Investigation of the neurochemical, molecular and behavioural properties of the putative lithium-mimetic, ebselen

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Abstract

Lithium is the first choice drug for the treatment of bipolar disorder and it is also used as supplementary therapy for treatment-resistant depression. A leading candidate target of lithium is inositol monophosphatase (IMPase) which is critically involved in signalling via the phosphoinositide cycle (PI). Ebselen, first developed as an anti-inflammatory and anti-oxidant drug, has recently been shown to inhibit IMPase and therefore is a putative lithium-mimetic.

This project aimed to investigate the neuropharmacological effects of ebselen in mouse models relevant to depression. Initial studies examined the function of 5-HT_{2A} and 5-HT_{2C} receptors, which signal through PI cycle. Ebselen attenuated the responses of systemically administered 5-HT_{2A} agonists at the behavioural and molecular level; specifically the induction of head twitches and ear scratches and increased immediate early gene (IEG) expression. Lithium produced similar effects to ebselen in the same experiments. In addition, an IMPase inhibitor but not a GSK-3 inhibitor mimicked the effect of ebselen at the behavioural model of 5-HT_{2A} receptor function. Ebselen also reduced 5-HT_{2C} receptor function, as assessed using IEG expression, and lithium had a similar effect. Ebselen was also found to increase 5-HT synthesis in mouse brain regions as previously reported for lithium. In microdialysis studies, ebselen tended to enhance the increase in extracellular 5-HT levels induced by the SSRI citalopram in hippocampus. This finding was consistent with the observation that ebselen and citalopram increased IEG-expression when administered together but not when administered separately. Lastly, ebselen when administered repeatedly increased the abundance of a variety of gene markers of neuronal plasticity in mouse brain regions. Lithium also increased the abundance of markers of neuronal plasticity.

Overall, the present study found that ebselen attenuated 5-HT_2 receptor function and partially augmented the effects of citalopram, while it increased markers of neuronal plasticity. These effects of ebselen were largely mimicked by lithium. These findings show that ebselen has similar neuropharmacological effects to lithium across a range of mouse models relevant to depression. The antidepressant potential of ebselen is discussed.

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I would like to conclude with a poem that kept inspiring me throughout this first step in the world of science:

The first step

The young poet Evmenis
complained one day to Theocritos:
“I have been writing for two years now
and I have composed just one idyll.
It’s my only completed work.
I see, sadly, that the ladder of Poetry
is tall, extremely tall;
and from this first step I now stand on
I will never climb any higher.”
Theocritos replied: “Words like that
are improper, blasphemous.
Just to be on the first step
should make you happy and proud.
To have come this far is no small achievement:
what you have done is a glorious thing.
Even this first step
is a long way above the ordinary world.
To stand on this step
you must be in your own right
a member of the city of ideas.
And it is a hard, unusual thing
to be enrolled as a citizen of that city.
Its councils are full of Legislators
no charlatan can fool.
To have come this far is no small achievement:
what you have done already is a glorious thing.

K. P. Kavafis

Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine
Arc	Activity-regulated cytoskeleton
BCA	Bicinchoninic acid
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
Cg	Cingulate cortex
cpm	Counts per minute
CPU	Caudate putamen
DAG	Diacylglycerol
DAT	Dopamine transporter
Den	Endopiriform nucleus
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DOI	1-(2,5-dimethoxy-4-iodophenyl)-propan-2-amine
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Egr-2	Early growth response-2
ESR	Ear scratch response
FST	Forced swim test
G 6-P	Glucose-6-phosphate
GSK-3	Glycogen synthase kinase 3
HMIT	*H+ myo*- inositol transporter
HPLC	High performance liquid chromatography
HTR	Head twitch response

IC50	Half maximal inhibitory concentration
icv	Intracerebral
IDO	Indoleamine 2,3-dioxygenase
IEG	Immediate early gene
IMPase	Inositol monophosphatase
IP1	Inositol monophosphate
IP2	Inositol bisphosphate
IP3	Inositol 1, 4, 5 trisphosphate
IP3R	Inositol trisphosphate receptor
MAO	Monoamine oxidase inhibitor
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
NCAN	Neurocan
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Phosphoinositide
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase C
Ro 60-0175	6-Chloro-5-fluoro-α-methyl-1*H*-indole-1-ethanamine fumarate
SMIT	Sodium myo-inositol transporter
SSC	Saline sodium citrate
SSC	Somatosensory cortex
SSRIs	Serotonin reuptake inhibitors
TdT	Terminal deoxynucleotidyl transferase
VGLuT	Vesicular glutamate transporter

Publications/Conference proceedings

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2. **Antoniadou I**, Johal E, Kouskou M, Sharp T (2013) Effect of lithium and ebselen, a novel inhibitor of inositol monophosphatase on molecular markers of neuronal plasticity in the mouse brain. Proceedings of the British Pharmacological Society

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Chapter 1

General Introduction

1 General Introduction

1.1 Bipolar disorder

Bipolar disorder (BD) is a chronic and debilitating illness, characterised by mood fluctuations between mania and depression. According to current diagnostic criteria (DSM IV) a manic episode is characterised by extreme, abnormally irritable or euthymic mood that leads the patient to actions with potentially unwanted and harmful consequences. The severity of the manic episodes varies and can lead to occupational and social impairment or even hospitalisation. The mean age of onset of BD is 21 years old. BD has a global prevalence of 0.6-1.4%, there is similar morbidity between females and males, and there are no regional differences according to the World Health Organisation (Merikangas *et al.*, 2011). Patients with BD experience a high relapse rate of 50% within 2 years of recovery of a manic or depressed episode (Perlis *et al.*, 2006). The financial burden of BD is large: for example, in the United States it is estimated to cost 150.0 billion dollars annually (Dilsaver, 2011). First-degree relatives of BD individuals have an increased risk (4-24%) of developing BD. Whilst many gene mutations have been associated with BD, recent meta-analysis studies converge on three loci. As thoroughly reviewed by Craddock *et al.*, strong evidence suggests a link between BD and variations in i) CACNA1C gene, which encodes for the L-type calcium channel, ii) the ANK3 gene, which encodes for ankyrin-G, a protein highly expressed in nodes of Ranvier and involved in the actin-cytoskeleton organisation and iii) the recently reported NCAN gene, which encodes for neurocan, an extracellular matrix glycoprotein which is involved in cell adhesion and migration ((Cichon *et al.*, 2011; Liu *et al.*, 2011; Muhleisen *et al.*, 2012; Craddock *et al.*, 2013). However, strong evidence from a recent, large genome-wide association study of approximately 24,000 patients and

controls suggests that there are several additional risk loci which contribute to BD susceptibility highlighting the heterogeneity of genetic background of the illness (Muhleisen *et al.*, 2014).

There are four subtypes of BD: BD type I which is characterised by at least one episode of mania and episodes of depression; BD type II which is characterised by at least one hypomanic episode (but no manic episodes) and several protracted depressive episodes; cyclothymic disorder which is characterised by hypomanic episodes and depressive symptoms which do not fall to the category of depressive episodes; and BD not otherwise specified which is characterised by hypomanic-like and depressive symptoms that cannot be categorised in any of the aforementioned disorders (Phillips *et al.*, 2013). BD is frequently misdiagnosed as it is difficult to differentiate it from unipolar depression especially when the patient presents during a depressive episode or has not experienced a clear episode of mania (Phillips *et al.*, 2013). The identification of specific biomarkers for BD would help accurate diagnosis of the illness. Emerging evidence from neuroimaging studies demonstrate distinct brain circuity abnormalities in emotion processing neural networks of BD patients suggesting that neural circuit biomarkers might be possible to be identified in BD (Phillips *et al.*, 2013).

1.1.1 Treatment of bipolar disorder

Current therapeutic guidelines for BD suggest lithium as the first line treatment for maintenance but lithium is also used to treat acute manic and depressed episodes (Yatham *et al.*, 2005) (Fountoulakis *et al.*, 2012) (Kessing *et al.*, 2011) (Geddes *et al.*, 2013) (Nivoli *et al.*, 2011). Additional agents used in the maintenance treatment of BD patients include the anticonvulsant lamotrigine and the antipsychotics olanzapine, quetiapine, risperidone and aripiprazole (Yatham *et al.*, 2013) (Fountoulakis *et al.*, 2012). For the treatment of the depressive state of BD patients, the use of lithium in combination with lamotrigine, quetiapine, olanzapine or selective serotonin reuptake inhibitors (SSRIs) in combination with lithium are suggested by current guidelines (Fountoulakis *et al.*, 2012) (Yatham *et al.*, 2013). The anticonvulsants valproic acid and carbamazepine and the antipsychotics asenapine, haloperidol, risperidone, olanzapine, quetiapine, aripiprazole, ziprasidone and paliperidone are amongst the drugs used for the treatment of acute mania (Yatham *et al.*, 2013) (Fountoulakis *et al.*, 2012) (Cipriani *et al.*, 2011).

Meta-analysis of randomized controlled trials clearly demonstrated that lithium is effective in relapse prevention of manic and depressive episodes, and lithium remains the most effective antisuicidal agent (Cipriani *et al.*, 2005) (Geddes *et al.*, 2004). However, shortcomings of lithium treatment arise from its narrow therapeutic index 0.6-1.5 mEq/L which requires regular monitoring of plasma levels. Additionally, prolonged treatment with lithium increases the risk of renal failure, hypothyroidism and hyperparathyroidism, and is associated with weight gain (meta-analysis by (McKnight *et al.*, 2012)).

The mood stabilising effects of lithium in BD patients were first discovered 60 years ago and since 1970 lithium has been approved for use in BD, the pharmacological target mediating the therapeutic effect of lithium remains unclear (Cade, 1949; Shorter, 2009). Currently, the two most popular theories propose that lithium exerts its therapeutic actions either through inhibition of inositol monophosphatase (IMPase) or glycogen synthase kinase 3 (GSK-3). These theories are discussed separately below.

1.2 Properties of IMPase

IMPase is a cytosolic enzyme that catalyses the dephosphorylation of inositol monophosphate (IP1) to inositol (Figure 1) (Takimoto *et al.*, 1985). Inositol is the precursor of the membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP2), which is hydrolysed by phospholipase C (PLC), to generate two signalling molecules, inositol 1, 4, 5 trisphosphate (IP3) and diacylglycerol (DAG) (Figure 1). IMPase is encoded by two genes in mammals, IMPase1 on human chromosome 8q21.13–21.3 (Sjoholt *et al.*, 1997) and IMPase2 on human chromosome 18p11.2 (Yoshikawa *et al.*, 1997). In the mouse it has been found that the two isoforms of IMPase, show several differences. Compared to IMPase1, IMPase2 has lower dephosphorylation activity, is less sensitive to lithium, and its mRNA is found in great abundance in the heart. IMPase1 mRNA is found in higher abundance in the brain, the spleen and testis (Ohnishi *et al.*, 2007). IMPase1 protein is also found to be more abundant in the brain compared to IMPase2 (Ohnishi *et al.*, 2007). IMPase1 mRNA is not equally distributed in the brain, and in mouse higher abundance was found in the olfactory bulb, hippocampus and cerebellum while medium abundance was found in the caudate

putamen, thalamus and hypothalamus. In this thesis I will refer to IMPase1 as IMPase, as the vast majority of studies on the brain have investigated this isoform of the enzyme.

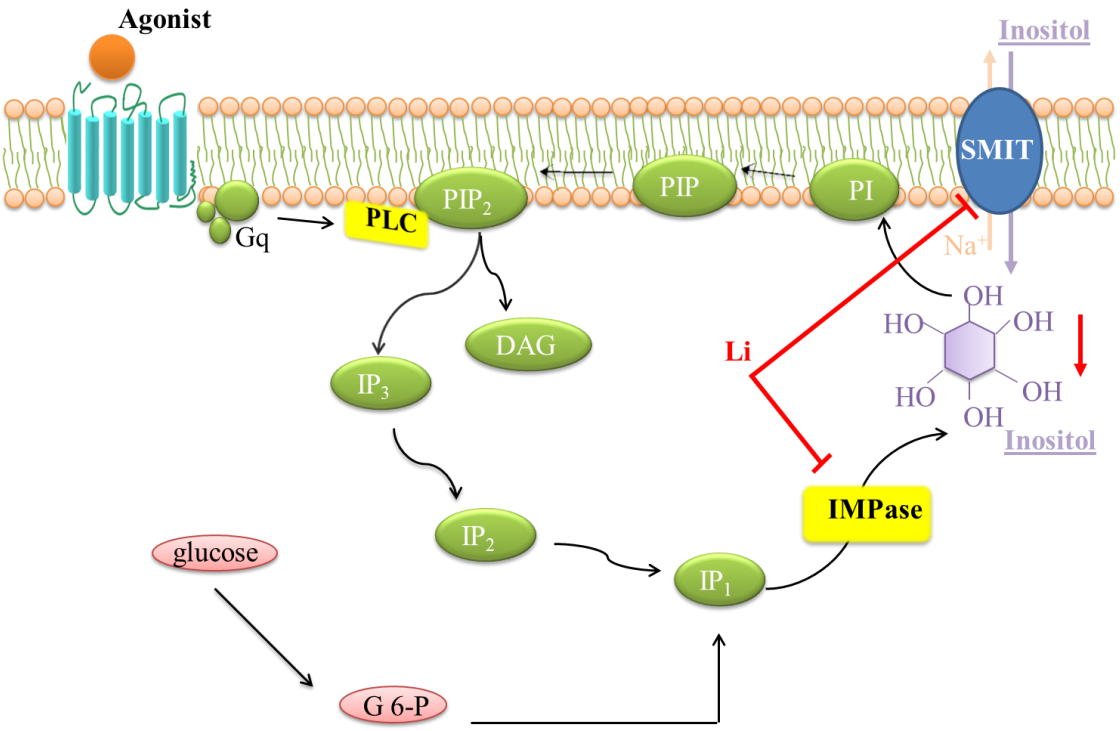

Figure 1: Phosphoinositide signalling. Agonist stimulation of a Gq receptor linked to PI signalling activates phospholipase C (PLC) to hydrolyse phosphatidylinositol 4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP3). IP3 is dephosphorylated to inositol bisphosphate (IP2), then inositol monophosphate (IP1) and finally to inositol. IMPase catalyses the dephosphorylation of IP1 to inositol. Inositol is then phosphorylated to the phosphatidylinositols PI, then PIP and finally PIP2. Extracellular inositol can enter the cell via the sodium myo-inositol transporter (SMIT). Inositol can be also synthesized *de novo* by glucose-6-phosphate (G 6-P). Lithium inhibits IMPase and SMIT and as a result inhibits all known pathways of intracellular inositol sources.

1.3 Sources of intracellular inositol

Intracellular inositol levels are not solely dependent on recycling from IP1 through IMPase. Inositol can be synthesized *de novo* by glucose-6-phosphate that is converted to IP1 by the enzyme myo-inositol-1-phosphate synthase (Naccarato *et al.*, 1974). However, in this pathway IMPase is also required to catalyse the dephosphorylation of IP1 to inositol (Figure 1). Therefore, inhibition of IMPase by lithium leads to inhibition of the *de novo* synthesis of inositol, too. Inositol can also be imported into the cell from the extracellular environment by a sodium *myo*-inositol transporter (SMIT) and a H^+ *myo*-inositol transporter (HMIT) (Kwon *et al.*, 1992; Uldry *et al.*, 2001). Lithium has been also shown to inhibit SMIT of human astrocytic cells at therapeutic concentrations (Lubrich *et al.*, 1999).

1.4 Inositol depletion hypothesis of lithium action

In the CNS, inositol levels are dependent on synthesis and recycling of inositol, as inositol crosses the blood brain barrier through a low capacity, saturable system (Spector, 1988). Lithium inhibits IMPase at concentrations within the therapeutic range; 0.6-1.5 mEq/L with the IC_{50} of IMPase inhibition by lithium around 0.8 mM (Hallcher *et al.*, 1980; Hopkins *et al.*, 2000). The first indication that lithium inhibits IMPase came in 1971 from an *in vivo* experiment in rats, where acute systemic lithium administration was found to decrease brain levels of inositol. The greatest reduction (30%) of inositol occurred 12 h following lithium administration and started recovering 12 h later (Allison *et al.*, 1971; Allison *et al.*, 1980). A subsequent experiment showed that lithium administration also increased IP1 in cortical

regions (Allison *et al.*, 1976). These findings suggested that lithium interacts with the recycling of inositol possibly by inhibiting IMPase, which metabolizes IP1 to inositol. Later experiments confirmed that lithium inhibits IMPase by occupying the binding site of magnesium which is required for the dissociation of the enzyme from the phosphate group of IP1 (Atack *et al.*, 1995).

IMPase has two metal binding sites which are normally occupied by magnesium. Upon substrate binding to IMPase two magnesium ions bind to the metal binding sites of the enzyme and inositol is released, leaving the phosphate group bound to the two metals. One magnesium is then released, before phosphate group is released from the enzyme and then the second magnesium is also released. However, when lithium is present, it occupies the second magnesium binding site which does not allow the release of the phosphate group from the enzyme, and this essentially leads to inhibition of the enzyme (reviewed by (Atack *et al.*, 1995)).

In 1989 Berridge formulated the inositol depletion hypothesis to explain the mechanism of action of lithium (Berridge *et al.,* 1989). This hypothesis postulated that lithium attenuated receptor signalling in the brain by decreasing the generation of second messengers generated by receptor stimulation. Specifically, it was proposed that lithium inhibited IMPase and reduced inositol levels which then diminished the PIP2 pool and the subsequent generation of the second messengers IP3 and DAG. Notably, lithium was found to inhibit IMPase in an uncompetitive manner (Sherman *et al.*, 1981), meaning that the higher the substrate levels the greater the inhibition of the enzyme. Therefore, it was suggested that lithium would be even more effective in hyperactive PI-linked receptors which was hypothesized to be the

case in BD. In the brain several neutrotransmitters signal through the PI pathway (see table 1).

Ligand	Receptor
5-Hydroxytryptamine	$5\text{-}HT_{2A}$
	$5\text{-}HT_{2B}$
	$5\text{-}HT_{2C}$
Acetylcholine	M_1
	M_3
	M_5
Adenosine	A_{2B}
	A_3
Adrenaline	α_{1A}
	α_{1B}
	α_{1D}
Glutamate	$mGluR_1$
Histamine	H_1
Oxytocin	OXTR
Substance P	NK1R
Vasopressin	V_{1A}
	V_{1B}

Table 1: Example of neurotransmitters that signal through the PI pathway.

1.4.1 Evidence for the inositol depletion hypothesis

In support of the inositol depletion hypothesis, lithium was found to decrease PIP2 levels in cultured cells, following muscarinic Gq-linked receptor stimulation with carbachol (Jenkinson *et al.*, 1994). Additionally, it was found that lithium decreased carbachol-induced increase of IP3 levels in cultured cells (Varney *et al.*, 1992; Jenkinson *et al.*, 1995). Lithium application also attenuated carbachol induced elevation of IP3 in rat cerebral slices (Kennedy *et al.*, 1989; Kennedy *et al.*, 1990; Varney *et al.*, 1992). The attenuation in IP3 response following agonist stimulation by lithium, was also confirmed in isolated cerebral slices from rats treated *in vivo* with lithium (Varney *et al.*, 1992). In the same study it was also reported that calcium release following carbachol stimulation was attenuated in cultured cells pre-treated with lithium (Varney *et al.*, 1992). More recently, pharmacological inhibition of IMPase with the selective inhibitor L-690,330 and its prodrug L-690,448 was also found to attenuate carbachol-induced increase of IP3 in cultured cells in a way similar to lithium (Atack *et al.*, 1994; Atack, 1997). Overall, these studies demonstrated that lithium attenuated Gq receptor signalling in cell cultures and cortical slices, and that this effect was mediated through IMPase inhibition.

The reduction of inositol induced by lithium treatment *in vivo* as reported in 1971 by Alison *et al.* was further validated in more recent studies which also showed that the decrease in inositol by lithium is maintained following repeated administration. Specifically, lithium treatment of rats for 2-4 weeks was found to decrease inositol in whole brain extracts but also in selective brain regions, as measured by nuclear magnetic resonance spectroscopy or high performance liquid chromatography (Lubrich *et al.*, 1997; O'Donnell *et al.*, 2000;

McGrath *et al.*, 2006). Additionally, repeated lithium treatment of mice for 2-4 weeks was also found to increase IP1 levels in the brain (O'Donnell *et al.*, 2000; Pettegrew *et al.*, 2001). Interestingly, lithium increased IMPase mRNA in hippocampus and cortex of mice treated repeatedly with lithium, and this effect was considered to reflect adaptation to repeated IMPase inhibition by lithium (Parthasarathy *et al.*, 2003; Shamir *et al.*, 2003).

Evidence to support the inositol depletion hypothesis also comes from behavioural studies. Early studies found that treatment of rats with lithium prior to pilocarpine produced limbic seizures which was accompanied by an increase in IP1 levels in the brain (Honchar *et al.*, 1983). Moreover, it has consistently been reported that inositol reverses the augmentation of pilocarpine-induced seizures by lithium. In particular, intracerebral (icv) injection of inositol has been found to increase the seizure latency in animals receiving combination of lithium and pilocarpine (Kofman *et al.*, 1991; Kofman *et al.*, 1993; Belmaker *et al.*, 1998). This finding was replicated in other studies which found a high dose of inositol (11 µmol) to completely inhibit the proconvulsive effect of lithium (Tricklebank *et al.*, 1991). A later study also showed that peripheral administration of a high dose of inositol to rats increased inositol levels in the brain and attenuated the proconvulsive effect of lithium on pilocarpine-induced seizures (Agam *et al.*, 1994).

The ability of inositol to reverse behavioural effects of lithium in rodents has been investigated in other models. In particular, lithium injection to rats decreased rearing and this effect was reversed by icv administration of inositol (Kofman *et al.*, 1990). Additionally, lithium enhanced the 5-HT behavioural syndrome induced by the 5-methoxy-

N,*N*-dimethyltryptamine and this effect of lithium was reversed by acute icv administration of inositol (Kofman *et al.*, 1995).

Some studies have compared the behavioural effects of lithium with those of the selective IMPase inhibitor L-690,330. Like lithium, L-690,330 administered icv was found to enhance pilocarpine-induced seizures. In addition, L-690,330 decreased immobility time in the forced swim test (FST) in a way similar to lithium (Shtein *et al.*, 2013). In the FST, inositol administration through the icv route, did not reverse the decrease in immobility time induced by lithium (Toker *et al.*, 2013). Lastly, inhibition of calbidin D-28k which is a protein that increases IMPase activity, was found to decrease immobility time in the FST in a way similar to lithium (Shamir *et al.*, 2005) (Levi *et al.*, 2013).

1.4.2 Genetic knockout of IMPase and SMIT

The inositol depletion hypothesis *in vivo* has also been tested using genetic knockout mice for IMPase and SMIT. IMPase1 knockout mice are not viable and die at the ninth day in the uterus however, inositol supplementation in diet allows the animals to survive (Cryns *et al.*, 2008). Although IMPase1 homozygous knockout mice show a reduction of IMPase activity, the levels of inositol between wild type and transgenic animals did not differ. Interestingly, IMPase knockout animals showed increased sensitivity to the pilocarpine-induced seizures and a decrease in the immobility time in the FST, effects similar to lithium (Cryns *et al.*, 2008). In comparison, IMPase2 knockout mice, did not show any reduction in inositol levels compared to wild type mice and did not resemble lithium in the pilocarpine seizure or the amphetamine-induced hyperactivity models (Cryns *et al.*, 2007). However, IMPase2

knockout female mice were found to exhibit decreased immobility time in the FST, which is similar to the effect of lithium (Cryns *et al.*, 2007). Lastly, it was recently reported that IMPase1 mutant mice generated with the N-ethyl-N-nitrosourea mutagenesis technique, die perinatally. The IMPase1 mutants were rescued with inositol supplementation, but showed severe developmental defects. These mutant IMPase1 mice behaved similar to lithium in the FST, but were not tested in the pilocarpine-induced seizures or amphetamine-induced hyperactivity models (Ohnishi *et al.*, 2014). Overall, these findings suggest that genetic loss of the IMPase1 gene is associated with lithium-like effects in the pilocarpine-induced seizure model and FST.

In mice genetic knockout of SMIT is lethal and animals die at birth due to apnoea (Berry *et al.*, 2003). However, the phenotype is rescued by supplementation of inositol in the drinking water of pregnant mice. Compared to wild type controls, SMIT knockout mice had decreased inositol levels in hippocampus and frontal cortex. Interestingly, homozygous SMIT knockout mice had increased sensitivity to pilocarpine-induced seizures and decreased immobility time in the FST, effects similar to those produced by lithium (Bersudsky *et al.*, 2008a). SMIT heterozygous mice whilst having decreased inositol levels in hippocampus and frontal cortex, did not mimic the effects of lithium in the pilocarpine-induced seizure, FST or amphetamine-induced hyperactivity models (Shaldubina *et al.*, 2006; Shaldubina *et al.*, 2007). The latter finding raised the question of whether by itself, reduction of brain inositol levels is sufficient to produce lithium-like effects (Agam *et al.*, 2009).

1.4.3 Preclinical and clinical studies of inositol depletion hypothesis

Studies in humans have not been successful in drawing firm conclusions regarding the inositol depletion hypothesis. Platelets derived from BD patients were found to have decreased PIP2 levels following lithium treatment (Soares *et al.*, 2000). Additionally, IMPase mRNA was found to be increased in lymphocytes of BD patients under lithium treatment, possibly indicating an adaptive response to inhibition of IMPase by lithium (Nemanov *et al.*, 1999). *In vivo* studies using magnetic resonance spectroscopy (MRS) are not conclusive due to both clinical and technical limitations (reviewed by (Silverstone *et al.*, 2005). To be more specific, the metabolite changes in MRS studies, are expressed as ratio of an internal standard which is a brain metabolite that is assumed not to be altered between controls and patients. This metabolite is usually creatinine however, studies have shown that creatinine can be also altered in pathological and treatment conditions. Additionally, the studies suffer from small sample sizes, while there are large differences in patients tested in MRS studies in terms of sex, mood, age or other comorbidities that makes drawing conclusions more difficult (Silverstone *et al.*, 2005; Yildiz-Yesiloglu *et al.*, 2006; Silverstone *et al.*, 2009). Review of the current findings, cannot exclude or strongly support the inositol depletion hypothesis. More consistent findings seem to occur in BD patients in the depressed state, treated with lithium, in who lithium is found to increase phosphomonoester levels, such as IP1 (Silverstone *et al.*, 2005; Silverstone *et al.*, 2009).

1.4.4 Limitations to inositol depletion hypothesis

Although there are several lines of evidence in support of the inositol depletion hypothesis as a mechanism of action of lithium in BD, confirmation of the hypothesis is hindered by the lack of suitable pharmacological and genetic tools to manipulate IMPase. The available IMPase inhibitors (L-690,330 and its prodrug L-690,448) suffer from low bioavailability and IMPase knockout mice require inositol supplementation in order to survive (Cryns *et al.*, 2008) (Atack, 1997). Regarding the available IMPase inhibitors, although they have been very useful in investigating the effects of IMPase inhibition in cell models, they were not as useful *in vivo* as they were found to have low bioavailability (Atack, 1997). IMPase genetic knockout mice, were found to die in uterus, unless pregnant mice were supplemented with inositol and as a result the offspring did not have reduced inositol levels, which is one of the fundamental effects of lithium (Cryns *et al.*, 2008). Another important question, is whether the effects of lithium on IMPase occur globally in the brain, or whether there are region specific changes that actually mediate the therapeutic effects. For example IMPase shows distinct distribution in the mouse brain and is not equally abundant in all brain regions (Ohnishi *et al.*, 2007). Lastly, it should be considered whether reduction of inositol as a result of IMPase inhibition is the actual mediator of the effects of lithium, or whether IMPase inhibition triggers downstream effects, that could lead to more long term changes, associated with the clinical effects. For example, lithium has been found to increase neuronal plasticity in bipolar disorder patients and a limited number of experiments has investigated the mechanism through which lithium might exert this effect.

1.4.5 Neuroplasticity changes in BD

Relatively modern theories suggest that mood disorders result from maladaptive changes at the level of synapse which result in structural abnormalities in the brain presented as reduced volume of brain regions including the prefrontal cortex and hippocampus and synaptic deficits, which are reversed by psychotropic agents that enhance synaptogenesis through the activation of a gene expression programme (Sharp, 2013) (Duman *et al.*, 2012). Focusing in BD, a mega-analysis of individual neuroimaging data revealed structural brain abnormalities in non-treated BD patients, and enhanced hippocampal and amygdala volumes associated with lithium treatment (Hallahan *et al.*, 2011). Magnetic resonance imaging studies have found that repeated lithium treatment resulted in grey matter volume increase in patients with bipolar disorder (Moore *et al.*, 2000; Lyoo *et al.*, 2010). This volumetric increase was linearly correlated to the duration of treatment and was associated to mood improvement (Lyoo *et al.*, 2010). At the molecular level, lithium has been shown to increase brain-derived neurotrophic factor (BDNF) in BD patients (discussed in detail in Chapter 5).

In relevant animal studies, lithium has been shown to increase neurogenesis in the dentate gyrus of mice (Chen *et al.*, 2000). In addition, lithium was found to enhance BDNF in rodents (discussed in detail in Chapter 5). The mechanism underlying the effects of lithium on neuronal plasticity has been little investigated and evidence supports that inositol depletion might play a fundamental role. In particular, lithium has been found to increase growth cone area and reduce cone collapse of primary cultures derived from rat dorsal root ganglia and these effects were reversed by inositol, indicating that lithium and consequently

inositol depletion has neurotrophic effects (Williams *et al.*, 2002). Lithium has been also found to increase synapse formation in rat primary neuronal cultures and this effect was reversed by inositol supplementation (Kim *et al.*, 2009). In *c. elegans*, lithium has also been found to play a pivotal role in synaptic vesicle localisation which is reversed by inositol supplementation (Tanizawa *et al.*, 2006).

1.5 GSK-3 hypothesis of lithium action

A recent hypothesis proposes that lithium exerts its therapeutic action through inhibition of glycogen synthase kinase-3 (GSK-3). GSK-3 is a cytosolic protein found in two isoforms (GSK-3α and GSK-3β). GSK-3 has a plethora of substrates and consists a convergence point of several different pathways, such the Wnt, insulin and neurotrophin signalling pathways (review by (Jope *et al.*, 2004) (Gould *et al.*, 2005). Lithium was found to inhibit GSK-3 in the concentration range 1-2 mM (Klein *et al.*, 1996; Stambolic *et al.*, 1996). It was later shown that lithium inhibited GSK-3 in a competitive way by binding to a magnesium site on the enzyme (Ryves *et al.*, 2001). Although at therapeutic levels lithium (0.6-1.2 mEq/l) would only lead to a partial inhibition of GSK-3 (IC_{50}=1-2 mM) it was suggested that lithium further decreased GSK-3 activity indirectly. Thus, it has been shown that lithium increases the phosphorylation of the protein kinase Akt, which leads to phosphorylation and therefore inactivation of GSK-3 (reviewed by (O'Brien *et al.*, 2009) (Gould *et al.*, 2005).

The involvement of GSK-3 inhibition in behavioural effects of lithium has been investigated in the FST and amphetamine-induced hyperactivity model. A range of GSK-3 inhibitors

(indirubin, alsterpaullone, TDZD-8, AR-A014418, SB-216763 and SB-627772) have been shown to decrease amphetamine-induced hyperactivity in mice in a way similar to lithium (Kalinichev *et al.*, 2011) (O'Brien *et al.*, 2011). However, this effect was not replicated by a recent study in which two selective GSK-3 inhibitors (AZ1080 and compound A) were found to have no effect on amphetamine-induced hyperactivity in mice (Caberlotto *et al.*, 2013). Similarly, studies using heterozygous GSK-3 knockout mice have not consistently shown a decreased locomotor response to amphetamine as is consistently seen with lithium (Bersudsky *et al.*, 2008b) (Beaulieu *et al.*, 2008) (Beaulieu *et al.*, 2004). On the other hand, mice overexpressing GSK-3 show increased locomotor activity (Prickaerts *et al.*, 2006). However, it has not been investigated whether GSK-3 inhibitors or lithium stabilises the hyperactivity observed.

The effect of lithium on locomotion has been also tested in dopamine transporter (DAT) knockout mice (Beaulieu *et al.*, 2004) which have increased dopamine release resembling that induced by amphetamine. DAT knockout mice also have decreased phosphorylated levels of Akt and GSK-3. Lithium was shown to decrease locomotor activity in DAT knockout mice and to increase phosphorylation of Akt and GSK-3. Moreover, these effects were mimicked by GSK-3 inhibitors (Beaulieu *et al.*, 2004). Akt and GSK-3 have been shown to form a complex with β-arrestin, which leads to inactivation of Akt but activation of GSK-3 (O'Brien *et al.*, 2011). In β-arrestin knockout mice, lithium failed to induce phosphorylation of Akt and GSK-3 and did not reduce amphetamine-induced hyperactivity, suggesting that this complex is implicated in the effect of lithium in this behavioural model (O'Brien *et al.*, 2011) (Beaulieu *et al.*, 2008). However, a selective GSK-3 inhibitor decreased amphetamine-induced hyperlocomotion in β-arrestin knockout mice, raising the

concern of the association between GSK-3 inhibition and the effects of lithium in this model (O'Brien *et al.*, 2011). One of the numerous substrates of GSK-3 is β-catenin, which is shown to increase following GSK-3 inhibition (Kaidanovich-Beilin *et al.*, 2004). Therefore, overexpression of β-catenin would be expected to mimic the effects of lithium. However, amphetamine administration to mice overexpressing β-catenin elicited a similar response to control animals (Gould *et al.*, 2007). When these mice received amphetamine continuously for 5 days they showed a decrease in locomotion compared to controls, and this effect was mimicked by lithium (Gould *et al.*, 2007).

In the rat FST, a selective GSK-3 inhibitor was shown to decrease immobility time in a way similar to lithium (Gould *et al.*, 2004). Additionally, icv injection of a peptide inhibitor of GSK-3 was shown to decrease immobility time in the mouse FST (Kaidanovich-Beilin *et al.*, 2004). GSK-3β heterozygous knockout mice have been shown in one study to spent less time immobile, but this effect was not replicated by a second study (Bersudsky *et al.*, 2008b) (O'Brien *et al.*, 2004). β-catenin knockout mice show reduced immobility time in the FST (Gould *et al.*, 2007).

Overall, both IMPase and GSK-3 are targets of lithium and there is plausible evidence to link the inhibition of these enzymes to lithium's therapeutic effect. Interestingly, a recent study in yeast found that mutation of GSK-3 lead to inositol depletion, suggesting that the two pathways might converge, but this finding needs to be confirmed (Azab *et al.*, 2007).

1.6 5-HT hypothesis of lithium action

As well as being an important treatment of BD, lithium is also used as augmentation agent in treatment-resistant depression as discussed further in Chapter 4 (Price *et al.*, 1994) (Wood *et al.*, 1987) (Price *et al.*, 1990b). It has been suggested that lithium is effective in unipolar depression by increasing the function of the 5-HT system. In early key studies in rats, lithium treatment was found to increase 5-HT and 5-HIAA levels in brain tissue and also increase the release of 5-HT (Treiser *et al.*, 1981) (Sheard *et al.*, 1970). Lithium was also reported to increase 5-HT turnover in a similar way to l-tryptophan, while administration of lithium and l-tryptophan augmented the effect of each treatment alone (Broderick *et al.*, 1982). Additionally, in electrophysiological studies lithium was found to increase 5-HT function at the post-synaptic level (Sharp *et al.*, 1990) (Blier *et al.*, 1985). Furthermore, lithium was found to produce the 5-HT syndrome in rats when lithium was administered in combination with a monoamine oxidase inhibitor (MAO) such as was observed using a combination of l-tryptophan and MAO inhibitor. The latter effect of lithium was inhibited when a tryptophan hydroxylase inhibitor was administered prior to lithium and MAO inhibitor (Grahame-Smith *et al.*, 1974).

