A substantial number of patients do not respond sufficiently to antidepressant drugs and are therefore often co-medicated with lithium as an augmentation strategy. Also inhibitors of nitric oxide synthase (NOS) have been used as an augmentation strategy, while inhibitors of NOS exhibit antidepressant-like properties in various animal models. Therefore, we hypothesized that modulation of NOS may be involved in the long-term effects of antidepressants and lithium, and studied the influence of acute and chronic administration of citalopram, alone or in combination with lithium, on NOS activity in hippocampus, cerebellum, and frontal cortex, by determination of L-citrulline being formed. We found that administration of acute or chronic citalopram (5 mg/kg and 20 mg/kg/24h, respectively) alone or in combination with subchronic lithium (60 mmol/kg chow pellet) did not influence the activity of NOS \textit{ex vivo} in all regions compared to control. In contrast, high doses of lithium caused a significant decrease in NOS activity \textit{in vitro}. We conclude that basal conditions are unsuitable for the study of antidepressant effects on NOS, and that the neurochemistry of nitric oxide remains unaltered following chronic citalopram or subchronic lithium under normal physiological conditions.

\textbf{Key Words:} augmentation strategy, nitric oxide, lithium, citalopram.
INTRODUCTION

Lithium has been used in the treatment of bipolar disorder since 1949 with substantial evidence for efficacy. Originally, lithium was assumed to have a poor antidepressant effect, although convincing evidence to the contrary has been provided by several controlled clinical studies (1). Lithium is also one of the best documented drugs to add when choosing alternative strategies for non-responder patients (2), and with respect to efficacy, lithium-augmentation has been considered the first-choice procedure for patients who fail to respond to antidepressant monotherapy (3).

The mechanisms underlying the action of lithium, alone or as augmentatory agent, have been extensively studied. Although various molecular sites of action have been proposed for the action of lithium, the ion appears to exert its major effects by targeting intracellular signaling cascades, particularly inhibition of inositol monophosphatases, the regulation of the phosphoinositide/protein kinase C signaling cascade, and the modification of receptor-mediated G protein activation (4,5). The indoleamine, serotonin (5-HT), however, seems to play a key role in the pharmacological response of antidepressants (6). Studies based on the microdialysis technique have shown that lithium has distinct effects on 5-HT, increasing 5-HT levels in some areas of the brain but not in others (7).

Earlier studies, however, have also found that lithium may exert actions on the nitric oxide (NO)-cGMP transduction system (8-11), while NO itself may exert profound effects on brain 5-HTergic pathways (12,13). Although NO plays a modulatory role in 5-HT function, NOS is primarily activated by the glutamate N-methyl-D-aspartate (NMDA) receptor which catalyzes the synthesis of the NO from the amino acid L-arginine (L-Arg; (14,15). Interestingly, the nonselective inhibitor of soluble guanylyl cyclase (sGC) and nitric oxide synthase (NOS), methylene blue (MB), has been found to be effective as mono-therapy and augmentation strategy in depressed patients (16,17).

NO has been implicated in the regulation of various behavioural, cognitive, and emotional processes, e.g. learning, aggression, locomotion, anxiety, and depression (18,19). Furthermore, it has been demonstrated that patients suffering from depression have reduced numbers of NOS containing neurons in the hypothalamus (20), while depressed patients have increased plasma levels of nitrite and nitrate (NOx), the end-product of NO metabolism (21). Recent studies have also shown that chemically distinct NOS inhibitors produce antidepressant-like effect in the forced swim test in mice and rats (22,23). NO also interacts with other classical transmitters that have a regulatory role on mood, particularly the monoamines, as well as glutamate and GABA (13,24,25).

While antidepressants may indeed influence the NOS signalling pathway in vitro (26, 27), pre-clinical ex vivo studies (28,27) are not supportive of the clinical data where, for example, paroxetine has been found to decrease NOx in patients with depression (26). While these data suggest that modulation of NOS by
antidepressants and lithium may explain their antidepressant efficacy, but also any augmentatory actions, further clarification on the conditions of study are needed.

Thus, the aims of the present study were to firstly determine the effects of lithium on NOS activity in vitro, and then to clarify the effects of chronic citalopram and subchronic lithium, alone or in combination, on NOS activity in hippocampus, frontal cortex and cerebellum measured ex vivo. Characterization of the effects of acute/chronic citalopram and/or lithium therapy on NOS activity in the brain could have several neurobiological implications regarding the onset of antidepressant action. Moreover, it would also consolidate the current pre-clinical data that appear to dissociate a putative role of NOS in antidepressant drug action.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (MB Breeding Centre, Denmark) weighing 280-350 g were used. They were housed individually at 20±2°C in a 12h light/dark cycle (light on at 7.00 a.m.). Tap water and chow pellets were available ad libitum. The animals were kept for at least two weeks in the animal colony before entering experiments. All animal procedures were accepted by the Danish National Committee for Ethics in Animal Experimentation (2002/561-585).

