imipramine so that any improved response in the FST over imipramine after sub-chronic treatment would not be due to bias introduced by a higher dose. Data were analysed by means of one-way analysis of variance (ANOVA) across all groups, and were subsequently subjected to the Newman-Keuls multiple comparison test. Data were expressed as the mean \pm SEM with statistical significance defined at the 95% ($p < 0.05$) level. Descriptive statistics are provided in the accompanying tables.

4.2.1 The effect of chronic treatment with methylene blue and methylene green in the FST

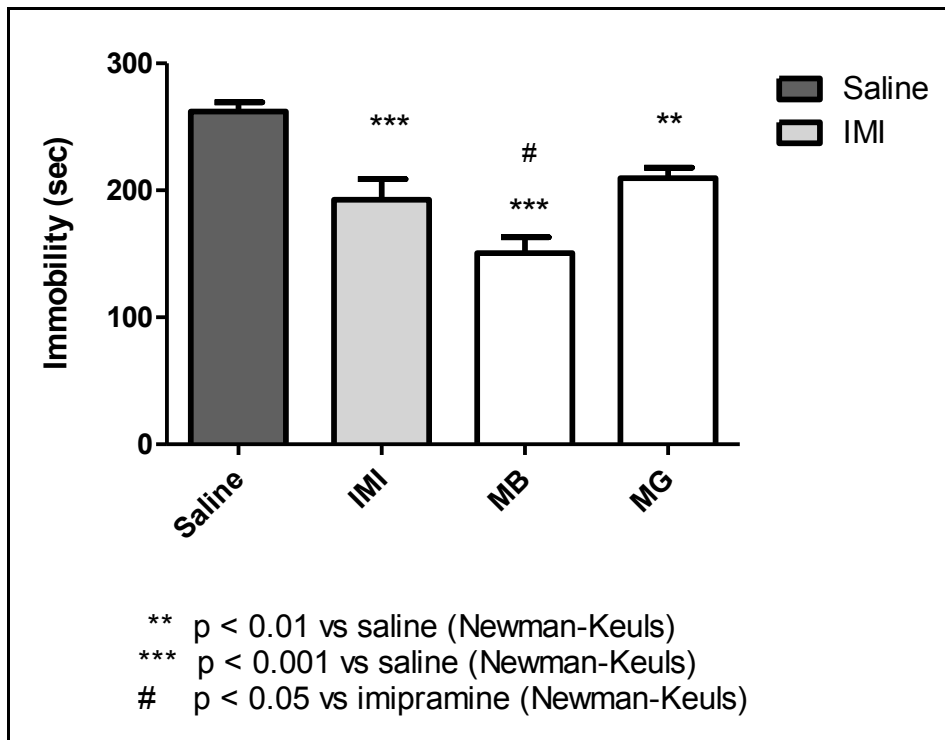


Figure 4-22: Effect of chronic drug treatment, as indicated, on the duration of immobility in the FST, compared to saline-treated animals (n = 10/group).

One way analysis of variance of the immobility data revealed a significant effect of treatment [F(3;36)=15.42;p=0.0001]. Post-hoc analysis of the data (Table 4-29) revealed a significant decrease in immobility in imipramine versus saline treated rats (decreasing immobility from 262 ± 7.348 to $192.5 \pm 16.45^{***}$, $^{***}p < 0.001$; Figure 4-22). Methylene blue and methylene green also significantly decreased immobility time versus saline treated rats (decreasing immobility from 262 ± 7.348 to $150.5 \pm 12.62^{***}$, $^{***}p < 0.001$; and 262 ± 7.348 to $209.5 \pm 8.252^{**}$, $^{**}p < 0.01$ respectively; Figure 4-22). Furthermore, methylene blue revealed a greater decrease in immobility time when compared to imipramine treated rats (decreasing immobility from 192.5 ± 16.45 to $150.5 \pm 12.62^{\#}$, $^{\#}p < 0.05$; Figure 4-22).

Table 4-29: The effect of chronic drug treatment, as indicated, on the immobility of rats in the FST, where $p < 0.05$, $**p < 0.01$ and $***p < 0.01$ vs saline-treated animals and $^{\#} p < 0.05$ vs imipramine treated animals (n=10). (MB=methylene blue; MG=methylene green)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	262 \pm 7.348 sec
Imipramine	192.5 \pm 16.45 ^{***} sec
MB	150.5 \pm 12.62 ^{***, #} sec
MG	209.5 \pm 8.252 ^{**} sec

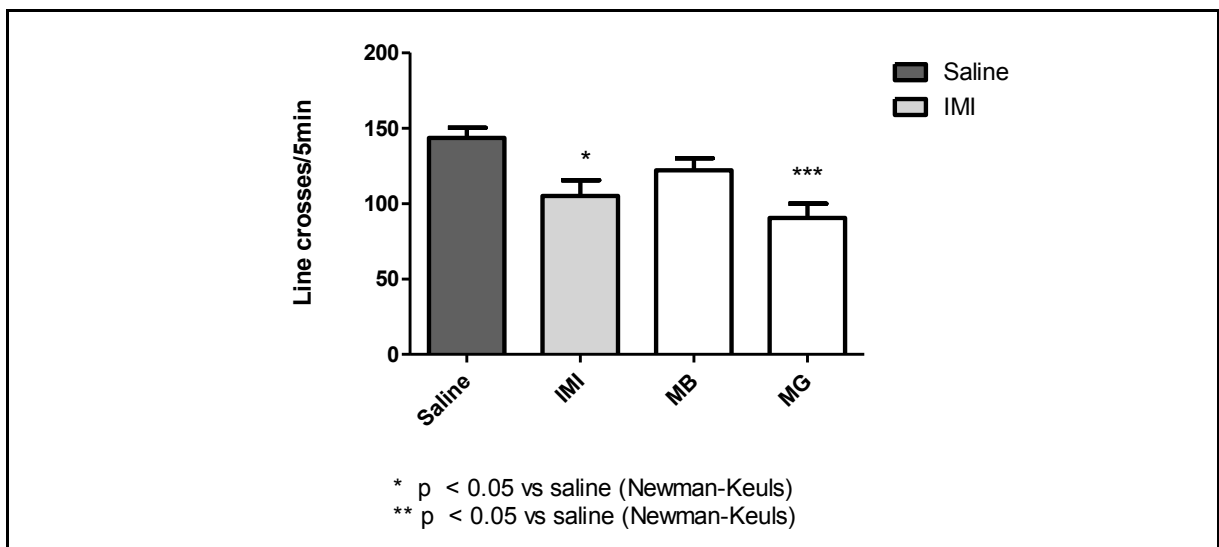


Figure 4-23: The effect of chronic drug treatment, as indicated, on locomotor activity as measured in the open field test, where $* p < 0.05$, $*** p < 0.001$ vs saline-treated animals (n=10).

One way analysis of variance of the locomotor data revealed a significant effect of treatment [F(3;36)=6.808;p=0.0009]. Post-hoc analysis of these data (Table 4-30) revealed there was a significant decrease in locomotor activity in the imipramine versus saline treated groups (from 143.5 \pm 6.756 to 105 \pm 10.53^{*}, $*p < 0.05$; Figure 4-23). Methylene blue treated rats revealed a slight but insignificant decrease in locomotor activity versus saline, whereas

methylene green significantly decreased locomotor activity when compared to saline treated rats (from 143.5 ± 6.756 to $90.5 \pm 9.351^{***}$, $^{***}p < 0.001$; Figure 4-23).

Table 4-30: The effect of chronic drug treatment, as indicated, on locomotor activity in the open field test, where $p < 0.05^*$, $^{***}p < 0.01$ vs saline-treated animals (n=10). (MB=methylene blue; MG=methylene green)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	143.5 ± 6.756 sec
Imipramine	$105 \pm 10.53^*$ sec
MB	122.1 ± 7.875 sec
MG	$90.5 \pm 9.351^{***}$ sec

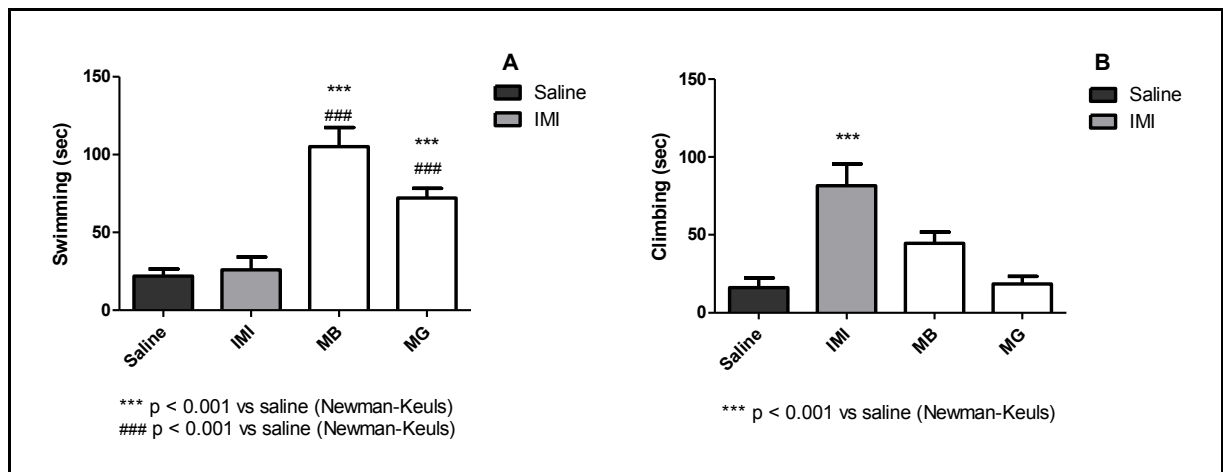


Figure 4-24: Effect of chronic drug treatment, as indicated, on (A) swimming behaviour and (B) climbing behaviour in the FST, compared to saline-treated animals (n = 10/group).