In human studies, lithium was also shown to increase 5-HT function. In particular lithium treatment was found to increase the levels of 5-HIAA in the cerebrospinal fluid of BD patients (Shiah *et al.*, 2000) (Price *et al.*, 1994). Additionally, lithium was shown to increase 5-HT function in neuroendocrine challenge studies. Specifically, lithium enhanced the prolactin response to l-tryptophan in depressed patients (Cowen *et al.*, 1990) (Price *et al.*,

1990a). The latter findings are consistent with the above animal data suggesting that lithium increases pre-synaptic 5-HT function.

1.6.1 Effect of lithium on 5-HT$_2$ receptors

Lithium is suggested to increase 5-HT neurotransmission and subsequently augment the effects of antidepressants through the increase of 5-HT$_{1A}$ and the decrease of 5-HT$_2$ post-synaptic receptor function. Lithium is suggested to enhance post-synaptic 5-HT$_{1A}$ receptor function supported by the observations that it augmented the effects of 5-HT$_{1A}$ receptor agonists in the serotonin syndrome and the behavioural model of forepaw trading (Uchitomi *et al.*, 1993) (Uchitomi *et al.*, 1987) (Goodwin *et al.*, 1986a) and augmented the disinhibition of CA3 pyramidal neurons induced by antidepressants, through enhancement of 5-HT$_{1A}$ function (Haddjeri *et al.*, 2000). These effects of lithium on post-synaptic 5-HT$_{1A}$ receptor function have been suggested to be involved in the antidepressant effects of the drug as it has been shown for other antidepressants and electroconvulsive treatment (Savitz *et al.*, 2009). However, the present project is mainly focused on the effects of lithium on 5-HT$_2$ receptor function.

The effects of lithium on 5-HT$_2$ receptor function in both animal models and humans is discussed in Chapters 2 and 3. Briefly, lithium has been reported to attenuate 5-HT$_2$ receptor-induced behavioural effect, that of head twitch response, in various studies (Goodwin *et al.*, 1986b; Hotta *et al.*, 1986). Additionally, in a sleep model of 5-HT$_2$ function

in humans, lithium has also been shown to attenuate the function of the 5-HT_2 receptor by increasing slow wave sleep (Friston *et al.,* 1989).

The 5-HT_2 receptors are members of the G-protein coupled receptor family of proteins coupled to PLC-phosphoinositide signalling. Three subtypes have been identified the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (Barnes *et al.*, 1999). Recently, 5-HT_{2A} and 5-HT_{2C} receptors have attracted attention regarding their possible role in antidepressant augmentation in treatment-resistant depression. As reviewed by Celada *et. al* and Quesseveur *et al.* this recent interest in these receptors arises in part from observations that atypical antipsychotics that possess antagonistic properties for the 5-HT_{2A} and 5-HT_{2C} receptors are effective in combination with SSRIs in treatment-resistant depressed patients (Celada *et al.*, 2004) (Quesseveur *et al.*, 2012). It is postulated that the utility of 5-HT_{2A} and 5-HT_{2C} receptor antagonists in SSRI augmentation relates to the role of these receptors in the negative feedback control of 5-HT neuronal firing (Sharp *et al.,* 2007).

Evidence from animal studies, suggest that activation of 5-HT_{2A} and 5-HT_{2C} receptors decreases 5-HT neuronal activity. In particular, *in vivo* electrophysiological studies demonstrated that systemic administration of 5-HT_{2A} and 5-HT_{2C} receptor agonists in rats decreased the firing rate of 5-HT neurons in the dorsal raphe (Wright *et al.*, 1990) (Garratt *et al.*, 1991) (Kidd *et al.*, 1991) (Martin-Ruiz *et al.*, 2001) (Boothman *et al.*, 2003) (Queree *et al.*, 2009) (Boothman *et al.*, 2006a) (Aghajanian *et al.*, 1970), the effects being reversed by selective 5-HT_{2A} and 5-HT_{2C} antagonists respectively (Martin-Ruiz *et al.*, 2001) (Boothman *et al.*, 2003) (Queree *et al.*, 2009) (Boothman *et al.*, 2006a). These observations are consistent with data obtained from rat microdialysis studies showing that systemic

administration of a 5-HT_{2A} agonist decreased 5-HT release in the frontal cortex (Wright *et al.*, 1990) (Garratt *et al.*, 1991) (Martin-Ruiz *et al.*, 2001), an effect also antagonised by 5-HT_{2A} receptor blockade (Martin-Ruiz *et al.*, 2001). Furthermore, administration of 5-HT_2 antagonists in combination with SSRIs has been reported to augment the increase in 5-HT levels evoked by SSRIs (Gobert *et al.*, 2000; Cremers *et al.*, 2004; Boothman *et al.*, 2006b). Collectively, these findings suggest that 5-HT_{2A} and 5-HT_{2C} receptors are involved in the negative feedback control of 5-HT neurons and that inhibition of these receptors leads to augmentation of the effects of SSRIs; since lithium also reduces 5-HT_2 function it could also enhance the effects of antidepressants in this way.

1.7 IMPase inhibitors and the repurposing of ebselen

Attempts to develop selective IMPase inhibitors using medicinal chemistry have resulted in useful pharmacological tools as discussed above. Specifically, Merck developed the selective inhibitor L-690,330 (IC_{50}=0.33 μM) based on the structure of IP1. However, L-690,330 suffered from weak cell permeability and as a result the administration of high doses was required to achieve IMPase inhibition both in cell cultures and *in vivo* (Atack *et al.*, 1993). Synthesis of an esterified prodrug of L-690,330 (L-690,488) did not improve bioavailability of the drug (Atack, 1997).

A recent attempt has been made to identify IMPase inhibitors using ‘Drug repurposing’, which is essentially the identification of new uses for old drugs that have been in clinical trials but lacked efficacy. Drug repurposing has attracted much attention because it offers

the possibility of fast-tracking drugs into clinical development compared to traditional medicinal chemistry based drug discovery approaches (Mullard, 2012). Specifically, one major advantage of drug repurposing is that the pharmacodynamic/pharmacokinetic profile and the toxicity and safety of the drugs is known, allowing for accelerated investigation of drugs in human subjects. Singh *et al.* searched a NIH drug database of 450 compounds approved for phase I-III clinical trials, for molecules with inhibitory actions at IMPase and found that the drug ebselen (Figure 2) inhibited IMPase with good potency (IC_{50}=1.5 μM) (Singh *et al.*, 2013).

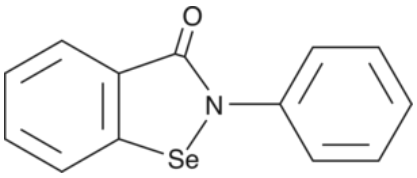

Figure 2: Chemical structure of ebselen

Ebselen was originally developed to mimic glutathione peroxidase and thus act as an anti-oxidant. The drug also has anti-inflammatory properties as it inhibits the production of prostaglandins (for reviews (Parnham *et al.*, 1991; Parnham *et al.*, 2013) (Schewe, 1995). Ebselen was also demonstrated to have neuroprotective effects in animal models of ischemic damage (Dawson *et al.*, 1995). In clinical studies ebselen was tested in patients with ischemic injury and stroke but found to lack efficacy (Yamaguchi *et al.*, 1998) (Saito *et al.*, 1998) (Ogawa *et al.*, 1999). In all these studies ebselen showed no significant adverse effects. Current studies are recruiting patients for the testing of ebselen in noise-induced hearing loss.

With the view that anti-oxidants might have antidepressant properties studies have investigated ebselen in antidepressant models and found that it is active in the FST and also has antimanic properties as demonstrated by the attenuation of amphetamine-induced hyperactivity model (Posser *et al.*, 2009) (Singh *et al.*, 2013)

The recent Singh *et al.* study showed that ebselen inhibited IMPase in lower concentrations compared to lithium in enzyme assays, and it was also found that ebselen inhibited IMPase in the brain *ex vivo* after administration in mice. Additionally, ebselen was shown to attenuate amphetamine-induced hyperactivity in mice and this effect was reversed by intracerebral injection of inositol suggesting IMPase inhibition as the mechanism of action (Singh *et al.*, 2013). Following on from this study, the aim of the present project was to investigate the neuropharmacological effects of ebselen in models relevant to depression in which lithium was active. The main aims are detailed below:

1.8 Aims

1. The aim of the experiments described in Chapter 2 was to investigate the effect of ebselen on the function of the central 5-HT_{2A} receptor (which signals via the PI cycle) at the behavioural and molecular level, and to evaluate the involvement of IMPase inhibition. Effects of ebselen were compared to lithium and inhibitors of IMPase and GSK-3.

2. The aim of the experiments described in Chapter 3 was investigate the effect of ebselen on the function of the central 5-HT_{2C} receptor, which also signals via the PI pathway. 5-HT_{2C} receptor function was investigated at the behavioural and molecular level and compared to lithium.

3. The aim of the experiments described in Chapter 4 was to examine the effect of ebselen on central 5-HT synthesis and release. This was complimented by studies on the effect of ebselen in combination with an SSRI, at the neurochemical and molecular level

4. The aim of the experiments described in Chapter 5 was to investigate the effect of ebselen on genetic markers of neuronal plasticity that are linked to antidepressant effects. The effect of ebselen was compared to that of lithium.

Chapter 2

Investigation of the effects of ebselen on central 5-HT_{2A} receptor function of the mouse

2 Investigation of the effects of ebselen on central 5-HT_{2A} receptor function of the mouse

2.1 Introduction

As noted in the General Introduction, the central 5-HT_{2A} receptor is an important target of lithium and is linked to bipolar disorder and major depression. Notably, the 5-HT_{2A} receptor is a major target of some antipsychotic agents and in particular atypical antipsychotics such as amperozide, clozapine, risperidone, zotepine, olanzapine and quetiapine (Meltzer *et al.*, 2011). Current studies support the use of antipsychotics in the treatment of bipolar disorder and major depression (for reviews see (Derry *et al.*, 2007; Geddes *et al.*, 2013)). Experiments described in this chapter examine the effect of ebselen on 5-HT_{2A} receptor function and compare to lithium.

The 5-HT_{2A} receptor belongs to the Gq family of receptors and signals through the PI cycle (for review see (Barnes *et al.*, 1999)). The 'inositol depletion hypothesis' of Berridge predicts that lithium, by inhibiting IMPase, would attenuate signalling of Gq-linked 5-HT_{2A} receptors (Berridge *et al.*, 1989). Evidence for this comes from a plethora of experiments in human and animal cell lines. These studies show that lithium causes IPx accumulation following 5-HT_{2A} receptor agonist administration to the cells (Briddon *et al.*, 1998; Rabin *et al.*, 2002; Kurrasch-Orbaugh *et al.*, 2003). In agreement with these findings, lithium attenuates 5-HT_2 receptor function *in vivo* in humans but also in animal models (discussed in more detail below) (Goodwin *et al.*, 1986b; Friston *et al.*, 1989; Basselin *et al.*, 2005). The effect of ebselen on 5-HT_{2A} receptor function has never been explored. There are well

validated models to study 5-HT_{2A} receptor function (see below) and lithium has been shown to be effective in these models. Thus it would be interesting to explore the effects of ebselen in such models.

The models of 5-HT_{2A} receptor function rely on the pharmacological selectivity of the available 5-HT_{2A} drug tools. The 5-HT_{2A} receptor shares high degree of homology with the 5-HT_{2C} receptor and available agonists have variable selectivity and efficacy for these receptors (Newton *et al.*, 1996). For example, DOI ($pK_{i\,(2A)}$ = 9.19, $pK_{i\,(2C)}$ = 8.27) and LSD ($pK_{i\,(2A)}$ = 9.12, $pK_{i\,(2C)}$ = 8.96) are partial 5-HT_{2A} agonists (McClue *et al.*, 1989; Nichols *et al.*, 1994; Knight *et al.*, 2004). However, available antagonists have high selectivity for either 5-HT_{2A} or 5-HT_{2C} receptor. For example, the antagonists MDL 100,907 ($pK_{i\,(2A)}$ = 9.07, $pK_{i\,(2C)}$ = 7.06) and ketanserin ($pK_{i\,(2A)}$ = 8.5, $pK_{i\,(2C)}$ = 6.7) show selectivity for 5-HT_{2A} receptors while SB242084 ($pK_{i\,(2A)}$ = 6.8, $pK_{i\,(2C)}$ = 9) also shows significant selectivity for 5-HT_{2C} receptors (Bonhaus *et al.*, 1995; Barnes *et al.*, 1999; Damjanoska *et al.*, 2003).

Agonists at the 5-HT_{2A} receptor elicit a range of behavioural and physiological effects in animals and much evidence indicates the involvement of 5-HT_{2A} receptor. For example, early studies used a two-lever drug discrimination paradigm to explore the pharmacology of the 5-HT_{2A} receptor. In this paradigm rats are trained to discriminate saline from a 5-HT_{2A} agonist. With the use of antagonists one can explore the pharmacology of the training drug (Nichols, 2004). Although time-consuming this paradigm has been used to great effect. For example, the stimulus effects of LSD were inhibited by a variety of 5-HT_{2A} antagonists and there was a correlation between the 5-HT_{2A} affinities of these antagonists and their potency to block the effects of LSD. No such correlation was shown for the 5-HT_{2C} receptor

antagonists in this model (Fiorella *et al.*, 1995). Similar results to LSD were obtained for DOI (Schreiber *et al.*, 1994).

A more straightforward and well validated behavioural model of 5-HT_{2A} receptor function is the head-twitch response (HTR), which is elicited by all hallucinogenic 5-HT_{2A} receptor agonists. HTR is a transient, side-to-side movement of the head of the animal. It is an unnatural, stereotypical behaviour, induced by 5-HT_{2A} agonists and may be linked to the hallucinogenic properties of such drugs. The HTR has been shown to be mediated through the 5-HT_{2A} receptor in a plethora of studies. In particular, it has been shown that DOI-induced HTR is abolished by 5-HT_{2A} antagonists, but not by 5-HT_{2C} antagonists (Schreiber *et al.*, 1995; Willins *et al.*, 1997). It has also been shown that the affinity of antagonists for the 5-HT_{2A} receptor and not 5-HT_{2C} receptor correlates to their potency to inhibit DOI-induced HTR (Schreiber *et al.*, 1994; Schreiber *et al.*, 1995). It should be noted that 5-HT_{2C} receptor knockout mice demonstrate an attenuated HTR to DOI (Canal *et al.*, 2010). This might reflect genetic compensations resulting from the ablation of the receptor since 5-HT_{2C} agonists do not evoke HTR. The later result is consistent with evidence that the mPFC a limbic region abundant in 5-HT_{2A} receptors is involved in the regulation of HTR, since local application of DOI in the mPFC elicits HTR, an effect inhibited by a 5-HT_{2A} but not by a 5-HT_{2C} receptor antagonist (Willins *et al.*, 1997). Furthermore, 5-HT_{2A} knockout mice do not elicit HTR when administered DOI, but genetic restoration of the receptor in cortical regions rescues the effect of DOI on HTR (Gonzalez-Maeso *et al.*, 2007).

Another behavioural effect elicited by certain 5-HT_{2A} receptor agonists is the ear-scratch response (ESR). The ESR is a rapid stereotypical response where the animal scratches their ear with one or two paws. Although the pharmacology of this effect of 5-HT_{2A} receptor

agonists has not been examined as thoroughly as the HTR, the evidence suggest that ESR is also mediated by the 5-HT_{2A} receptor. In particular, DOI dose-dependently increases ESR in mice and this effect is inhibited by a 5-HT_{2A} receptor antagonist (Darmani *et al.*, 1990). Moreover, in 5-HT_{2A} receptor knockout mice the ESR to DOI is abolished but then restored when 5-HT_{2A} receptor expression is genetically rescued in the cortex (Gonzalez-Maeso *et al.*, 2007).

At the molecular level, 5-HT_{2A} agonists have been shown to induce an acute increase in the expression of a variety of genes, an effect evidently mediated through 5-HT_{2A} receptor activation. Initial microarray studies, replicated by PCR, revealed that LSD produces a characteristic molecular fingerprint by increasing the expression of several genes including the immediate early genes (IEGs) c-fos, Arc and egr2 in the rat brain (Nichols *et al.*, 2002). A follow-up study from the same group showed that this effect of LSD on c-fos, Arc and egr2 was abolished when animals were pre-treated with a selective 5-HT_{2A} antagonist (Nichols *et al.*, 2003). Additional studies, found that DOI increased the expression of the same genes. For example, DOI was shown to increase the levels of c-fos (Scruggs *et al.*, 2000) and Arc (Pei *et al.*, 2004) in cortical regions of the rat. In both studies these effects of DOI were abolished only when animals were pre-treated with 5-HT_{2A} antagonists, but not with 5-HT_{2C} antagonists. Similar data for c-fos and Arc have been obtained from mouse studies (Jennings *et al.*, 2008). Most importantly, in mice lacking the 5-HT_{2A} receptor, DOI failed to increase mRNA of c-fos or egr2 (Gonzalez-Maeso *et al.*, 2003). Moreover, it was shown that rescue of 5-HT_{2A} expression in cortical regions, reinstated the ability of DOI to trigger the expression of these genes (Gonzalez-Maeso *et al.*, 2007). These data strongly suggest that the molecular effects of 5-HT_{2A} agonists involve the activation of 5-HT_{2A} and not 5-HT_{2C} receptor.

Evidence suggests that 5-HT_{2A} receptor agonists activate not only PLC but also PLA_2 signalling pathway in *in vitro* experiments. In particular, in cell lines expressing the human 5-HT_{2A} receptor, a variety of 5-HT_{2A} agonists tested, stimulated both pathways (Berg *et al.*, 1998; Moya *et al.*, 2007). However, compelling evidence coming from *in vivo* experiments suggest that the behavioural and molecular effects of hallucinogens strongly involve the PI-PLC pathway. In particular, pharmacological inhibition of PLC and genetic knockout of the Ga_q protein completely inhibits DOI-induced IEG expression in the mouse (Garcia *et al.*, 2007; Gonzalez-Maeso *et al.*, 2007). Furthermore, a recent study, demonstrated that local infusion of a PLC inhibitor into the mPFC of a rabbit, completely abolishes the effect of DOI on HTR (Schindler *et al.*, 2013). Overall, these findings suggest that although in *in vitro* models 5-HT_{2A} agonists equally activate PLC or PLA_2, the *in vivo* effects of these agonists are likely to be mediated through PLC activation.

Lithium has been shown to attenuate 5-HT_{2A} receptor function in relevant animal paradigms (Jitsuiki *et al.*, 2000; Kitamura *et al.*, 2002; Basselin *et al.*, 2005), which is consistent with inhibition of IMPase by lithium and a consequent decrease in PI signalling. Specifically, long-term lithium treatment decreases 5-HTP induced HTR in both rats and mice (Goodwin *et al.*, 1986b; Hotta *et al.*, 1986). However, in the only two studies that have been conducted in rats to investigate the effect of lithium on 5-HT_{2A} receptor function at the molecular level, lithium was found to enhance the effects of DOI. In particular, lithium treatment of rats augmented the DOI-induced increase in c-fos expression in various brain regions (Leslie *et al.*, 1993; Moorman *et al.*, 1998).

The aim of the present chapter was to investigate the effects of ebselen in models of 5-HT_{2A} function and to compare with lithium. It was hypothesized that since 5-HT_{2A} receptor

signalling is linked to PI cycle, ebselen would attenuate the responses of a 5-HT_{2A} agonist at the behavioural (HTR and ESR) and the molecular (IEG expression) level, through IMPase inhibition. In some experiments selective inhibitors of IMPase and GSK-3 were also tested to help evaluate the mechanisms underlying the effects of ebselen and lithium.

2.2 Methods

2.2.1 Experimental animals

Adult, male C57BL/6 (7-9 weeks old) (Harlan, Bicester, UK) mice were housed in groups of 6 under controlled conditions of lighting (12 h light-dark cycle) and temperature (21±1 ^{o}C). Experiments were carried out during the light phase. Food and water was available *ad libitum*. At least one week acclimatisation in the animal facility was allowed before experiments were carried out. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and Home Office Guidelines.

2.2.2 Drugs and chemicals

The vehicle of lithium chloride (MP biomedicals LLC), 1-(2,5-dimethoxy-4-iodophenyl)-propan-2-amine (DOI; Sigma) and [1-(4-hydroxyphenoxy) ethylidene]bisphosphonic acid (L-690,330; Tocris) was saline. To improve solubility of 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen; Sigma), *N*-[(4-methoxyphenyl)methyl]-*N*'-(5-nitro-2-thiazolyl) urea (AR-A 014418; Tocris) and 3-[2-(dimethylamino)ethyl]-4-indolol (psilocin; LGC standards) their vehicles were modified. In particular the vehicle of ebselen was 4% (2-hydroxypropyl)-β-cyclodextrin and 0.4% (v/v) dimethyl sulfoxide (DMSO). For AR-A014418, vehicle consisted of 10% (2-hydroxypropyl)-β-cyclodextrin and 10% (v/v) DMSO. The psilocin vehicle was 0.05 mM tartaric acid, pH 6-7. All injections were administered i.p. except for L-690,330 that was injected s.c. The volume of injection was adjusted to weight (0.01 ml/g for all drugs except for ebselen, AR-A 014418 and their vehicles that were injected at a volume of 0.02 ml/g due to poor solubility).

2.2.3 Behavioural model of 5-HT_{2A} receptor function

The effect of acute or repeated ebselen and lithium treatment on 5-HT_{2A} receptor function was assessed by measuring HTR and ESR evoked by 5-HT_{2A} agonist administration. The effects of ebselen and lithium were compared in the same paradigm the selective IMPase inhibitor L-690,330 and selective GSK-3 inhibitor AR-A014418. Two 5-HT_{2A} receptor agonists were used; DOI which structurally belongs to the phenethylamine family of hallucinogens and psilocin, that belongs to the indole-ethylamine family of hallucinogens.

For the behavioural monitoring, mice were placed in individual, transparent, plexiglass chambers. In order to acclimatise, animals were placed in the chambers 1 h before agonist (DOI or psilocin) injection (Figure 1). DOI and psilocin were administered at a dose of 2 mg/kg in all experiments except for the dose response to DOI study, where DOI was administered at 2, 4 and 8 mg/kg. Behaviour was recorded with a video-camera 5 min after agonist injection, for 15 min. HTR and ESR were scored offline by two observers that were blind to the treatments administered to the mice. The results of the two observers were compared.

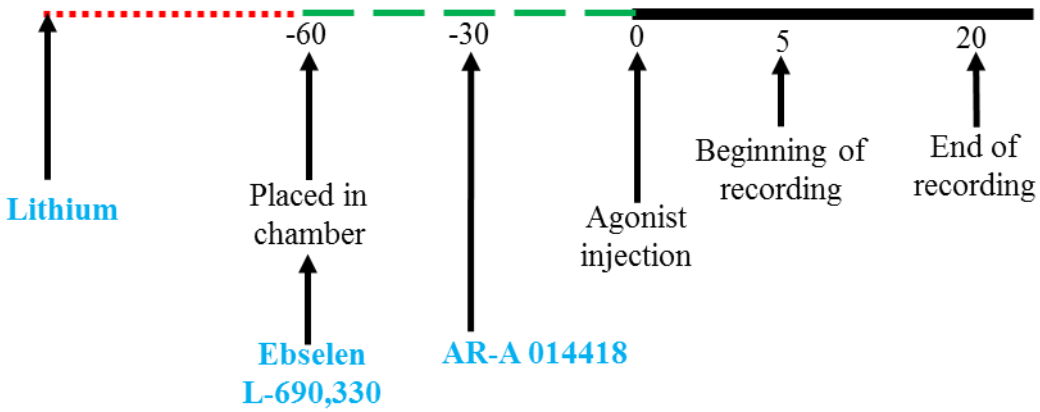

Figure 1: Experimental protocol used to assess the effects of ebselen, lithium, L-690,330 and AR-A014418 on 5-HT_{2A} receptor agonist evoked HTR and ESR.

The following experiments were carried out:

i. Dose response to DOI alone (2, 4 and 8 mg/kg)
ii. Acute ebselen (1, 5 or 10 mg/kg) plus DOI
iii. Acute ebselen (10 mg/kg) plus psilocin
iv. Repeated ebselen for 7 days (10 mg/kg, twice daily) plus DOI
v. Acute lithium (10 mmol/kg[1]) plus DOI
vi. Repeated lithium for 3 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3) plus DOI
vii. Repeated lithium for 7 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7) plus DOI
viii. Acute L-690,330 (150 or 240 mg/kg) plus DOI
ix. Acute AR-A 014418 (10 or 20 mg/kg) plus DOI

[1] 10 mmol/kg = 420 mg/kg

Animals were randomly allocated to the treatment groups (n = 6 per group except for the L-690,330 study where n = 8-13 per group).

Pre-treatment times for ebselen, lithium or the enzyme inhibitors (L-690,330 and AR-A014418) varied, depending on the pharmacokinetic properties of these agents (see Table 1 for a summary of experimental protocols).

treatment	**duration (days)**	**(last) injection before agonist**	**5-HT$_{2A}$ receptor agonist**
ebselen	0	1 h	DOI or psilocin
lithium	0	5 h	DOI
L-690,330	0	1 h	DOI
AR-A 014418	0	30 min	DOI
ebselen	7	1 h	DOI
lithium	3	18 h	DOI
lithium	7	18 h	DOI

Table 1: Summary table of pre-treatment times of different agents prior to 5-HT$_{2A}$ receptor agonist administration in the behavioural model.

2.2.4 Molecular model of 5-HT$_{2A}$ receptor function

The effect of acute or repeated ebselen and lithium treatment on the function of the 5-HT$_{2A}$ receptor was also tested at the molecular level (IEG expression). Similar to the behavioural studies, DOI and psilocin were used in the molecular studies.

Mice (n = 6 per group) were allowed to acclimatise in the procedure room where the injections were administered for 4 h before agonist administration. One hour post agonist

administration, animals were culled by cervical dislocation. In all studies DOI and psilocin were injected at a dose of 2 mg/kg. Brains were dissected, snap frozen in ice-cold isopentane, and stored in -80 ^{0}C until used.

The following experiments were carried out in the molecular paradigm:

i. Acute ebselen (10 mg/kg) plus DOI
ii. Acute ebselen (10 mg/kg) plus psilocin
iii. Repeated ebselen for 7 days (10 mg/kg, twice daily) plus DOI
iv. Repeated lithium for 3 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3) plus DOI
v. Repeated lithium for 7 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7) plus DOI

Pre-treatment times for ebselen and lithium varied, depending on their pharmacokinetic properties (see Table 2 for a summary of experimental protocols).

treatment	**duration (days)**	**(last) injection before agonist**	**5-HT$_{2A}$ receptor agonist**
ebselen	0	1 h	DOI or psilocin
ebselen	7	1 h	DOI
lithium	3	18 h	DOI
lithium	7	18 h	DOI

Table 2: Summary table of pre-treatment times of different agents prior to 5-HT$_{2A}$ receptor agonist administration in the molecular model.

2.2.4.1 Protocol for in situ hybridization

Tissue collection and preparation of sections

Cryostat cut coronal sections (12 µm) were collected onto gelatinised slides from the frontal cortex and caudate putamen (plates 7 and 23 respectively; (Paxinos *et al.*, 2007). Sections were stored at -80 ^{0}C until pre-treatment.

For pre-treatment, all solutions were treated with diethyl pyrocarbonate (DEPC) before being applied to the sections, except for the ethanol solutions that were made up using DEPC treated double distilled water (ddH_2O). In particular, in all solutions 1 ml/l of DEPC was added and left to stand for 2 h before being autoclaved. Ethanol solutions were prepared in autoclaved bottles.

Slides were allowed to defrost and then fixed in 4% (w/v) Paraformaldehyde (PFA)[2] in phosphate buffered saline (PBS), pH 7.5, for 5 mins. Then slides were immersed in PBS twice to rinse PFA before being acetylated in triethanolamine hydrochloride/acetic acid solution (TEA/AA)[3], (0.25% (v/v) acetic anhydride, 0.1 M triethanolamine hydrochloride and 0.15M NaCl, pH 8) for 10 min. This was followed by dehydration with increasing concentrations of alcohol solutions. Specifically, slides were placed in 70% (v/v) ethanol for 1 min, then 80% (v/v) ethanol for 1 min, 95% (v/v) ethanol for 2 min and 100% ethanol for 1 min. Sections were then treated for 10 min with chloroform for the lipids to be

[2] 4% (w/v) PFA was made just before use in DEPC treated PBS solution, 60 ^{0}C

[3] Acetic anhydride should be added shorty before use

removed, and then rehydrated in 100% then 95% (v/v) ethanol for 1 min each. Slides were then allowed to air dry overnight and stored at -20 ^{0}C until further used.

Radiolabelling and purification of oligonucleotide probes

Oligonucleotide probes complementary to c-fos (CTTCAGGGTAGGTGAAGACAAAGGAAGACGTGTAAGTAGTGCAGC), Arc (CTCGGTTGCCCATCCTCACCTGGCCCCCAAGACTGATATTGCTGA) and egr2 (GGATCATAGGAATGAGACCTGGGTCCATAGCTGGCTTGG) mRNA were purchased from Sigma Genosys. The probes were 3'-tail labelled with alpha-[^{35}S]-deoxyadenosine 5'-thiotriphosphate (Hartmann Analytic GmbH, Germany; specific activity 10 mCi/ml). For the labelling, the probe (2 µM) was incubated with [^{35}S]-dATP and terminal deoxynucleotidyl transferase (TdT) at 37 ^{0}C for 35 min in a water bath. The labelled oligonucleotide probe was then chromatographically separated from the unicorporated nucleotide using illustra NICK columns (GE Healthcare). Following the incubation, the radioactive mix was loaded on the column with 400 µl of tris- ethylenediaminetetraacetic acid (EDTA) buffer (1 M Tris-HCl, 0.1 mM EDTA, pH 8) and the eluent was collected. Two more eluents were collected with the addition of 400 µl Tris-EDTA solution each time. From each eluent 3 µl were mixed with 3ml of scintillation fluid (National Diagnostics) and the counts/minute (cpm) were measured by an automatic scintillation counter (Hidex 300 SL). The probe was used only when 70% or more of the total counts were found in the second eluent, otherwise the labelling was considered to be unsuccessful.

Before use, the probes were tested for specificity. Controls included using the sense orientation of the oligonucleotide and displacement with unlabelled probes.

In situ hybridization

Sections from selected brain regions were defrosted and placed in trays covered with filter paper (Whatman, Grade No.1) damped with 50 ml of tray buffer consisting of 25 ml formamide, 15 ml DEPC ddH_2O and 10 ml 20 x saline sodium citrate (SSC; 3 M NaCl, 0.3 M sodium citrate, pH 7). On each slide 200 µl of hybridization mix was added and the slides were cover slipped and incubated at 34 ^oC for 16 h. The hybridization mix consisted of the radioactive nucleotide (2.4 x 10^6 cpm/section), 50 mM dithiothreitol (DTT) and hybridization buffer. The hybridization buffer consisted of 50% deionised formamide, 4 x SSC (20 x stock), 25 mM sodium phosphate buffer, 1 mM sodium pyrophosphate, 5 x Denhart's (50 x stock; Sigma), 0.2 µg/ml boiled herring sperm (stock 10 µg/ml; Promega), 0.1 mg/ml poly adenilic acid (stock 5 mg/ml; Sigma), 120 µg/ml heparin (stock 120 mg/ml, 100 ku; Sigma), 0.1 g/ml Dextran powder (Sigma) to a final volume of 200 ml. The constituents were vigorously mixed overnight in an incubator at 50 ^0C. The hybridization buffer was stored at -20 ^0C for further use.

Following 16 h incubation of the sections with the hybridization mix, the cover slips were removed and the sections were washed with 1 x SSC, 50 ^0C for 20 min. The warm washes were repeated another 2 times and were followed by 2 times, 60 min washes at room temperature with 1 x SSC. Sections were then immersed in ddH_2O to facilitate removal of the SSC and allowed to dry overnight. Slides were then placed in cassettes and exposed to BioMax MR film (Kodak, Carestream) for 7 days. Autoradiographic films were exposed using an automatic X-ray film processor (Compact X4, X-ograph).

2.2.4.2 Image analysis of autoradiograms

The abundance of Arc, c-fos and egr2 mRNA was determined by measuring the optical density in selected brain regions (Figure 2) using MCID software. The optical density readings were converted into nCi/g of tissue by calibration using [^{14}C] microscales, which were co-exposed with the slides. Measurements of regions of interest were taken bilaterally from 3 sections per slide and the values for each region of interest were averaged.

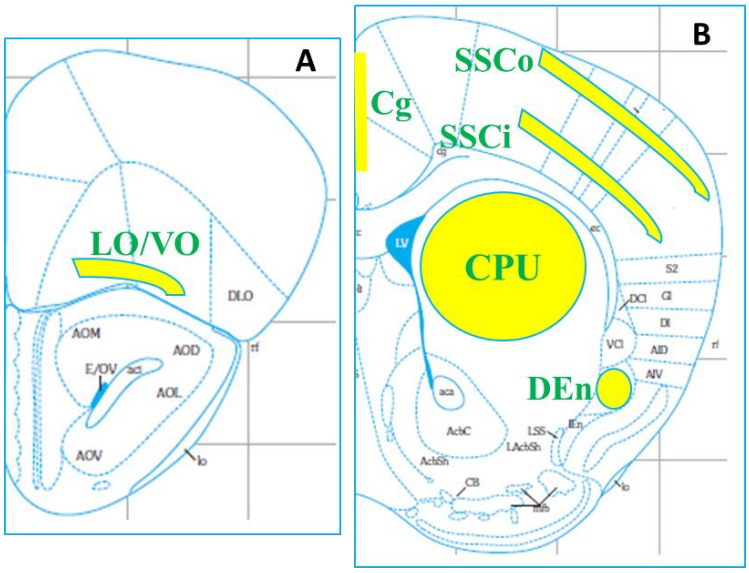

Figure 2: Coronal sections of the mouse brain at two levels; frontal cortex (A) and caudate putamen (B). Brain regions selected for gene expression analysis are highlighted in yellow. Abbreviations: LO/VO: Orbital cortex; Cg: Cingulate cortex; SSCo: Somatosensory cortex outer layer; SSCi: Somatosensory cortex inner layer; CPU: caudate putamen; Den: endopiriform nucleus. Image modified by (Paxinos *et al.*, 2007).

2.2.5 Protocol for 5-HT_{2A} receptor autoradiography

To investigate possible changes 5-HT_{2A} receptor abundance in frontal cortex following ebselen (acute or repeated) or lithium (repeated) treatment, measurement of [^{3}H]-ketanserin binding was conducted using a standard autoradiography protocol. Brain tissue from mice treated with acute or repeated ebselen and lithium described above, was used. Frontal cortex sections (12 µm) were cryostat cut and stored at -80 ^{0}C until further used.

Autoradiography

Sections were allowed to air dry for 30 min prior to incubating in Tris-HCl buffer (0.17 M, pH 7.7) for 20 min at room temperature. Following this step, sections were allowed to air dry for another 15 min and then 2 consecutive slides of each subject were incubated with 300 µl of either 2 nM [^{3}H]-ketanserin (PerkinElmer; specific activity: 53.4 Ci/mmol) to determine total binding, or 2 nM [^{3}H]-ketanserin with 10 µM methysergide to determine non-specific binding. Two hours later, sections were washed twice for 10 min with 4 ^{0}C Tris-HCl buffer (0.17 M, pH 7.7). Following this step, sections were rinsed in dd$H_2$0 at 4 ^{0}C. Slides were then allowed to air dry overnight before exposed to autoradiographic films (BioMax MR, Kodak, Carestream) for 12 weeks. Films were manually developed and fixed (D-19 developer; P6557 fixer Caresteam, Kodak). Abundance of binding was measured by analysing the optical density of the autoradiographs at the frontal cortex (Figure 3), using MCID software in a similar way as described above.