Drugs

All chemicals were of reagent grade or higher and were obtained from Sigma (St. Louis, MO, USA), except lithium and Na-EDTA that were obtained from Merck (Darmstadt, Germany). Citalopram-HBr was kindly donated by H. Lundbeck A/S, Valby, Copenhagen. All chemicals used for animal experimentation were dissolved in isotonic saline.

Surgical procedures and treatment schedule

In the chronic studies, an osmotic minipump (ALZET, 2ML4, Durect Corp. Cupertino, USA) was implanted subcutaneously just below the neck on the backside of the rat using fentanyl-fluanisone (0.0945 and 0.3 mg/kg, respectively) + midazolam (0.25 mg/kg) anesthesia (Day 1). The osmotic pumps contained citalopram (20 mg/kg/24h) or vehicle (0.9% saline). From day 15 half of the rats in each group were orally treated with lithium (60 mmol per kg chow pellet). Furthermore, these animals had access to a 2.7% NaCl drinking solution to prevent lithium toxicity. At day 22 the animals were sacrificed, the brains were rapidly removed on ice and dissected into hippocampus, frontal cortex, and cerebellum. The brain pieces were stored at -80°C until measurements were carried out. Tail vein blood samples were collected on day 3, 11, 18 and 20 to ensure a steady state level of citalopram and lithium.

The studies of the acute effects of citalopram and/or subchronic lithium were carried out in naïve animals. Half of the animals were orally pre-treated with a lithium containing diet (60 mmol/kg chow pellet) during five days. Subsequently, all animals received injections of citalopram (5 mg/kg) or vehicle. All animals were decapitated exactly 2 h following the injection (citalopram
or vehicle), the brains were rapidly removed on ice and dissected into hippocampus, frontal cortex and cerebellum. The brain pieces were stored at -80°C until measurements were carried out.

Sample analysis

The NOS activity analytical method was based on measuring the amounts of \[ ^{3} \text{H} \]-L-citrulline as described previously (29). Briefly, brain pieces (hippocampus, frontal cortex, and cerebellum) were homogenized separately (1:10 w/v) in ice-cold TRIS-HCl buffer containing EDTA. After centrifugation the supernatants were removed and used immediately to measure NOS activity. The aliquots of supernatant were added to reaction buffer and incubated 15 min at 37°C. The blank samples received buffer without CaCl\textsubscript{2} and NADPH. The reaction was stopped by addition of 1 ml of ice-cold HEPES buffer containing EDTA and subsequently transferred to ice. \[^{3}\text{H} \]-L-citrulline was separated using Dowex AG50WX-8 and quantified by the liquid scintillation spectroscopy (Tri-Carb 1900TR, Packard Instruments Ltd., Meriden, CT, USA). Protein concentrations were measured according to the method of Lowry using bovine serum albumin as standard (30).

Determination of serum-citalopram was carried out using a UV based HPLC system (Perkin Elmer 235C, CT, USA) with Hypersil BDS C18 column (Thermo Hypersil-Keystone, Cheshire, UK). The mobile phase was composed of 15 mM KH\textsubscript{2}PO\textsubscript{4}, 500 µl/l triethylamine and 325 ml/l acetonitrile with pH adjusted to 3.5.

Serum lithium was measured by standard flame photometry (FLM3, Radiometer, Copenhagen, Denmark).

Statistics

Statistical analyses (SPSS version 9.0) on NOS activity data were analyzed by comparing drug-treated and control animals for significant differences using analysis of variance. Corrections for multiple comparisons were carried out using the bonferroni procedure. Differences were considered statistically significant when \( P \) was less than 0.05. All data are ± standard error of the mean (SEM). The number of animals in each group is given in figure legends.

RESULTS

The effect of Lithium on the hippocampal NOS activity in vitro (Fig. 1)

In the first series of experiments, we studied the effects of lithium on NOS activity in vitro (Figure 1). We found that the NOS activity was inhibited by lithium in a concentration-dependent manner (\( p<0.001 \), control group not shown on the figure), although only the highest dose was significantly lower than control (\( P<0.05 \); bonferroni post-hoc test). This concentration is markedly higher than the concentrations used clinically. In the dose range of lithium used clinically (approx 1mM), the NOS activity in vitro was unaffected. We calculated the IC\textsubscript{50} value to be 138 mM. Interestingly, also citalopram caused a decrease in NOS activity compared to the control group (\( p<0.05 \), control group not shown on the figure). Also this concentration is markedly higher than the concentrations used clinically. In the dose range of citalopram used clinically (approx 300 nM), the NOS activity in vitro was unaffected. We calculated the IC\textsubscript{50} value of citalopram to be 608 µM.
As expected, the positive control (the NOS inhibitor NG-Nitro-L-arginine) caused a significant decrease in the NOS activity (p<0.001) with a significant reduction compared to control observed for all three doses (p<0.05; bonferroni post-hoc test). The IC$_{50}$ value was 28.5 µM.