When considering the effect of these treatments on swimming and climbing behaviour in the FST, one way analysis of variance of the swimming data revealed a significant effect of treatment [$F(3;36)=21.95;p=0.0001$]. Post-hoc analysis of these data (Table 4-31) found no differences in swimming behaviour between saline and imipramine treated rats, although methylene blue significantly increased swimming behaviour compared to saline treated rats (from 22 ± 4.55 to $105 \pm 12.47^{***}$, $^{***}p < 0.001$; Figure 4-23, A) as well as versus imipramine treated rats (from 26 ± 8.29 to $105 \pm 12.47^{###}$, $^{###}p < 0.001$; Figure 4-24, B). Similarly, MG

significantly increased swimming behaviour compared to saline treated rats (from 22 ± 4.55 to $72 \pm 6.42^{***}$, $^{***}p < 0.001$; Figure 4-24, A) as well as versus imipramine treated rats (from 26 ± 8.29 to $72 \pm 6.42^{###}$, $^{###}p < 0.001$; Figure 4-24, B). 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 (Table 4-32) found that imipramine significantly increased climbing behaviour compared to saline treated rats (from 16 ± 6.49 to $81.5 \pm 14^{***}$, $^{***}p < 0.001$; Figure 4-24, B), although both methylene blue and methylene green were without effect.

Table 4-31: The effect of chronic drug treatment, as indicated, on swimming behaviour in the FST, where $^{***}p < 0.001$ vs saline-treated animals and $^{###}p < 0.01$ vs imipramine treated animals (n=10).

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	22 ± 4.55 sec
Imipramine	26 ± 8.29 sec
MB	$105 \pm 12.47^{***, ###}$ sec
MG	$72 \pm 6.42^{***, ###}$ sec

Table 4-32: The effect of chronic drug treatment, as indicated, on the climbing behaviour in the FST, where $^{***}p < 0.001$ vs saline-treated animals (n=10). (MB=methylene blue; MG=methylene green)

Treatment group	Mean \pm SEM, immobility time (sec)
Saline	16 ± 6.49 sec
Imipramine	$81.5 \pm 14^{***}$ sec
MB	44.5 ± 7.28 sec
MG	18.5 ± 4.78 sec

4.2.2 Summary and conclusions of the chronic FST studies

The chronic treatment FST study confirmed the antidepressant activity of imipramine. The locomotor activity in imipramine-treated rats was slightly decreased, but does not refute the reduction in immobility time observed with this drug. Methylene blue significantly reduced immobility time as compared to saline-treated rats, and proved to be even more effective than imipramine, while at the same time not affecting locomotor activity, thus providing robust evidence that methylene blue possesses “true” antidepressant-like activity. Methylene green similarly significantly decreased immobility, with a slight but insignificant effect on locomotor performance, thus confirming its antidepressant-like effects. Of special note is that while acute treatment with imipramine, methylene blue and methylene green selectively bolstered noradrenergic-mediated behaviours in the FST (climbing), sub-chronic treatment with methylene blue and methylene green now selectively increased serotonergic-mediated

behaviours (swimming) with minimal noradrenergic responses observed. Interestingly, imipramine behaved similarly in both acute and sub-chronic treatment regimes, selectively increasing noradrenergic behaviours. These behavioural data emphasize that the antidepressant efficacy of methylene blue and methylene green may be related to possible actions on MAO (Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007), although these antidepressant-like effects may be achieved via actions on the NOS system as well (Eroglu and Caglayan, 1997, Volke *et al.*, 1999). The subsequent sections will closely address whether either or both these two mechanisms may explain the antidepressant activity of MB and MG.

4.3 Recombinant human MAO-A and MAO-B inhibition studies

Since earlier studies have found that methylene blue presents with non-specific inhibitory actions on MAO-A and B (Ehringer *et al.*, 1961; Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007), and which would also contribute towards an antidepressant-like profile, all seven test compounds including methylene blue, were evaluated for their potential to inhibit recombinant human MAO-A. Imipramine, a known antidepressant will also be used in this study. However, only those compounds that expressed antidepressant-like activity in the FST will be tested for their inhibitory potential on recombinant human MAO-B in order to determine their selectivity. Since MAO-A is the principle MAO involved in mood regulation and in antidepressant action (see Section 2.1.4.1), we have not studied MAO-B inhibitory activity if not proven active as an MAO-A inhibitor. The IC_{50} values (concentration of the inhibitor that produces 50% inhibition) for the inhibition of MAO by each test compound were determined, and presented below.

4.3.1 Methylene blue

The first compound to be tested for MAO inhibitory activity was methylene blue (Figure 4-25), which is known to have effects on MAO (Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007). Its chemical structure, in its oxidised state, is depicted below:

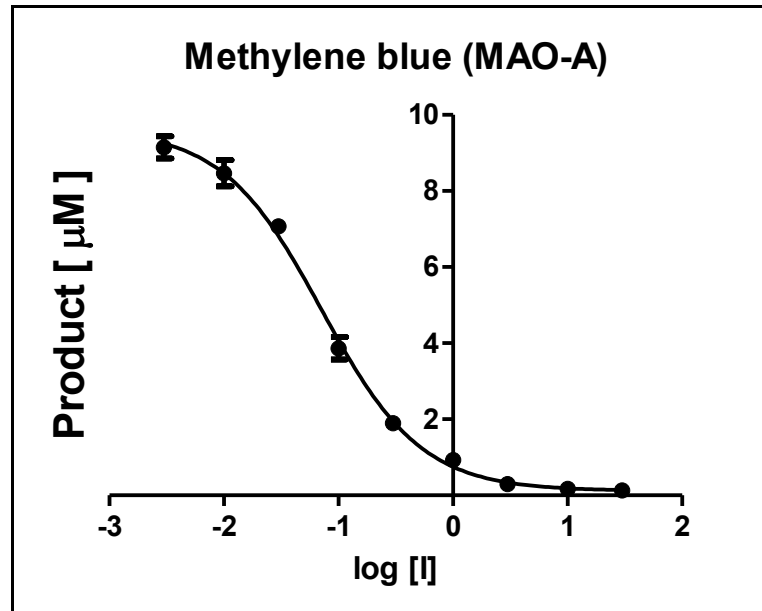
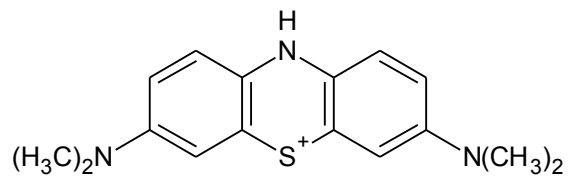


Figure 4-25: The dose response curve for the inhibition of MAO-A by methylene blue.

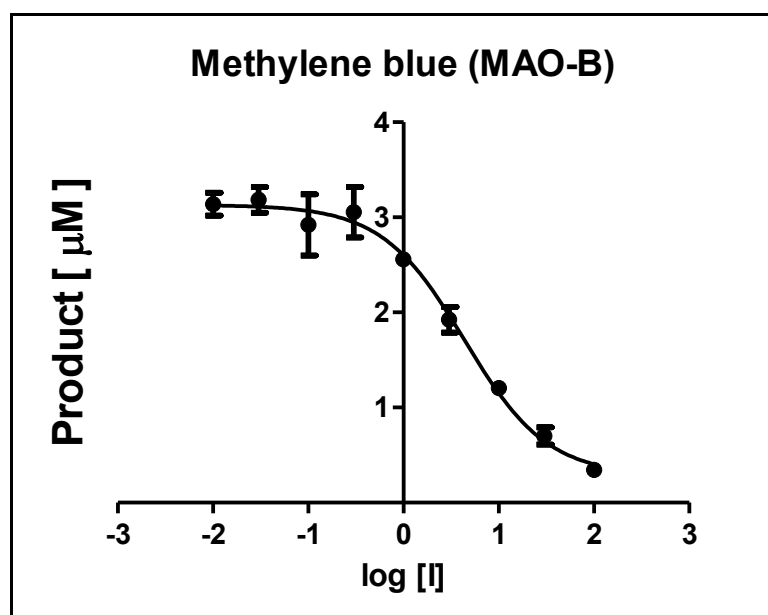
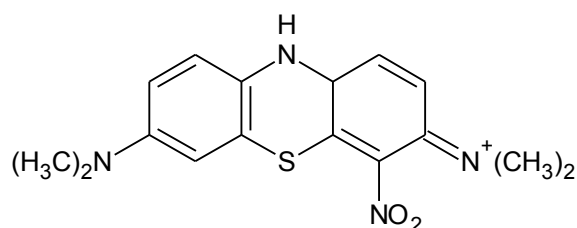


Figure 4-26: The dose response curve for the inhibition of MAO-B by methylene blue.

The dose response curve depicted in Figure 4-26 indicates that methylene blue is a highly potent inhibitor of MAO-A, with an IC_{50} value of $0.07 \pm 0.17 \mu\text{M}$. Further, data from Figure 4-26 suggest that methylene blue is also a moderate inhibitor of MAO-B, with an IC_{50} value of $4.37 \pm 0.14 \mu\text{M}$.