Measurements from FC (Figure 3) were taken bilaterally, from three sections per slide and the values were averaged. Specific binding was calculated by subtracting non-specific

binding from total binding. The system was calibrated by using ^{3}H-microscales which were co-exposed with the sections to allow conversion of optical density to nCi/g of tissue.

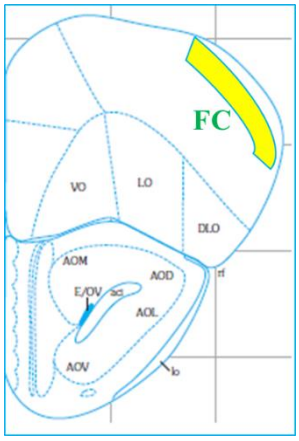

Figure 3: Coronal section of the mouse brain at the level of the frontal cortex. The abundance of the 5-HT_{2A} receptor was analysed in the FC, which is highlighted in yellow.

2.2.6 Data analysis

Data were analysed using IBM SPSS Statistics, version 20. Data were expressed as percentage of controls (DOI-Veh group for HTR and ESR studies; Veh-Veh group for IEG studies) and presented as mean ± SEM values. Statistically significant effects were considered when $p < 0.05$.

For the behavioural study, the effect of each treatment was compared to controls using Student's unpaired *t*-test or one-way ANOVA followed by LSD *post-hoc* test, as appropriate.

For the molecular study, the effect of each treatment was compared statistically, using one-way ANOVA followed by LSD *post-hoc* test. Each region was analysed independently.

For the 5-HT_{2A} receptor abundance study, the effect of each treatment compared to vehicle control was analysed using Student's unpaired t-test.

2.3 Results

2.3.1 Dose-response to DOI-evoked HTR

In order to establish a dose of DOI that induced HTR in mice a dose response study was conducted. In particular, 3 doses of DOI (2, 4 or 8 mg/kg) were administered acutely, and HTR was scored. All three doses elicited a statistically significant increase in HTR compared to vehicle controls ($F(3,15) = 63.746$, 2 mg/kg versus veh: $p<0.001$; 4 mg/kg versus veh: $p<0.001$; 8 mg/kg versus veh:$p<0.001$) (Table 3). There was no difference in the magnitude of the effect between the doses. Based on these findings a dose of 2 mg/kg DOI was used in further experiments. Previous studies found this dose of DOI be completely inhibited by 5-HT_{2A} antagonists (Schreiber *et al.*, 1995).

Treatment	HTR
Vehicle	3±1
DOI (2)	43±2 *
DOI (4)	48±2 *
DOI (8)	48±5 *

Table 3: Effect of different doses of DOI (2, 4 and 8 mg/kg) on HTR. Data shown are mean ± SEM value (4 animals/group). *p<0.001 treatment vs vehicle controls. One-way ANOVA followed by LSD post-hoc.

2.3.2 Effect of different doses of ebselen on DOI-evoked HTR and ESR

Administration of DOI (2 mg/kg) significantly increased HTR (F (4,29) = 31.903, p<0.001) and ESR (F (4,29) = 7.118, p<0.001) compared to vehicle treated controls. Pretreatment with ebselen (1, 5 or 10 mg/kg) prior to DOI, led to a dose-dependent attenuation on the effect of DOI (Figure 4). In particular, the lower dose of ebselen did not have an effect on either HTR or ESR. However, 5 mg/kg ebselen, showed a strong trend to decrease HTR (p=0.057). The highest dose of ebselen (10 mg/kg) significantly decreased both HTR (p<0.001) and ESR (p=0.035). At this highest dose ebselen caused a 50% decrease in the effect of DOI (both HTR and ESR), but did not completely block it.

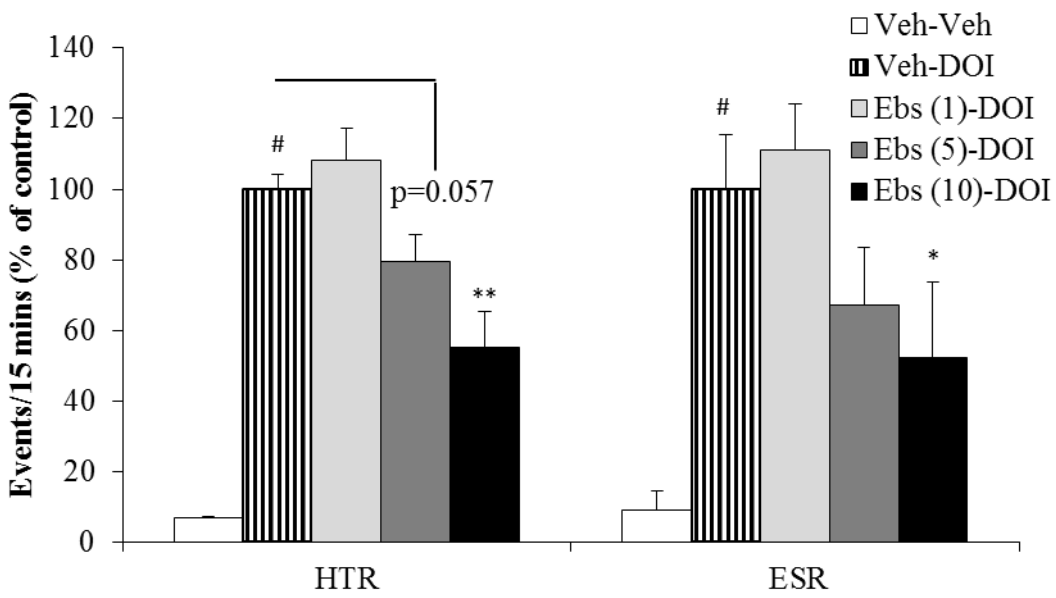

Figure 4: Effect of different doses of ebselen (1, 5 or 10 mg/kg) on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage response to that of DOI (6 animals/group), $^{\#}$p<0.001 Veh-DOI vs Veh-Veh, *p<0.05, **p<0.001 Veh-DOI vs Ebs-DOI. One-way ANOVA followed by LSD post-hoc.

2.3.3 Effect of acute ebselen on psilocin-evoked HTR

In the previous experiment it was shown that 10 mg/kg ebselen attenuated DOI-evoked behaviour. The same dose of ebselen was tested using a different 5-HT_{2A} agonist, psilocin. Psilocin (2 mg/kg) elicited a statistically significant increase in HTR ($F(2,17) = 33.499$, $p<0.001$) compared to vehicle controls but did not have an effect on ESR (Figure 5). Psilocin elicited less HTR events compared to DOI. Importantly, ebselen (10 mg/kg) decreased psilocin-induced HTR by 40% ($p=0.001$) (Figure 5).

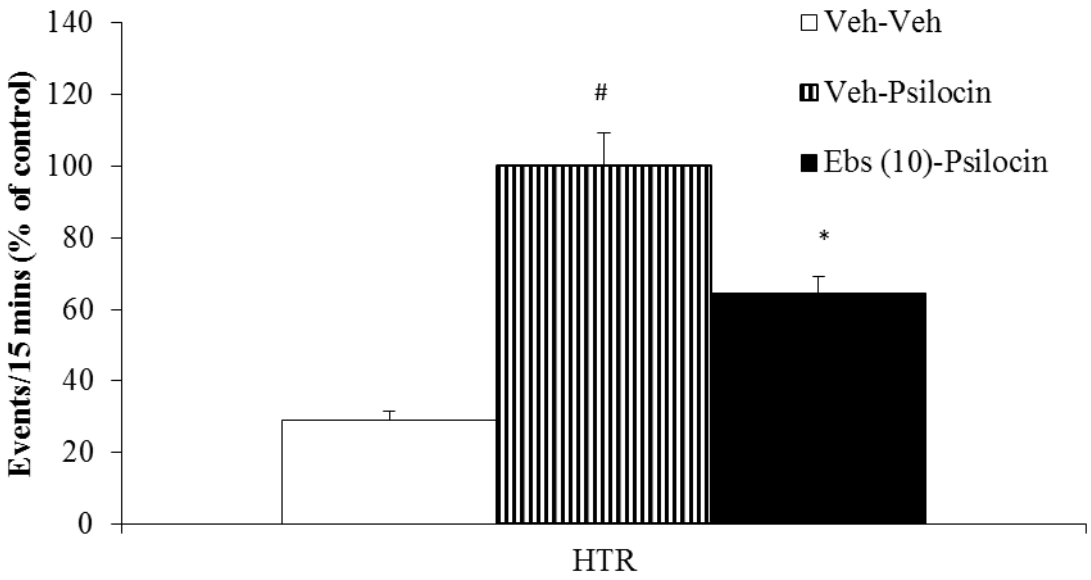

Figure 5: Effect of ebselen (10 mg/kg) on psilocin (2 mg/kg) evoked HTR. Data shown are mean ± SEM percentage of response to that of psilocin (6 animals/group), $^{\#}p<0.001$ Veh-Psilocin vs Veh-Veh, $^{*}p<0.005$ Veh-Psilocin vs Ebs-Psilocin. One-way ANOVA followed by LSD post-hoc.

2.3.4 Effect of repeated ebselen on DOI-evoked HTR and ESR

The above experiments established that acute ebselen administration attenuated 5-HT_{2A} agonist induced behavioural effects. To investigate whether this effect of ebselen persisted following repeated treatment, 10 mg/kg ebselen was administered for 7 days. Ebselen treatment alone, did not induce HTR or ESR compared to vehicle controls, as expected (data not shown). DOI administration significantly increased HTR ($F(3,23) = 31.351$, $p < 0.001$) and ESR ($F(3,22) = 31.273$, $p < 0.001$) compared to vehicle controls. Furthermore, repeated ebselen administration attenuated the effect of DOI on HTR ($p=0.017$) and ESR ($p=0.003$) by 30% and 40% respectively (Figure 6).

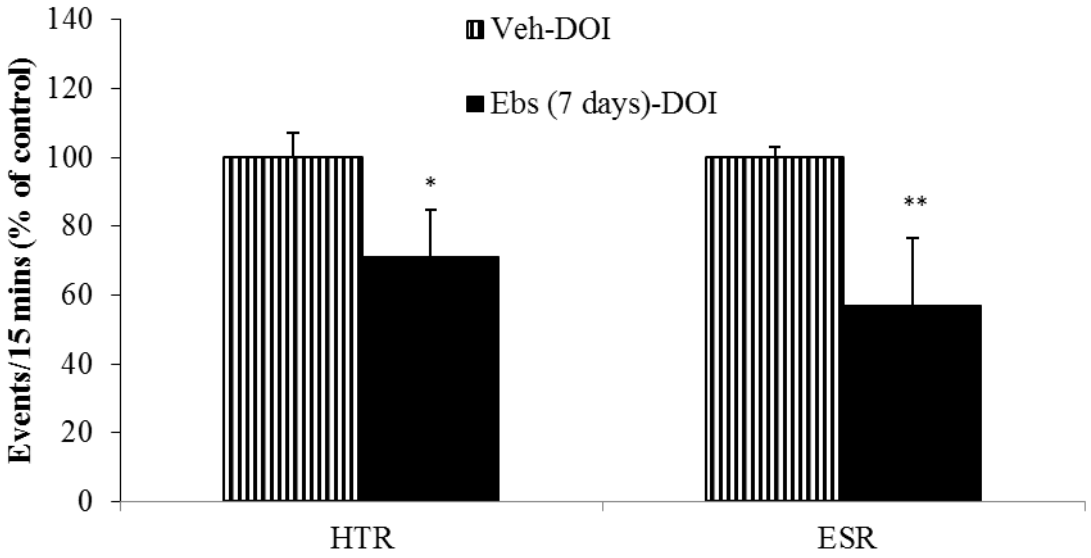

Figure 6: Effect of repeated ebselen (10 mg/kg, twice daily, for 7 days), on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage of response to that of DOI (6 animals/group), *p<0.05, **p<0.005 Veh-DOI vs Ebs-DOI. One-way ANOVA followed by LSD post-hoc (Veh-Veh and Ebs-Veh groups not shown).

2.3.5 Effect of acute lithium on DOI-evoked HTR and ESR

The effect of ebselen in the behavioural model of 5-HT_{2A} receptor function was compared to lithium. DOI administration elicited HTR and ESR as expected. Pretreatment with lithium (10 mmol/kg) attenuated DOI-evoked ESR (t (11) = 3.774, *p*=0.003) by 50% but did not have an effect on DOI-induced HTR (Figure 7).

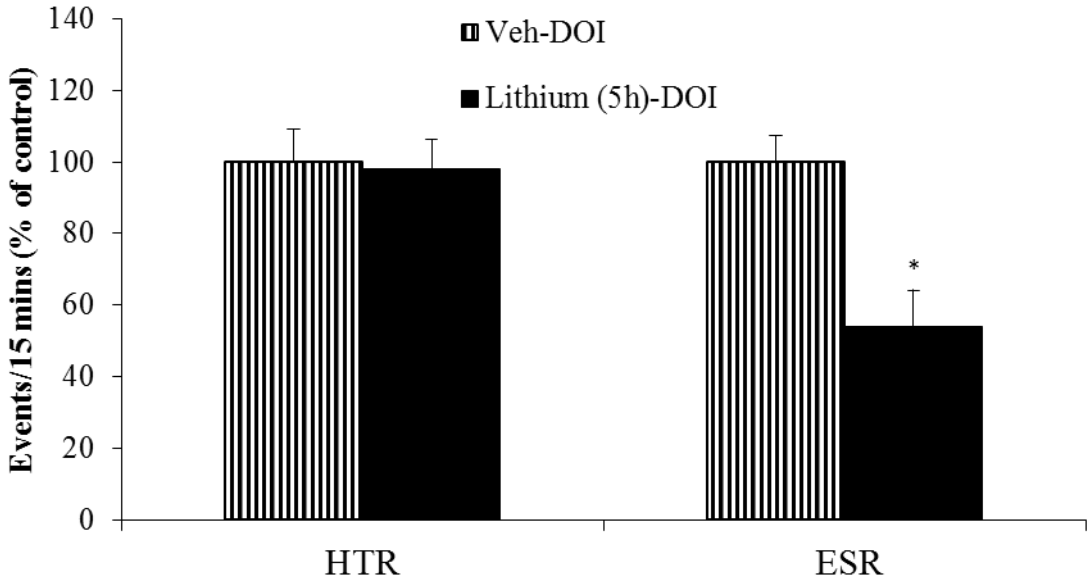

Figure 7: Effect of acute lithium (10 mmol/kg) on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage of response to that of DOI (6 animals/group), *p<0.005 Veh-DOI vs Lithium-DOI. Student's unpaired t-test.

2.3.6 Effect of repeated lithium treatment on DOI-evoked HTR and ESR

Since acute lithium administration reduced DOI-evoked ESR but not HTR, lithium was administered repeatedly. DOI injection evoked HTR and ESR as expected. Treatment with lithium for 3 days decreased DOI-induced HTR (t (8) = 3.038, *p*=0.016) and ESR (t (8) = 2.632, *p*=0.030) by 25% and 40%, respectively (Figure 8).

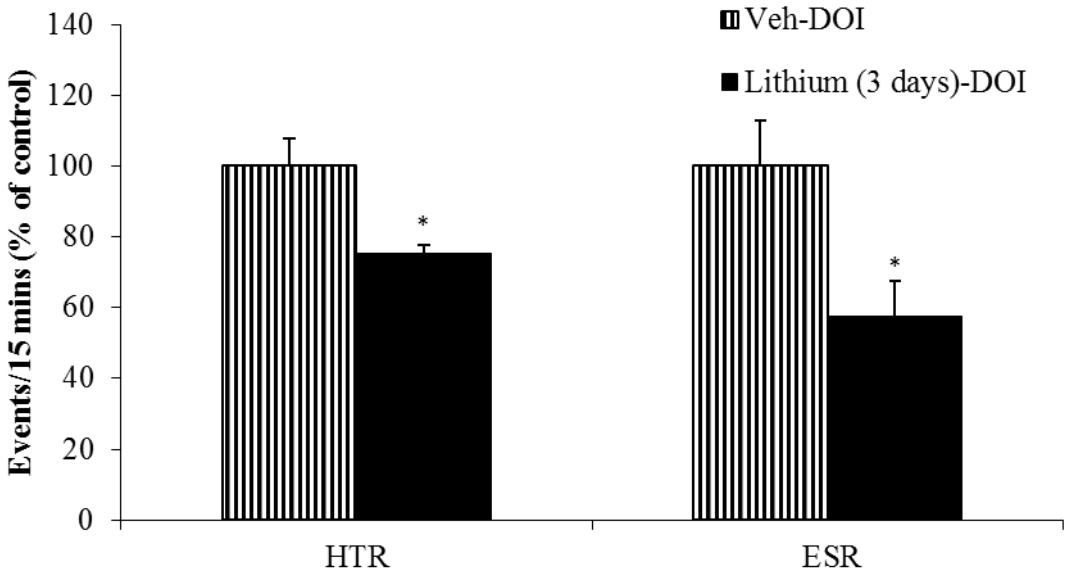

Figure 8: Effect of 3 days treatment with lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage response to that of DOI (6 animals/group), *p <0.05, Veh-DOI vs Lithium-DOI. Student's unpaired t-test.

In a further experiment, lithium was administered for 7 days. Lithium treatment alone for 7 days did not induce HTR or ESR (data not shown). DOI increased the number of HTR ($F(3,23) = 112.949$ $p<0.001$) and ESR ($F(3,23) = 71.608$, $p<0.001$) compared to vehicle controls. Treatment with lithium for 7 days, attenuated DOI-evoked HTR ($p=0.023$) and ESR ($p<0.001$) by 20% and 60%, respectively (Figure 9).

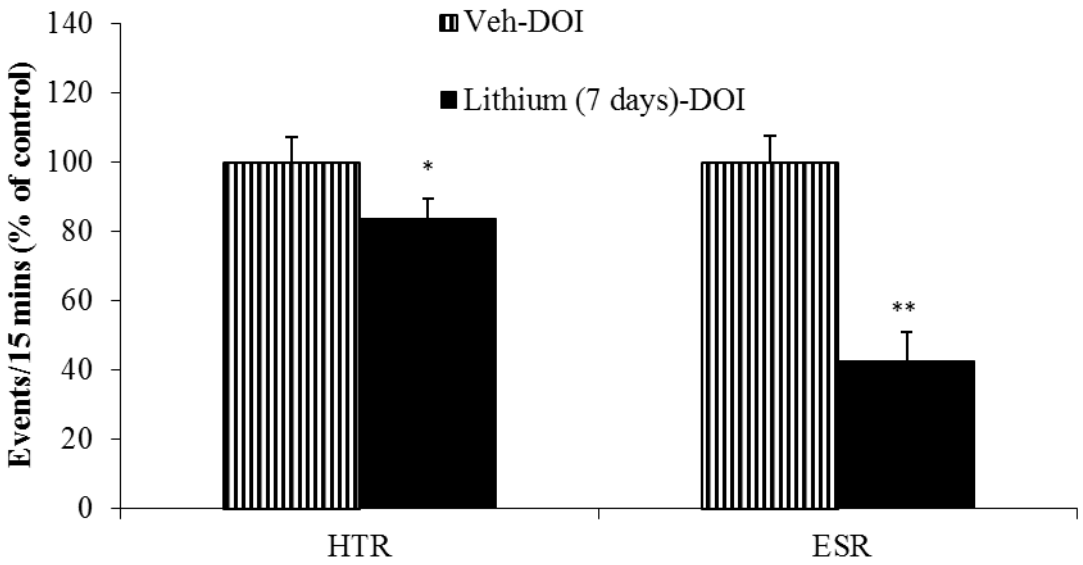

Figure 9: Effect of 7 days treatment with lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7), on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage of response to that of DOI (6 animals/group), *p<0.05, **p<0.001 Veh-DOI vs Lithium-DOI. One-way ANOVA followed by LSD post-hoc (Veh-Veh and Lithium-Veh groups not shown).

2.3.7 Effect of inhibitors of IMPase and GSK-3 on DOI-evoked HTR and ESR

The effect of ebselen and lithium in the behavioural model of 5-HT_{2A} receptor function was compared to that of inhibitors of IMPase (L-690,330) and GSK-3 (AR-A014418). Pretreatment with L-690,330 (150 or 240 mg/kg) attenuated in a dose-dependent way, DOI-induced HTR ($F(2,28) = 3.244$, $p=0.052$) and ESR ($F(2,28) = 5.457$, $p=0.003$) by 25% and 40% respectively, (Figure 10).

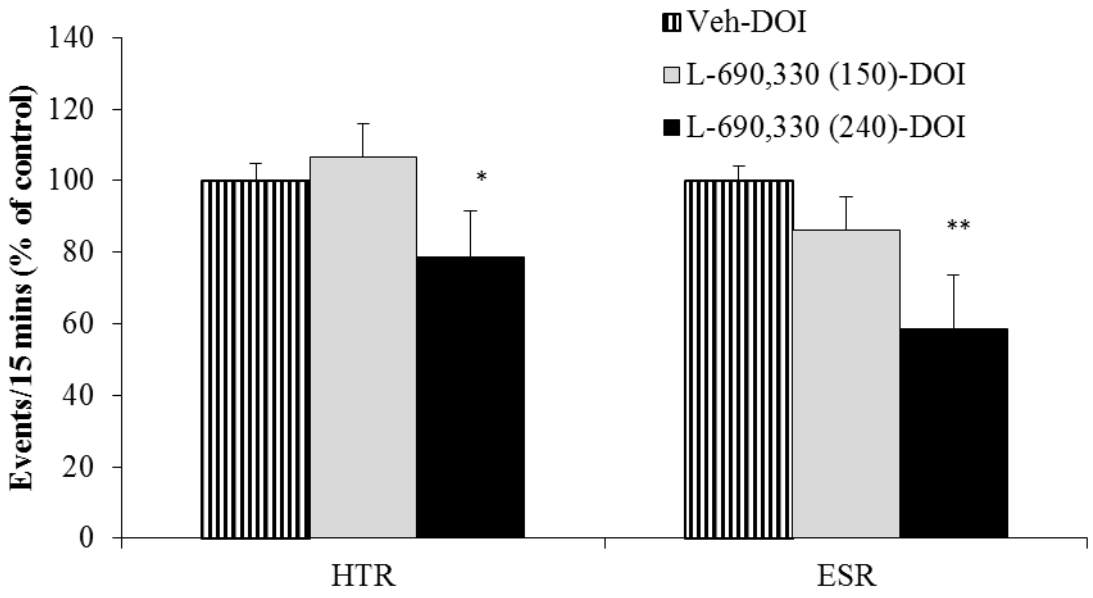

Figure 10: Effect of acute L-690,330 (150 or 240 mg/kg), on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage of response to that of DOI (8-13 animals/group). *p<0.05, **p<0.005 L-690,330-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc.

Pretreatment with AR-A014418 (10 or 20 mg/kg) did not have an effect on either HTR (F (2,16) = 0.011, p=0.989) or ESR (F (2,16) = 0.008, p=0.992) evoked by DOI (Figure 11).

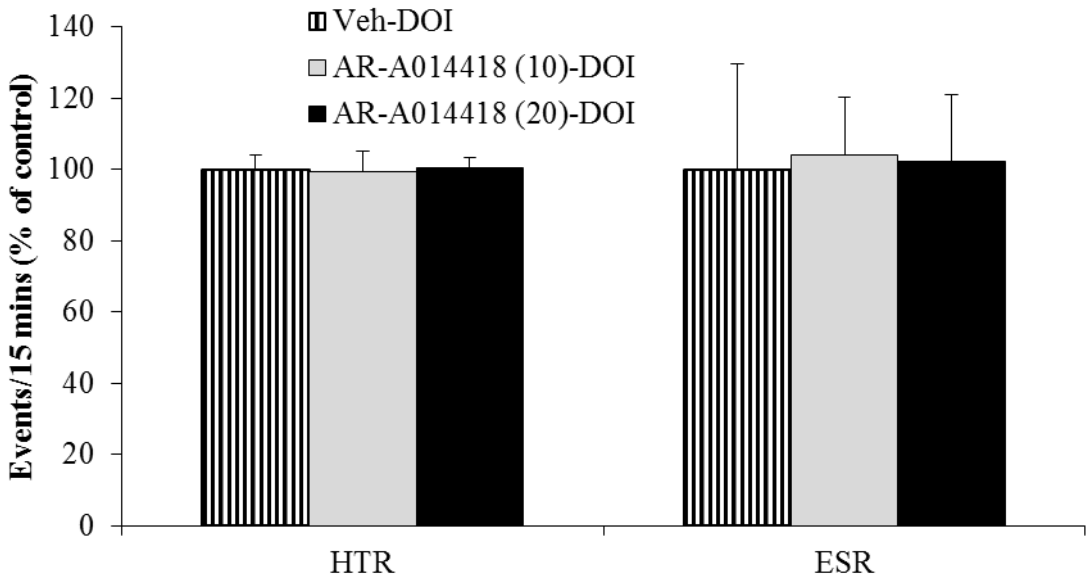

Figure 11: Effect of AR-A014418 (10 or 20 mg/kg), on DOI (2 mg/kg) evoked HTR and ESR. Data shown are mean ± SEM percentage of response to that of DOI (6 animals/group). One-way ANOVA followed by LSD post-hoc.

2.3.8 Effect of acute ebselen on DOI-evoked IEG expression

The distributions of Arc, c-fos and egr2 mRNA are shown in Figure 12. Arc mRNA showed a robust expression and greater than that of c-fos mRNA. In comparison, expression of egr-2, was identified to be lower than both Arc and c-fos.

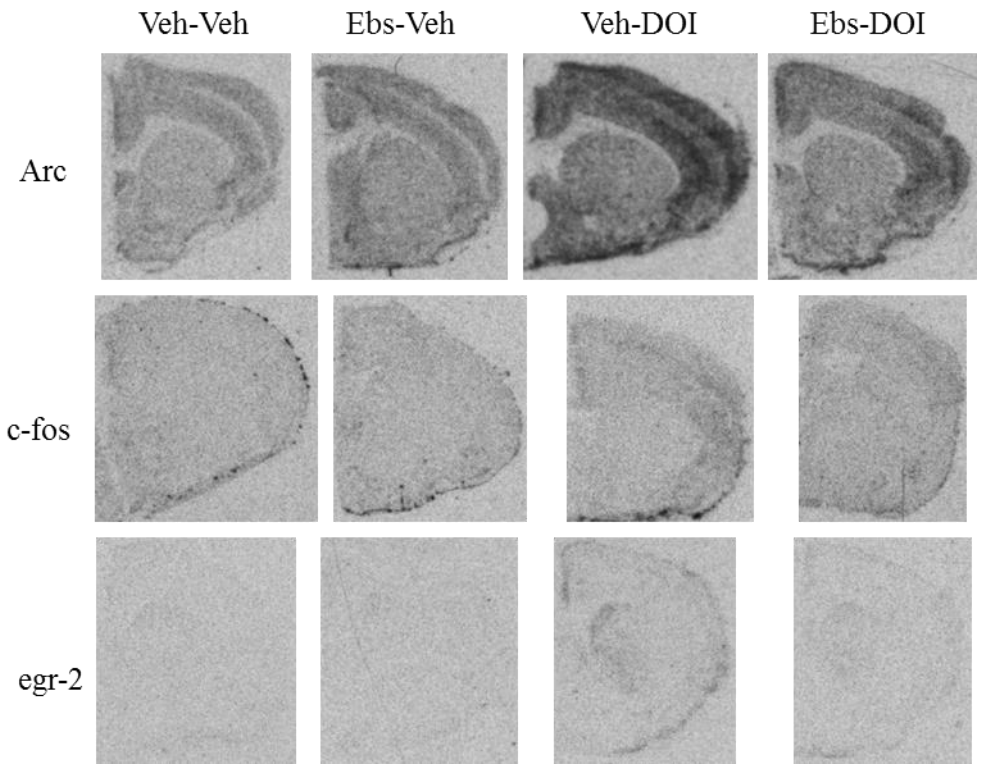

Figure 12: Representative autoradiograms showing distribution of Arc, c-fos and egr-2 mRNA in the caudate putamen of mice treated with either vehicle or ebselen (10 mg/kg, i.p.) followed by DOI (2 mg/kg, i.p.) or saline.

Arc mRNA: Administration of DOI (2 mg/kg) elicited an increase in Arc mRNA abundance compared to vehicle injected controls in all the regions investigated (LO/VO: F (3,20) = 37.272, $p<0.001$; SSCo: F (3,19) = 17.863, $p<0.001$; SSCi: F (3,21) = 16.727, $p<0.001$; Den: F (3,21) = 15.306, $p<0.001$; Cg: F (3,19) = 48.904, $p<0.001$). Treatment with ebselen (10 mg/kg) attenuated the DOI-induced increase in Arc mRNA in the majority of regions investigated (LO/VO: $p = 0.001$; Den: $p = 0.033$; Cg: $p = 0.001$). This effect of ebselen was of similar magnitude, approximately 60%, amongst these regions. Ebselen by itself, did not alter Arc mRNA in any of the regions investigated, with the exception of Cg (F (3,19) = 48.904, $p = 0.010$), where ebselen increased Arc mRNA compared to vehicle controls (Figure 13).

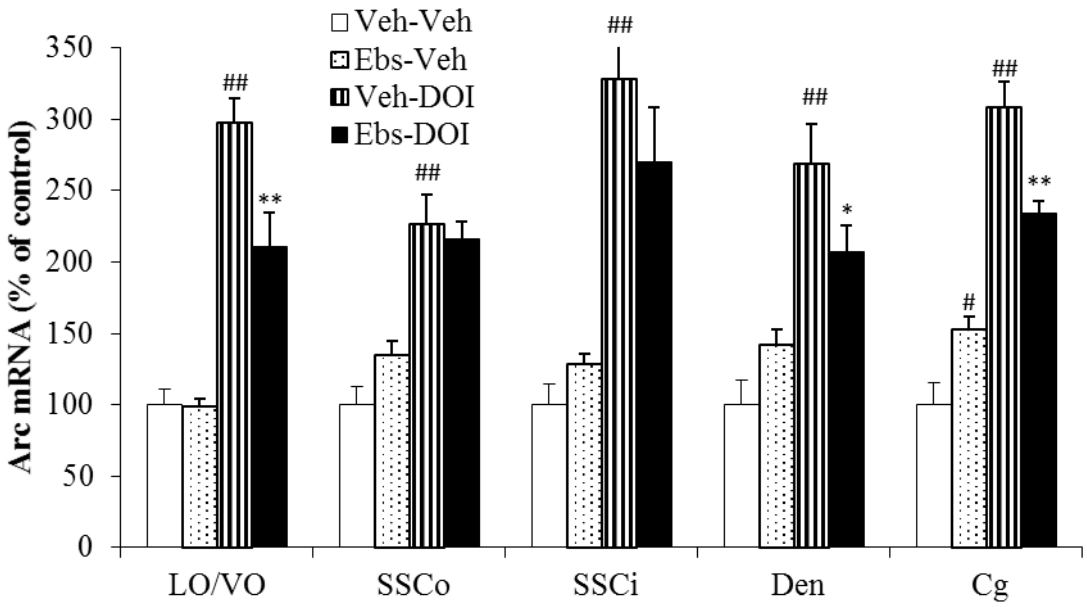

Figure 13: Effect of acute ebselen (10 mg/kg) treatment on DOI (2 mg/kg) induced increase of Arc mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.001$ treatment vs Veh-Veh, $*p<0.05$, $**p<0.005$ Ebs-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: When compared to vehicle treated animals, DOI increased c-fos mRNA across all regions investigated (LO/VO: F (3,21) = 32.161, $p<0.001$; SSCo: F (3,21) = 11.239, $p<0.001$; SSCi: F (3,22) = 6.768, $p=0.001$; Den: F (3,22) = 16.758, $p<0.001$; Cg: F (3,22) = 9.125, $p=0.001$). Pretreatment with ebselen (10 mg/kg) attenuated the effect of DOI on c-fos expression in the majority of the regions investigated (SSCo: $p=0.005$; Den: $p=0.004$; Cg: $p=0.008$). The greatest effect of ebselen was in the SSCo, where DOI-evoked increase in c-fos mRNA was reduced by 60%. Ebselen by itself did not alter c-fos mRNA abundance when compared to controls (Figure 14).

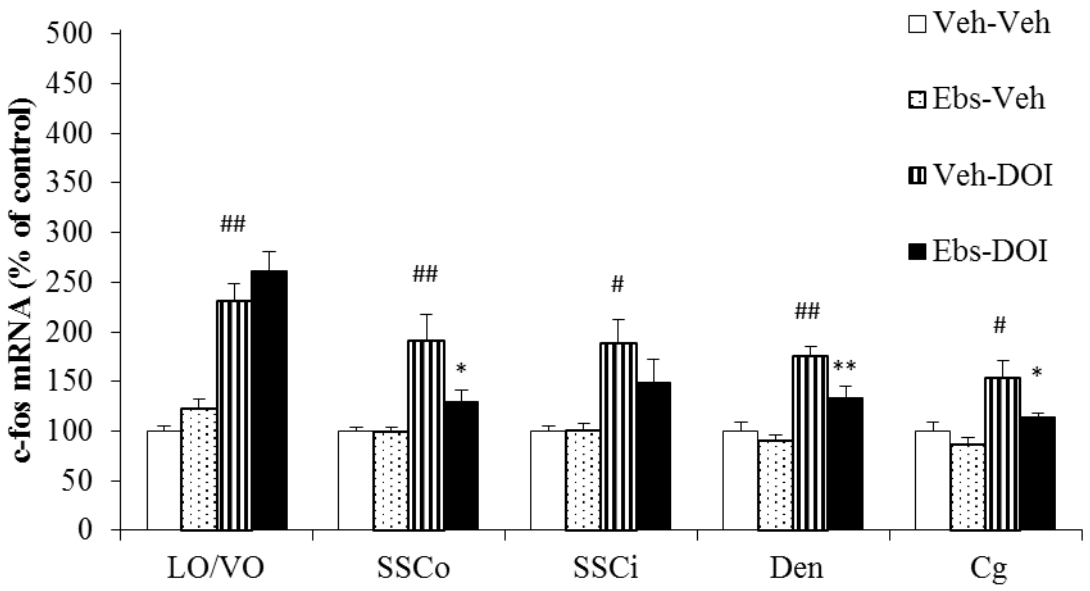

Figure 14: Effect of acute ebselen (10 mg/kg) treatment on DOI (2 mg/kg) induced increase of c-fos mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p<0.005$, $^{\#\#}p<0.001$ treatment vs Veh-Veh, $*p<0.05$, $**p<0.005$ Ebs-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

egr-2 mRNA: DOI (2 mg/kg) increased egr-2 mRNA abundance in all of the regions investigated compared to vehicle injected controls (LO/VO: F (3,18) = 11.444, $p<0.001$; SSCo: F (3,22) = 4.683, $p=0.006$; SSCi: F (3,22) = 6.631, $p=0.042$; Den: F (3,21) = 10.200, $p<0.001$; Cg: F (3,21) = 3.150, $p=0.048$; CPU: F (3,22) = 10.857, $p<0.001$). Ebselen decreased DOI-evoked increase in egr-2 mRNA in the majority of regions investigated (LO/VO: $p = 0.031$; Den: $p = 0.003$; CPU: $p = 0.005$). Ebselen alone did not alter egr-2 mRNA, compared to vehicle controls in any of the regions investigated (Figure 15).