Fig. 1. The hippocampal NOS activity in vitro following incubation with saline (control, n=3), lithium (n=3) and NG-Nitro-L-arginine (L-NA, n=3). An asterisk (*) indicates a statistical difference from control group (p<0.001). Values shown are means ± S.E.M.

Fig. 2. The NOS activity in frontal cortex, hippocampus, and cerebellum measured ex vivo following acute citalopram (5 mg/kg, n=4), Subchronic lithium (60 mmol/kg chow pellet, n=4), acute citalopram + subchronic lithium (5 mg/kg and 60 mmol/kg chow pellet, n=4), and vehicle (0.9% NaCl, n=4). No statistical difference from control group. Values shown are means ± S.E.M.
Effect of acute citalopram and/or subchronic lithium on brain NOS activity (Fig. 2)

In the next part of the experiments, we determined the effects of acute citalopram (5 mg/kg) and subchronic lithium (60 mmol/kg/chow pellet), alone or in combination on NOS activity in frontal cortex, hippocampus, and cerebellum. We found in all studied regions that the NOS activity measured *ex vivo* was unaffected by all treatments compared to controls.

Effect of chronic citalopram and/or subchronic lithium on brain NOS activity (Fig. 3)

In the last part of the experiments we determined the effects of chronic citalopram (20 mg/kg/24h) and subchronic lithium (60 mmol/kg/chow pellet), alone or in combination, on NOS activity in frontal cortex, hippocampus, and cerebellum.

In this part we also found that the NOS activity was unaffected by all treatments compared to controls. The lithium and citalopram levels were stable throughout the experiment (*Table 1*).

![Graph](image)

Fig. 3. The NOS activity in frontal cortex, hippocampus, and cerebellum measured *ex vivo* following chronic citalopram (20 mg/kg/24h, n=10), Subchronic lithium (60 mmol/kg chow pellet, n=14), Chronic citalopram + subchronic lithium (20 mg/kg/24h and 60 mmol/kg chow pellet, n=12), and vehicle (0.9% NaCl, n=9). No statistical difference from control group. Values shown are means ± S.E.M.
The main findings in the present study are that administration of acute citalopram, chronic citalopram or subchronic lithium alone or in combination did not change the NOS activity in hippocampus, frontal cortex, and cerebellum measured ex vivo. However, the NOS activity in vitro was significantly decreased following incubation at a high, non-pharmacological dose of lithium. These negative findings are at odds with clinical studies where depression (20,21), as well as antidepressant treatment in depression (26), has been found to involve actions on the NO pathway. However, the current data confirm and extend earlier pre-clinical studies. Thus, daily single injections for 14 days with citalopram or imipramine were found not to affect the NOS activity in the cerebellum, cortex, and hippocampus (28), while the findings of this study also extend previous work from our laboratory, where acute and chronic citalopram administration failed to influence the hippocampal NOS activity (27).

Considering the different conclusions derived from clinical and pre-clinical studies, it raises the question why these differences are evident, and how to best address the question of NO’s role in antidepressant action.

Concerning the possible effects of lithium on NOS and its downstream signaling pathway, several differences in results and methodology exist. The lack of effect of subchronic lithium on the ex vivo brain NOS activity reported here is also in line with previous reports. Thus, Bagetta and co-workers found no change in endogenous brain citrulline content following a single bolus of lithium 24 h before measurements (9,31). However, it is noteworthy that a very high non-