4.3.2 Methylene green

The neurobiological actions of methylene green have never before been studied. Methylene green has the chemical structure as depicted below:



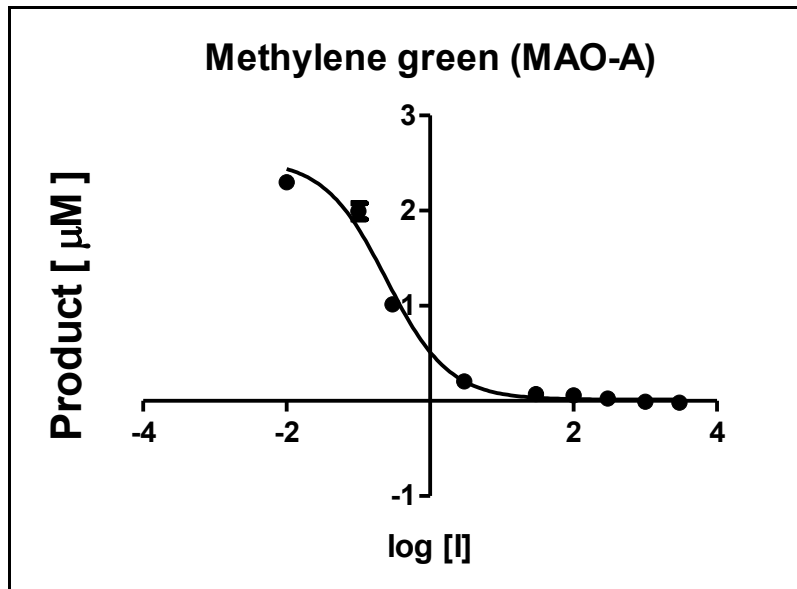


Figure 4-27: The dose response curve for the inhibition of MAO-A by methylene green.

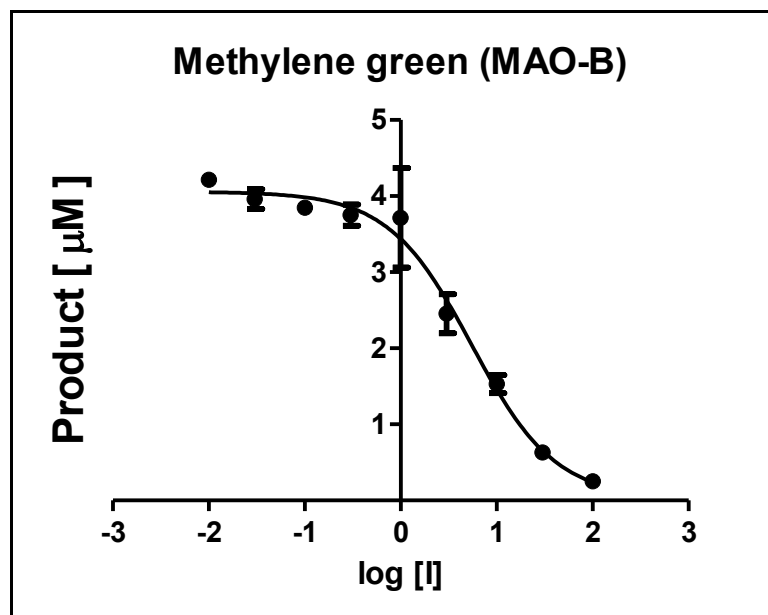


Figure 4-28: The dose response curve for the inhibition of MAO-B by methylene green.

The dose response curve depicted in Figure 4-27 indicates that methylene green is a very potent inhibitor of MAO-A with an IC_{50} value of $0.25 \pm 0.11 \mu\text{M}$. Moreover, it is a moderate inhibitor of MAO-B with an IC_{50} value of $5.5 \pm 0.23 \mu\text{M}$ (Figure 4-28).

4.3.3 Imipramine

Like the test compounds under scrutiny in this study, imipramine is also a tricyclic structure as depicted below. However, it is a recognised tricyclic antidepressant (TCA) compound that acts by inhibiting synaptic monoamine reuptake and is reported to have minimal effects on MAO (Egashira *et al.*, 1999).

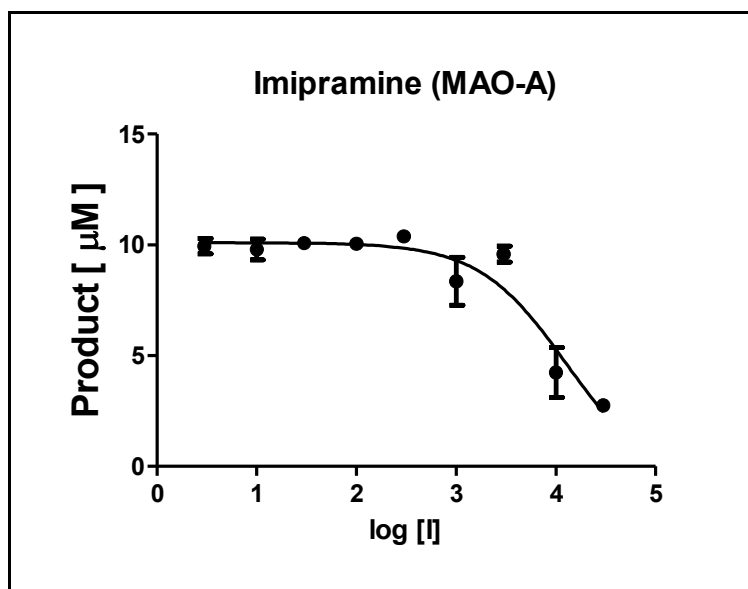
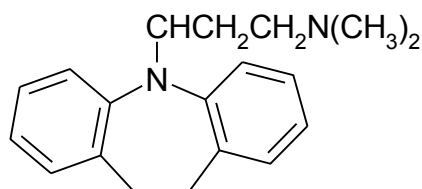


Figure 4-29: The dose-response curve for the inhibition of MAO-A by imipramine.

According to the dose response curve performed in Figure 4-29, imipramine is a very weak inhibitor of MAO-A, with an IC_{50} value of $12500 \pm 0.11 \mu\text{M}$. Considering its inactivity as a MAO-A inhibitor, we have not studied MAO-B inhibitory activity (see note above).

4.3.4 Phenthiazine

Like the test compounds under scrutiny in this study, including imipramine, phenthiazine is also a tricyclic structure as depicted below:

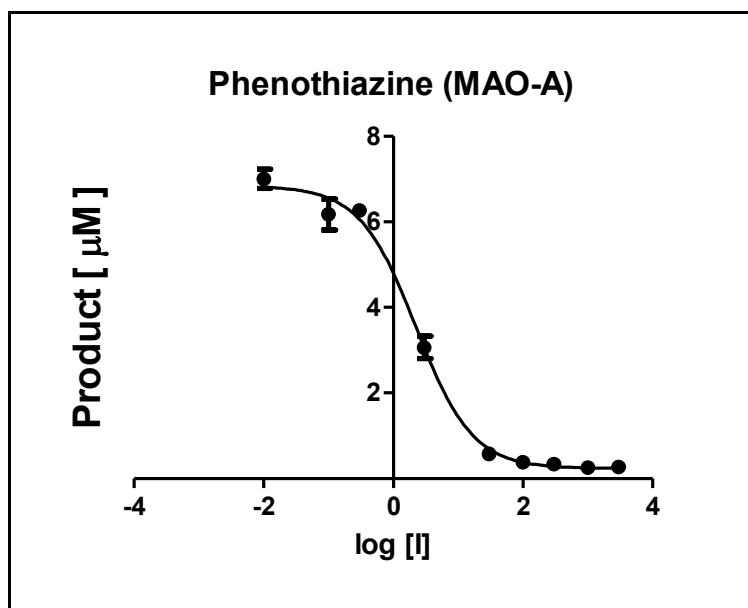
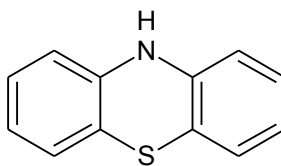
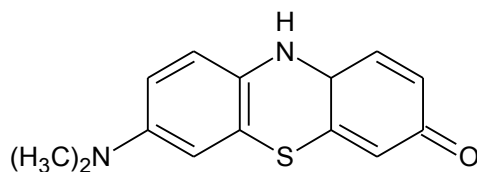


Figure 4-30: The dose-response curve for the inhibition of MAO-A by phenthiazine.

The dose-response curve described in Figure 4-30 indicates that phenthiazine is a moderate inhibitor of MAO-A, with an IC_{50} value of $2.25 \pm 0.15 \mu\text{M}$.

4.3.5 Methylene violet

The neurobiological action of methylene violet has never before been studied, and has the chemical structure as depicted below:



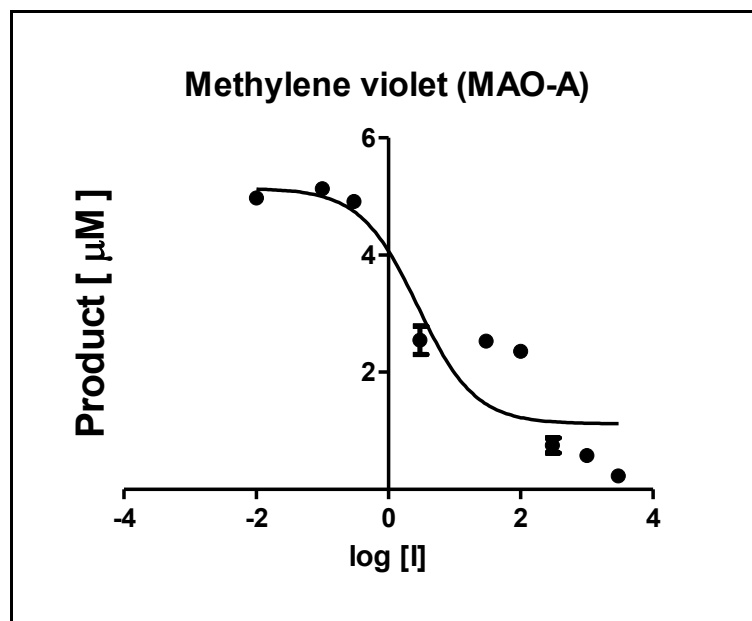
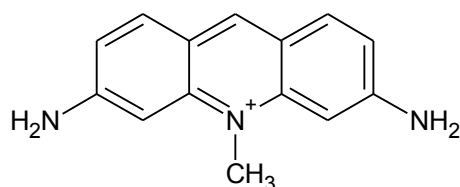


Figure 4- 31: The dose-response curve for the inhibition of MAO-A by methylene violet.