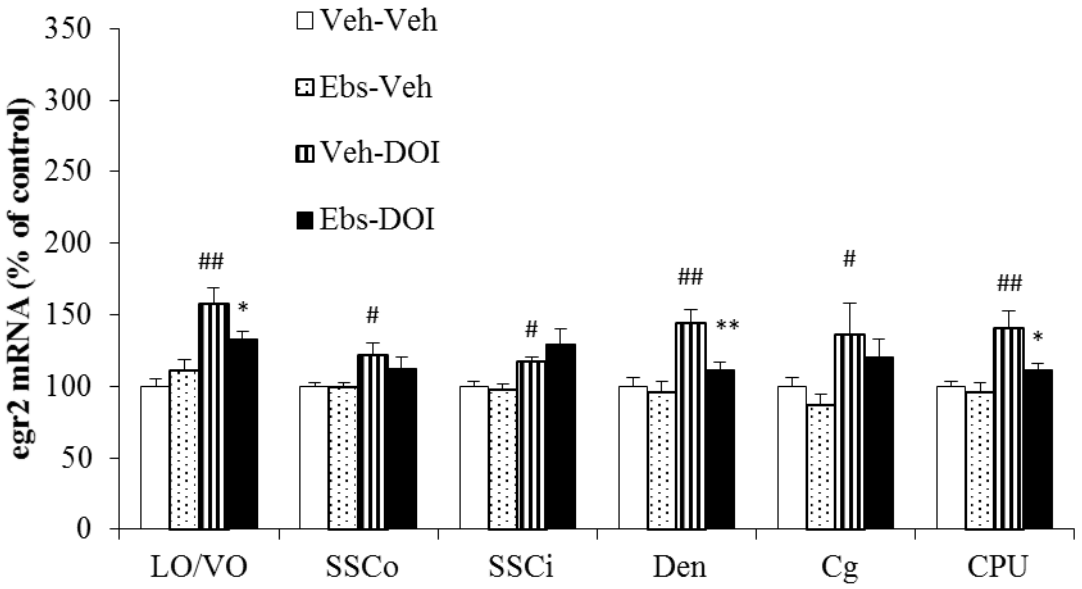

Figure 15: Effect of acute ebselen (10 mg/kg) treatment on DOI (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.001$ treatment vs Veh-Veh, $^{*}p<0.05$, $^{**}p<0.005$ Ebs-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

2.3.9 Effect of acute ebselen on psilocin-evoked IEG expression

In the previous experiments it was demonstrated that acute ebselen did not have an effect on IEG expression. Therefore, in order to reduce animal use, the ebselen group was not included in the following study.

Arc mRNA: Acute administration of psilocin (2 mg/kg) led to a statistically significant increase in Arc mRNA in all regions investigated (LO/VO: $F(2,13) = 8.071$, $p=0.006$; SSCo: $F(2,13) = 6.992$, $p=0.003$; SSCi: $F(2,13) = 7.594$, $p=0.003$; Den: $F(2,13) = 11.371$, $p=0.002$; Cg: $F(2,13) = 20.800$, $p<0.001$). Compared to DOI, psilocin increased Arc mRNA in the same regional pattern but the effect of DOI was greater. The greatest increase in Arc mRNA induced by psilocin was observed in LO/VO. Pretreatment with ebselen attenuated the psilocin-evoked increase of Arc mRNA in all regions investigated except for LO/VO (SSCo: $p=0.051$; SSCi: $p=0.034$; Den $p=0.002$; Cg: $p=0.001$) (Figure 16).

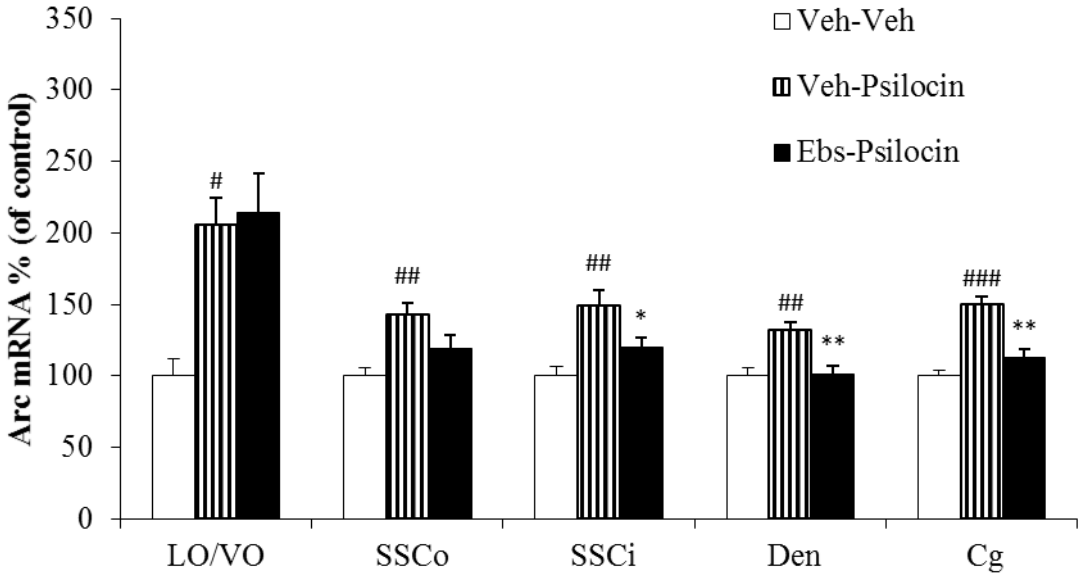

Figure 16: Effect of acute ebselen (10 mg/kg) treatment on psilocin (2 mg/kg) induced increase of Arc mRNA. Data shown are mean ± SEM percentage response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.005$, $^{\#\#\#}p<0.001$ treatment vs Veh-Veh, $^{*}p<0.05$, $^{**}p<0.005$ Ebs-Psilocin vs Veh-Psilocin. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: Psilocin (2 mg/kg) did not increase c-fos mRNA in any of the regions investigated. This is in contrast with the effect of DOI, which increased c-fos mRNA in all regions investigated Psilocin did not alter for c-fos mRNA in animals pretreated with ebselen (Figure 17).

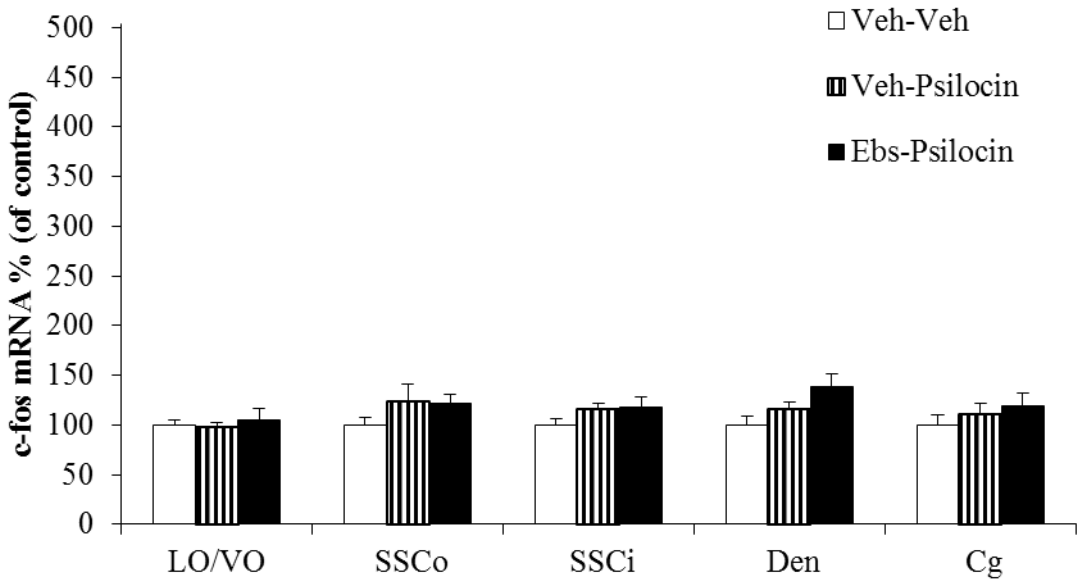

Figure 17: Effect of acute ebselen (10 mg/kg) and psilocin (2 mg/kg) on c-fos mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

egr-2 mRNA: Psilocin (2 mg/kg) elicited a small increase in egr-2 mRNA in the SSCi and CPU of 20% and 40% respectively (SSCi: F (2,13) = 6.834, p=0.014; CPU: F (2,14) = 24.812, p<0.001). Pre-treatment with ebselen, attenuated the effect of psilocin on egr-2 mRNA in the CPU (p=0.001) but not in the SSCi (Figure 18).

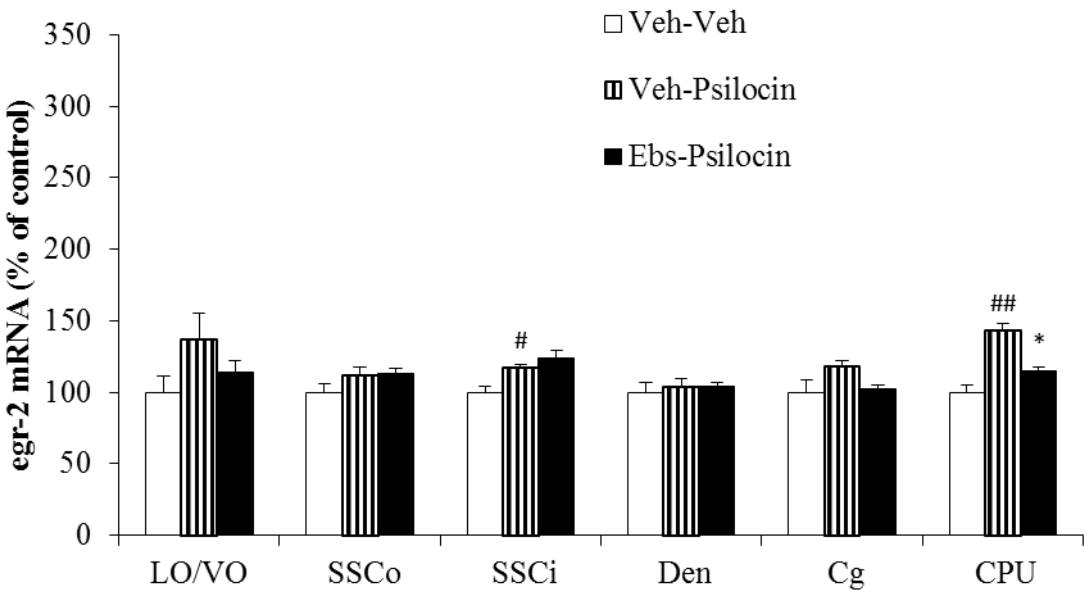

Figure 18: Effect of acute ebselen (10 mg/kg) treatment on psilocin (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.001$ treatment vs Veh-Veh, *p<0.005 Ebs-Psilocin vs Veh-Psilocin. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

2.3.10 Effect of repeated ebselen on DOI-evoked IEG expression

Experiments were carried out to determine whether the inhibition of DOI-evoked IEG expression by ebselen, was maintained with repeated administration of ebselen (10 mg/kg, i.p. for 7 days).

Arc mRNA: Acute DOI (2 mg/kg) increased Arc mRNA in all regions investigated except for Cg (LO/VO: F (3,23) = 18.568, $p<0.001$; SSCo: F (3,23) = 4.669, $p=0.007$; SSCi: F (3,23) = 9.002, $p=0.001$; Den: F (3,23) = 6.204, $p=0.001$), although the magnitude of the effect of DOI was less than previous experiments. Importantly, repeated ebselen attenuated DOI-evoked increase in Arc mRNA (LO/VO: $p=0.004$; SSCi: $p=0.012$; Den: $p=0.015$). Repeated treatment of animals for 7 days with ebselen alone, did not affect the abundance of Arc mRNA in any of the regions investigated (Figure 19).

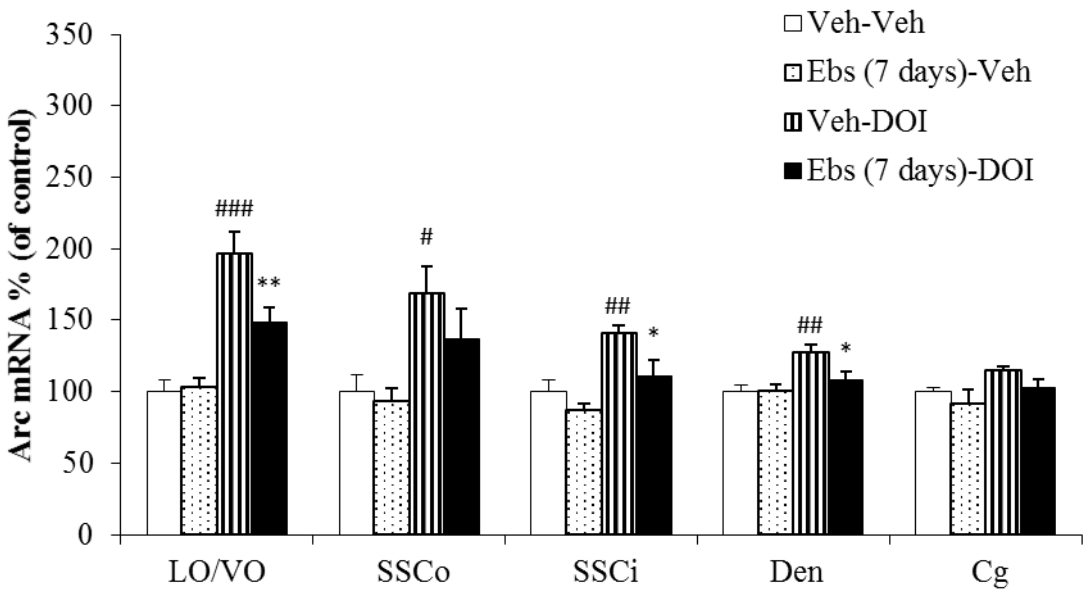

Figure 19: Effect of repeated ebselen (10 mg/kg, twice daily, for 7 days) on DOI (2 mg/kg) induced increase of Arc mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.005$, $^{\#\#\#}p<0.001$ treatment vs Veh-Veh, $^{*}p<0.05$, $^{**}p<0.005$ Ebs-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: DOI elicited a statistically significant increase of c-fos mRNA in all regions investigated (LO/VO: F (3,23) = 20.552, $p < 0.001$; SSCo: F (3,23) = 12.354, $p < 0.001$; SSCi: F (3,23) = 5.038, $p = 0.008$; Den: F (3,23) = 5.347, $p = 0.018$; Cg: F (3,23) = 5.162, $p = 0.008$). Repeated treatment with ebselen also decreased the DOI-evoked increase of c-fos mRNA but this effect was statistically significant only in the LO/VO (p=0.029). Repeated treatment with ebselen alone did not affect c-fos mRNA abundance in any of the regions investigated. (Figure 20).

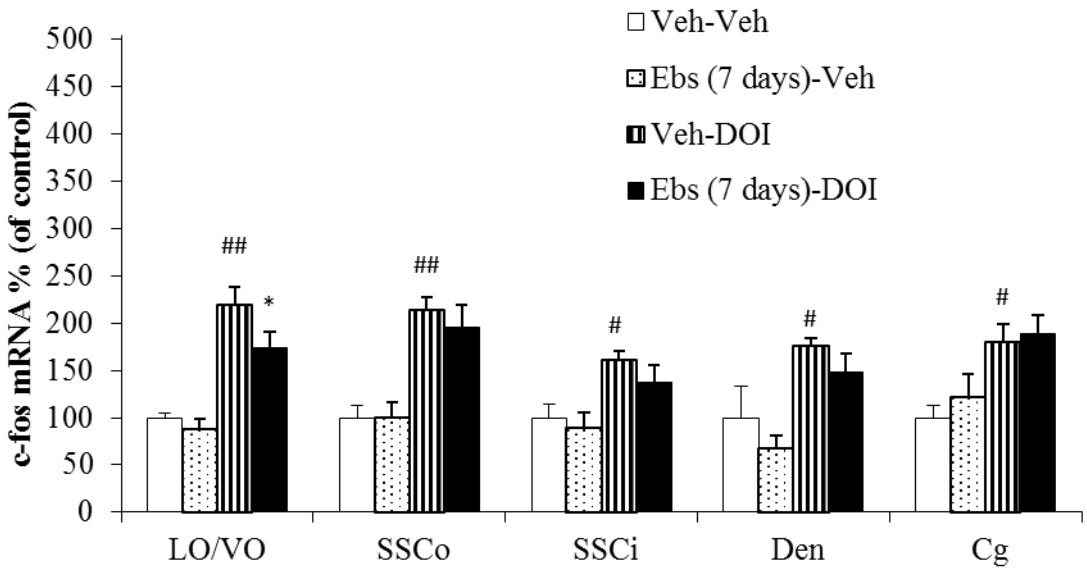

Figure 20: Effect of repeated ebselen (10 mg/kg, twice daily, for 7 days) on DOI (2 mg/kg) induced increase of c-fos mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.001$ treatment vs Veh-Veh, *p<0.05 Ebs-DOI vs Veh-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

egr2 mRNA: DOI weakly increased egr-2 mRNA but the effect was statistically significant only in Den (F (3,23) = 6.622, *p*=0.009). Ebselen did not have an effect on DOI-induced increase of egr-2 in the Den. Repeated treatment with ebselen alone did not alter egr-2 mRNA (Figure 21).

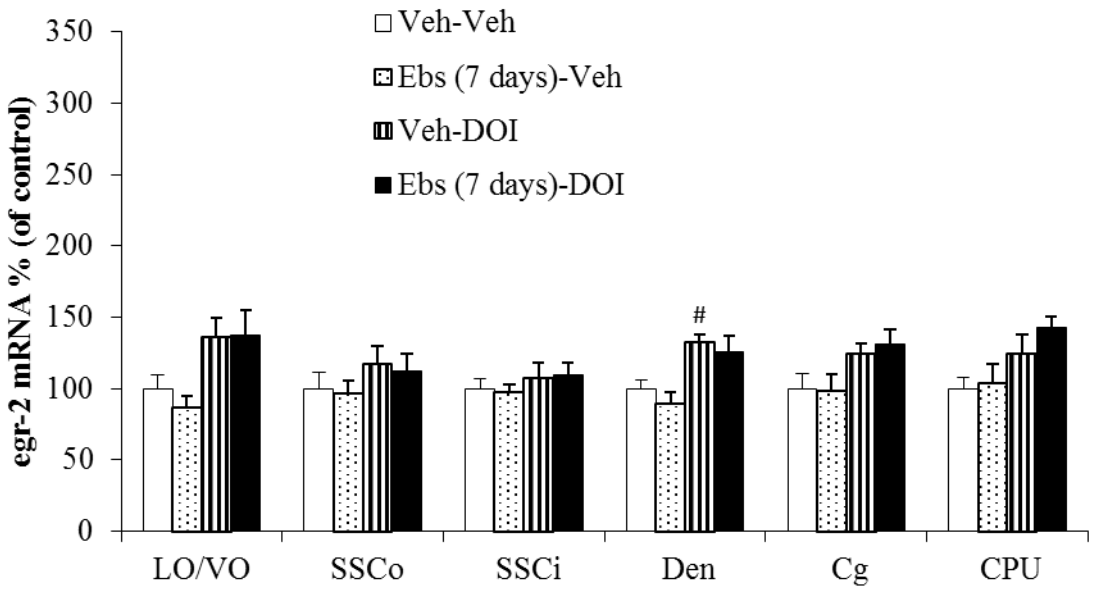

Figure 21: Effect of repeated ebselen (10 mg/kg, twice daily, for 7 days) on DOI (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$ treatment vs Veh-Veh. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

2.3.11 Effect of repeated lithium on DOI-evoked IEG expression

Acute treatment with lithium, only attenuated DOI-evoked ESR and not HTR. Therefore, the effect of acute lithium was not investigated in the molecular model. Two studies were performed in order to explore the effect of 3 and 7 days lithium treatment on DOI-evoked IEG expression.

Representative autoradiograms showing the distribution of Arc, c-fos and egr-2 mRNA are shown in Figure 22.

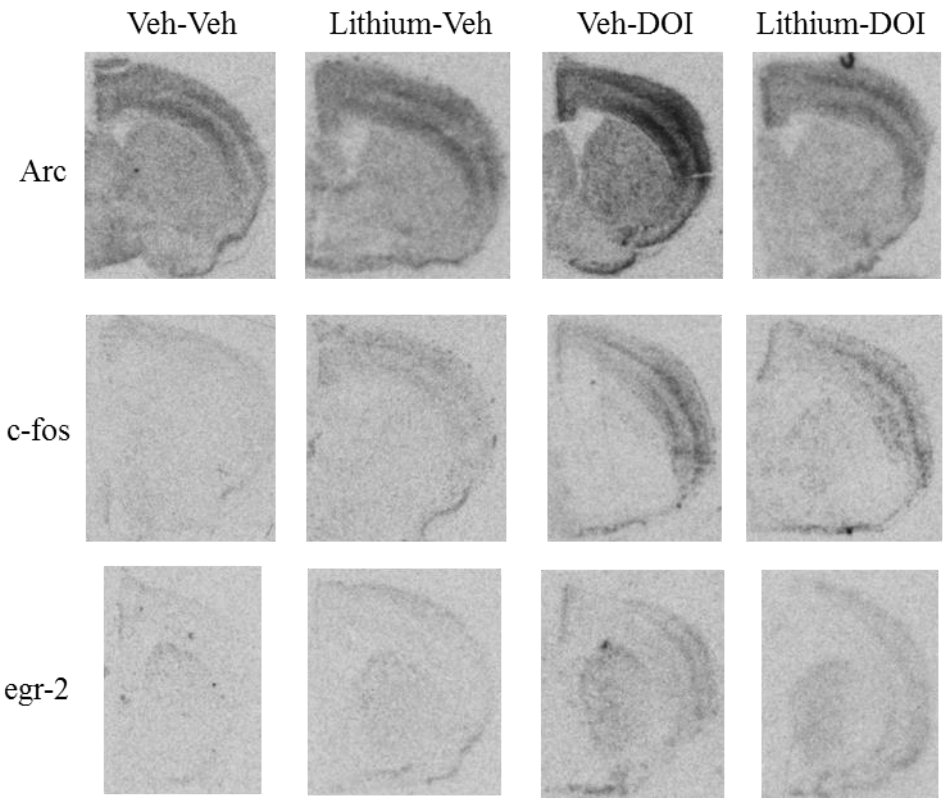

Figure 22: Representative autoradiograms showing distribution of Arc, c-fos and egr-2 mRNA in the caudate putamen of mice treated with either vehicle or lithium (10 mmol/kg on day1, 3 mmol/kg twice daily on days 2-3, i.p.) followed by DOI (2 mg/kg, i.p.) or saline.

Arc mRNA: As seen previously DOI (2 mg/kg) increased Arc mRNA in all regions investigated (LO/VO: F (3,21) = 4.623, p = 0.009; SSCo: F (3,23) = 24.404, p < 0.001; SSCi: F (3,23) = 15.115, p = 0.001; Den: F (3,22) = 14.019, p = 0.026; Cg: F (3,22) = 13.662, p = 0.010). Treatment with lithium for 3 days, decreased the Arc response to DOI except for LO/VO (SSCo: F (3,23) = 24.404, p=0.002; SSCi F (3,23) = 15.115, p=0.014; Den: F (3,22) = 14.019, p=0.002; Cg: F (3,22) = 13.662, p=0.008). Repeated lithium by itself elicited a statistically significant increase to the levels of Arc mRNA in the Den (F (3,22) = 14.019, p=0.031) with an upwards trend in other regions (Figure 23).

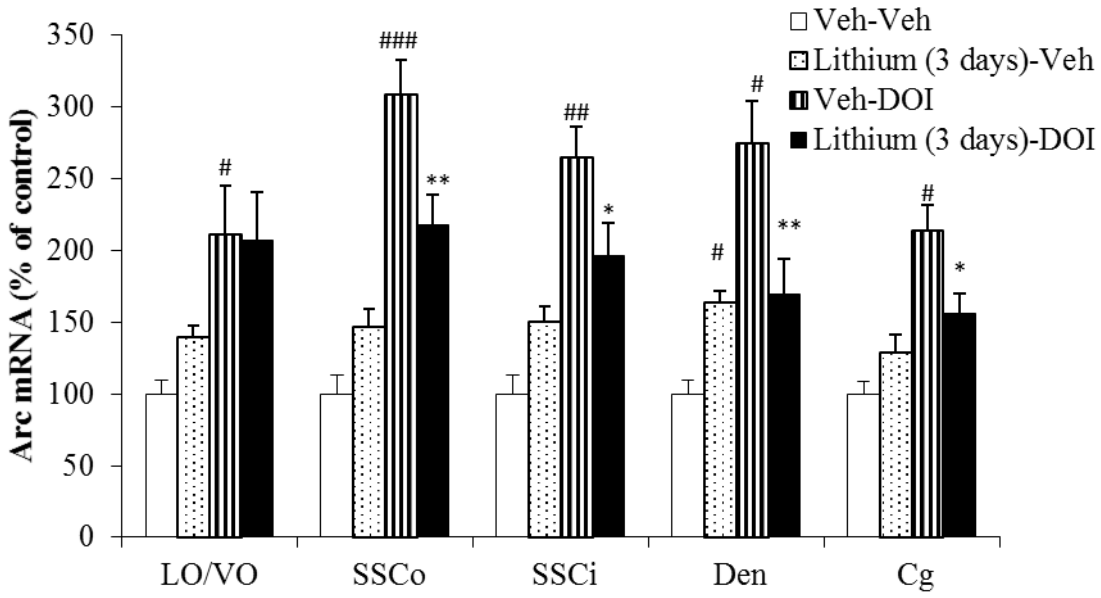

Figure 23: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on DOI (2 mg/kg) induced increase of Arc mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$, $^{\#\#}p<0.005$, $^{\#\#\#}p<0.001$ treatment vs Veh-Veh. $^{*}p<0.05$, $^{**}p<0.005$ Veh-DOI vs Lithium-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: DOI (2 mg/kg) increased c-fos mRNA in all of the regions investigated (LO/VO: F (3,22) = 12.912, $p<0.001$; SSCo: F (3,23) = 13.858, $p<0.001$; SSCi: F (3,23) = 7.642, $p<0.001$; Den: F (3,22) = 17.471, $p<0.001$; Cg: F (3,22) = 7.433, $p<0.001$) and this effect was also attenuated by lithium treatment for 3 days in all regions investigated (LO/VO: $p = 0.009$; SSCo: $p = 0.001$; SSCi: $p = 0.028$; Den: $p = 0.001$; Cg: $p = 0.009$). Lithium alone did not alter significantly c-fos mRNA (Figure 24).

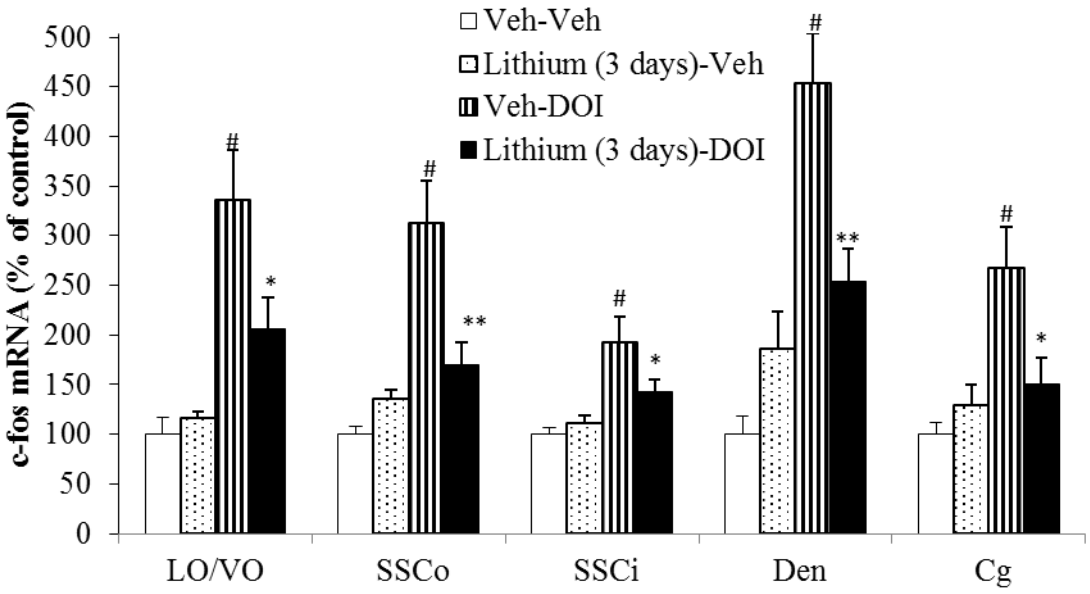

Figure 24: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on DOI (2 mg/kg) induced increase of c-fos mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.001$ treatment vs Veh-Veh. *p<0.05, **p <0.005 Veh-DOI vs Lithium-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

egr-2 mRNA: DOI (2 mg/kg) also increased egr-2 mRNA in all of the regions investigated (LO/VO: F (3,21) = 8.510, p=0.001; SSCo F (3,22) = 19.244, p<0.001; SSCi: F (3,22) = 11.901, p<0.001; Den: F (3,19) = 13.395, p<0.001; Cg: F (3,22) = 36.452, p<0.001; CPU: F (3,21) = 22.809, p<0.001) and this effect was attenuated by pretreatment with lithium for 3 days in all the regions investigated, with the exception of LO/VO (SSCo: p=0.008; SSCi: p=0.007; Den: p=0.045; Cg: p<0.001; CPU: p=0.023). Lithium treatment for 3 days did not have an effect on egr-2 mRNA in any of the regions investigated (Figure 25).

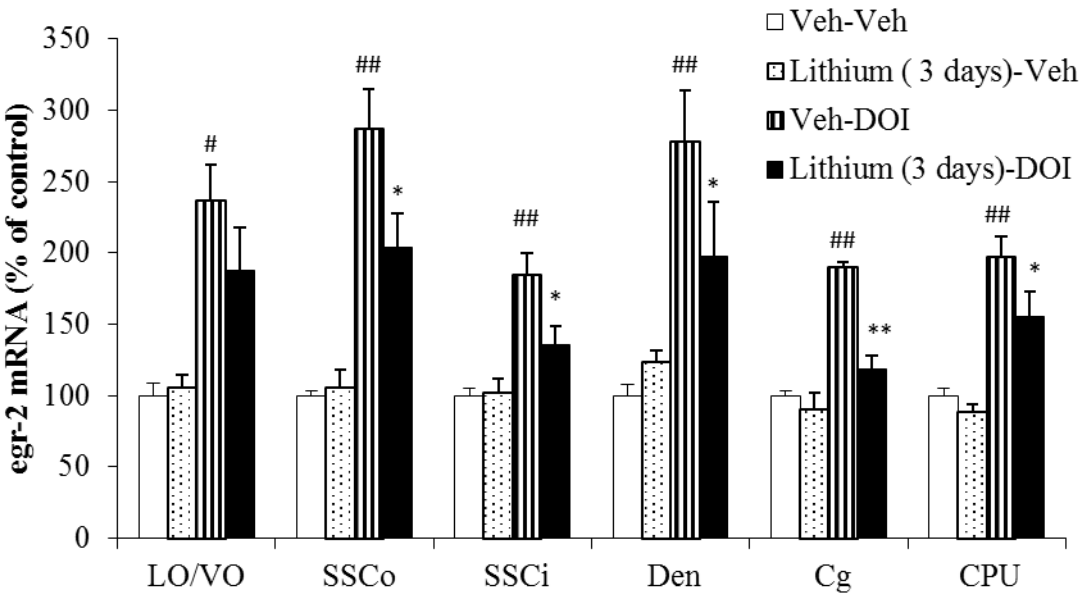

Figure 25: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on DOI (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.005$, $^{\#\#}p<0.001$ treatment vs Veh-Veh. *p<0.05, **p<0.001 Veh-DOI vs Lithium-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

These experiments on the effect of 3 days treatment with lithium were followed up with a study using 7 days treatment.

Arc mRNA: Surprisingly, in this experiment DOI (2 mg/kg) had a weak effect of Arc mRNA and the effect was statistically significant in only one region, the LO/VO ($F(3,23) = 3.883$, $p=0.046$). Treatment with lithium for 7 days did not alter Arc response to DOI in the LO/VO. In addition, 7 days treatment with lithium alone did not alter Arc mRNA levels (Figure 26).

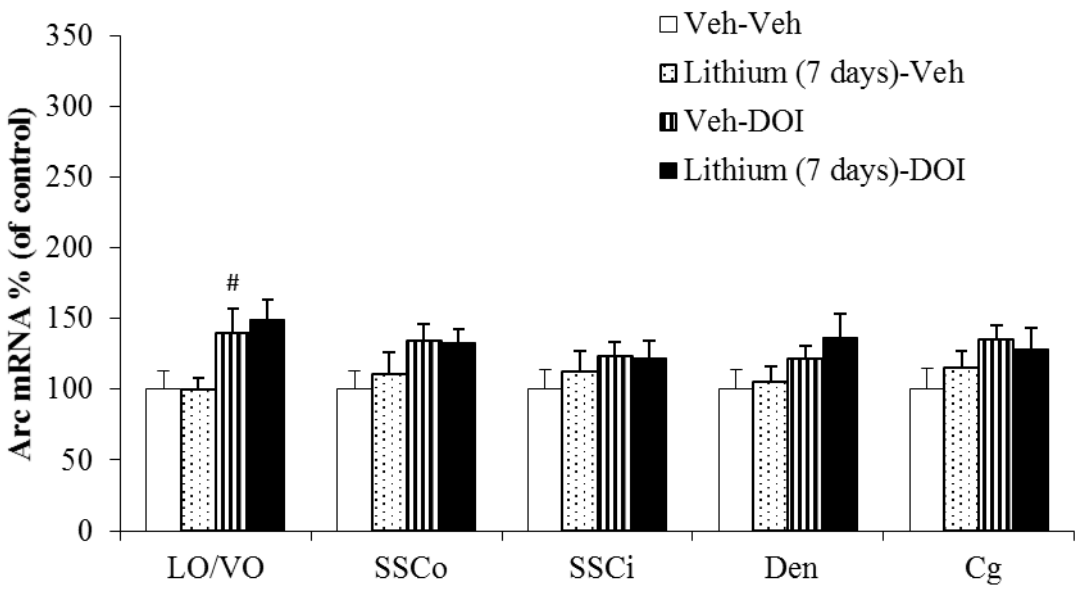

Figure 26: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7), on DOI (2 mg/kg) induced increase of Arc mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$ treatment vs Veh-Veh. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: In contrast to Arc, DOI caused a clear increase in c-fos expression (LO/VO: F (3,23) = 9.883, $p = 0.004$; SSCo: F (3,23) = 15.027, $p < 0.001$; Den: F (3,23) = 11.497, $p < 0.001$; Cg: F (3,23) = 10.719, $p < 0.001$) and treatment with lithium for 7 days decreased the c-fos response to DOI in Cg with a baseline effect in the SSCo (Figure 27).