**Table 1.** Serum values of citalopram (20 mg/kg/24h) and lithium (60 mmol/kg chow pellet). Citalopram samples collected on day 3, 11, 18 and 20. No significant differences between the groups. Lithium samples collected on day 18 and 20. No significant differences between the groups. Values shown are means ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day 3</th>
<th>Day 11</th>
<th>Day 18</th>
<th>Day 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram</td>
<td>10</td>
<td>344.7 ± 27.7</td>
<td>302.8 ± 30.5</td>
<td>337.2 ± 37.3</td>
<td>337.4 ± 32.5</td>
</tr>
<tr>
<td>Citalopram + Lithium</td>
<td>12</td>
<td>344.6 ± 16.1</td>
<td>292.7 ± 16.4</td>
<td>331.6 ± 26.2</td>
<td>323.1 ± 18.6</td>
</tr>
<tr>
<td>Lithium</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>0.78 ± 0.03</td>
<td>0.82 ± 0.05</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Lithium (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram + Lithium</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Lithium</td>
<td>13</td>
<td>-</td>
</tr>
</tbody>
</table>
therapeutic dose of lithium (12 mEq/kg) was used in the latter studies, which clearly limits any conclusions as to their therapeutic relevance. The same is true for the current data on the effects of lithium on NOS activity in vitro, where the inhibition of NOS was present only in non-therapeutic concentrations above 5 mM. Harvey and co-workers also failed to demonstrate any affect of clinically relevant lithium plasma levels on plasma nitrogen oxides (NOx), the end-products of the NO metabolism, following chronic (3 weeks) oral lithium therapy (32). Interestingly, these authors also observed that an effect of chronic lithium on NOx could be realized in the presence of increased glutamatergic activity (32). Since NOS activation is a down-stream event for glutamatergic pathways, this suggests that effects on NOS may only ensue under conditions where NOS is under a state of tonic activation.

Indeed, the effect on NO estimation and metabolism in all the above studies may reflect an action of lithium on the NMDA receptor complex. Lithium has been shown to modulate the NMDA receptor in cultured rat cortical, cerebellar and hippocampal neurons (33). Moreover, lithium treatment in animals modulates synaptic uptake of glutamate in vitro (34) and prevents brain damage in rats following stroke, possibly via NMDA receptor actions (35). Indeed, the evidence in support of a causal role for NMDA receptor dysfunction in depression and antidepressant action is now irrevocable (36-38).

This possible effect of the NMDA receptor complex is also reflected in studies examining the effects of lithium therapy on cGMP formation, believed to be the major downstream signalling pathway of NO (39). Thus, chronic lithium treatment was found to increase cortical cGMP levels (10,32), while Jope and co-workers (1992) found that acute and chronic lithium treatment (4 weeks) had no influence on cortical and hippocampal cGMP levels (55).

In vitro studies, on the other hand, have shown that single administration of lithium inhibits cGMP synthesis in brain homogenates and various cell cultures (40-44). These data, although varied, indicate that lithium may exert distinct effects on cGMP formation, the mechanism of which remains illusive. However, it is of major interest that several studies indicate an important role for cGMP in the regulation of synaptic monoamine levels (25,45), which is also influenced by lithium (7,46).

Both lithium (3) and inhibitors of NOS and sGC (16) have been used as antidepressant augmentatory agents, as have inhibitors of the NMDA receptor (37,47). While the antidepressant efficacy of serotonergic agents, such as the SRI's, implicates diminished 5-HT function in depressive illness (6), significant evidence has accumulated in support of a role for glutamate in depression as well as antidepressant action (37,38). The possible existence of functional cross-talk between glutamate and 5-HT in depression is of significant interest. In this regard, increased NOS activity is associated with animal models of serotonin depletion (48), while serotonin depletion is also associated with long-lasting increases in glutamatergic activity (49). Our results reported here did not find any direct effect on NOS activity when combining citalopram and lithium in normal
unstressed rats. However, we have previously reported that long term citalopram and lithium therapy caused a significant increase in the basal 5-HT level, and that co-administration of chronic citalopram and lithium further potentiated this increase (7).

These and previous studies emphasize the complexity of the neuronal circuits involved in mood regulation (50). Although the current data have provided some information on the actions of citalopram and lithium on brain NOS function, it must be underlined that the circumstances of the current study are not analogous to those of major depressive disorder. Depression is associated with impairments of structural plasticity and cellular resilience, and various studies have demonstrated that signalling pathways involved in regulating cell survival and cell death are important long-term targets for antidepressant action (51). Since neurotoxic elevations of glutamatergic and nitrergic activity have been proposed to underlie the neurodegenerative pathology documented in the hippocampus of depressed patients (52), the actions of antidepressants and lithium on NOS may be realized only under pathological conditions. Indeed, lithium-induced increases in NOx only occur upon activation of glutamatergic circuits (32), while we have recently found that stress evokes a significant increase in hippocampal NOS, together with hippocampal NMDA receptor changes (53). Since stress, and particulary antidepressant action, involves NMDA receptor modulation, it seems reasonable to deduce that actions on NOS may only ensue in situations where NMDA receptors and/or NOS are activated.

In conclusion, we find no evidence that citalopram and lithium alone, or in combination, directly affect NOS activity under basal conditions. Nevertheless, since the NMDA receptor plays a vital role in brain NOS activation (54), secondary actions on NOS could occupy a central role in the action of antidepressants and lithium, especially in pathological states such as anxiety and depression. Further studies evaluating the effects of antidepressants in pathological versus basal states are clearly warranted.

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