As depicted in Figure 4-31, the dose-response curve found methylene violet to be a moderate inhibitor of MAO-A, with an IC_{50} value of $2.76 \pm 0.42 \mu\text{M}$. Its action on MAO-B was not studied, for reasons noted earlier.

4.3.6 Acriflavine

The neurobiological action of acriflavine has never before been studied. Acriflavine has the chemical structure as depicted below:



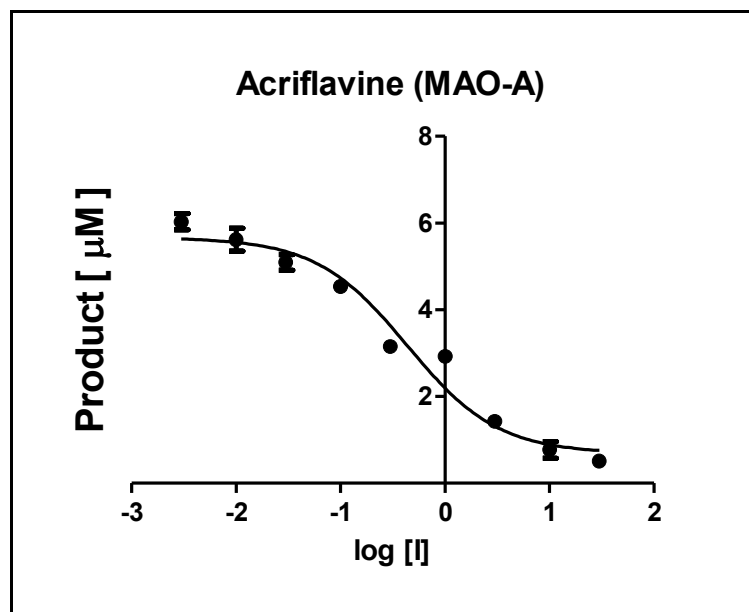
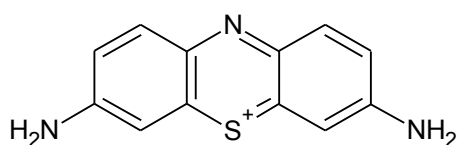


Figure 4-32: The dose-response curve for the inhibition of MAO-A by acriflavine.

As outlined in the dose-response curve depicted in Figure 4-32, we found acriflavine to be a very potent inhibitor of MAO-A, with an IC_{50} value of $0.43 \pm 0.25 \mu\text{M}$. However, due to its lack of effect in the acute FST, we did not perform an analysis of MAO-B activity.

4.3.7 Thionin acetate

The neurobiological action of thionin acetate has never before been studied. Thionin has the chemical structure as depicted below:



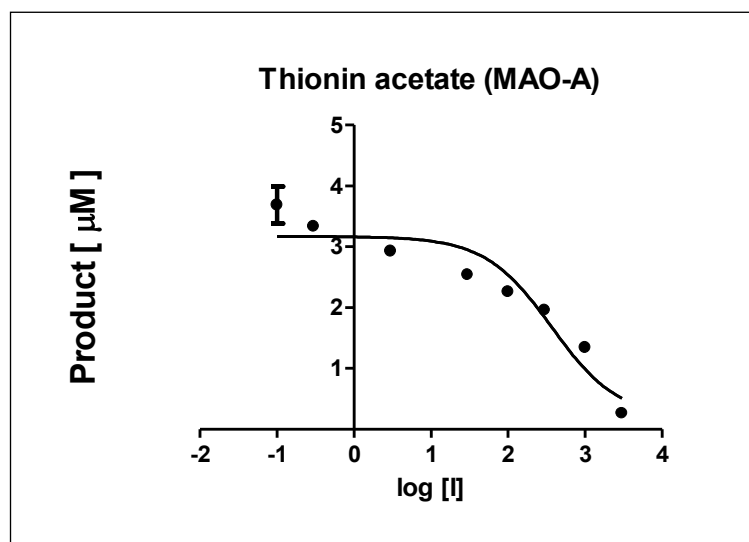
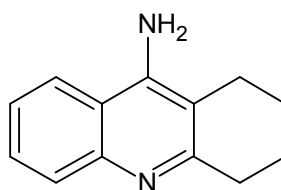


Figure 4- 33: The dose-response curve for the inhibition of MAO-A by thionin acetate.

As highlighted in Figure 4-33, thionin acetate is a very weak inhibitor of MAO-A, with an IC_{50} value of $371.8 \pm 0.36 \mu\text{M}$.

4.3.8 Tacrine

Tacrine is a recognised acetylcholine esterase inhibitor (Fang *et al.*, 2008) and has been used for the treatment of Alzheimer's disease (Kozikowski *et al.*, 1992). It has the chemical structure as depicted below:



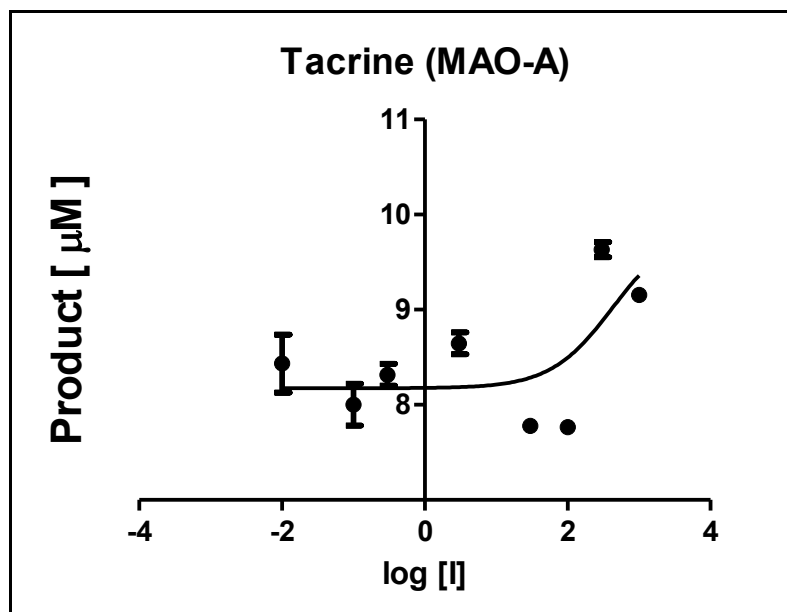


Figure 4-34: The dose-response curve for the inhibition of MAO-A by tacrine.

As depicted in the dose-response curve in Figure 4-34, tacrine was found to be a very weak inhibitor of MAO-A (Figure 4-31) with an IC_{50} value of $424.5 \pm 1.22 \mu\text{M}$. We thus did not perform studies on MAO-B.

In summary, the IC_{50} values of each compound tested for MAO-A, and MAO-B if required, are listed in Table 4-33

Table 4-33: The IC₅₀ values for the inhibition of recombinant human MAO-A and –B by methylene blue and its analogues.

^aThe IC₅₀ values are expressed as the mean ± SEM of duplicate determinations. Lower IC₅₀ values suggest a more potent inhibitor (DND=did not determine).

Compound	MAO-A	MAO-B
	IC₅₀ value^a (μM)	IC₅₀ value^a (μM)
Methylene blue	0.07 ± 0.17 μM	4.37 ± 0.14 μM
Methylene green	0.25 ± 0.11 μM	5.5 ± 0.23 μM
Imipramine	12500 ± 0.11 μM	DND-
Phenothiazine	2.25 ± 0.15 μM	DND
Methylene violet	2.76 ± 0.42 μM	DND -
Acriflavine	0.43 ± 0.25 μM	DND -
Thionin acetate	371.8 ± 0.36 μM	DND -
Tacrine	424.5 ± 1.22 μM	DND -

4.4 Nitric oxide fluorometric assay

As has been mentioned earlier, the antidepressant-like effects of methylene blue may be related to effects on MAO or nitric oxide. While the effects on MAO were determined *in vitro* in Section 4.3, the effects on NO were determined *ex vivo* following 7 days treatment in rats with the selected analogues. According to the rationale for the selection of compounds for the chronic treatment studies (see Section 4.2), only imipramine, methylene blue and methylene green were tested for actions on NO. In this regard, the accumulation of the

inactive, stable metabolite of NO, viz. NO_2^- and NO_3^- , in the hippocampus were analysed by fluorometric analysis. The hippocampus was selected for this analysis because a number of studies have demonstrated the inhibitory effects of antidepressants on hippocampal NOS activity (Wegener *et al.*, 2003; Harvey *et al.*, 2006), including methylene blue (Volke *et al.*, 1999), while this brain region plays a critical role in the neurobiology of depression (MacQueen *et al.*, 2003; Wegener *et al.*, 2010).

After sub-chronic treatment, as described in Section 4.4.2.2., rats were decapitated and the hippocampus removed and assayed separately. Upon establishing their positive antidepressant response in the chronic FST study, the goal of this study was to establish whether methylene blue and methylene green exert any noteworthy effects on the accumulation of nitrites, after conversion of tissue NO_2^- and NO_3^- to NO_2^- , in the cortico-limbic regions of the rat brain after sub-chronic treatment.