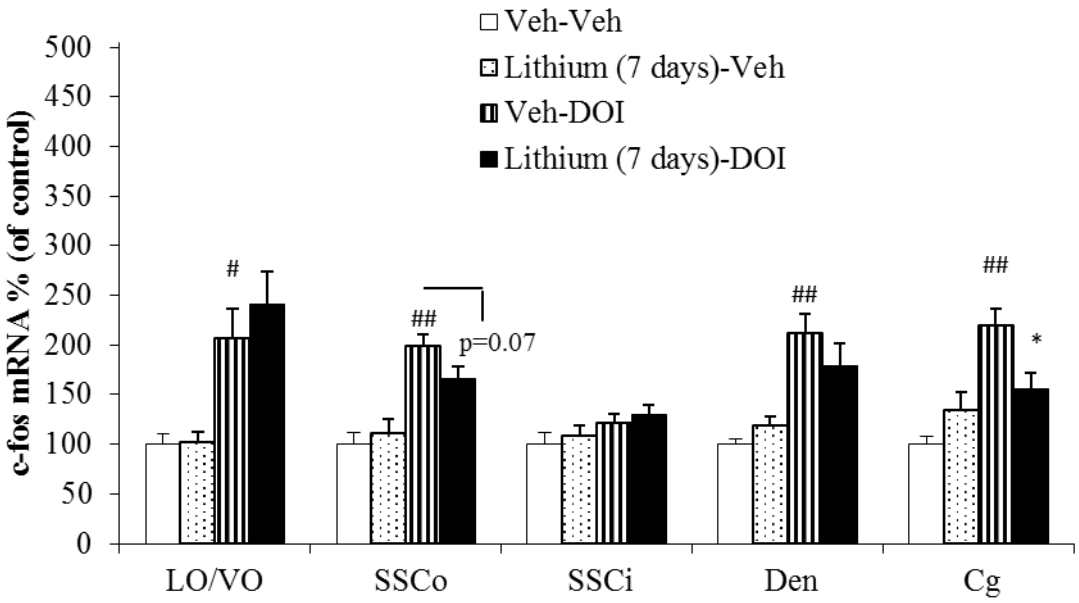

Figure 27: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7), on DOI (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.005$, $^{\#\#}p<0.001$ treatment vs Veh-Veh. $^{*}p<0.05$ Veh-DOI vs Lithium-DOI. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

egr-2 mRNA: In this experiment, DOI also increased egr-2 mRNA (LO/VO: F (3,22) = 4.383, *p* = 0.035; SSCo: F (3,22) = 3.593, *p*=0.026; Den: F (3,22) = 6.486, *p*=0.002; Cg: F (3,22) = 3.242, *p*=0.026) but the effect was not clearly altered by treatment with lithium for 7 days (Figure 28).

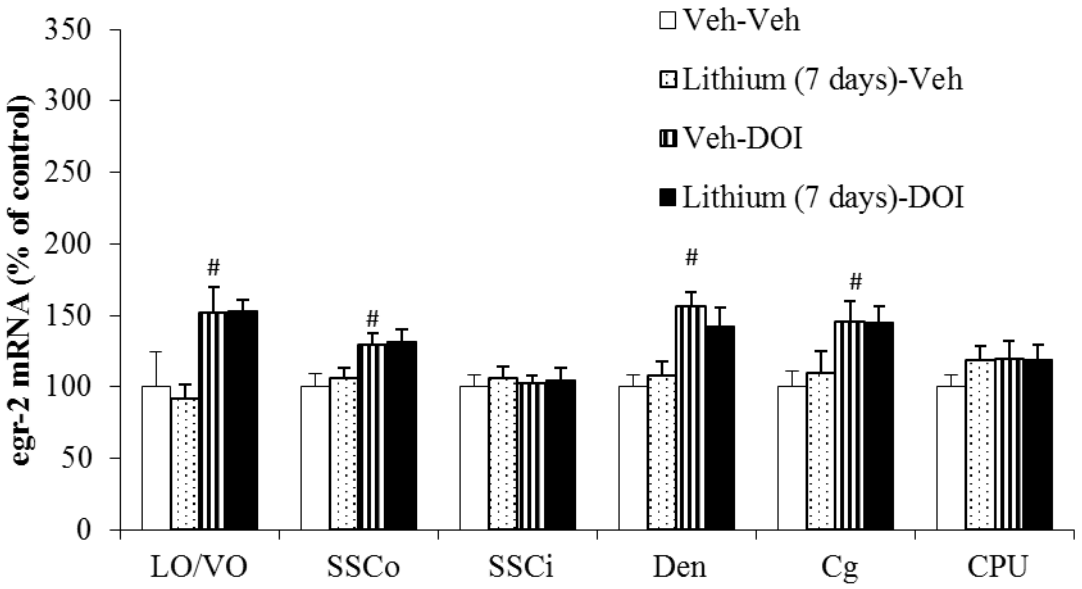

Figure 28: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on DOI (2 mg/kg) induced increase of egr-2 mRNA. Data shown are mean ± SEM percentage of response to Veh-Veh (6 animals/group). $^{\#}p<0.05$ treatment vs Veh-Veh. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

2.3.12 Effect of ebselen and lithium on 5-HT_{2A} receptor abudance

To investigate whether attenuation of 5-HT_{2A} receptor function by ebselen and lithium as detected in the behavioural and molecular models involved downregulation of the levels of the receptor, 5-HT_{2A} receptor abundance was measured by autoradiography using [^{3}H] ketanserin. Sections from animals treated with either ebselen (acute or repeated) or lithium (7 days) were investigated. There was no statistically significant effect of treatment with either ebselen or lithium on [^{3}H] ketanserin binding in frontal cortex (Figures 29 and 30).

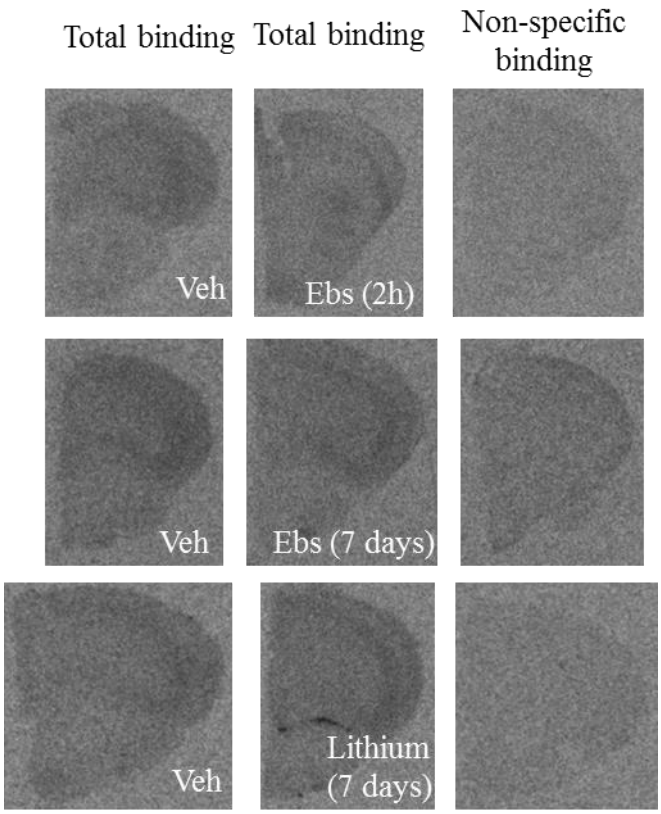

Figure 29: Representative autoradiograms showing distribution of [^{3}H] ketanserin binding sites in the frontal cortex of mice treated with vehicle, acute ebselen (10 mg/kg, i.p.), repeated ebselen (10 mg/kg twice daily for 7 days) or repeated lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-7, i.p.).

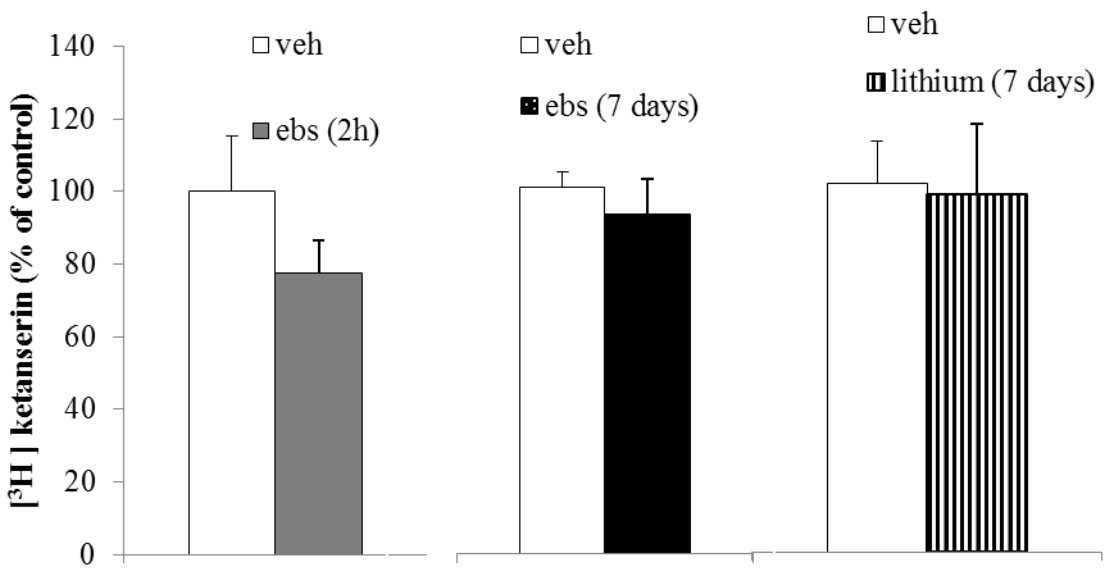

Figure 30: Effect of acute ebselen (10 mg/kg) (A), 7 days ebselen (B) or 7 days lithium (C) treatment on [^{3}H] ketanserin binding sites in the FC. Data shown are mean ± SEM percentage of treatment to Veh-Veh (6 animals/group). Student's unpaired t-test. Abbreviation as in methods section.

2.4 Discussion

The aim of the experiments described in this chapter was to compare the neuropharmacological effects of ebselen and lithium in mouse models of 5-HT_{2A} receptor function. The present data demonstrate that ebselen attenuated behavioural responses elicited by the 5-HT_{2A} agonists DOI and psilocin. Ebselen also reduced IEG expression evoked by DOI and psilocin. In comparison, lithium also reduced 5-HT_{2A} agonist-evoked behavioural and molecular responses. Interestingly, the IMPase inhibitor L-690,330, produced similar results to ebselen and lithium, in that it also decreased behavioural responses elicited by DOI, while the GSK-3 inhibitor AR-A 014418 did not. Neither ebselen nor lithium, altered the levels of 5-HT_{2A} receptor binding in cortical regions. Overall, these data indicate resemblances between ebselen and lithium in terms of their inhibiting effect of 5-HT_{2A} receptor function, and suggest IMPase inhibition as the mechanism involved.

2.4.1 Ebselen, lithium and L-690,330 but not AR-A 014418 attenuated 5-HT_{2A} agonist-induced HTR and ESR

The inhibitory effect of ebselen on 5-HT_{2A} receptor function seemed clear in the behavioural model. The behavioural data demonstrated that acute ebselen dose-dependently attenuated DOI-induced HTR and ESR. This attenuation of 5-HT_{2A} agonist-induced HTR was further confirmed with the use of psilocin. Moreover, the effect of ebselen on DOI-induced HTR was maintained after repeated treatment for 7 days.

Early studies suggested that the 5-HT_{2A} agonist-induced HTR is mediated through 5-HT_{2A} receptors located in the frontal cortex (Willins *et al.*, 1997). Specifically, it was shown that bilateral injection of DOI into the medial prefrontal cortex of rats elicited a HTR which was inhibited by peripheral administration of selective 5-HT_{2A} receptor antagonists (Willins *et al.*, 1997). These findings are corroborated by more recent data demonstrating the loss of 5-HT_{2A} agonist-induced HTR in mice with a conditional knockout of forebrain 5-HT_{2A} receptors (Gonzalez-Maeso *et al.*, 2007).

Here, the effect of lithium on DOI-induced HTR and ESR was evaluated using three different lithium dosing paradigms; acutely or repeatedly for 3 or 7 days. In comparison to ebselen, acute and repeated lithium similarly attenuated the ESR, although only repeated and not acute lithium attenuated the HTR. These findings are in agreement with previous experiments showing that only repeated (3 and 14 days) and not acute lithium treatment attenuated 5-HTP induced HTR in mice and rats (Goodwin *et al.*, 1986b; Hotta *et al.*, 1986).

None of ebselen or lithium, and L-690,330 completely blocked the HTR and ESR. The doses of L-690,330 administered in the present study (150 and 240 mg/kg) have previously been shown to inhibit central IMPase *in vivo* in the mouse as demonstrated by an increase in brain levels of IP1, the precursor of inositol (Atack *et al.*, 1993). Although the involvement of the PI cycle in the HTR/ESR to 5-HT_{2A} receptor agonist administration has not been proven, this seems likely given recent findings that the administration of a PLC inhibitor in the mPFC of rabbits abolishes DOI-induced HTR (Schindler *et al.*, 2013). It is possible that the incomplete block of 5-HT_{2A} agonist evoked HTR is due to the contribution of 5-HT_{2A} receptor mediated signalling that is independent of the PI cycle, such as the arachidonic acid pathway (Kurrasch-Orbaugh *et al.*, 2003).

To investigate the role of GSK-3 inhibition, a popular putative therapeutic target of lithium, the GSK-3 inhibitor AR-A 014418 was compared in the behavioural paradigm of 5-HT_{2A} receptor function. In contrast to the effects of ebselen, lithium and L-690,330, administration of AR-A 014418 did not attenuate DOI-induced HTR and ESR at either of the doses used (10 and 20 mg/kg). These doses of AR-A 014418 have been shown previously to rapidly cross the blood brain barrier and lead to concentrations in the brain sufficient to inhibit GSK-3 and also attenuate amphetamine-induced hyperactivity in the mouse (Gould *et al.*, 2004; Kalinichev *et al.*, 2011). Specifically, peripheral administration of 30 µmol/kg AR-A 014418 (equivalent of 9.2 mg/kg) resulted in a brain concentration of 1.2 µM in rats, while the K_i of GSK-3 inhibition is 38 nM (Bhat *et al.*, 2003; Gould *et al.*, 2004). Therefore, the present findings suggest that GSK-3 inhibition is not likely to mediate the effects of ebselen or lithium on 5-HT_{2A} receptor function.

Collectively, the experiments in the behavioural model of 5-HT_{2A} receptor function indicate that both ebselen and lithium attenuate 5-HT_{2A} agonist-induced behaviour, and that this effect is likely to be mediated through IMPase and not GSK-3 inhibition. This concords with the idea that IMPase inhibition by ebselen and lithium results in an attenuation of 5-HT_{2A} receptor signalling via G_q receptors, linked to PI cycle (Berridge *et al.*, 1989). Since all three IMPase inhibitors (ebselen, lithium and L-690,330) had a greater effect on ESR than HTR, ESR might be a more sensitive model of PI signalling of 5-HT_{2A} receptor than HTR. Interestingly, neither ebselen nor lithium completely blocked 5-HT_{2A} agonist evoked HTR and ESR. This may be due to incomplete inhibition of IMPase (discussed below in more detail).

2.4.2 Ebselen and lithium attenuated 5-HT_{2A} agonist-evoked IEG expression

In order to further test evidence that ebselen and lithium reduced 5-HT_{2A} receptor function at the behavioural level, a molecular model of 5-HT_{2A} receptor function was utilised, that of agonist-evoked regional brain IEG expression. As with the behavioural response ebselen attenuated molecular responses to DOI and psilocin. Thus, acute ebselen reduced DOI-evoked c-fos mRNA in many regions tested and this result was confirmed with two additional IEGs, Arc and egr-2. Acute ebselen also reduced IEG response to a second agonist, psilocin (Arc and egr-2; c-fos was not increased by psilocin) in the majority of the regions investigated. Repeated administration of ebselen for 7 days also attenuated DOI-evoked IEG expression but this was statistically significant in fewer regions for Arc and c-fos while egr2 was not affected. Ebselen alone was generally without effect on IEG expression. These findings indicate that ebselen reduced 5-HT_{2A} function at the molecular level although the effects were more robust after acute than chronic treatment.

As with ebselen, lithium attenuated 5-HT_{2A} function at the molecular level. Thus, 3 days treatment with lithium clearly attenuated DOI-evoked mRNA encoding for c-fos, Arc and egr-2 in most regions investigated. Repeated administration of lithium for 7 days also reduced DOI-induced c-fos but in fewer regions and did not affect Arc or egr-2 mRNA.

The present findings are not in agreement with previous studies which reported that lithium enhanced the effect of DOI on c-fos mRNA abundance (Leslie *et al.*, 1993; Moorman *et al.*, 1998). This discrepancy might reflect methodological differences. Thus, those studies used rats and lithium was administered for 3 weeks through diet. Additionally, in these studies lithium was administered at a lower dose (approximately 50 mg/kg) compared to the present

one (250 mg/kg). The dose used in the present study has been previously reported to result in plasma and brain homogenate concentrations of lithium within the therapeutic range that inhibit IMPase and reduce inositol levels in the brain (Allison *et al.*, 1971; Goodwin *et al.*, 1986b; Hillert *et al.*, 2012). It should also be pointed out that the dose of DOI used by the earlier work (8 mg/kg), was high and could lead to both toxic and non 5-HT_{2A} mediated effects. Indeed, the authors report that DOI induced convulsions in some animals (Moorman *et al.*, 1998).

Overall, both ebselen and lithium attenuated 5-HT_{2A} function at the molecular level and this effect was maintained although less robust after repeated treatments. The role of IMPase and PI cycle in the IEG effects of lithium and ebselen was not explored here. However, it was previously found that the increase in IEG expression following 5-HT_{2A} agonist administration is G_q-PLC-β signalling dependent, as U73122 a selective PLC-β inhibitor eliminated the 5-HT_{2A} agonist-induced IEG expression in primary cortical neurons of the mouse (Gonzalez-Maeso *et al.*, 2007). Since PI signalling is necessary for 5-HT_{2A} agonist-induced IEG expression, IMPase inhibition and the subsequent attenuation of PI signalling is likely to contribute to the decrease of 5-HT_{2A} agonist-induced IEG expression by ebselen and lithium.

Although ebselen and lithium consistently reduced 5-HT_{2A} agonist-induced IEG expression, the effects were not always statistically significant in the same regions. Additionally, no specific regional pattern of reduction of 5-HT_{2A} agonist induced IEG expression was identified for either ebselen or lithium. In hindsight this may be due to an under-powered study design which made it difficult to detect small reductions compared to controls.

The reduction of 5-HT_{2A} receptor function at both the behavioural and molecular level by ebselen and lithium, was generally weaker following longer term treatment, which could reflect adaptation of PI signalling. For example, it might reflect compensatory increase of inositol which offsets the inositol depletion evoked by ebselen and lithium. Results presented later in this thesis (chapter 5) show that repeated treatment with ebselen or lithium for 14 days induced an increase in IMPase and sodium *myo*-inositol transporter mRNA in several brain regions, which could potentially lead to increased biosynthesis of inositol and elevated import of inositol from the extracellular environment.

The inhibitory effects of ebselen and lithium on 5-HT_{2A} receptor function is unlikely to be mediated through down-regulation of the 5-HT_{2A} receptor, since neither agent decreased 5-HT_{2A} binding in cortical regions. This in agreement with previous studies showing that lithium treatment for 14 days in the same doses used here did not change the abundance of 5-HT_{2A} receptors in the frontal cortex of mice (Goodwin *et al.*, 1986b).

Neither ebselen nor lithium completely abolished the behavioural and molecular effects of 5-HT_{2A} receptor agonists, which could result from incomplete inhibition of IMPase. Ebselen has been shown to inhibit IMPase *ex vivo* in the same dose that was used in the present study (10 mg/kg), however the inhibition was partial (Singh *et al.*, 2013). Lithium, has also been shown to decrease but not completely deplete inositol levels in rats in the doses administered in the present study, indicating incomplete IMPase inhibition too (Allison *et al.*, 1971). It would be interesting to explore whether higher doses of ebselen in the same models of 5-HT_{2A} receptor function, can completely inhibit the effects of 5-HT_{2A} agonists. However, this was not possible here since ebselen has poor solubility and higher doses could not be injected. However, ebselen could be administered orally in chow in higher doses in future

studies. With regards to lithium, a further increase in dose used results in toxic effects. Although complete inhibition of IMPase may be possible to achieve pharmacologically, it might not be desirable since genetic knockout of IMPase in mice is lethal. These mice have substantially depleted inositol levels and require dietary supplementation of inositol to survive (Cryns *et al.*, 2008).

In summary, ebselen was found to attenuate central 5-HT_{2A} function in both behavioural and molecular models in the mouse. Lithium showed striking similarities to ebselen in the same models. The effects of ebselen and lithium were mimicked by an IMPase inhibitor suggesting inhibition of IMPase as the likely mechanism. The latter conclusion was supported by the finding that a selective GSK-3 inhibitor was not effective in the behavioural model. To further compare the neuropharmacological effects of ebselen and lithium the next chapter describes experiments using a molecular and behavioural model of 5-HT_{2C} receptor function; the 5-HT_{2C} receptor also couples to the PI cycle via Gq signalling.

Chapter 3

Investigation of the effects of ebselen on central 5-HT_{2C} receptor function in the mouse

3 Investigation of the effects of ebselen on central 5-HT_{2C} receptor function in the mouse

3.1 Introduction

In Chapter 2 it was shown that ebselen attenuated the function of central 5-HT_{2A} receptors in the mouse and that this effect was likely to be mediated through IMPase inhibition. In comparison, lithium attenuated the function of 5-HT_{2A} receptor in a way similar to ebselen. To further investigate the effects of ebselen on the central 5-HT system ebselen and lithium were compared in a behavioural and molecular model of 5-HT_{2C} receptor function, which is also G_q linked to PLC signalling (Barnes *et al.*, 1999). The 5-HT_{2C} receptor is coupled to PLC signalling and activation of the receptor has been shown to increase PI hydrolysis (Sanders-Bush *et al.*, 1988; Newton *et al.*, 1996). Lithium has been shown to inhibit IMPase linked to 5-HT_{2C} signalling in human and animal cell lines in numerous experiments (Newton *et al.*, 1996; Berg *et al.*, 1998; Briddon *et al.*, 1998). For example, in xenopus oocytes lithium attenuated Ca^{2+} release and Ca^{2+}-induced chloride currents evoked by 5-HT stimulation of 5-HT_{2C} receptors (Matsuoka *et al.*, 1997). Interestingly, in the same study lithium was shown to selectively inhibit 5-HT_{2C} versus muscarinic receptor activated Ca^{2+} mobilisation (Matsuoka *et al.*, 1997).

Lithium has also been shown to attenuate 5-HT_{2C} receptor function in a human model (Friston *et al.*, 1989). Slow wave sleep in humans is increased by blockade of central 5-

HT_{2C} receptors using drugs such as ritanserin, in a dose-dependent manner and this increase is maintained following repeated treatment (Idzikowski *et al.*, 1987; Solomon *et al.*, 1989; Sharpley *et al.*, 1990; Sharpley *et al.*, 2000). Lithium has also been shown to increase slow wave sleep in healthy volunteers (Friston *et al.*, 1989). Surprisingly, the effect of lithium on 5-HT_{2C} receptor function in animal models has not been explored. Since ebselen attenuated 5-HT_{2A} receptor function, it was expected that it should have similar effects to other G_q receptors, including the 5-HT_{2C}.

The 5-HT_{2C} receptor is an important target of psychotropic drugs and regulates a variety of physiological functions. The 5-HT_{2C} receptor is widely distributed within the brain in particular in cortical regions, the limbic system and basal ganglia (Pompeiano *et al.*, 1994; Barnes *et al.*, 1999). 5-HT_{2C} receptor mRNA undergoes editing that leads to at least 14 functionally distinct isoforms with different sensitivity to 5-HT and isoform expression is differentially altered by stress (Fitzgerald *et al.*, 1999; Niswender *et al.*, 2001; Jensen *et al.*, 2010). The 5-HT_{2C} receptor exerts a tonic and phasic inhibition on mesolimbic dopamine systems which might contribute to antidepressant effect of SSRIs (for review see (Serretti *et al.*, 2004; di Giovanni *et al.*, 2011). The 5-HT_{2C} receptor controls neuroendocrine secretion of oxytocin, prolactin and corticosterone (Bagdy *et al.*, 1992; Bagdy *et al.*, 1995; Heisler *et al.*, 2007a).

Several antidepressant and antipsychotic agents show affinity for the 5-HT_{2C} receptor. Specifically, antipsychotics such as clozapine, chlorpromazine, risperidone, thioridazine, fluphenazine, spiperone and haloperidol have been shown to have affinity for the 5-HT_{2C} receptor (Canton *et al.*, 1994). The antidepressants doxepin, mianserin and trazodone also show affinity for the 5-HT_{2C} receptor (Barnes *et al.*, 1999).

Additionally, the novel antidepressant agomelatine, which has unique pharmacological profile compared to typical antidepressants, as well as being an agonist at melatonin receptors is an antagonist at the 5-HT_{2C} receptor (Loo *et al.*, 2002).

A number of drug tools show selectivity for the 5-HT_{2C} receptor. The 5-HT_{2C} agonist Ro 60-0175 ($pK_{i(2C)}$ = 8.8, $pK_{i(2A)}$ = 6) and antagonist SB242084 ($pK_{i(2C)}$ = 9, $pK_{i(2A)}$ = 6.8), have been important tools in this aspect (Kennett *et al.*, 1997; Barnes *et al.*, 1999; Dekeyne *et al.*, 1999). However, early studies often used mCPP, a non-selective 5-HT_{2C} agonist ($pK_{i(2C)}$ = 7.2, $pK_{i(2A)}$ = 6.4) (Dalton *et al.*, 2004). Additional agonists with selectivity for the 5-HT_{2C} receptor include MK212, WAY161503, lorcaserin, CP809,101, Ro 60-0332 and antagonists SDZ SER 082, SB-206,553.

Much evidence links the 5-HT_{2C} receptor from both pharmacological and genetic manipulations to anxiety. For example, 5-HT_{2C} receptor agonists have anxiogenic properties while 5-HT_{2C} receptor antagonists induce anxiolytic effects in the elevated plus maze and social interaction test (Kennett *et al.*, 1997; Martin *et al.*, 2002; Moya *et al.*, 2011). The antidepressant agomelatine, has also been reported to exert anxiolytic effects mediated through antagonism at the 5-HT_{2C} receptor (Millan *et al.*, 2005). Lastly, genetic knockout or overexpression of the 5-HT_{2C} receptor in mice lead to anxiolytic or anxiogenic behaviour respectively (Heisler *et al.*, 2007b; Kimura *et al.*, 2009).

In addition to anxiety, 5-HT_{2C} receptor activation has been linked to number of other behavioural effects. In particular, various agonists including mCPP and Ro 60-0175

have been shown to decrease food intake in rodents, an effect which is inhibited by SB242084 and other 5-HT_{2C} antagonists. The anorectic effect of 5-HT_{2C} agonists is completely abolished in 5-HT_{2C} knockout mice (Kennett *et al.*, 1997; Hewitt *et al.*, 2002; Dalton *et al.*, 2006; Fletcher *et al.*, 2009). In rodents, 5-HT_{2C} receptor agonists also dose-dependently increase penile erections and 5-HT_{2C} antagonists block this effect (Bagdy *et al.*, 1992; Millan *et al.*, 1997; Higgins *et al.*, 2001). Reduced locomotor activity is another well characterised effect of 5-HT_{2C} receptor activation. For instance, mCPP and Ro 60-0175 dose-dependently decrease locomotor activity in rats and mice and this effect is reversed by SB242084, and abolished in 5-HT_{2C} genetic knockout mice (Kennett *et al.*, 1997; Higgins *et al.*, 2001; Martin *et al.*, 2002; Fletcher *et al.*, 2009).

The function of the 5-HT_{2C} receptor has also been investigated at the molecular level using the IEG approach described in the previous chapter to model 5-HT_{2A} function. In particular, it has been shown that mCPP and Ro 60-0175 induce the expression of c-fos protein in a variety of cortical and limbic regions (De Deurwaerdere *et al.*, 2000; Singewald *et al.*, 2000; Singewald *et al.*, 2003; Varcoe *et al.*, 2003; Stark *et al.*, 2006; Beyeler *et al.*, 2010; Kadiri *et al.*, 2012; Navailles *et al.*, 2013). Additionally, Ro 60-0175 has been shown to increase the expression of Period1 gene, which is related to circadian oscillations, in the suprachiasmatic nucleus (Varcoe *et al.*, 2003).

The aim of the present chapter was to investigate the effect of ebselen on 5-HT_{2C} receptor function in comparison to lithium. The effects of ebselen and lithium were investigated both at the behavioural and the molecular level. Specifically, the 5-HT_{2C} receptor function was assessed through the hypolocomotor and IEG responses to administration of Ro 60-0175. It was hypothesized that both ebselen and lithium would

attenuate the function of the 5-HT_{2C} receptor in a way similar to that observed for the 5-HT_{2A} receptor.

3.2 Methods

3.2.1 Experimental animals

Adult, male C57BL/6 (7-9 weeks old) (Harlan, Bicester, UK) mice were housed in groups of 6 under controlled conditions of lighting (12 h light-dark cycle) and temperature (21±1 ^{o}C). Experiments were carried out during the light phase. Food and water was available *ad libitum*. One week acclimatisation in the animal facility was allowed before the experiments were carried out. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and Home Office Guidelines.

3.2.2 Drugs and chemicals

The vehicle of lithium chloride (MP biomedicals LLC) and (α*S*)-6-Chloro-5-fluoro-α-methyl-1*H*-indole-1-ethanamine fumarate (Ro 60-0175; Tocris) was saline. To improve solubility of 2-Phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen; Sigma) and 6-Chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1*H*-indole-1-carboxyamide dihydrochloride (SB 242084; Tocris) their vehicles were modified. In particular the vehicle of ebselen consisted of 4% (2-hydroxypropyl)-β-cyclodextrin, 0.4% (v/v) dimethyl sulfoxide (DMSO). For SB 242084, vehicle consisted of 25 mM citric acid in 10% (2-hydroxypropyl)-β-cyclodextrin, pH 7. All injections were administered i.p. The volume of injection was adjusted to weight (0.01 ml/g for all drugs except for ebselen and its vehicle that were injected at a volume of 0.02 ml/g due to poor solubility).

3.2.3 Behavioural model of 5-HT_{2C} receptor function

The effect of ebselen and lithium treatment on 5-HT_{2C} receptor function was assessed using Ro 60-0175-evoked hypolocomotion. Initial experiments established a dose response to Ro 60-0175. To confirm that the effect of Ro 60-0175 was mediated through the 5-HT_{2C} receptor a second set of experiments was carried out using pretreatment with the selective 5-HT_{2C} antagonist, SB 242084.The effect of ebselen and lithium was then investigated in the same model (see list of experiments below).

In all studies, mice received Ro 60-0175 at a dose of 6 mg/kg, except for the dose-response study, where additional doses of 2 and 3 mg/kg were administered. Immediately after Ro 60-0175 injection mice were placed in individual, transparent, plexiglass chambers (42 x 21 x 21 cm) and the number of horizontal beam breaks was monitored for 1 h using an automated system (AM548 IR, Linton Instrumentation, Norfolk, UK or PAS System, SanDiego Instruments, San Francisco). Animals were randomly allocated to treatment groups (n = 3-6 per group).

The following experiments were carried out:

i. Dose response to Ro 60-0175 (2, 3 and 6 mg/kg)
ii. Acute SB 242084 (0.5 and 1 mg/kg) plus Ro 60-0175
iii. Acute ebselen (10 mg/kg) plus Ro 60-0175
iv. Repeated lithium for 3 days (10 mmol/kg[4] on day 1, 3 mmol/kg, twice daily, on days 2-3) plus Ro 60-0175

[4] 10 mmol/kg = 420 mg/kg

v. Repeated lithium for 7 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-7) plus Ro 60-0175)

Pretreatment times for with ebselen, lithium and SB 242084 were varied according to pharmacokinetic properties of these agents (see Table 1 for a summary of experimental protocols).

treatment	duration (days)	(last) injection before agonist
SB 242084	0	30 min
ebselen	0	1 h
lithium	3	18 h
lithium	7	18 h

Table 1: Summary table of pre-treatment times of different agents prior to Ro 60-0175 (6 mg/kg) administration in the behavioural model.

3.2.4 Molecular model of 5-HT_{2C} receptor function

The effect of acute or repeated ebselen and lithium treatment on the function of the 5-HT_{2C} receptor was also tested at the molecular level (IEG expression). Similar to the behavioural studies, initial experiments were carried out to test the dose response effect of Ro 60-0175 and then the role of the 5-HT_{2C} receptor was investigated using SB 242084. The effect of ebselen and lithium was then investigated in the same model (see a list of experiments below).

In all studies animals (n = 6 per group) were allowed to acclimatise in the procedure room where the injections were administered for 4 h before agonist administration. One hour post

agonist administration, animals were culled by cervical dislocation. In these studies Ro 60-0175 was injected at a dose of 6 mg/kg. Brains were dissected, snap frozen in ice-cold isopentane and stored in -80 ^{0}C until used.

The following experiments were carried out:

x. Dose response to Ro 60-0175 (2, 3 and 6 mg/kg)

xi. Acute SB 242084 (0.5 mg/kg) plus Ro 60-0175

xii. Acute ebselen (10 mg/kg) plus Ro 60-0175

xiii. Repeated lithium for 3 days (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3) plus Ro 60-0175

Pretreatment times for ebselen, lithium or SB 242084 are summarised in Table 2.

treatment	**duration (days)**	**(last) injection before agonist**
SB 242084	0	30 min
ebselen	0	1 h
lithium	3	18 h

Table 2: Summary table of pre-treatment times of different agents prior to Ro 60-0175 (6 mg/kg) administration in the molecular model.

3.2.4.1 Protocol for in situ hybridization

Coronal sections (12 µm) were collected onto gelatinised slides at the level of the caudate putamen (Figure 23 in (Paxinos *et al.*, 2007). Sections were stored in -80 ^{0}C until pre-treatment. Sections were pre-treated using standards protocols, as it was described in Chapter 2. Briefly, slides were defrosted, fixed in paraformaldehyde, acetylated in acetic anhydride in triethanolamine hydrochloride, dehydrated in increasing concentrations of alcohols, delipidated in chloroform and rehydrated in increasing concentration of alcohols. Sections were then left to air dry and stored in -20 ^{0}C.

Oligonucleotide probes complementary to c-fos (CTTCAGGGTAGGTGAAGACAAAGGAAGACGTGTAAGTAGTGCAGC), Arc (CTCGGTTGCCCATCCTCACCTGGCCCCCAAGACTGATATTGCTGA) and egr2 (GGATCATAGGAATGAGACCTGGGTCCATAGCTGGCTTGG) mRNA were 3'-tailed labelled with [^{35}S]-dATP (see Chapter 2 for the detailed protocol). Briefly, 2 µM of each oligonucleotide probe was incubated with [^{35}-S]-dATP and terminal deoxynucleotidyl transerase (TdT) at 37 ^{0}C for 35 min. The unincorporated oligonucleotide probe was then separated from the labelled using illustra NICK column.

Sections from selected brain regions were defrosted and incubated overnight at 34 ^{0}C with the labelled probe in hybridization buffer (2.4x10^{6} cpm/section) containing 50 mM DTT (see Chapter 2 for detailed protocol). Sections were washed 16 h post incubation, in 1 x SSC for 3 x 20 min at 55 ^{0}C followed by 2 x 60 min at room temperature. Sections

were allowed to dry overnight and exposed for 7 days to BioMax MR film. Films were developed using an automatic X-ray film processor.

Image analysis of autoradiograms

The mRNA abundance of Arc, c-fos and egr2 was calculated by densitometric quantification of selected brain regions (see Figure 1) on the autoradiograms using MCID software as described in Chapter 2. In particular, the regions of interest were the cingulate (Cg), somatosensory cortex outer (SSCo) and inner (SSCi) layer, caudate putamen (CPU) and endopiriform nucleus (Den).

The optical density readings were converted in nCi/g of tissue by calibration with the use of [^{14}C] microscales, which were co-exposed with the slides. Measurements of regions of interest were taken bilaterally from 3 sections per slide and the values for each region of interest were averaged.

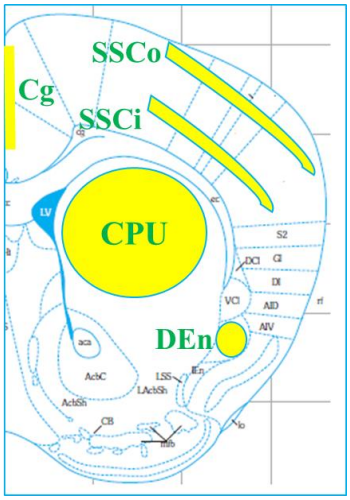

Figure 1: Coronal section of the mouse brain at the level of caudate putamen. Brain regions selected for gene expression analysis are highlighted in yellow. Cg: cingulate cortex; SSCo: somatosensory cortex outer layer; SSCi: somatosensory cortex inner layer; CPU: caudate putamen; Den: endopiriform nucleus. Image modified by (Paxinos *et al.*, 2007).