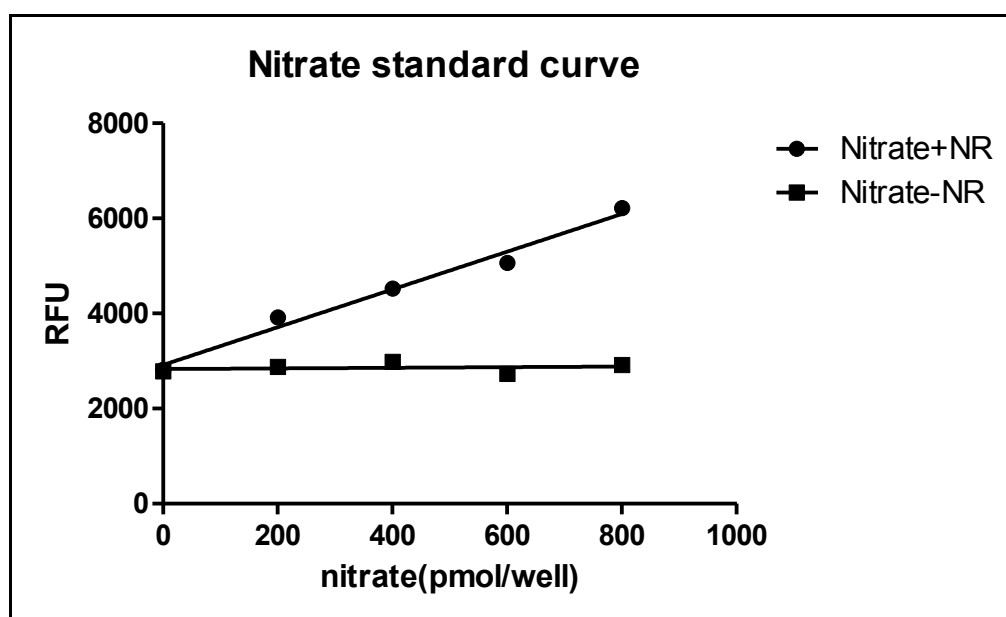


Figure 4-35: A representative NO_3^- calibration curve used for the analysis of sample NO_3^- after conversion to NO_2^- by nitrate reductase (NR). Nitrate + NR measures sample NO_2^- , while nitrate – NR measures sample NO_3^- , and acts as a negative control.

Using the assay methods described in section 4.2.2.2, a suitable calibration curve was constructed (Figure 4-35). The r^2 value obtained was 0.9724. The assay measures NO_2^- as a stable surrogate marker of formed NO in the tissue, as explained in Section 3.4.2.2. From the nitrate + NR curve, sample NO_2^- is extrapolated. Data were analysed by means of one-way

analysis of variance (ANOVA) across all groups, and were subsequently subjected to the Dunnett's post test. Data were expressed as the mean \pm SEM with statistical significance defined at the 95% ($p < 0.05$) level. Descriptive statistics are provided in the accompanying tables.

4.4.1 Effect of drug treatment on the accumulation of NO_3^- in the rat hippocampus

One way analysis of variance of the data described no effect of treatment on hippocampal nitrates ($F(3,36)=2.014;p=0.1294$). Post-hoc analysis of these data (Table 4-31) revealed that methylene blue and methylene green decreased nitrate concentrations in this brain region, compared to saline (from $1338 \pm 118.2 \mu\text{M}$ to $1006 \pm 157 \mu\text{M}$ and $1030 \pm 112.8 \mu\text{M}$ respectively; Figure 4-36), although this change was not significant. Imipramine on the other hand was without effect, in this regard (Figure 4-36).

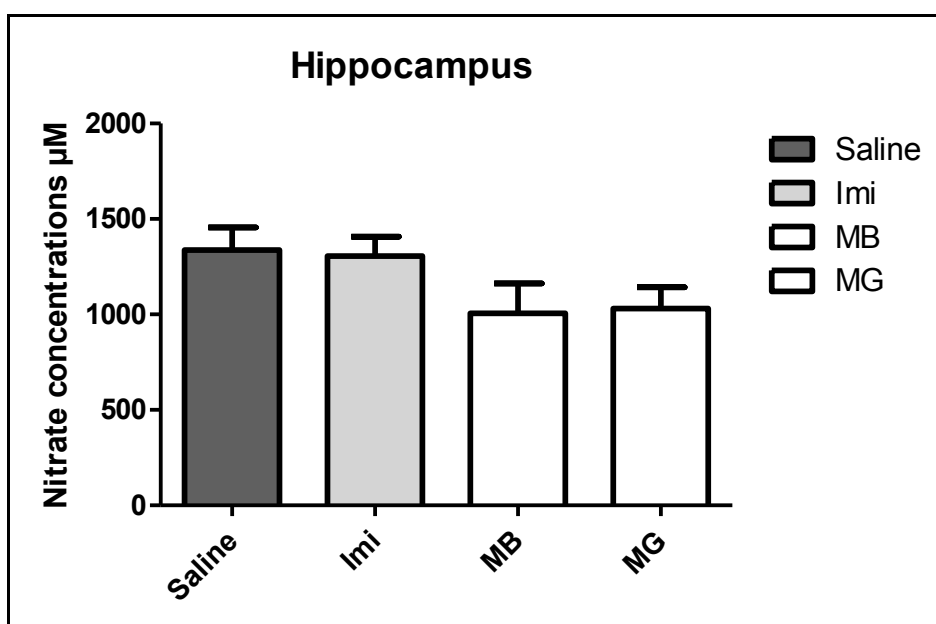


Figure 4-36: Nitrate concentrations in the rat hippocampus after chronic drug treatment, as indicated ($n=5$). IMI=imipramine, MB=methylene blue, MG=methylene green

Table 4-34: The effect of drug treatment, as indicated, on nitrate levels in the rat hippocampus (n=5). IMI=imipramine, MB=methylene blue, MG=methylene green

Compound	Mean \pm SEM, Concentration nitrate
Saline	1338 \pm 118.2 μ M
Imipramine	1306 \pm 101.9 μ M
MB	1006 \pm 157 μ M
MG	1030 \pm 112.8 μ M

This chapter will discuss and interpret the experimental results described in chapter 4. The study is divided into two experimental procedures:

- **Experimental procedure 1:** The acute *in vivo* study aims to confirm the antidepressant action of methylene blue in the rat forced swim test (FST), and to determine whether any of the selected analogues of methylene blue also possess antidepressant-like activity. Furthermore the effect of the methylene blue and its analogues will be investigated with respect to monoamine oxidase (MAO) inhibitory activity in an *in vitro* preparation containing recombinant human MAO-A and B and following acute administration of each analogue.

Experimental procedure 2: The chronic treatment study aims to determine if methylene blue, as well as any analogues that have demonstrated antidepressant-like action in the acute studies, also possess antidepressant-like activity in a chronic treatment protocol. In this experiment, the effects of the test compounds on the NO/cGMP pathway will also be investigated following chronic drug treatment.

5.1 The effect of acute treatment with methylene blue and its analogues in the rat forced swim test (FST)

5.1.1 Effect of acute methylene blue treatment in the FST

Methylene blue significantly reduced immobility time in rats compared to animals receiving saline (Figure 4-1), suggesting that the animals present with an increased drive to escape the water. A similar antidepressant-like response was noted with imipramine, a known antidepressant compound (Figure 4-1). Important to note here is that the acute FST is sensitive to MAO-I's (Porsolt *et al.*, 1977; Bourin, 1990), of which methylene blue is one (Ehringer *et al.*, 1961; Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007). Interestingly, the antidepressant effect of methylene blue in the FST was evident only at a dosage range between 15-60 mg/kg. Methylene blue treated rats also demonstrated a significant decrease in locomotor activity vs saline treated rats in the open field test (Figure

4-2). It can thus be concluded that the observed decrease in immobility time suggests antidepressant activity. The decrease in locomotor activity induced by the drug confirms that the antidepressant effect was unlikely a result of the drug stimulating locomotion. These results are in agreement with data obtained by Eroglu and Caglayan (1997), although these latter authors found methylene blue to be an effective antidepressant at a dosage range of 15 and 30 mg/kg, but ineffective at 60 mg/kg. They proposed that methylene blue displays a U-shaped response curve. However, data obtained in the present study confirms the antidepressant effect of methylene blue even at a dose of 60 mg/kg. This discrepancy warrants further study, but could be due to differences in study conditions.

Imipramine is known to be a more effective inhibitor of norepinephrine reuptake (Heninger & Charney, 1987; Barker & Blakely, 1995), thus potentiating catecholaminergic responses. This assumption was confirmed in the present study, where imipramine was found to significantly increase climbing behaviour, as depicted in Figure 4-3, B, and which is indicative of an increase in catecholaminergic activity (Cryan *et al.*, 2002). Imipramine did not bolster swimming activity, a known response in the FST to increased serotonergic activity (Cryan *et al.*, 2002). The increased climbing behaviour thus confirmed a dominant catecholaminergic response to this agent. Methylene blue treated rats demonstrated a slight increase in swimming behaviour, although this was not significant (Figure 4-3, A). This would suggest some action on serotonin, in agreement with earlier studies (Ramsay *et al.*, 2007; BK *et al.*, 2008). However, methylene blue increased climbing behaviour significantly (Figure 4-3, B), indicating that this compound more powerfully potentiates catecholaminergic neurotransmission during acute treatment. Thus methylene blue evokes a very similar behavioural response in the acute FST as does imipramine. These actions very likely explain their similar antidepressant-like responses.

5.1.2 Effect of acute methylene green treatment in the FST

Acute methylene green treatment decreased immobility time, compared to saline treated animals in the FST at doses of 7.5, 15, 25, and 40 mg/kg (Figure 4-4). Rats receiving 15 mg/kg methylene green evoked a trend towards reduced locomotor activity in the open field test (Figure 4-5), with doses of 15 mg/kg and 60 mg/kg significantly reducing locomotor activity. However, it can be concluded that the reduction in immobility time engendered by methylene green in the acute FST is not a result of an increase in locomotor activity, which would have negated an argument for an antidepressant-like response. These data therefore

support the hypothesis that methylene green possess antidepressant-like activity, at least in rodents.

Methylene green treatment gradually increased swimming behaviour in rats compared to saline treated animals, but only of significance at a dose of 25 mg/kg (Figure 4-6, A). The treated animals also gradually increased climbing behaviour, but this was observed at doses of 7.5 and 25 mg/kg (Figure 4-6, B). These observations suggested that methylene green may potentiate both catecholaminergic, and to a lesser degree serotonergic, neurotransmission, which may explain its pronounced antidepressant-like response.

5.1.3 Effect of acute acriflavine treatment in the FST

Acute acriflavine treatment failed to evoke a noteworthy decrease in immobility time compared to saline treated animals (Figure 4-7) and thus we can conclude that the compound does not display antidepressant-like activities in the FST. However, there is a significant decrease in the locomotor activity in acriflavine-treated rats compared to saline treated rats at doses of 3.75, 7.5, 15, 30 and 40 mg/kg (Figure 4-8). Even if the compound may have had inherent antidepressant-like properties that were not revealed by the acute FST, this decrease in locomotor activity would obscure any possible antidepressant-like effect and may explain why acriflavine is ineffective as an antidepressant over the dosage range tested. The fact that these pronounced effects were evident even at relatively low dosages argues against the possibility that this compound may have any clinically relevant antidepressant actions. Nevertheless, further studies over a wider dosage range may have revealed a different picture. Interestingly, the swimming and climbing data suggest that acriflavine increases catecholaminergic neurotransmission, albeit only significantly at higher dose of 40 mg/kg (Figure 4-9, B). Consequently, even if acriflavine does have antidepressant-like effects at doses outside the range tested here, the side effect burden of this compound on the general level of functioning of the animal (or patient) would far outweigh any possible therapeutic benefit. It did not affect swimming behaviour. As is typical of antidepressants like imipramine (discussed above), increased catecholaminergic activity may mediate an antidepressant-like response. However, any such therapeutic effect is very likely going to be abrogated by the severe locomotor toxicity of the drug.

5.1.4 Effect of acute tacrine treatment in the FST

Tacrine treated animals did not demonstrate any change in immobility time in the acute FST compared to animals treated with saline (Figure 4-10), thus arguing against the compound having any viable antidepressant-like activity. Animals treated with tacrine demonstrated a significant decrease in locomotor activity in the open field test compared to saline treated animals, at doses of 1, 2.5, 3.75, 5, and 7.5 mg/kg (Figure 4-11). This progressive decrease in locomotor activity may explain why tacrine treatment does not have an antidepressant effect in rats over the dosage range tested. As with acriflavine, it is nevertheless possible that the compound may have inherent antidepressant-like properties that are not revealed by the acute FST. However, the pronounced adverse effects on locomotor performance that are evident even at relatively low dosages argue against the possibility that this compound may have any clinically relevant or useful antidepressant actions.

Tacrine treated animals gradually increased their climbing behaviour, attaining significance at doses of 3.75 mg/kg and 7.5 mg/kg as compared to the saline treated rats (Figure 4-12, B). As with imipramine, a known inhibitor of norepinephrine reuptake that bolsters noradrenergic neurotransmission, these data indicate that tacrine may also bolster catecholaminergic neurotransmission at selected doses. Unfortunately, these possible benefits are abrogated by its significant locomotor toxicity. Moreover, the fact that tacrine also suppresses swimming behaviour, and hence serotonergic behaviour (Figure 4-12, A), raises the thought that this compound may suppress serotonergic function, which will also counter any antidepressant-like effects. However, further studies in this regard are required to validate this claim.

5.1.5 Effect of acute methylene violet treatment in the FST

Acute treatment with methylene violet resulted in a slight decrease in immobility time as the dose increased compared to saline treated rats, but not significantly so (Figure 4-13). It would thus appear then that methylene violet is capable of reducing immobility in the FST after acute dosing but that this only occurs at higher doses. However, due to problems with solubility we were unable to fully explore the upper dosage ranges of this compound, which could be considered for future studies. At the doses tested, methylene violet does not show any noteworthy antidepressant-like effects. Methylene violet treated animals also demonstrated a gradual decrease in locomotor activity in the open field test, but only

reaching significance at a dose of 7.5 mg/kg, compared to saline treated animals (Figure 4-14)

Methylene violet gradually increased the swimming behaviour, but not of any significance (Figure 4-15, A). Considering Figure 4-15, B, by bolstering climbing behaviour at a dose of 5 mg/kg, methylene violet may potentiate catecholaminergic neurotransmission. Interestingly, this same dose evoked a near significant reduction in immobility in the FST (Fig 4-13), suggesting that an increase in catecholaminergic activity may be responsible for this effect. Further studies are needed to explore and confirm these findings. However, the gradual escalation of locomotor toxicity with this compound suggests that it may not be viable to explore higher doses in the hope of finding useful antidepressant activity.

5.1.6 Effect of acute thionin acetate treatment in the FST

Treatment of animals with acute thionin acetate resulted in a slightly decreased immobility time, albeit not significantly, as compared to saline treated animals (Figure 4-16). The locomotor activity of thionin acetate treated animals versus saline treated animals did not differ significantly (Figure 4-17). Although adverse effects on locomotor behaviour cannot be used to explain the lack of antidepressant-like effects as with some of the compounds described above, it is of interest that thionin acetate did not bolster serotonergic (Figure 4-18, A) or adrenergic (Figure 4-18, B) transmission, thus possibly explaining its obvious lack of antidepressant-like effects at the doses tested.

5.1.7 Effect of acute phenothiazine treatment in the FST

Acute phenothiazine treatment in rats did not alter the immobility time as compared to saline treated rats (Figure 4-19), suggesting a lack of noteworthy antidepressant-like activities in the FST, at least at the doses tested in this study. Considering effects on locomotor activity, phenothiazine treated rats demonstrated a slight increase as compared to saline treated rats, but this change was not significant (Figure 4-20). Unlike imipramine, phenothiazine did not drastically alter (increase) noradrenergic (Figure 4-22, B) or serotonergic (Figure 4-22, A) mediated behaviour, which were in keeping with its lack of antidepressant-like effects in the FST.

5.2 Chronic treatment study

Apart from methylene blue itself, the only other methylene blue related compound with noteworthy antidepressant-like effects in the acute FST was methylene green. These two compounds were subsequently tested in the chronic FST, together with saline and imipramine. The known tricyclic antidepressant, imipramine, significantly decreased immobility time as compared to saline treated rats after a 7 day chronic treatment period (Figure 4-21). Methylene blue treated rats similarly demonstrated a significant decrease in immobility time as compared to saline treated rats (Figure 4-21). Importantly, methylene blue was found to be more effective than imipramine in decreasing immobility time (Figure 4-21). This latter observation is exciting as it suggests an improved efficacy for methylene blue in the treatment of depression, which today is a highly sought after attribute in an antidepressant. The pharmacological basis for this apparent improved efficacy versus a gold standard such as imipramine definitely requires further study.

Recent studies have emphasised that multiple sites of action in a single molecule (Van der Schyf & Youdim, 2009; Millan 2009) or combining more than one antidepressant with different modes of action, offer distinct therapeutic advantages over traditional approaches where only one neurobiological target is selected, for example with the SSRI's. The powerful MAO-A inhibitory actions of methylene blue presented in this study and elsewhere (Ehringer *et al.*, 1961; Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007) as well as its inhibitory effects on the NO system, partly demonstrated in this study but with robust efficacy noted elsewhere (Eroglu and Caglayan, 1997; Volke *et al.*, 1999), are both well known mechanisms that mediate antidepressant effects on their own (See Sections 2.1.4.1 and 2.1.4.6 respectively). However, the combination of these two properties may represent a more effective approach to treating disorders of mood, as well as representing a novel approach to improved antidepressant drug design. Likewise methylene green also significantly decreased immobility time compared to saline treated rats in the FST (Figure 4-21), with a similar response to that of imipramine. The locomotor activity tests performed in the open field found that both imipramine and methylene green significantly decreased locomotor activity compared to saline treated animals (Figure 4-22), while methylene blue engendered the same response, but not significantly (Figure 4-22). It can thus be concluded that the observed decrease in locomotor activity in the Open field test (Figure 4-22) cannot explain the decrease in immobility observed in the FST for these three compounds, suggesting a true anti-depressant-like response.

Imipramine is a known inhibitor of norepinephrine reuptake and thus will bolster noradrenergic neurotransmission (Figure 4-23, B). Analysis of the swimming and climbing behaviours in the chronic treatment study indicated that imipramine induced an increase in climbing behaviour, confirming a pronounced catecholaminergic response. Methylene blue following chronic treatment was also found to significantly increase swimming behaviour, while slightly (albeit insignificantly) increasing the climbing behaviour, as compared to saline treated rats (Figure 4-23, B). Thus methylene blue bolsters serotonergic, and to a lesser extent noradrenergic, mechanisms that may underlie its psychotropic actions. Its bolstering effect on serotonergic responses explains its ability to evoke a serotonin syndrome when combined with other serotonergic agents (Ramsay *et al.*, 2007; Stanford *et al.*, 2009). As with methylene blue, methylene green significantly increased swimming behaviour, while only slightly increasing climbing behaviour in the FST compared to saline treated rats, although to a lesser extent than methylene blue.