3.2.5 Data analysis

All data were analysed using IBM SPSS Statistics, version 20. Data were expressed as percentage of controls and presented as mean ± SEM values. Effects were considered significant when $p < 0.05$.

The effect of each treatment was evaluated against vehicle controls using one-way ANOVA followed by LSD *post-hoc* test. For the IEG expression data, each region was analysed separately.

3.3 Results

3.3.1 Effect of different doses of Ro 60-0175 on hypolocomotion

In order to establish an effective dose of Ro 60-0165 that elicits hypolocomotor effects a dose-response study was conducted. As shown in Figure 2 compared to vehicle controls 2, 3 and 6 mg/kg Ro 60-0175 resulted in a reduction in locomotor activity of 49%, 54% and 64% respectively ($F(3,10) = 14.644$, Veh compared to 2 mg/kg: $p = 0.004$; Veh compared to 3 mg/kg: $p = 0.001$, Veh compared to 6 mg/kg: $p < 0.000$). There was no statistically significant difference between the doses of Ro 60-0175.

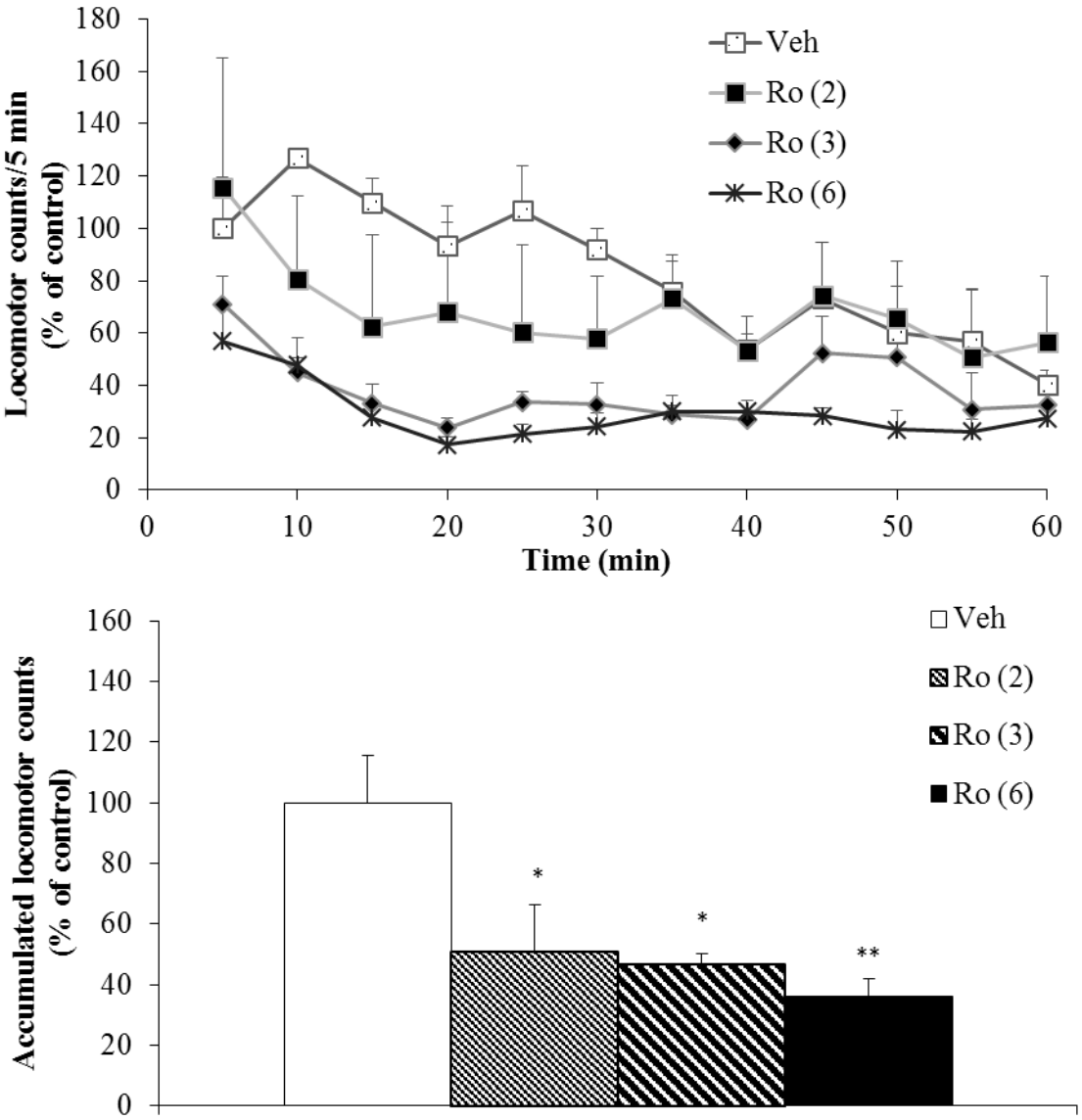

Figure 2: Effect of different doses of Ro 60-0175 (2, 3 and 6 mg/kg) on locomotor activity over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (3 animals/group), *p<0.005, **p <0.001 treatment vs Veh. One way ANOVA followed by LSD post-hoc.

3.3.2 Effects of SB242084 on Ro 60-0175-evoked hypolocomotion

In order to establish that the inhibitory effect of Ro 60-0175 on hypolocomotion was mediated through the 5-HT_{2C} receptor, mice were pretreated with the selective 5-HT_{2C} receptor antagonist SB242084.A dose of 6 mg/kg Ro 60-0175 and two doses of SB242084 were used (0.5 and 1 mg/kg).

Compared to vehicle controls, Ro 60-0175 6 mg/kg decreased locomotor activity (SB242084 0.5 mg/kg: $F(3,17) = 6.948$, $p = 0.021$; SB242084 1 mg/kg: $F(3,17) = 10.114$, $p = 0.032$) but this effect was completely abolished by SB242084 (SB242084 0.5 mg/kg: $p = 0.001$; SB242084 1 mg/kg: $p < 0.000$) (Figures 3 and 4). SB242084 (0.5 and 1 mg/kg) alone had no effect on locomotor activity compared to vehicle controls but animals treated with 1 mg/kg but not 0.5 mg/kg of SB242084 plus Ro 60-0175 had increased locomotor activity levels compared to vehicle controls ($p = 0.018$) (Figures 3 and 4).

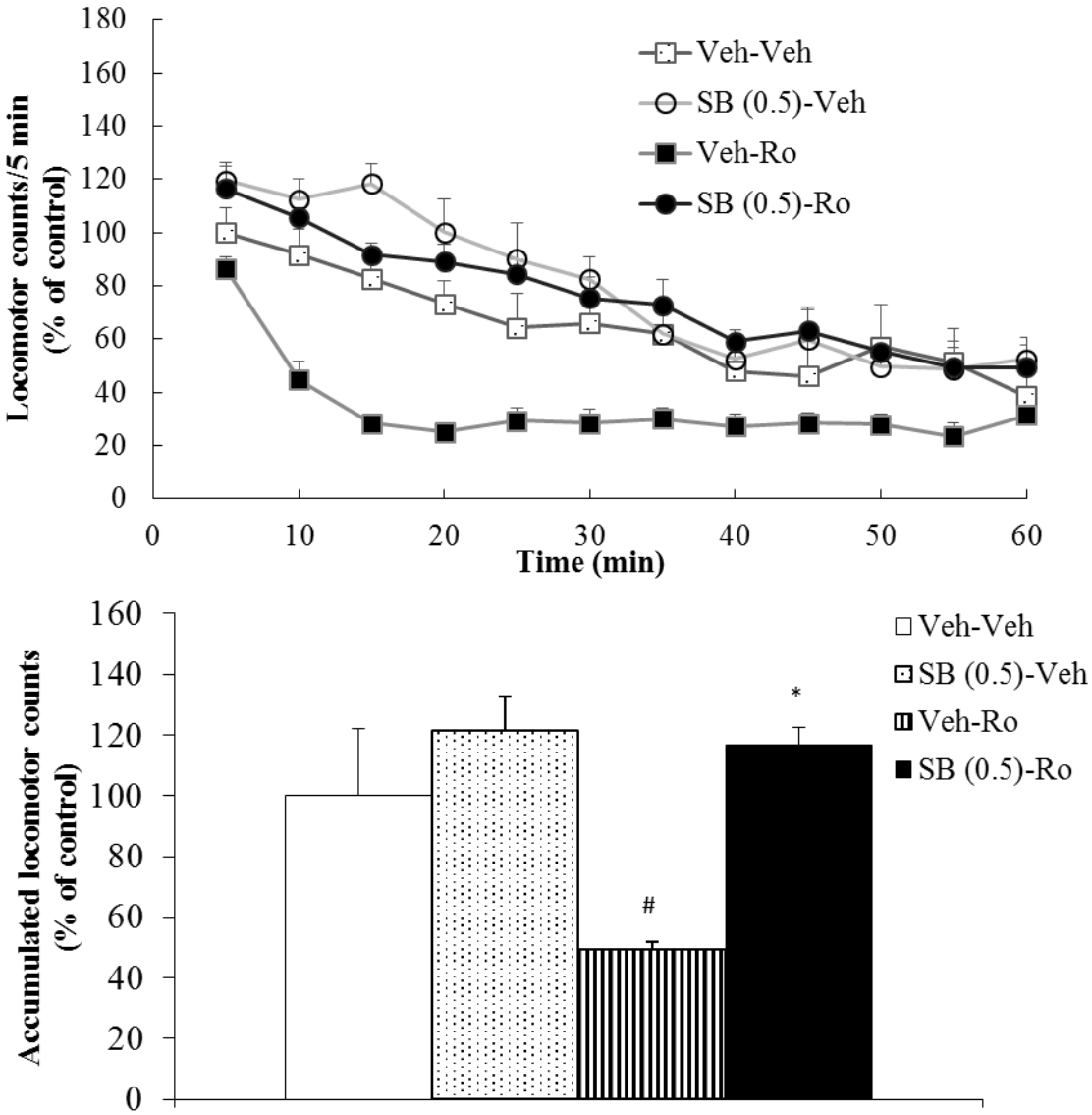

Figure 3: Effect of SB242084 (0.5 mg/kg) on Ro 60-0175 (6 mg/kg) induced hypolocomotion over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (6 animals/group), $^{\#}p < 0.05$ treatment vs Veh-Veh, $^{*}p < 0.005$ treatment vs Veh-Ro. One way ANOVA followed by LSD post.

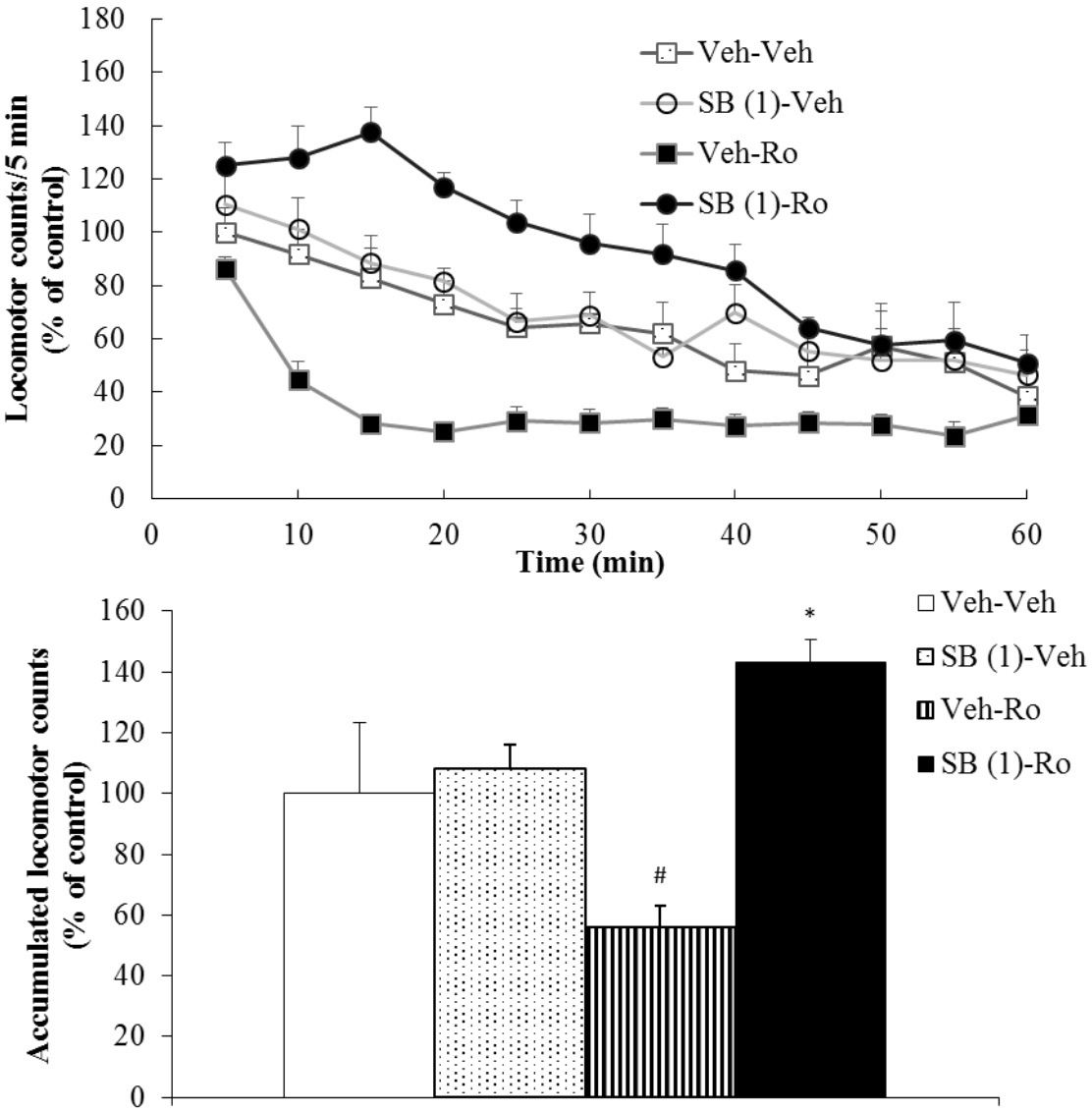

Figure 4: Effect of SB242084 (1 mg/kg) on Ro 60-0175 (6 mg/kg) induced hypolocomotion over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (6 animals/group), $^{\#}p < 0.05$ treatment vs Veh-Veh, $*p < 0.005$ treatment vs Veh-Ro. One way ANOVA followed by LSD post.

3.3.3 Effect of ebselen on Ro 60-0175-evoked hypolocomotion

The above experiments show that Ro 60-0175 elicited hypolocomotion through a 5-HT_{2C} receptor mechanism. Next, animals received ebselen prior to Ro 60-0175. Compared to vehicle controls, Ro 60-0175 (6 mg/kg) reduced locomotor activity by approximately 50% ($F(3,20) = 7.414$, $p = 0.003$). However, ebselen (10 mg/kg) alone also decreased locomotion compared to vehicle controls ($p = 0.001$). Ebselen did not inhibit the effect of Ro 60-1075 on hypolocomotion (Figure 5).

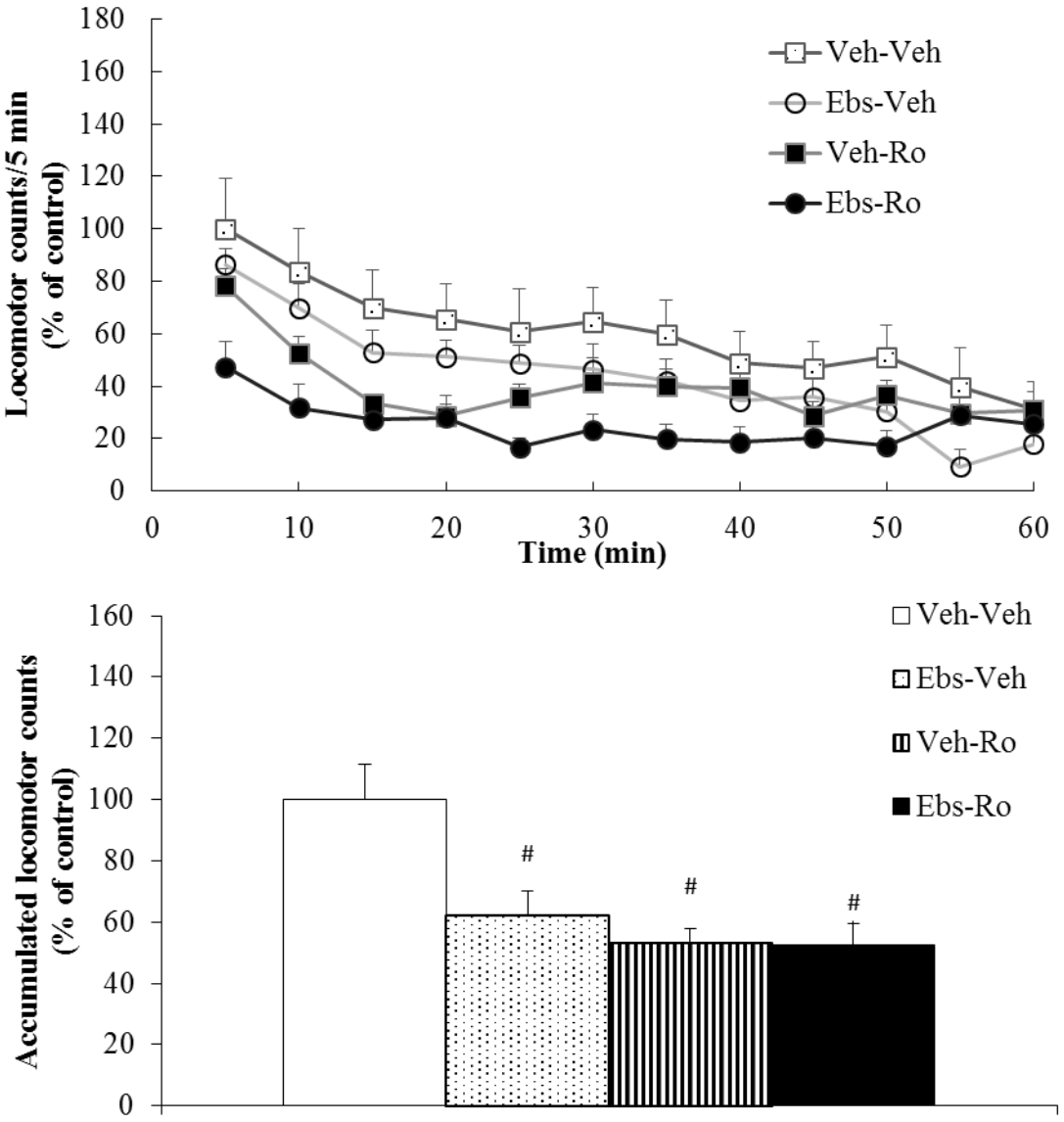

Figure 5: Effect of ebselen (10 mg/kg) on Ro 60-0175 (6 mg/kg) induced hypolocomotion over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (6 animals/group), $^{\#}p < 0.005$ treatment vs Veh-Veh. One way ANOVA followed by LSD post.

3.3.4 Effect of lithium on Ro 60-0175-evoked hypolocomotion

The effects of pretreatment with lithium on Ro 60-0175-evoked hypolocomotion was tested. Lithium was administered for 3 days prior to Ro 60-0175 (6 mg/kg). Compared to vehicle controls, Ro 60-0175 (6 mg/kg) decreased locomotor activity by approximately 50% ($F(3,19) = 14.485$, $p = 0.001$) (Figure 6). Lithium treatment did not have an effect on Ro 60-0175-evoked hypolocomotion. Additionally, lithium alone did not alter locomotion compared to vehicle controls (Figure 6).

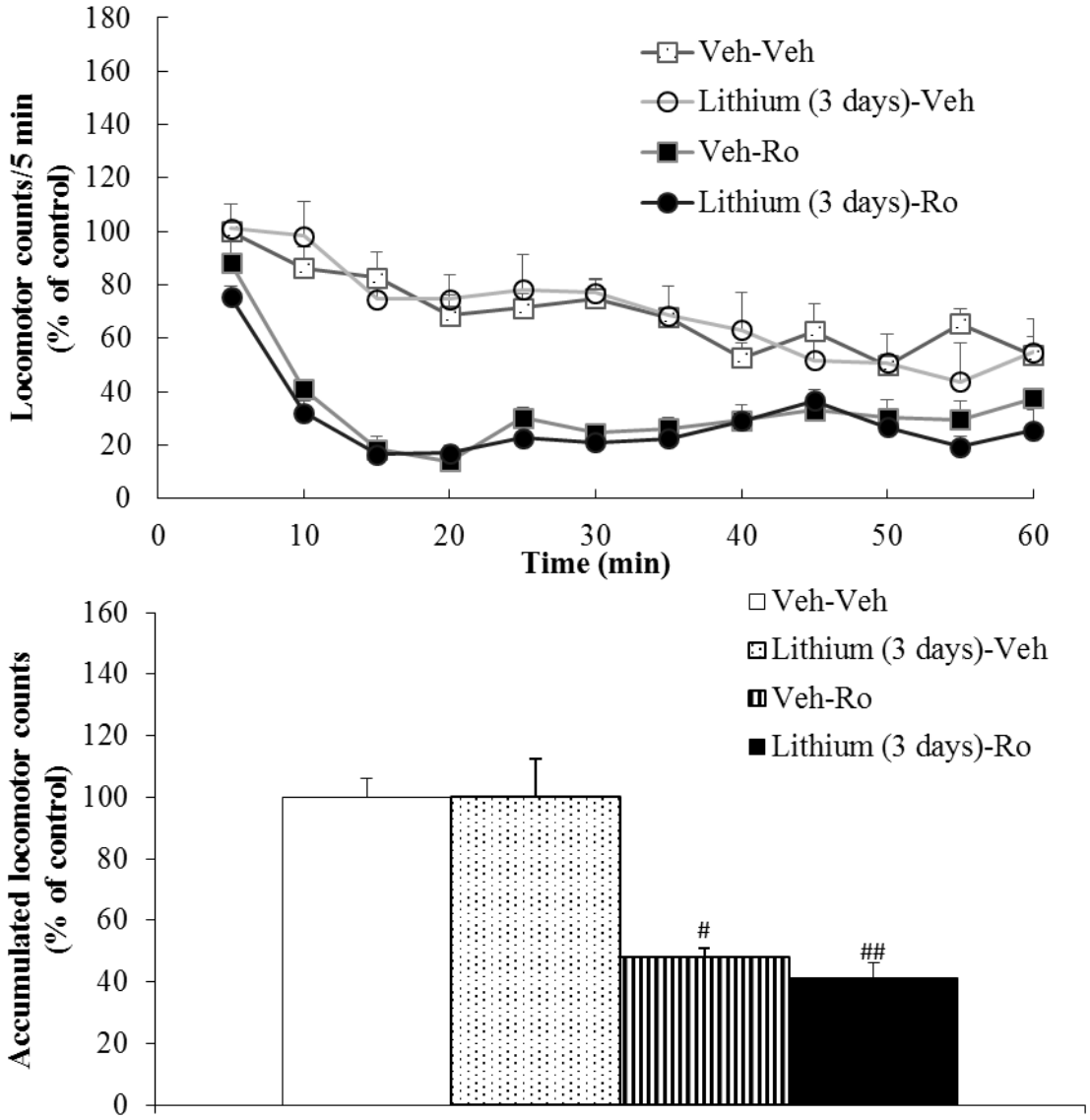

Figure 6: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3) on Ro 60-0175 (6 mg/kg) induced hypolocomotion over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (6 animals/group), $^{\#}p < 0.005$ treatment vs Veh-Veh. One way ANOVA followed by LSD post.

Since 3 days lithium treatment were not effective on Ro 60-0175-evoked hypolocomotion, a second study was carried out, in which lithium was administered for 7 days. Compared to

vehicle controls, Ro 60-0175 decreased locomotor activity by approximately 50% ($F(3,19) = 22.211$, $p < 0.000$). However, pretreatment with lithium for 7 days did not affect Ro 60-0175-evoked hypolocomotion nor alter locomotion by itself (Figure 7).

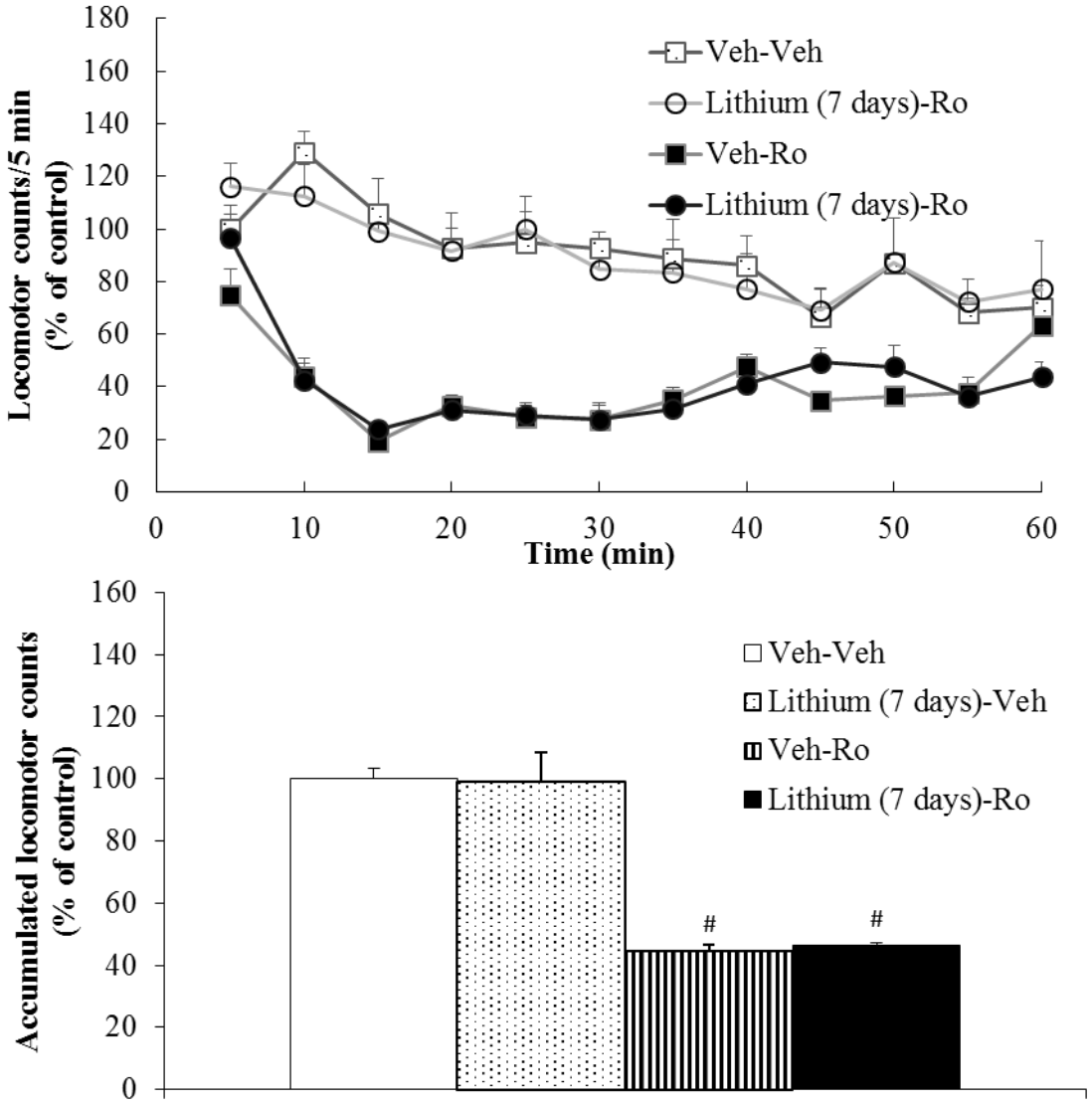

Figure 7: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-6) on Ro 60-0175 (6 mg/kg) induced hypolocomotion over 60 min. Data shown are mean ± SEM percentage of first 5 min beam breaks of control (upper graph) or total beam breaks of control (lower graph) (6 animals/group), $^{\#}p < 0.001$ treatment vs Veh-Veh. One way ANOVA followed by LSD post.

3.3.5 Effect of different doses of Ro 60-0175 on IEG expression

Given that the action of ebselen in the 5-HT_{2C} hypolocomotion model was confounded by ebselen lowering locomotion when administered alone, attempts were made to investigate the effect of ebselen in a molecular model of 5-HT_{2C} function. To this end, the effect of Ro 60-0175 on IEG expression was examined. For representative autoradiograms see Figure 10.

Arc mRNA: Compared to vehicle controls, Ro 60-0175 (2, 3 and 6 mg/kg) increased Arc mRNA abundance in the SSCo ($F(3,11) = 15.372$, 2 mg/kg: $p = 0.007$; 3 mg/kg: $p = 0.001$; 6 mg/kg: $p < 0.000$). The effect of Ro 60-0175 was dose related: 2 mg/kg elicited an approximately 30% increase of Arc mRNA while Ro 60-0175 6 mg/kg, increased Arc mRNA by 70% in the SSCo. In the Cg, Ro 60-0175 (6 mg/kg) increased Arc mRNA by approximately 35%, but this effect was not statistically significant compared to vehicle injected controls (Figure 8).

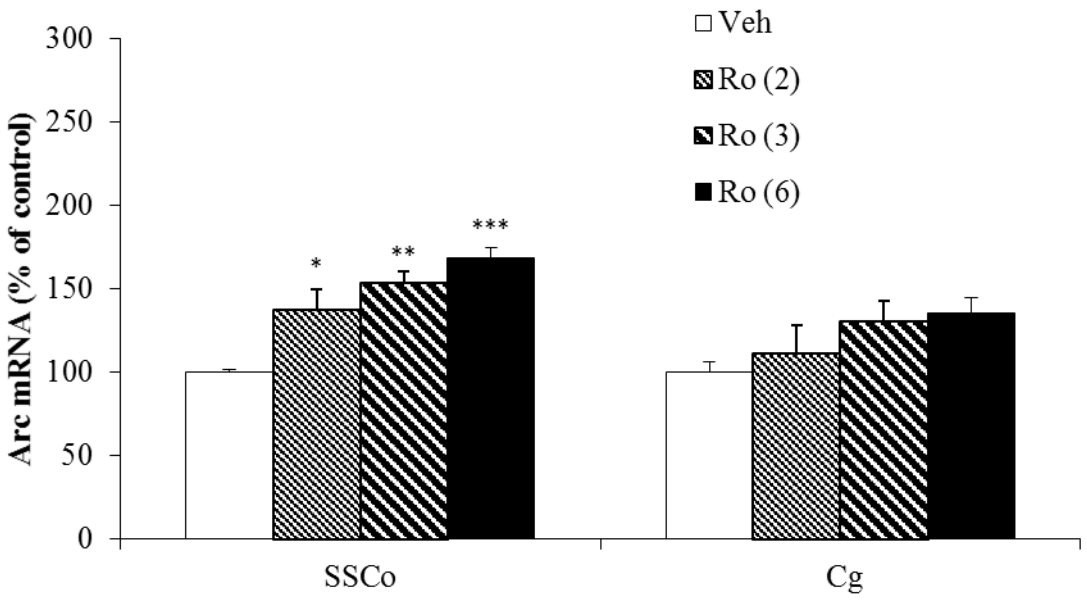

Figure 8: Effect of different doses of Ro 60-0165 (2, 3 and 6 mg/kg) on Arc mRNA. Data shown are mean ± SEM percentage of Veh (3 animals/group). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ treatment vs Veh. One-way ANOVA followed by LSD *post-hoc*. Abbreviations: as in methods section.

c-fos mRNA: Compared to vehicle controls Ro 60-0175 increased the expression of c-fos mRNA in the SSCo and the Cg and this effect was dose dependent (Figure 9). In the SSCo 2, 3 and 6 mg/kg of Ro 60-0175 elicited a statistically significant increase of c-fos mRNA of approximately 30%, 50% and 60% respectively compared to vehicle injected controls (F (3,11) = 31.808, 2 mg/kg: $p = 0.003$; 3 mg/kg: $p < 0.000$; 6 mg/kg: $p = 0.000$). Similarly, 2, 3 and 6 mg/kg of Ro 60-0175 increased c-fos mRNA in the Cg by 35%, 50% and 75%, respectively compared to vehicle controls (F (3,11) = 15.958, 2 mg/kg: $p = 0.012$; 3 mg/kg: $p = 0.001$; 6 mg/kg: $p < 0.000$).

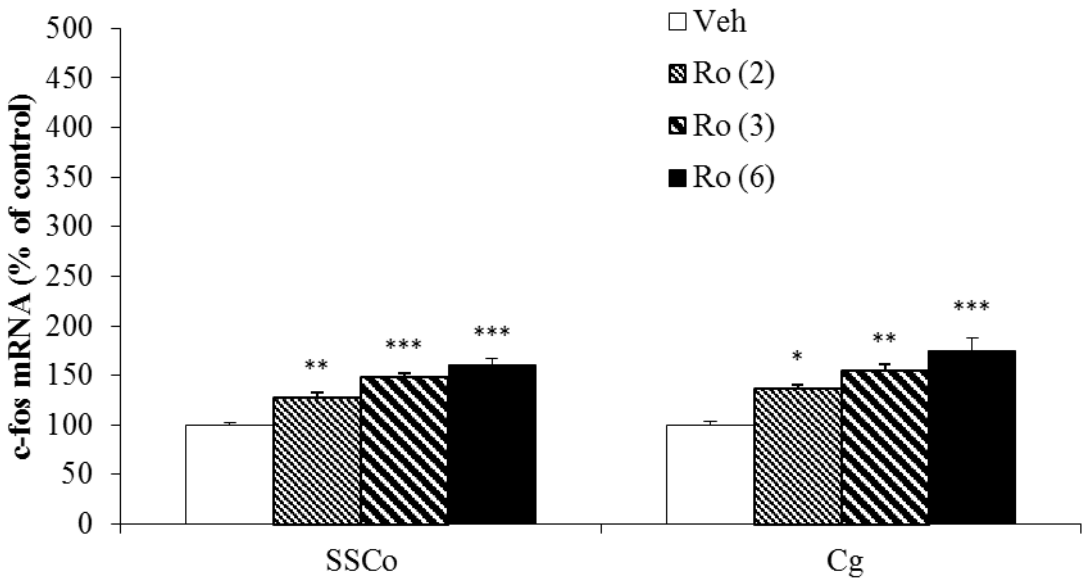

Figure 9: Effect of different doses of Ro 60-0165 (2, 3 and 6 mg/kg) on c-fos mRNA. Data shown are mean ± SEM percentage of Veh (3 animals/group). *p < 0.05, **p < 0.005, ***p < 0.001 treatment vs Veh. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

3.3.6 Effects of SB242084 on Ro 60-0175-evoked IEG expression

A second set of experiments was carried out to evaluate the effect of SB240284 on Ro 60-0175-evoked IEG expression. Autoradiograms of Arc, c-fos and egr-2 mRNA expression are shown in Figure 10.

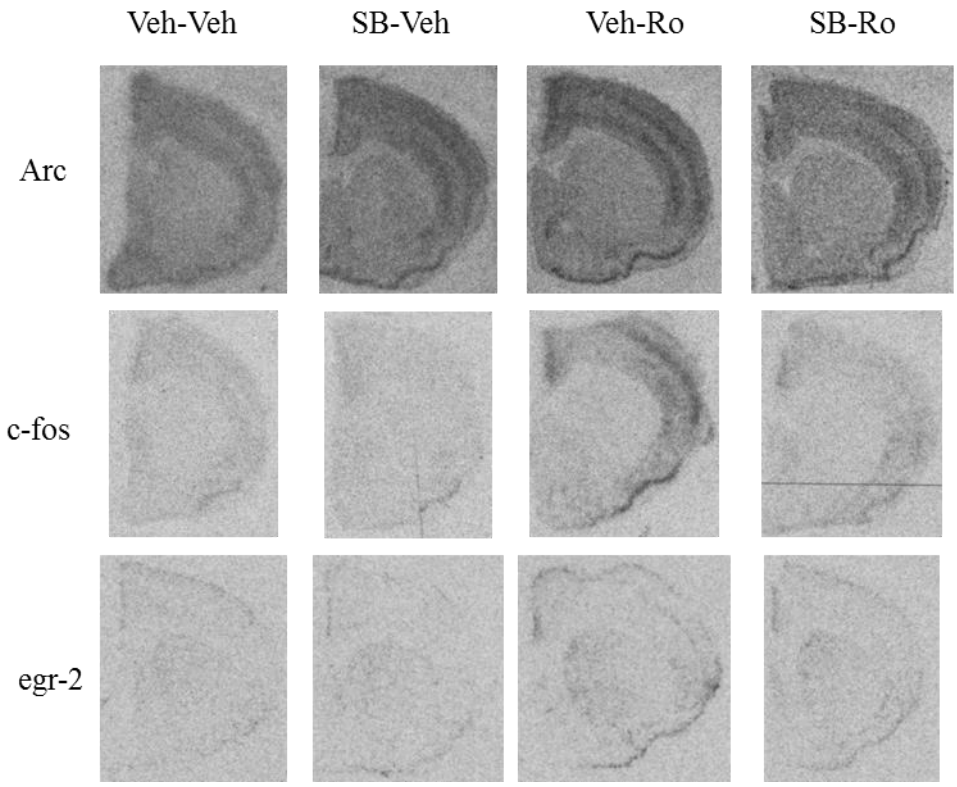

Figure 10: Representative autoradiograms showing distribution of Arc, c-fos and egr-2 mRNA in mice treated with either vehicle or SB 242084 (0.5 mg/kg, i.p.) followed by Ro 60-0175 (6 mg/kg, i.p.) or saline.

Arc mRNA: As observed above Ro 60-0175 (6 mg/kg) increased Arc mRNA compared to vehicle injected controls (SSCo: F (3,23) = 11.326, $p < 0.000$; SSCi: F (3,23) = 13.708, $p < 0.000$; Cg: F (3,23) = 24.686, $p < 0.000$; Den: F (3,23) = 36.083, $p < 0.000$). Pretreatment of animals with SB242084 (0.5 mg/kg), inhibited Ro 60-0175-evoked Arc mRNA in all of the regions investigated except for the SSCi (SSCo: $p = 0.006$; Den: $p < 0.000$; Cg: $p = 0.001$). This inhibition by SB242084 was not complete, possibly in part due to SB242084 increasing Arc mRNA when administered alone (Den: $p = 0.010$; Cg: $p = 0.029$) (Figure 11).

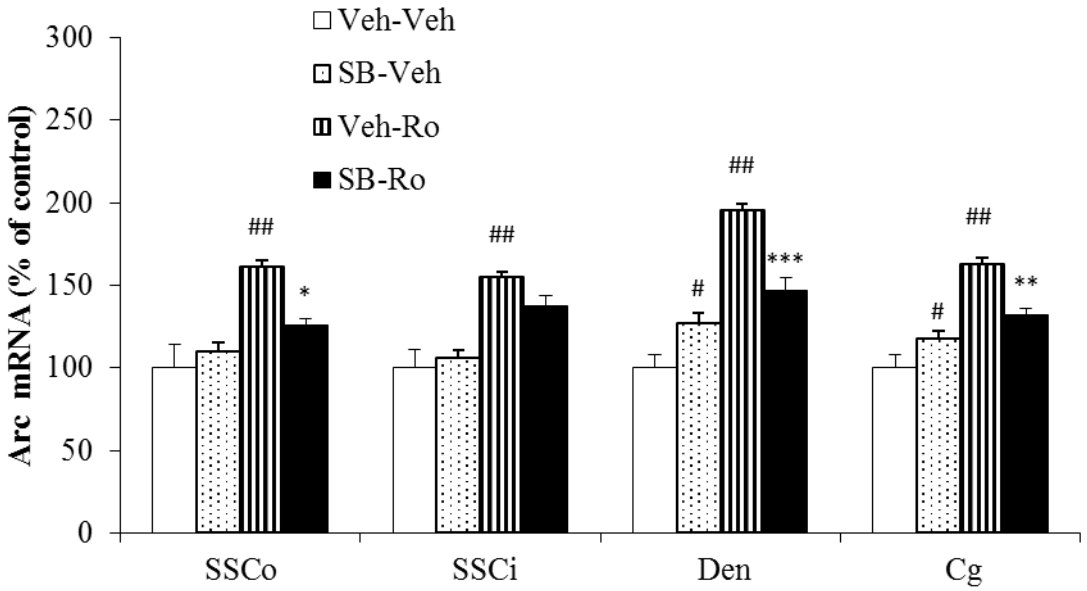

Figure 11: Effect of SB242084 (0.5 mg/kg) on Ro 60-0165 (6 mg/kg) induced Arc mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.001$ treatment vs Veh-Veh, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations: as in methods section.

c-fos mRNA: Similarly to Arc mRNA, compared to vehicle controls Ro 60-0175 increased the expression of c-fos mRNA (SSCo: F (3,23) = 9.605, $p = 0.001$; SSCi: F (3,23) = 4.647, $p = 0.013$; Cg: F (3,23) = 37.682, $p < 0.000$; Den: F (3,23) = 31.173, $p < 0.000$). Pretreatment with SB242084 attenuated Ro 60-0175-evoked c-fos mRNA in all of the regions investigated (SSCo: $p = 0.044$; SSCi: $p = 0.044$; Den: $p < 0.000$; Cg: $p = 0.001$) and this effect was complete in the SSCo and SSCi but not in the Den and Cg (SB242084-Ro compared to veh: SSCo: $p = 0.069$; SSCi: $p = 0.565$; Den: $p = 0.001$; Cg: $p = 0.005$). In the Cg SB242084 decreased c-fos mRNA when administered alone ($p = 0.004$) (Figure 12).

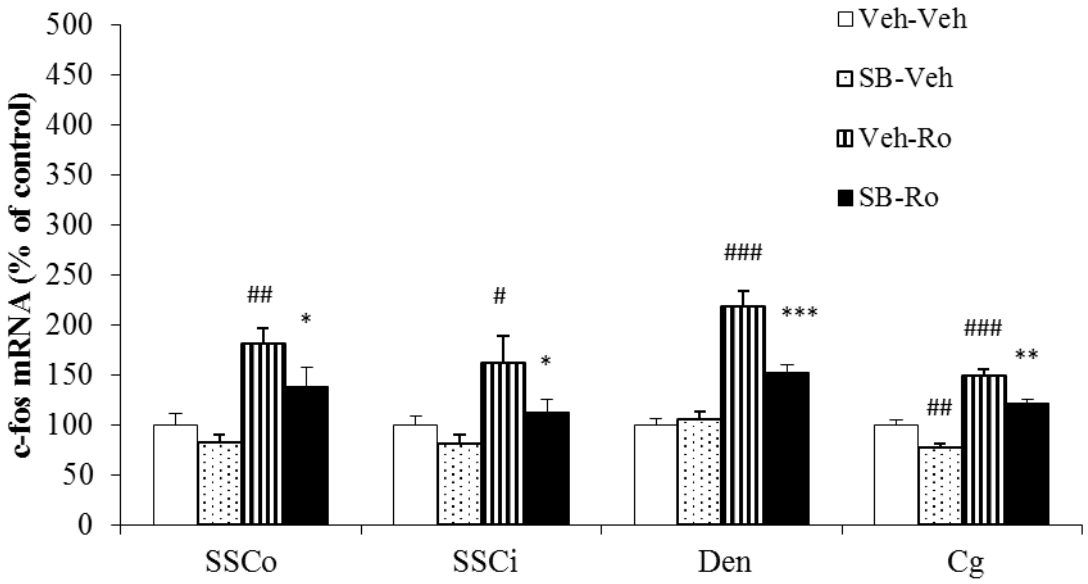

Figure 12: Effect of SB242084 (0.5 mg/kg) on Ro 60-0165 (6 mg/kg) induced c-fos mRNA. Data shown are mean ± SEM percentage of Veh-Veh.(6 animals/group) $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.001$ treatment vs Veh-Veh, $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

egr-2 mRNA: Ro 60-0175 also elicited a clear increase in the abundance of egr-2 mRNA (SSCo: F (3,23) = 7.205, $p = 0.001$; SSCi: F (3,23) = 3.340, $p = 0.014$; Den: F (3,22) = 31.472, $p < 0.000$; Cg: F (3,23) = 5.241, $p = 0.065$; CPU: F (3,23) = 18.390, $p < 0.000$) and this effect was abolished by pretreatment with SB242084 (SSCo: $p = 0.003$; SSCi: $p = 0.033$; Den: $p = 0.003$; CPU: $p = 0.002$). SB242084 did not alter egr-2 mRNA when administered alone (Figure 13).

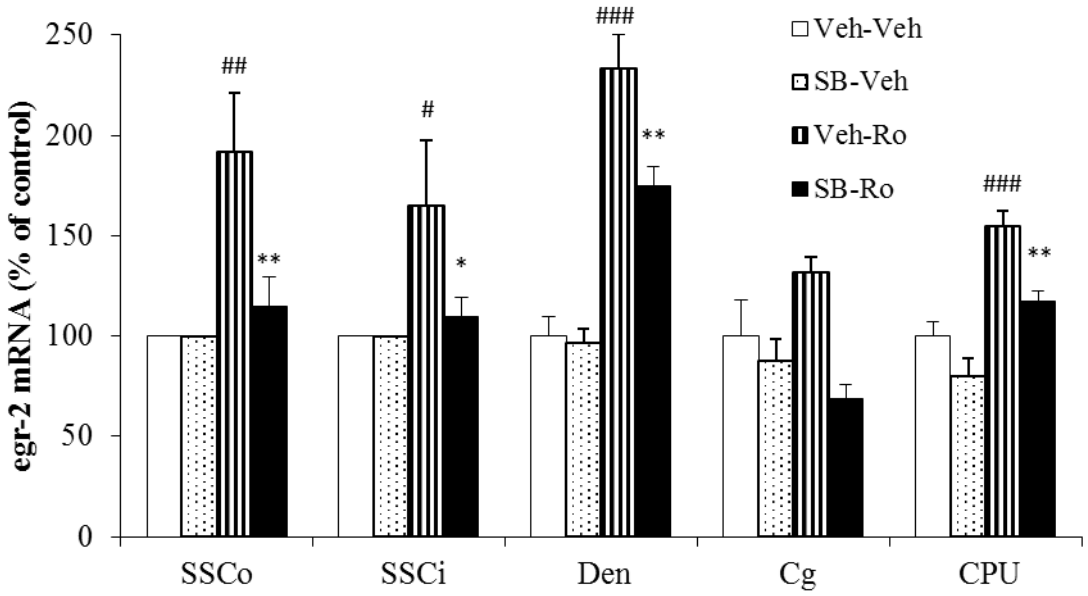

Figure 13: Effect of SB242084 (0.5 mg/kg) on Ro 60-0165 (6 mg/kg) induced egr-2 mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.001$ treatment vs Veh-Veh, $^{*}p < 0.05$, $^{**}p < 0.005$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

3.3.7 Effect of ebselen on Ro 60-0175-evoked IEG expression

The above experiments, establish that Ro 60-0175-induced IEG expression involves the activation of the 5-HT_{2C} receptor, and therefore is a valid model of 5-HT_{2C} receptor function at the molecular level. The effect of Ro 60-0175-induced IEG expression was tested in animals pretreated with ebselen.

Autoradiograms representative of Arc, c-fos and egr-2 distribution are show in Figure 14. All genes, showed similar baseline patterns of distribution as described in the above studies.

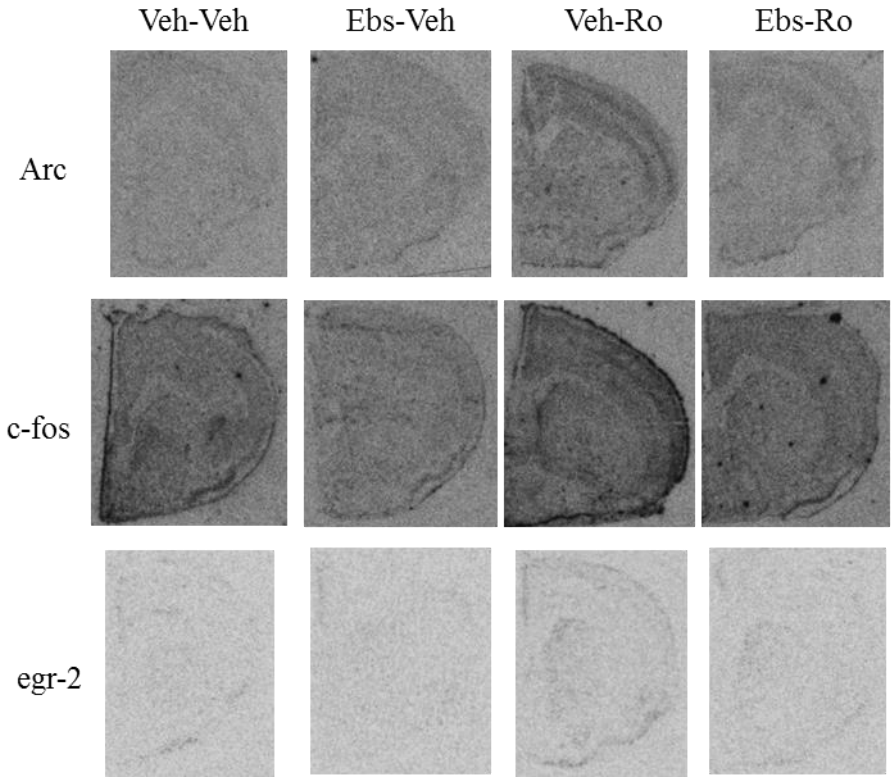

Figure 14: Representative autoradiograms showing distribution of Arc, c-fos and egr-2 mRNA in mice treated with either vehicle or ebselen (10 mg/kg, i.p.) followed by Ro 60-0175 (6 mg/kg, i.p.) or saline.

Arc mRNA: Ro 60-0175 (6 mg/kg) eliced a two-fold increase of Arc mRNA compared to vehicle controls in SSCo, Den and Cg (SSCo: F (3,23) = 13.914, $p < 0.000$; Cg: F (3,23) = 41.462, $p < 0.000$; Den: F (3,23) = 6.470, $p = 0.001$). Ebselen, (10 mg/kg) decreased the Arc response to Ro 60-0175 and this effect was statistically significant in the SSCo ($p = 0.010$) and Cg ($p < 0.000$). When administered alone ebselen did not affect on Arc mRNA compared to vehicle controls (Figure 15).

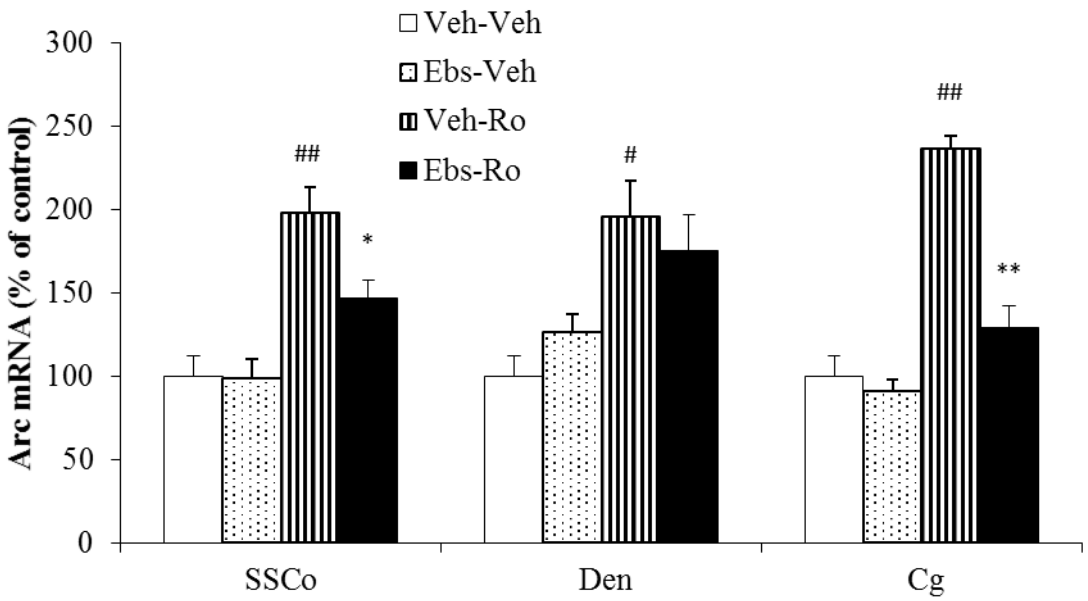

Figure 15: Effect of ebselen (10 mg/kg) on Ro 60-0165 (6 mg/kg) induced Arc mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.005$, $^{\#\#}p < 0.001$ treatment vs Veh-Veh, $^{*}p < 0.05$, $^{**}p < 0.001$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

c-fos mRNA: Ro 60-0175 also increased c-fos mRNA (SSCo: F (3,23) = 5.166, p = 0.021; SSCi: F (3,23) = 4.368, p = 0.027; Cg: p = 0.002) and this was reduced by pretreatment with ebselen (SSCo: p = 0.014; SSCi: p = 0.026; Den: p = 0.050; Cg: p = 0.006). Ebselen administration did not alter the levels of c-fos mRNA (Figure 16).

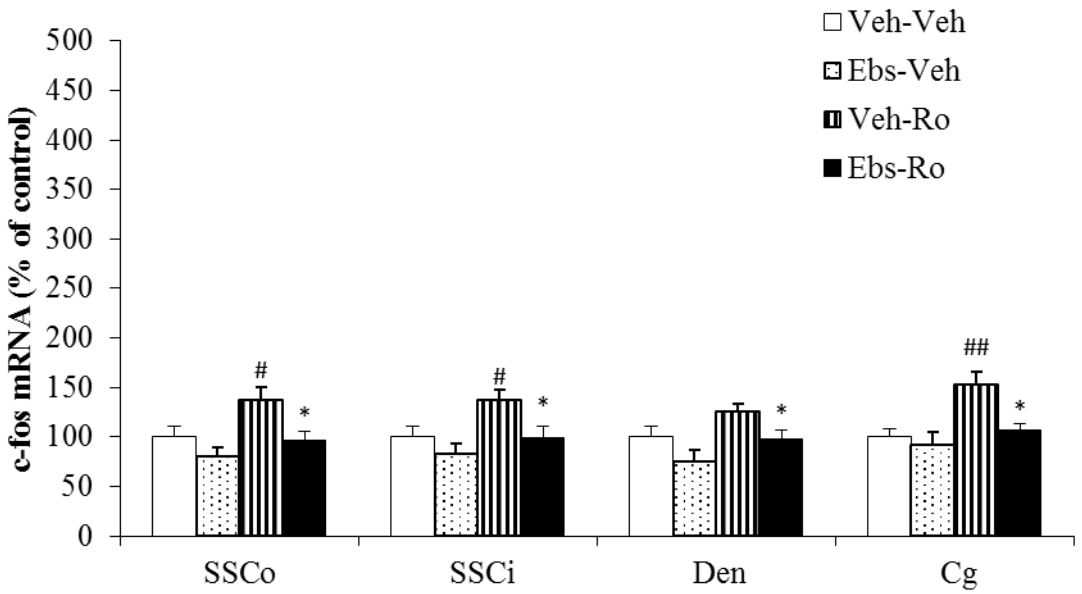

Figure 16: Effect of ebselen (10 mg/kg) on Ro 60-0165 (6 mg/kg) induced c-fos mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$ treatment vs Veh-Veh, $*p < 0.05$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

egr-2 mRNA: Ro 60-0175 also increased egr-2 mRNA (SSCo: F (3,21) = 6.631, p = 0.001; SSCi: F (3,21) = 13.127, p = 0.015; Den: F (3,21) = 5.099, p = 0.040; Cg: F (3,21) = 6.964, p = 0.002; CPU: p = 0.002) and this effect was attenuated by ebselen (SSCo: p = 0.009; SSCi: p = 0.024; Cg: p = 0.042; CPU: p = 0.042). Similar to Arc and c-fos mRNA, ebselen did not have an effect on the abundance of egr-2 mRNA when administered alone (Figure 17).

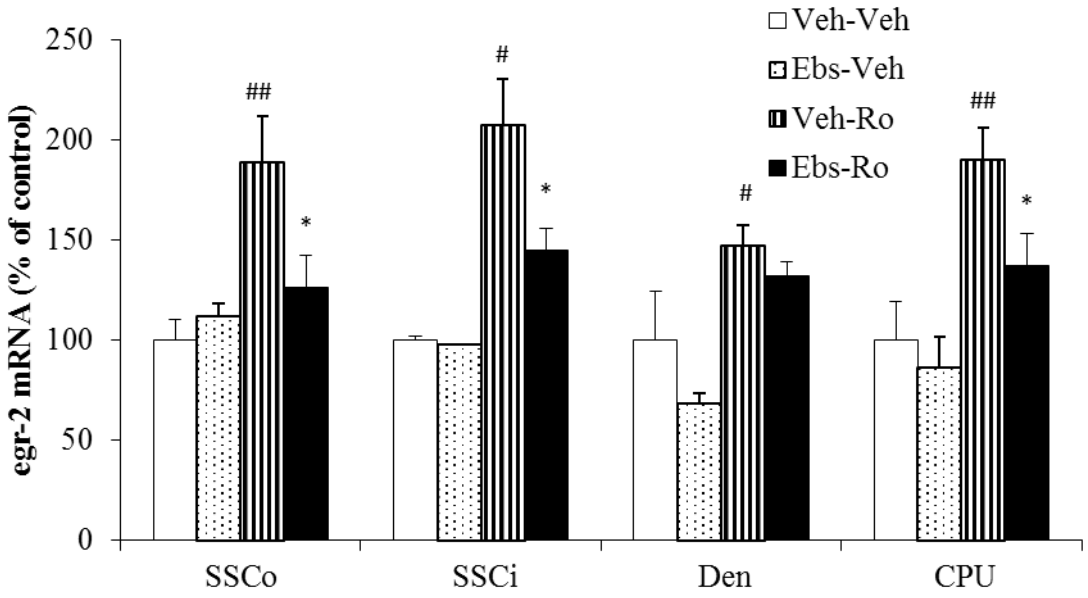

Figure 17: Effect of ebselen (10 mg/kg) on Ro 60-0165 (6 mg/kg) induced egr-2 mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$, $^{\#\#}p < 0.005$ treatment vs Veh-Veh, $*p < 0.05$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section

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3.3.8 Effect of lithium on Ro 60-0175-evoked IEG expression

The effect of lithium on Ro 60-0175 induced IEG expression was tested for comparison with ebselen.

Autoradiograms, representative of Arc, c-fos and egr-2 distribution are shown in Figure 18. The expression of all three genes showed a similar pattern of distribution compared to the above studies.

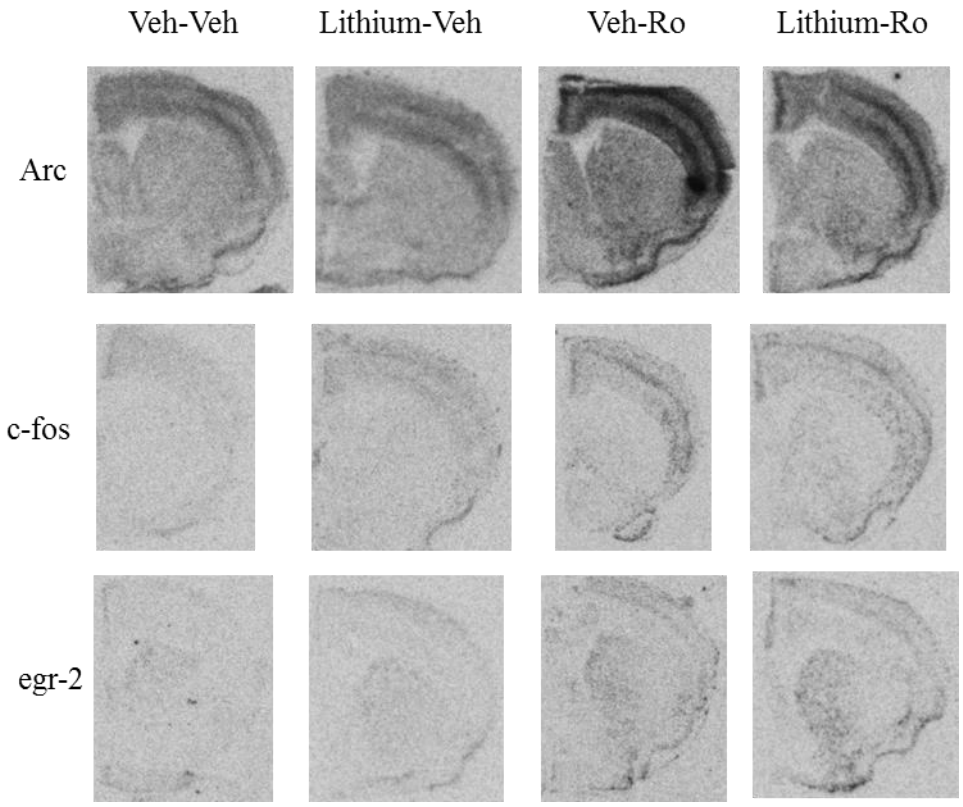

Figure 18: Representative autoradiograms showing distribution of Arc, c-fos and egr-2 mRNA in the caudate putamen of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3) followed by Ro 60-0175 (6 mg/kg, i.p.) or saline.

Arc mRNA: As observed above Ro 60-0175 increased the levels of Arc mRNA (SSCo: F (3,21) = 24.300, $p = 0.000$; Den: F (3,22) = 13.557, $p = 0.000$; Cg: F (3,22) = 11.316, $p < 0.000$). In mice receiving lthium for 3 days the Arc response to Ro 60-0175 was attenuated in the majority of regions examined (SSCo: $p = 0.035$; Den: $p = 0.002$). By itself lithium treatment for 3 days increased Arc mRNA in the SSCo and Den but not in Cg (SSCo, $p = 0.038$; Den: $p = 0.012$) (Figure 19).

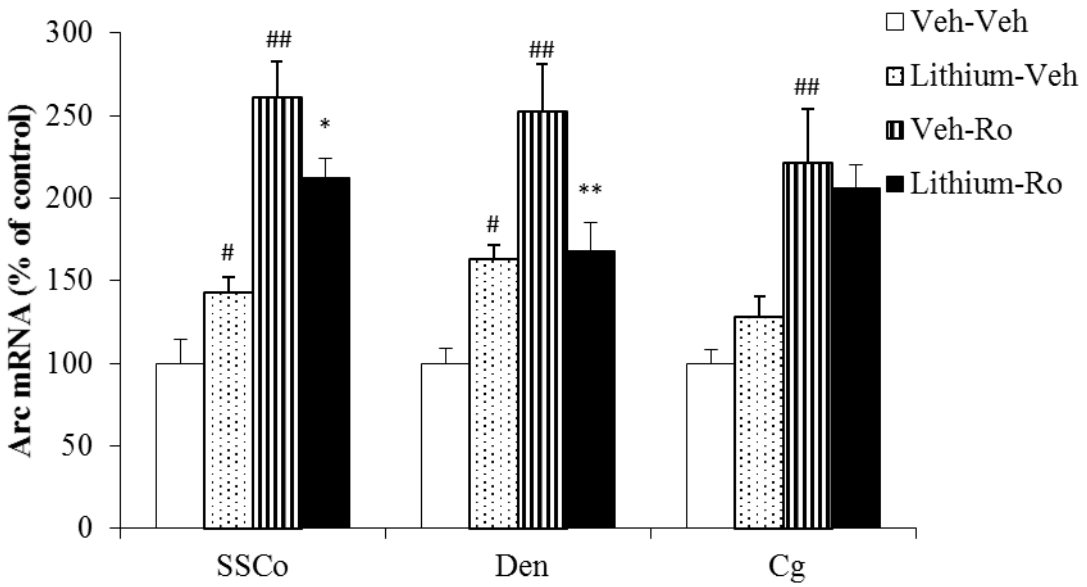

Figure 19: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on Ro 60-0175 (6 mg/kg) induced Arc mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ treatment vs Veh-Veh, $^{*}p < 0.05$, $^{**}p < 0.005$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

c-fos mRNA: Ro 60-0175 increased c-fos mRNA (SSCo: $F(3,23) = 22.648$, $p < 0.001$; SSCi: $F(3,23) = 7.426$, $p < 0.001$; Den: $F(3,23) = 22.489$, $p < 0.001$; Cg: $F(3,23) = 13.165$, $p < 0.001$) and this effect was decreased by treatment with lithium for 3 days (SSCo: $p = 0.005$; SSCi: $p = 0.047$; Den: $p = 0.004$) which by itself increased c-fos mRNA in the SSCo and Den (SSCo: $p = 0.011$; Den: $p = 0.039$) (Figure 20).

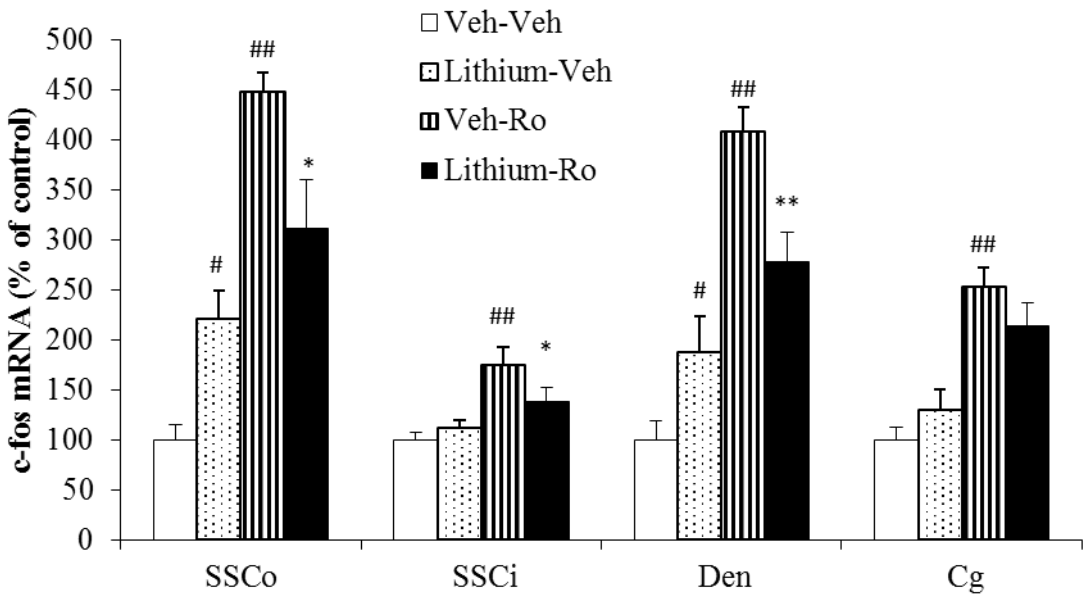

Figure 20: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on Ro 60-0175 (6 mg/kg) induced c-fos mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.001$ treatment vs Veh-Veh, $^{*}p < 0.05$, $^{**}p < 0.005$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

egr-2 mRNA: Ro 60-0175 also increased egr-2 mRNA (SSCo: F (3,19) = 24.735, $p < 0.000$; SSCi: F (3,22)= 8.549, $p < 0.000$; Den: F (3,22) = 21.566, $p < 0.000$) and as with Arc and c-fos, this effect was reduced by pretreatment with lithium for days (SSCo: $p = 0.056$; SSCi: $p = 0.021$; Den: $p = 0.039$). Similarly to Arc and c-fos mRNA, lithium treatment for 3 days induced an increase in egr-2 mRNA in the SSCo ($p = 0.041$) (Figure 21).

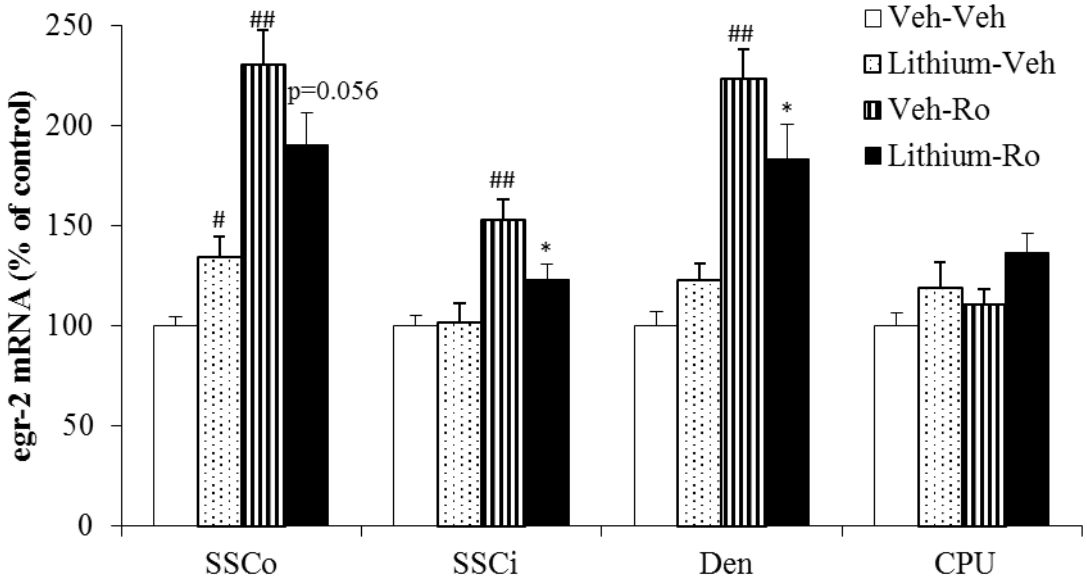

Figure 21: Effect of repeated lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily, on days 2-3), on Ro 60-0175 (6 mg/kg) induced egr-2 mRNA. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$ $^{\#\#}p < 0.001$ treatment vs Veh-Veh, $*p < 0.05$ treatment vs Veh-Ro. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

3.4 Discussion

The aim of the experiments in this chapter was to compare the effects of ebselen and lithium in molecular and behavioural models of 5-HT_{2C} receptor function. It was initially established that the increase of IEG expression and reduction in locomotor activity induced by the 5-HT_{2C} agonist Ro 60-0175 were mediated through the 5-HT_{2C} receptor, since effects were blocked by the selective 5-HT_{2C} antagonist SB242084. Subsequently it was demonstrated that ebselen attenuated the IEG response to Ro 60-0175, and that lithium had a similar effect. Neither ebselen nor lithium altered the hypolocomotion response to Ro 60-0175. However, interpretation of the latter result was complicated by the finding that ebselen alone reduced locomotion. Overall, these data suggest that ebselen and lithium share similar neuropharmacological properties in that both agents attenuated 5-HT_{2C} receptor function at the molecular level as shown by the IEG expression experiments.

3.4.1 Ro 60-0175-induced IEG expression is mediated through the 5-HT_{2C} receptor

A novel molecular model of 5-HT_{2C} receptor function was established, by investigating the effect of Ro 60-0175 on IEG expression and specifically the 3 genes (Arc, c-fos, egr2) shown in the previous chapter to be sensitive to 5-HT_{2A} receptor activation. Ro 60-0175 dose-dependently increased the expression of Arc, c-fos and egr2 mRNA. This is the first time that the effect of Ro 60-0175 on Arc, c-fos and egr-2 mRNA is investigated in the mouse brain although previous studies in rats report that c-fos protein is increased by administration of the 5-HT_{2C} agonists mCPP and Ro 60-0175 (De

Deurwaerdere *et al.*, 2000; Singewald *et al.*, 2000; Singewald *et al.*, 2003; Stark *et al.*, 2006; Beyeler *et al.*, 2010; Kadiri *et al.*, 2012; Navailles *et al.*, 2013).