Of interest is that these chronic treatment data are in contrast to the results obtained from the acute study (Figure 4-3, A and B; Figure 4-24, B). As discussed in Section 2.1.4.1, the onset of antidepressant activity has been linked to late onset changes in serotonin and noradrenalin receptor density (Leonard, 1984; Blier & de Montigny, 1999). Blier (2003) suggests that conventional antidepressants acutely increase the availability of 5HT and/or NE in the synapse, which in turn triggers a negative feedback mechanism that effectively reduces transmitter release and “brakes” the onset of antidepressant activity. With continued stimulation of these receptors, the negative feedback mechanism becomes desensitized so that over time the levels of 5HT and/or NE are allowed to increase eventually culminating in the onset of antidepressant activity and the improvement in symptoms of depression. This may explain why acute responses of antidepressants on 5-HT and NA will differ depending on the duration of treatment (Blier, 2003). Since antidepressant efficacy requires long-term and not acute treatment, we conclude that the chronic FST data using methylene blue or methylene green are a more accurate reflection of their antidepressant properties, which our data suggests occurs through a bolstering of serotonergic neurotransmission. The discrepancy displayed here highlights the possible erroneous and misleading information that can be obtained by only considering behavioural data from the acute FST, especially since the true reflection of antidepressant efficacy is only attainable after a chronic treatment period (Detke *et al.*, 1997; Porsolt *et al.*, 2000).

5.3 Recombinant human MAO-A and MAO-B inhibition studies

5.3.1 Methylene blue

Methylene blue was found to be a potent inhibitor of MAO-A and a moderate inhibitor of MAO-B (Figure 4-25 and 4-26). The acute FST is sensitive to MAO inhibitors (Porsolt *et al.*, 1977; Bourin, 1990), and this potent action by methylene blue on the principle metabolising enzyme for monoamines, especially 5HT and NA, provides a strong construct for explaining the antidepressant activity of methylene blue evident in both the acute and chronic FST studies (Figure 4-1; Figure 4-21). This also can be corroborated with its potentiation of noradrenergic mediated behaviour in the acute FST (Figure 4-24), and the increased serotonergic neurotransmission noted after chronic treatment (Figure 4-25). These findings are in agreement with earlier studies describing methylene blue as an inhibitor of MAO-A (Ehringer *et al.*, 1961; Aeschlimann *et al.*, 1996; Oxenkrug *et al.*, 2007; Ramsay *et al.*, 2007). Moreover, the MAO-A inhibitory activity of methylene blue also explains the induction of central nervous system serotonin toxicity in conjunction with any other serotonergic agent (Ramsay *et al.*, 2007; Stanford *et al.*, 2009).

5.3.2 Methylene green

Our studies found methylene green to be a very potent inhibitor of MAO-A (Figure 4-26) and a moderate inhibitor of MAO-B (Figure 4-27). In this study we show for the first time that methylene green displays significant antidepressant-like properties in both the acute and chronic FST protocols (Figure 4-4 and Figure 4-21), which may indeed be explained by its MAO-A and MAO-B inhibitory actions. These results are also congruent with its potentiation of noradrenergic and serotonergic mediated behaviour in the acute and chronic FST (Figure 4-6 and 4-23).

5.3.3 Imipramine

Imipramine is a very weak inhibitor of MAO-A (Figure 4-28). Imipramine is a highly effective antidepressant in animal models as well as clinically (see Section 2.1.4.1). Its mode of

action, however, is ascribed to its powerful inhibition of monoamine reuptake sites in the synaptic cleft resulting in an elevation in NA and 5HT (Section 2.1.4.1). Our MAO studies confirm that the antidepressant activity of imipramine cannot be related to an inhibitory effect on MAO-A.

5.3.4 Phenothiazine

Phenothiazine was found to be a moderate MAO inhibitor (Figure 4-29). While phenothiazine presents with notable effects on a number of important neuro-receptors, including dopamine and histamine (Horn & Snyder, 1971), we have demonstrated that phenothiazine does not present with any antidepressant-like effects in the FST (Figure 4-19), which is congruent with what is known about the clinical profile of this drug (Section 2.1.3.1). The moderate degree of MAO inhibitory action evident with phenothiazine described here is apparently not sufficient for antidepressant effects. Studies on locomotor activity also suggest a slight increase in locomotor activity, but this does not explain the lack of mobility of rats in the FST. It should however be borne in mind that this study only studied this aspect in the acute FST, and it is plausible that chronic treatment may have evoked an antidepressant-like response.

5.3.5 Methylene violet

In this study we show for the first time that methylene violet displays moderate MAO-A inhibition (Figure 4-30), although our earlier behavioural work has demonstrated that the compound presents with minimal antidepressant-like properties in the acute FST (Figure 4-13), again concluding, as with phenothiazine, that its MAO inhibitory properties is below the required threshold for clinically relevant efficacy.

5.3.6 Acriflavine

The powerful inhibitory action of acriflavine on MAO-A (Figure 4-31) suggests that this drug should present with noteworthy antidepressant-like effects in the FST. However, this is not the case (Figure 4-7). For this reason we did not analyse MAO-B activity. The possible

explanation for the apparent discrepancy between its potent MAO-A inhibitory actions but lack of antidepressant-like efficacy in the FST can be explained by the performance of the compound in the open field test (Figure 4-8). Here we found that acriflavine significantly decreased locomotor activity, which would effectively suppress any psychological benefits that the compound may have in engendering a drive to escape. Indeed, these pronounced effects on locomotor activity were evident even at relatively low dosages. Consequently, these results suggest that, even if acriflavine does have antidepressant-like effects, possibly at doses outside the range tested here, the side effect burden of this compound on the general level of functioning of the animal (or patient) would far outweigh any possible therapeutic benefit. This study also highlights that antidepressant activity in the FST appears not to be exclusively dependant on the inhibition of MAO-A, since compounds that are good MAO-A inhibitors, such as acriflavine, can fail to exhibit antidepressant activity in the FST. Since the FST is sensitive to MAO-I's, a possible explanation is that acriflavine may not be able to cross the blood brain barrier to exert an antidepressant action.

5.3.7 Thionin acetate

Thionin acetate was found to be a weak inhibitor of MAO-A (Figure 2-32). These data are in agreement with our behavioural data showing a complete lack of antidepressant-like effects in the acute FST (Figure 4-16).

5.3.8 Tacrine

Tacrine is a very weak inhibitor of MAO-A (Figure 2-33), which very likely explains its lack of antidepressant-like effects in the FST (Figure 4-10). It is possible that the compound may have antidepressant-like effects following chronic treatment, although this was not investigated.

In summary, all the drugs tested were found to inhibit MAO-A *in vitro* to a greater or lesser extent, yet only those with potent inhibitory effects could be correlated with significant antidepressant-like effects in both the acute and chronic FST. The weakest MAO inhibitors were imipramine and tacrine, while thionin acetate was also deemed to be a very weak

inhibitor. Phenothiazine and methylene violet were found to be moderate inhibitors of MAO-A, with methylene blue, methylene green and acriflavine noted as potent MAO-A inhibitors. However, methylene blue and methylene green were the only two drugs, apart from imipramine, to display powerful antidepressant-like effects in both versions of the FST.

While both methylene blue and methylene green may exert antidepressant-like effects via inhibition of MAO-A and to some extent through inhibition of MAO-B, methylene blue has also been noted for its significant effects on the NO-cGMP signalling cascade (Section 2.3). In order to ascertain whether their antidepressant-like efficacy in the FST can also be linked to actions on the NO-cGMP pathway, the action of methylene blue and methylene green on this pathway was studied following sub-chronic drug treatment for 7 days. This was followed by subsequent fluorometric analysis of nitrite in rat hippocampus, a brain region intimately involved in mood regulation and antidepressant action.

5.4 Nitric oxide fluorometric assay

5.4.1 Effect of drug treatment on accumulation of NO₃⁻ in the hippocampus

Neither imipramine, nor methylene blue or methylene green, significantly altered hippocampal nitrite concentrations (Figure 4-34). However, both methylene blue and methylene green did decrease nitrate concentrations, albeit not significantly. These observations are somewhat disappointing since similarly designed studies have previously found chronic imipramine treatment to significantly reduce hippocampal NOS activity (Harvey et al, 2006), while methylene blue has been found to inhibit hippocampal NOS *in vivo* using an *in vivo* microdialysis technique (Volke et al, 1999). However, an inhibitory action of imipramine on NOS hasn't always been reproducible. In fact, chronic imipramine treatment (for 30 days) has been found to increase nitric oxide synthase gene expression in the brain, followed by augmented NO production (Suzuki *et al.*, 2003). On the other hand, Jopek and colleagues found increased NOS activity after electroconvulsive treatment, but no effect after chronic imipramine or citalopram treatment (Jopek *et al.*, 1999). This may be the result of treatment-induced adaptive changes in the NMDA receptor complex (Jopek *et al.*, 1999). On the other hand, treatment duration, route of administration and dose of imipramine may also explain these discrepancies. Thus the current study used a dose of 15 mg/kg x 1 week ip,

while our earlier study (Harvey *et al.*, 2006) used a dose of 15 mg/kg x 3 weeks ip. In fact, that methylene blue evoked a trend towards suppression of hippocampal nitrites does suggest that a longer duration of treatment and/or a higher dose may have realised a similar result to that observed by Harvey and colleagues (2006) and Volke and colleagues (1999) who used a completely different treatment protocol. This is the first study using methylene green, but a similar explanation as above may also apply. Further studies are the only means to clarify these questions, and are recommended.

5.5 Structure-activity relationships (SAR)

An important secondary outcome for this study is to enable the development of a new class of antidepressants based on the molecular structure of methylene blue, and to consider the structure and efficacy (or lack thereof) of the various methylene blue analogues tested. This study indicates that the antidepressant activity of imipramine does not involve hippocampal NOS activity or actions on MAO. However, the antidepressant action of methylene blue and methylene green can be directly ascribed to inhibition of MAO, with some but limited involvement of NO.

Considering the results obtained from the acute FST and MAO inhibition studies, a number of suggestions for future development for chemical synthesis can be proposed.

For the inhibition of MAO-A, a permanent charge on the molecule is a requirement, since those structures carrying a charge, such as methylene blue, methylene green and acriflavine, are all potent MAO-A inhibitors. Those structures that were neutral, such as imipramine, phenothiazine, methylene violet, tacrine and thionin acetate, were found to be weak MAO-A inhibitors.

For antidepressant action in the FST, a charged structure seems to be a requirement in this series, since all of the active compounds, viz. methylene blue and methylene green, are charged, with imipramine the exception. However, the lack of a charge on the molecular structure of imipramine probably makes it more amenable to inhibiting the monoamine reuptake protein and not MAO. The reverse applies to methylene blue and methylene green which may not be effective inhibitors of monoamine uptake, although such studies still need to be performed.

A dimethylamine substituent at C-3 and C-7 also appears to be a requirement for antidepressant activity since methylene blue and methylene green are the only compounds that have these substituents.

Also of interest is the observation that methylene blue and methylene green are the only compounds that were found to be inhibitors of MAO-B. Since these are also the only compounds with dimethylamine substituents at both at C-3 and C-7 of a fused-ring tricyclic system, it can be concluded that this disubstitution is a requirement for MAO-B inhibition. A possible explanation for this observation is that the dimethylamine substituents interact by hydrophobic interactions with the apolar binding pockets within the MAO-B active site (Binda *et al.*, 2001).

The potencies by which methylene blue (Figure 5-1) and methylene green inhibit MAO-B inhibition are however 62 and 33 fold, respectively, lower than their inhibition potencies towards MAO-A. This difference in affinities for the two MAO isoforms may be dependent on the positive charge of the fused-ring system. It is known that an important interaction between the aromatic moieties of a reversible inhibitor and the active site of MAO-A involves a π - π interaction between the aromatic system and the amide functional group of Gln 215 (Son *et al.*, 2008). This interaction may be especially significant with an aromatic moiety containing a delocalized positive charge. Although aromatic systems of reversible inhibitors are also stabilized in the active site of MAO-B by π - π interactions, the results of this study suggest that the interaction between MAO-B and a charged aromatic system is relatively weaker compared to those in the MAO-A active site. It can therefore be concluded that a fused-ring aromatic moiety containing a delocalized positive charge may be a structural characteristic that confers selective binding to MAO-A in preference to the B isoform.

With the exception of imipramine, tacrine is the weakest MAO-A inhibitor evaluated in this study. The relatively low MAO-A inhibition potency of tacrine can most likely be attributed to the presence of the sp^3 carbons in the tricyclic ring structure which abolishes the aromaticity and planarity of the fused-system (Figure 5-1). It is known that planar aromatic structures bind preferably in the relatively narrow active site cavities of the MAO enzymes and those structures with fused-ring systems that deviate from planarity are less well accommodated in the MAO active sites (Gnerre *et al.*, 2000).

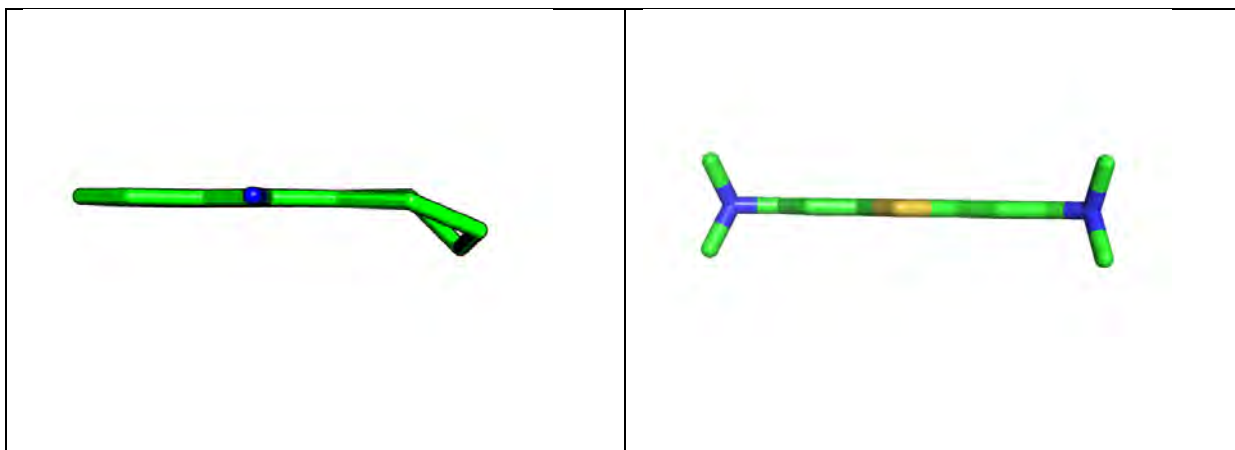


Figure 5-1: The respective energy minimized conformations of tacrine (left) and methylene blue (right). Carbons are depicted in green, nitrogen in blue and sulphur in yellow.

An interesting observation is that thionin acetate was found to be a weak inhibitor of MAO-A, even though it is a close structural analogue of methylene blue which was found to be a potent MAO-A inhibitor. The absence of the dimethylamine substituents in the structure of thionin acetate does not account for this finding since acriflavine proved to be a potent MAO-A inhibitor even though the structure of acriflavine is devoid of this substitution. The reason for this observation is not well understood and requires further investigation.

The antidepressant activity in the FST appears not to be exclusively dependent on the inhibition of MAO-A, since compounds that are good MAO-A inhibitors such as acriflavine, may be poor antidepressants in the FST.

Although methylene blue and methylene green did not demonstrate marked abilities to modify NOS in rat brain in the current study, the combined ability of these compounds to modulate both MAO and NOS may represent an important structural characteristic of these compounds with respect to improved antidepressant activity. This is especially relevant considering the superior antidepressant-like effects of methylene blue compared to imipramine in the chronic FST described in this study, but also in lieu of other experimental evidence that an improved antidepressant response can be achieved by combining monoaminergic and nitrenergic active drugs (Harkin *et al.*, 2004).

Despite the wide array of commercially available antidepressants, more effective drugs with a quick onset of action and minimal side effects are still eluding current drug manufacturers. In this study we have aimed to correlate the structures of methylene blue and selected analogues with antidepressant activity, as determined in the acute and chronic versions of the FST, and have related this to their measured efficacy to inhibit MAO-A and -B, as well as to target hippocampal NOS.

The following principle conclusions can be drawn from this study:

- Six structurally similar analogues of methylene blue were identified, but only methylene blue and methylene green were found to be comparable to imipramine in the acute FST study.
- Methylene blue and methylene green were also found to have comparable antidepressant-like properties to imipramine during a 7 day chronic treatment FST protocol.
- Methylene blue specifically, was found to be superior in antidepressant-like efficacy than imipramine in the chronic FST.
- Methylene blue and methylene green, but not imipramine, proved to be very potent inhibitors of MAO-A as well as moderate inhibitors of MAO-B.
- The antidepressant activity of imipramine does not involve actions on MAO, while contrary to earlier studies; it did not inhibit hippocampal NOS activity in a 7 day treatment protocol.
- The antidepressant activity of methylene blue and methylene green in the chronic FST protocol is strongly related to its ability to inhibit MAO, with some but limited action on NO, although further in-depth studies in this regard are needed.

- Of interest, methylene blue and methylene green increase catecholaminergic activity when assessed in the acute FST, but increase serotonergic activity when assessed in the chronic FST.

Minor conclusions:

Acriflavine was found to be a very potent inhibitor of MAO-A, but induces pronounced deficits in locomotor performance that may explain the compounds lack of antidepressant activity.

Phenothiazine and methylene violet were found to be moderate inhibitors of MAO-A, but were devoid of antidepressant activity. Phenothiazine slightly increased locomotor activity which cannot explain the lack of antidepressant activity. On the other hand, methylene violet slightly decreased locomotor activity which may contribute to its apparent lack of antidepressant activity.

Tacrine and thionin acetate were found to be very weak inhibitors of MAO-A which is consistent with the lack of antidepressant activity found in the acute FST. Tacrine significantly reduced locomotor ability which may also explain the lack of antidepressant activity. However, thionin acetate did not reduce locomotor activity which cannot explain the lack of antidepressant activity.

The following structure activity relationships (SAR) were apparent in this study:

- The inhibition of MAO-A requires a molecule with a permanent charge.
- For antidepressant action in the FST, a charged structure seems to be a requirement of the molecule, although this may not be obligatory.
- A dimethylamine substituent at C-3 and C-7 may also be a requirement for antidepressant activity.
- Being a potent MAO-A inhibitor does not necessary imply the drug will be an effective antidepressant in the FST.

6.1 Future recommendations and future studies

In the current study we provide evidence that methylene green is a novel antidepressant in the rat FST, and that charged tricyclic structures akin to methylene blue may be novel lead antidepressant compounds. This finding warrants further investigation using different animal

models of depression, as well as other preclinical studies to clarify the complete pharmacological and toxicological profile of this compound.

Based on the outcome of the current study, several strategies for future studies are proposed:

- Studying the effects of methylene blue and methylene green in animal models that simulate more closely the chronic disease, such as the olfactory bulbectomy model (Song and Leonard, 2005) or a genetic model of depression such as the Flinders Sensitive Line (FSL) rats (Overstreet, 1993).
- The determination of regional monoamine concentrations in the brain of animals treated chronically with methylene blue and/or methylene green.
- Studying monoamine, GABA and glutamate release following acute challenge with the above compounds or after chronic treatment, using *in vivo* microdialysis.
- Re-analysis of the effects of methylene blue and methylene green on brain NOS.
- Studying the long-term effects of methylene blue and methylene green on neuroreceptor binding.
- Studying the effects of methylene blue and methylene green on markers of cellular resilience, such as BDNF, CREB etc
- Studying the ability of methylene blue and methylene green to augment existing antidepressant treatment in animals. A similar study using methylene blue is a viable option in humans as well since it is a registered medicine in most countries.
- Chemical synthesis of compounds based on the methylene blue/methylene green structure and performing subsequent pharmacological and behavioural studies on these compounds.

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