Pretreatment with SB242084 attenuated the IEG response to Ro 60-0175 indicating that it is mediated via 5-HT_{2C} receptor activation. SB242084 abolished the effect of Ro 60-0175 on egr2 but not completely in the case of Arc and c-fos. These findings suggest that egr-2 might be a more sensitive molecular marker of 5-HT_{2C} receptor stimulation compared to Arc and c-fos. The Ro 60-0175 evoked increase in Arc and c-fos mRNA expression might be partially mediated through 5-HT_{2A} or 5-HT_{2B} activation, receptors for which the drug has comparable affinity ($pK_{i(2A)}$ = 6.8, $pK_{i(2B)}$ = 7, $pK_{i(2C)}$ = 9) (Kennett *et al.*, 1997) and this could be tested by the use of relevant 5-HT_{2A} and 5-HT_{2B} selective antagonists.

In some cases SB242084 administered alone caused small increases in the expression of Arc and decreases in the expression of c-fos, but had no effect on egr-2. There is no certain explanation for these changes in c-fos and Arc mRNA abundance following SB242084, however it could result from the disinhibition of dopaminergic and adrenergic pathways that 5-HT_{2C} receptors control (Millan *et al.*, 1998; Di Matteo *et al.*, 2000; Millan *et al.*, 2003).

Overall, the present study demonstrates evidence that Ro 60-0175 evoked IEG expression through 5-HT_{2C} receptor activation and provides a model for the pharmacological assessment of ebselen and lithium.

3.4.2 Ro 60-0175 induced IEG expression: effect of ebselen and lithium

The effect of ebselen on 5-HT_{2C} function was investigated using the IEG response to Ro 60-0175 administration. Notably, pretreatment with ebselen attenuated Ro 60-0175 induced expression of 3 IEGs investigated. The effect of ebselen was clear cut for egr-2 for which inhibition was complete, but the decreases in Ro 60-0175-evoked Arc and c-fos were also robust. Overall these data support the idea that ebselen reduces the function of 5-HT_{2C} receptors as shown in Chapter 2 for 5-HT_{2A} receptors. Indirect evidence in support of the idea that ebselen reduces 5-HT_{2C} function *in vivo* comes from experiments in which ebselen is shown to have antinociceptive properties in the formalin test in mice (Zasso *et al.*, 2005). That the latter effect might involve 5-HT_{2C} receptors is supported by a report that SB 242084 has been shown to dose-dependently produce analgesic effects in the formalin test in rats (Nakajima *et al.*, 2009).

As with ebselen, repeated lithium treatment, also attenuated the IEG response to Ro 60-0175 and this effect was apparent in the measurement of egr-2, c-fos and Arc expression in the majority of regions. Evidence from an *in vitro* study in xenopus oocytes, corroborates the present findings on lithium attenuating 5-HT_{2C} function. In particular, lithium was shown to attenuate 5-HT induced calcium release through 5-HT_{2C} receptor while it did not affect Ca^{2+} response to acetylcholine through muscarinic PI-linked receptors (Matsuoka *et al.*, 1997). Indirect evidence also suggest that lithium attenuates 5-HT_{2C} function in humans as assessed by slow wave sleep measurements (Friston *et al.*, 1989).

3.4.3 Ro 60-0175-induced hypolocomotion is mediated through the 5-HT_{2C} receptor

On the behavioural level, Ro 60-0175 dose-dependently decreased locomotor activity as previously reported for Ro 60-0175 and other 5-HT_{2C} receptor agonists (Kennett *et al.*, 1997; Heisler *et al.*, 2000; Higgins *et al.*, 2001; Dalton *et al.*, 2004; Fletcher *et al.*, 2009). SB 242084 (0.5 and 1 mg/kg) completely inhibited the effect of Ro 60-0175 on locomotor activity at both doses investigated. Paradoxically, SB242084 1 mg/kg combined with Ro 60-0175 increased locomotor activity compared to vehicle controls. This is in agreement with previous studies showing that Ro 60-01765 or administration of other 5-HT_{2C} agonists to 5-HT_{2C} knockout mice elicits an increase in locomotor activity compared to control animals. This increase in locomotor activity induced by 5-HT_{2C} agonists, when 5-HT_{2C} receptors are blocked or genetically modified has been attributed to activity of 5-HT_{2C} agonists at additional targets (Higgins *et al.*, 2001; Fletcher *et al.*, 2009).

3.4.4 Effect of ebselen on a behavioural model of 5-HT_{2C} receptor function; comparison with lithium

In contrast to the inhibitory effect of ebselen on Ro 60-0175-evoked IEG expression, ebselen did not inhibit the effect of Ro 60-0175 on hypolocomotion. However, this experiment was confounded by the finding that by itself ebselen reduced locomotion to levels similar to those induced by Ro 60-0175. The hypolocomotive effect of ebselen is unlikely to be associated with anxiogenic properties, since ebselen-treated mice did not show anxiogenic behaviour in the elevated plus maze (see appendix). Moreover, it is

unlikely that ebselen-induced hypolocomotion is mediated through agonism at the 5-HT_{2C} receptor, as ebselen should increase IEG expression in a similar way to Ro 60-0175 and this was not the case. Another possibility is that the hypolocomotor effect of ebselen involves the 5-HT_{2A} receptor. Drugs which reduce 5-HT_{2A} receptor function have been found previously to have hypolocomotor effects. In particular clozapine, which is a non-selective antagonist at the 5-HT_{2A} receptor, typically reduces locomotion. When clozapine was administered to 5-HT_{2A} knockout mice it did not reduce locomotion, but clozapine's hypolocomotive effects were restored when the 5-HT_{2A} receptor was restored in the cortex of 5-HT_{2A} knockout mice (McOmish *et al.*, 2012). As described in the previous chapter, ebselen attenuates the function of 5-HT_{2A} receptor, which could therefore account for the reduction in locomotion seen in the experiments described in this chapter. Against this hypothesis however, lithium did not have a hypolocomotor effect and yet like ebselen reduced 5-HT_{2A} function.

The finding that both ebselen and lithium reduced the IEG response to Ro 60-0175 but not the hypolocomotor effect raises the question of the link between these two models. Specifically, are the same signalling pathways involved in the IEG and hypolocomotor response? Plethora of reports demonstrate that calcium is necessary to induce gene expression in neuronal cells (Bading *et al.*, 1993; Matthews *et al.*, 1994; Dolmetsch *et al.*, 1998; Wiegert *et al.*, 2011). For example, elevation of cytoplasmic calcium levels following cell stimulation has been shown to control transcription of c-fos in cells (Hardingham *et al.*, 1997). It is therefore likely that dampening of PI signalling, through IMPase inhibition and inositol depletion would lead to attenuation of IP3-induced calcium release and subsequently attenuation of 5-HT_{2C} agonist induced gene expression. Since ebselen and lithium both inhibit IMPase, then the attenuation of 5-

HT_{2C} agonist evoked IEG expression could be due to dampened PI signalling through the Gq coupled 5-HT_{2C} receptor. Given that neither ebselen nor lithium attenuated Ro 60-0175-induced hypolocomotion, it is possible that the latter is mediated by a PI independent signalling pathway. In this regard it is interesting that the 5-HT_{2C} receptor is also linked to PLA2 signalling (Berg *et al.*, 2008). Activation of the PLA2 signalling pathway could therefore account for the hypolocomotor effects of 5-HT_{2C} agonist administration and that this is why neither ebselen nor lithium were effective in this model.

In summary, the present study validated a molecular (IEG expression) and a behavioural (locomotor activity) model of 5-HT_{2C} receptor function. Ebselen as well as lithium attenuated the function of the 5-HT_{2C} receptor in the molecular model as evident by a reduction in Ro 60-0175-induced IEG expression. However, neither ebselen nor lithium attenuated 5-HT_{2C} receptor function in the behavioural model. Although the mechanism underlying the effects of ebselen and lithium were not investigated, the similarities between the two agents, suggest IMPase inhibition as a putative mechanism for the molecular model of 5-HT_{2C} function and a IMPase-independent mechanism for the behavioural model. Overall, data in this Chapter and Chapter 2 indicate that ebselen reduces 5-HT_2 receptor function. 5-HT_2 receptor blockade has been associated with antidepressant augmenting action, an effect that is already clinically proven for lithium. Experiments in the next chapter investigate the antidepressant augmenting properties of ebselen.

Chapter 4

Investigation of the effects of ebselen on 5-HT function

4 Investigation of the effects of ebselen on presynaptic 5-HT function

4.1 Introduction

In Chapters 2 and 3 it was shown that ebselen attenuated the function of postsynaptic 5-HT_{2A} and 5-HT_{2C} receptors of the mouse, and this effect was likely to be mediated through IMPase inhibition. In comparison, lithium also attenuated 5-HT_{2A} and 5-HT_{2C} receptor function. Experiments in the current Chapter investigated whether ebselen might increase presynaptic 5-HT function since lithium has this effect and such an action might come from the attenuation of 5-HT_2 receptor function. Specifically experiments investigated whether ebselen might increase 5-HT synthesis as this is commonly reported for lithium and might link to its antidepressant augmentation properties. In addition experiments investigated the effect of ebselen alone and in combination with an SSRI in extracellular 5-HT (as well as correlated IEG expression) as 5-HT_2 antagonists augment SSRIs in this paradigm and this is also thought to relate to their SSRI enhancing properties in depressed patients.

Lithium is a well-documented augmentation treatment for patients who are refractory to antidepressants which is estimated to affect 30% of patients. Lithium is validated by several studies to be an effective first-line agent for the treatment of refractory depression (reviewed by (Carvalho *et al.*, 2014)). In addition, meta-analysis reveals that lithium augmentation elicits a significant amelioration of depression symptoms in 50% of refractory patients, and in 20% of the cases this effect of lithium is exerted within the first week of administration (Bauer *et al.*, 2003; Bschor *et al.*, 2006).

Although the mechanism underlying the antidepressant-augmenting properties of lithium is not known, experiments in rodents suggest that enhancement of 5-HT neurotransmission is an important contributor. Attenuation of 5-HT_{2A} and 5-HT_{2C} receptor function by lithium could mediate the antidepressant augmenting properties of lithium since 5-HT_{2A} and 5-HT_{2C} antagonists also have this effect. Many studies have investigated the effect of lithium on markers of presynaptic 5-HT function and in many cases increased function was detected. For example, lithium was found to increase post mortem tissue levels of 5-HT in several brain regions (Collard *et al.*, 1975; Berggren, 1985). Also, in *in vivo* microdialysis studies lithium was found to increase extracellular levels of 5-HT in the hippocampus and hypothalamus of rats (West *et al.*, 1991). Other microdialysis studies also found that lithium increased extracellular 5-HT but only when 5-HT neurons were stimulated electrically (Sharp *et al.*, 1991). The latter findings are in agreementwith *in vitro* studies showing that lithium treatment enhanced electrically evoked radiolabelled 5-HT from hippocampal, hypothalamic and cortical slices (Friedman *et al.*, 1988). These results are consistent with evidence that lithium increased 5-HT synthesis, as assessed by the 5-HTP accumulation method, possibly through increased brain levels of the 5-HT precursor (Berggren, 1987).

Microdialysis experiments also report that repeated lithium treatment has been found to enhance the increase in the extracellular levels of 5-HT induced by acute or repeated antidepressant treatment. In particular, repeated lithium treatment for 7 days enhanced acute citalopram-induced extracellular 5-HT in the frontal cortex of awake rats (Muraki *et al.*, 2001). In another study, lithium treatment for 7 days augmented the increase in extracellular 5-HT in rat frontal cortex induced by the antidepressant, milnacipran (Kitaichi *et al.*, 2005). Additionally, lithium treatment for 5 days enhanced the increase in extracellular 5-HT

induced by repeated (21 days) citalopram treatment in ventral hippocampus of the rat (Wegener *et al.*, 2003).

In accord with above neurochemical studies, early studies found that lithium evoked the 5-HT behavioural syndrome in rats treated with a monoxidase inhibitor (Grahame-Smith *et al.*, 1974). Acute lithium has also been found to shorten the decrease in immobility time in the forced swim test, elicited by several antidepressants, including citalopram (Nixon *et al.*, 1994). However, in another study, repeated lithium was found to have no effect on immobility time in the forced swim test when combined with citalopram, although in the same experiment, lithium enhanced citalopram-induced elevation of 5-HT in the hippocampus (Wegener *et al.*, 2003).

As with lithium, selective 5-HT_{2A} and 5-HT_{2C} antagonists are found to augment the acute effects of antidepressants (discussed in General Introduction). In microdialysis studies acute injection of MDL 100907, ketanserin or SB242084, which are antagonists at the 5-HT_{2A} and 5-HT_{2C} receptor respectively, elevated SSRI-induced increase in extracellular 5-HT *in vivo* in rats (Gobert *et al.*, 2000; Cremers *et al.*, 2004; Boothman *et al.*, 2006b). The latter work is complemented by IEG expression studies showing that combination of an SSRI with a selective 5-HT_2 antagonist leads to elevation of Arc mRNA in a variety of brain regions while SSRIs are without effect on Arc mRNA when administered alone as a single injection but increase Arc when administered repeatedly (Serres *et al.*, unpublished data). These experiments are based on studies showing that blockade of the 5-HT_{1A} autoreceptor augments the effect of an SSRI in microdialysis and IEG expression studies (Castro *et al.*, 2003; Pei *et al.*, 2003b; Tordera *et al.*, 2003).

Similarly to lithium, in preliminary studies it was found that acute ebselen increased the tissue levels of 5-HT in the frontal cortex, caudate putamen, brainstem and hippocampus of mice as shown in Table 1 (Fowler *et. al.*, unpublished data). Interestingly, this work might connect with evidence that ebselen inhibits indoleamine 2,3-dioxygenase (IDO) which might be expected to increase availability of tryptophan in the brain (Terentis *et al.*, 2010).

Treatment	**Brain region**			
	Frontal cortex	Caudate putamen	Hippocampus	Brainstem
Veh	100±13.2	100±11.6	100±15.9	100±9.4
Ebs (0.5)	184.4±18.9***	122.7±7.6	186.8±21.1**	159.7±16.15**
Ebs (1)	173.6±13.5**	172.7±19.0**	179.9±11.6**	195.8±14.1***
Ebs (5)	154.6±11.0*	136.5±9.7	140.7±17.9	137.2±8.8*

Table 1: Effect of different doses of ebselen (0.5, 1 or 5 mg/kg) on the concentration of 5-HT in discrete mouse brain regions as measured by high performance liquid chromatography. Data shown are mean ± SEM percentage of Veh *n*=6/group. $^{*}p<0.05$, $^{**}p<0.05$, $^{***}p<0.001$ Veh vs Ebs. One-way ANOVA followed by LSD post-hoc. Fowler *et. al.*, unpublished data.

The aim of the experiments described in this chapter was to determine the effect of ebselen on presynaptic 5-HT function, which is increased by lithium. Initial experiments measured the effect of ebselen on 5-HT synthesis, using the 5-HTP method of Carlsson (Carlsson *et al.*, 1972). Microdialysis experiments then also investigated the effect of ebselen on extracellular 5-HT. Additionally, since in Chapters 2 and 3 it was found that ebselen attenuated 5-HT_{2A} and 5-HT_{2C} receptor function it was predicted that ebselen would augment the effect of an SSRI on extracellular 5-HT, and this was studied. Finally,

experiments were also carried out examining the effect of ebselen alone and in combination with an SSRI on IEG expression.

4.2 Methods

4.2.1 Experimental animals

Adult, male C57BL/6 (7-9 weeks old) (Harlan, Bicester, UK) mice were housed in groups of 6 under controlled conditions of lighting (12 h light-dark cycle) and temperature (21±1 ^{o}C). Experiments were carried out during the light phase. Food and water was available *ad libitum*. At least one week acclimatisation in the animal facility was allowed before experiments were carried out. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and Home Office Guidelines.

4.2.2 Measurement of 5-HT synthesis

Groups of mice (n = 6 per group) received ebselen (0.5, 1 or 5 mg/kg) or vehicle (4% (2-hydroxypropyl)-β-cyclodextrin, 0.4% (v/v) DMSO). One hour later animals received the aromatic amino acid decarboxylase inhibitor NSD1015 (100 mg/kg) and were returned to their home cage. Mice were culled 30 min following the second injection by cervical dislocation and brain tissue was isolated and stored in -80 ^{0}C until used.

4.2.2.1 Sample preparation

The right hemisphere was used to isolate the frontal cortex, caudate putamen, somatosensory cortex, hippocampus and brainstem using a scalpel blade. Dissections were always carried out on ice and individual regions were placed in tubes containing 500 µl perchloric acid

(0.09 M) and homogenised using a polytron kinetic homogeniser (15000 rpm for 10 sec). Samples were then centrifuged (1000 rpm for 10 min) and the supernatant was transferred in a new eppendorf tube and kept on ice out of light for analysis using HPLC. Tissue debris was kept for protein quantification (see protocol below).

4.2.3 Microdialysis

Surgical procedure

Mice were anaesthetised in an isoflurane box (4% isoflurane delivered in oxygen) before moved to a stereotaxic frame (Kopf Instruments, Tujunga, USA). Once stably anaesthetised, isoflurane was maintained to 2%. Body temperature was maintained at 36 ^{0}C-37 ^{0}C using a homeothermic blanket attached to a rectal probe (Harvard Instruments). Skin was incised, connective tissue was cleared from the skull and lidocaine (20 mg/ml) was locally applied. A hole was drilled over the hippocampus and a guide cannula (shaft length: 5 mm, shaft outer diameter 0.2 mm, Royem Scientific Ltd) was stereotaxically lowered into the hippocampus (coordinates from bregma: AP -3.0, ML -3.3, DV -4.4 atlas of (Paxinos *et al.*, 2007)) and secured with resin dental cement (Duralay I, Henry Schein Minerva Dental Ltd). Mice were then removed from the stereotaxic frame and allowed to recover for 6-7 days in an individual cage.

On the day of the experiment, mice were briefly anaesthetised in an isoflurane box (4% isoflurane delivered in oxygen) and a microdialysis probe (2 mm 6K Da PES membrane, shaft length: 7 mm, Royem Scientific Ltd) was implanted through the guide cannula, into hippocampus. The microdialysis probe was connected to a perfusion pump (CMA/100,

CMA Microdialysis Ltd.) and was perfused continuously with artificial CSF (140 mM NaCl, 3.0 mM KCl, 1.2 mM Na_2HPO_4, 0.27 mM NaH_2PO_4, 1 mM $MgCl_2$, 2.4 mM $CaCl_2$ and 7.2 mM glucose) at a flow of 2 µl/min. Following 2 h of the probe insertion, perfusate samples were collected every 20 min and analysed immediately using high performance liquid chromatography (HPLC) with electrochemical detection (see below for protocol). At the end of each experiment, the brain tissue was collected and probe placement was confirmed by histological examination.

Drug administration

Once 3 consecutive, stable baseline samples were collected, mice received a single injection of ebselen (10 mg/kg) or vehicle (4% (2-hydroxypropyl)-β-cyclodextrin, 0.4% (v/v) DMSO). Citalopram (5 mg/kg) was administered 1 h later. Dialysates were collected for another 2 h, following citalopram injection. All drugs were administered i.p. and volume of the injection was adjusted to weight (0.01 ml/g for citalopram and saline; 0.02 ml/g for ebselen and vehicle).

4.2.4 High performance liquid chromatography

Dialysates (20 µl) and brain supernatants (50 µl) were analysed using HPLC (CC-4, Bioanalytical systems), connected with a silica-based, reversed phase column (3.0um ODS2, 4.6mmx100mm, Waters Ltd) coupled with electrochemical detection (LC-4C, Bioanalytical systems). Glass carbon working electrode (BASi MF 1000) and a Ag/AgCl reference electrode (BASi MF-2078) were used. The flow of the mobile phase was set at 1

ml/min. See Figure 1 for representative chromatograms. The system was calibrated daily with the use of standards (0.05 pmol for dialysates and 0.5 pmol for brain supernatants).

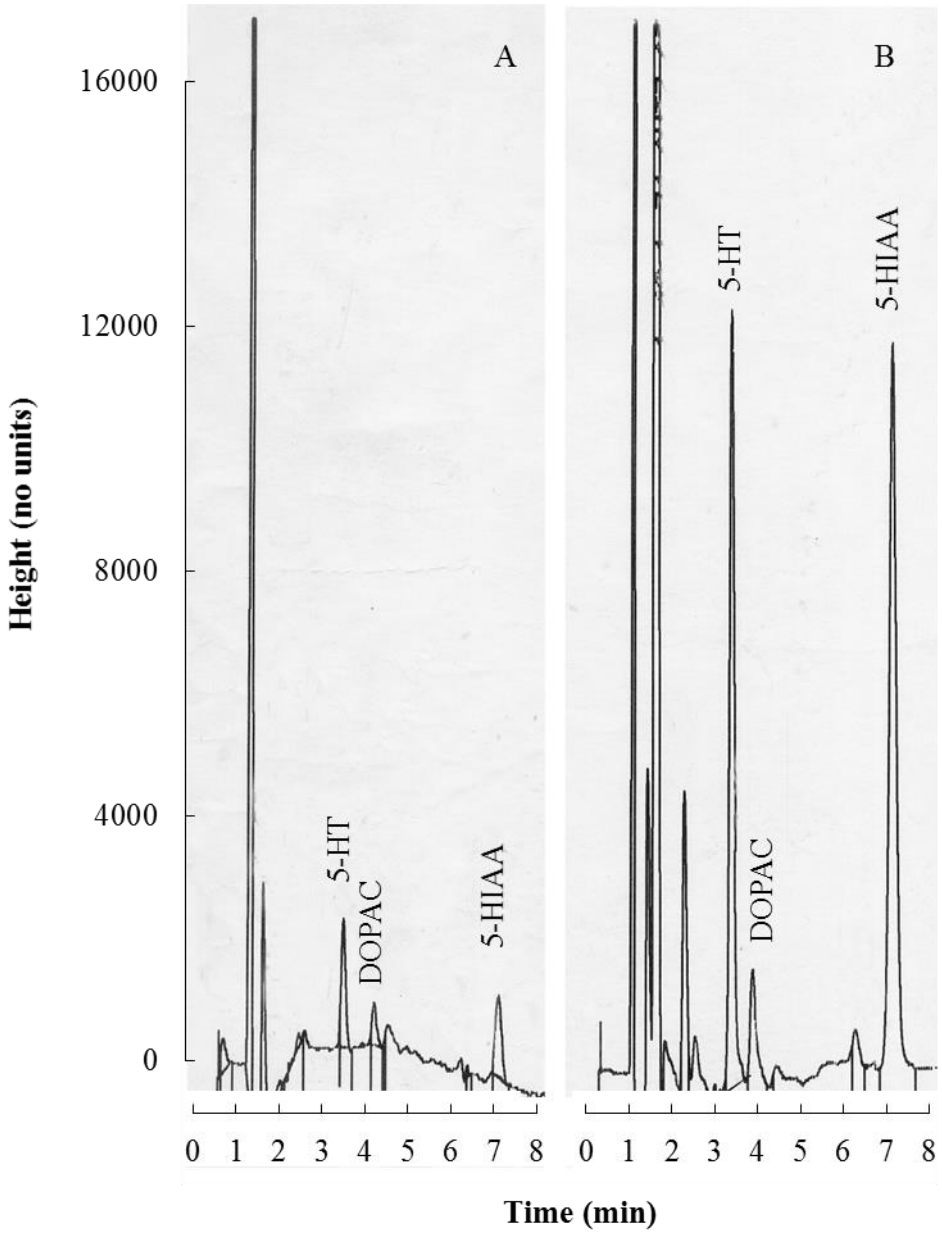

Figure 1: Example HPLC chromatograms for a 10^{-9} M mixed standard (A) and a dialysate sample (B). Height relates to the detector response (nano amps; nA).

Mobile phase for microdialysis experiment

0.13 M $NaHPO_4.H_2O$, 12.5% (v/v) methanol (HPLC grade), 0.85 mM EDTA, 0.025 mM octanyl sulphonic acid, pH 3.5

Mobile phase for 5-HTP experiment

0.12 M $NaHPO_4.H_2O$, 15% (v/v) methanol (HPLC grade), 2 mM NaCl, 0.1 mM EDTA, 2.5 mM octanyl sulphonic acid, pH 3.4

4.2.5 Protein quantification

The protein content of the samples was quantified with colorimetric detection following the use of bicinchoninic acid (BCA) protein assay kit (Pierce 23225). Tissue debris was suspended in 1 ml of BCA and 25 µl from this mixture was pipetted in 96 microwell plate wells. Standards (0-2000 µg/ml) were prepared with consecutive dilutions of BSA stock (2 mg/ml) in the same diluent as the samples, and 25 µl of each dilution were pipetted in the same plate as the samples. Then, 200 µl of working reagent (50 x BCA reagent A, 1 x BCA reagent B), was added in each well and the plate was covered and incubated at 37 ^oC for 30 min. Upon cooling to room temperature, the plate was put in a plate reader and absorbance was measured at 562 nm. This protocol was repeated three times.

Average reading from the blank wells was subtracted from average reading of each standard and standard curve was plotted. Using this standard curve, the protein concentration of each sample was calculated.

4.2.6 Measurement of IEG expression

Groups of mice (*n* = 6 per group) were randomly allocated to treatment groups. Mice initially received an injection of ebselen (10 mg/kg) or vehicle (4% (2-hydroxypropyl)-β-cyclodextrin, 0.4 % (v/v) DMSO) followed 1 h later by citalopram (5 mg/kg) or saline, and then mice were returned to their home cage. Animals were culled 1 h later by cervical dislocation and brains were dissected, snap frozen in ice-cold isopentane and stored in -80 ^{0}C. All drugs were administered i.p. and volume of the injection was adjusted to weight (0.01 ml/g for citalopram and saline; 0.02 ml/g for ebselen and vehicle).

4.2.6.1 Protocol for in situ hybridization

Tissue collection and preparation of sections

Cryostat cut coronal sections (12 µm) were collected onto gelatinised slides from the frontal cortex and caudate putamen (plates 7 and 23 respectively; (Paxinos *et al.*, 2007). Sections were stored at -80 ^{0}C until pre-treatment.

For pre-treatment, all solutions were treated with diethyl pyrocarbonate (DEPC) before being applied to the sections, except for the ethanol solutions that were made up using DEPC treated double distilled water (ddH_2O). In particular, in all solutions 1 ml/l of DEPC was

added and left to stand for 2 h before being autoclaved. Ethanol solutions were prepared in autoclaved bottles.

Slides were allowed to defrost and then fixed in 4% (w/v) Paraformaldehyde (PFA)[5] in phosphate buffered saline (PBS), pH 7.5, for 5 mins. Then slides were immersed in PBS twice to rinse PFA before being acetylated in triethanolamine hydrochloride/acetic acid solution (TEA/AA)[6], (0.25% (v/v) acetic anhydride, 0.1 M triethanolamine hydrochloride and 0.15M NaCl, pH 8) for 10 min. This was followed by dehydration with increasing concentrations of alcohol solutions. Specifically, slides were placed in 70% (v/v) ethanol for 1 min, then 80% (v/v) ethanol for 1 min, 95% (v/v) ethanol for 2 min and 100% ethanol for 1 min. Sections were then treated for 10 min with chloroform for the lipids to be removed, and then rehydrated in 100% then 95% (v/v) ethanol for 1 min each. Slides were then allowed to air dry overnight and stored at -20 ^{0}C until further used.

Radiolabelling and purification of oligonucleotide probes

Oligonucleotide probes complementary to Arc (CTCGGTTGCCCATCCTCACCTGGCCCCCAAGACTGATATTGCTGA) mRNA were purchased from Sigma Genosys. The probes were 3'-tail labelled with alpha-[^{35}S]-deoxyadenosine 5'-thiotriphosphate (Hartmann Analytic GmbH, Germany; specific activity 10 mCi/ml). For the labelling, the probe (2 µM) was incubated with [^{35}S]-dATP and terminal deoxynucleotidyl transerase (TdT) at 37 ^{0}C for 35 min in a water bath. The labelled

[5] 4% (w/v) PFA was made just before use in DEPC treated PBS solution, 60 ^{0}C

[6] Acetic anhydride should be added shorty before use

oligonucleotide probe was then chromatographically separated from the unicorporated nucleotide using illustra NICK columns (GE Healthcare). Following the incubation, the radioactive mix was loaded on the column with 400 µl of tris- ethylenediaminetetraacetic acid (EDTA) buffer (1 M Tris-HCl, 0.1 mM EDTA, pH 8) and the eluent was collected. Two more eluents were collected with the addition of 400 µl Tris-EDTA solution each time. From each eluent 3 µl were mixed with 3ml of scintillation fluid (National Diagnostics) and the counts/minute (cpm) were measured by an automatic scintillation counter (Hidex 300 SL). The oligonucleotide probe was used only when 70% or more of the total counts were found in the second eluent, otherwise the labelling was considered to be unsuccessful.

Before use, the oligonucleotide probes were tested for specificity. Controls included using the sense orientation of the oligonucleotide and displacement with unlabelled probes.

In situ hybridization

Sections from selected brain regions were defrosted and placed in trays covered with filter paper (Whatman, Grade No.1) damped with 50 ml of tray buffer consisting of 25 ml formamide, 15 ml DEPC ddH_2O and 10 ml 20 x saline sodium citrate (SSC; 3 M NaCl, 0.3 M sodium citrate, pH 7). On each slide 200 µl of hybridization mix was added and the slides were cover slipped and incubated at 34 ^oC for 16 h. The hybridization mix consisted of the radioactive nucleotide (2.4 x 10^6 cpm/section), 50 mM dithiothreitol (DTT) and hybridization buffer. The hybridization buffer consisted of 50% deionised formamide, 4 x SSC (20 x stock), 25 mM sodium phosphate buffer, 1 mM sodium pyrophosphate, 5 x Denhart's (50 x stock; Sigma), 0.2 µg/ml boiled herring sperm (stock 10 µg/ml; Promega), 0.1 mg/ml poly adenilic acid (stock 5 mg/ml; Sigma), 120 µg/ml heparin (stock 120 mg/ml,

100 ku; Sigma), 0.1 g/ml Dextran powder (Sigma) to a final volume of 200 ml. The constituents were vigorously mixed overnight in an incubator at 50 ^{0}C. The hybridization buffer was stored at -20 ^{0}C for further use.

Following 16 h incubation of the sections with the hybridization mix, the cover slips were removed and the sections were washed with 1 x SSC, 50 ^{0}C for 20 min. The warm washes were repeated another 2 times and were followed by 2 times, 60 min washes at room temperature with 1 x SSC. Sections were then immersed in ddH_2O to facilitate removal of the SSC and allowed to dry overnight. Slides were then placed in cassettes and exposed to BioMax MR film (Kodak, Carestream) for 7 days. Autoradiographic films were exposed using an automatic X-ray film processor (Compact X4, X-ograph).

Image analysis of autoradiograms

The abundance of Arc mRNA was determined by measuring the optical density in selected brain regions (Figure 2) using MCID software. The optical density readings were converted into nCi/g of tissue by calibration using [^{14}C] microscales, which were co-exposed with the slides. Measurements of regions of interest were taken bilaterally from 3 sections per slide and the values for each region of interest were averaged.

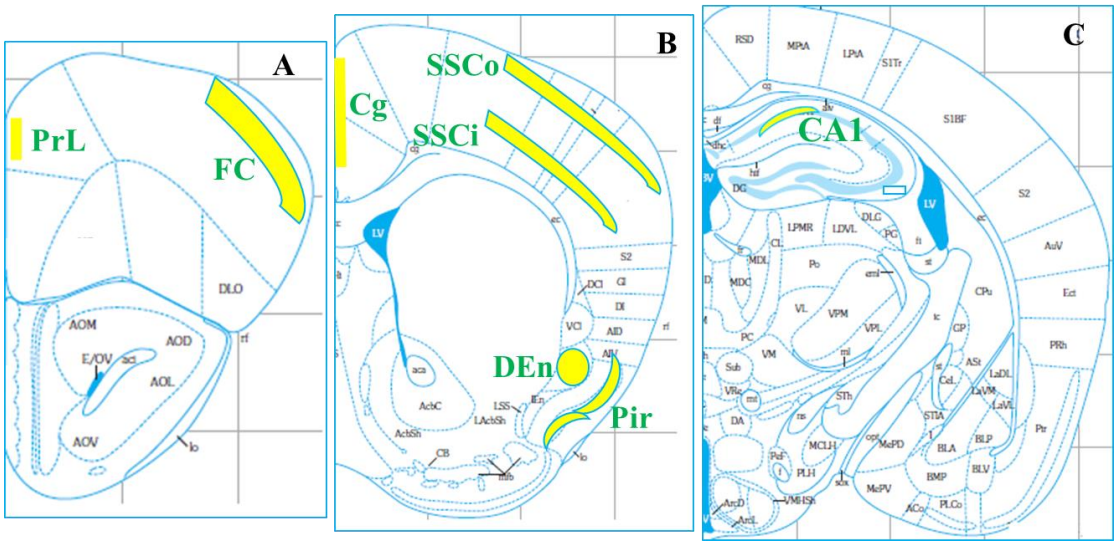

Figure 2: Coronal section of the mouse brain at the frontal cortex (A), caudate putamen (B) and hippocampus (C). Brain regions selected for gene expression analysis are highlighted in yellow. FC: Frontal cortex; PrL: Prilimbic cortex; Cg: Cingulate cortex; SSCo: Somatosensory cortex outer layer; SSCi: Somatosensory cortex inner layer; Pir: Piriform cortex; Den: Endopiriform nucleus. Image modified by (Paxinos *et al.*, 2007).

4.2.7 Data analysis

All data were analysed using IBM SPSS Statistics, version 20. For the 5-HT synthesis experiment, raw 5-HTP data are presented, for IEG data are expressed as percentage of controls, and microdialysis data are presented as percentage of the mean concentration of 5-HT contained in the samples collected immediately before administration of ebselen or vehicle injection. All data are presented as mean ± SEM values. Effects were considered statistically significant when $p < 0.05$.

For the 5-HT synthesis and IEG study the effect of each treatment was evaluated by using one-way ANOVA followed by LSD *post-hoc* test. For the IEG study, each region was

analysed separately. For the microdialysis study repeated measures ANOVA were performed to investigate the effect of the combination of ebselen with citalopram in comparison to ebselen or citalopram treatment alone.

4.3 Results

4.3.1 Effect of ebselen on 5-HT synthesis

Previous work in our lab showed that acute administration of ebselen increased the levels of 5-HT in various mouse brain regions as measured by HPLC (Fowler *et al.,* unpublished data). Based on this finding the mechanism underling this effect was investigated. 5-HT synthesis was studied by pharmacological inhibition of AADC using NSD1015 (Carlsson *et al.*, 1972). 5-HTP levels in a number of brain regions after administration of vehicle or different doses of ebselen followed by NSD1015 are shown in Figure 3. It is evident that in all brain regions tested except for brainstem, 5 mg/kg ebselen increased 5-HTP levels compared to vehicle treated animals (FC: $F(3,22) = 19.124$, $p < 0.001$; SSC: $F(3, 23) = 9.321$, $p < 0.001$; CPU: $F(3,23) = 7.379$, $p = 0.001$; Hippocampus: $F(3,23) = 7.984$, $p = 0.002$; Brainstem: $F(3,18) = 1.277$, $p = 0.813$). This effect of 5 mg/kg ebselen was robust and the increase ranged between 50% for hippocampus to 100% for other brain regions tested. On contrast, 0.5 and 1 mg/kg ebselen did not have a significant effect on 5-HTP compared to vehicle treatment. The increase in 5-HTP following 5 mg/kg ebselen suggests that the drug increased the rate of 5-HT synthesis, which likely explains the increase in 5-HT levels observed in earlier experiments.

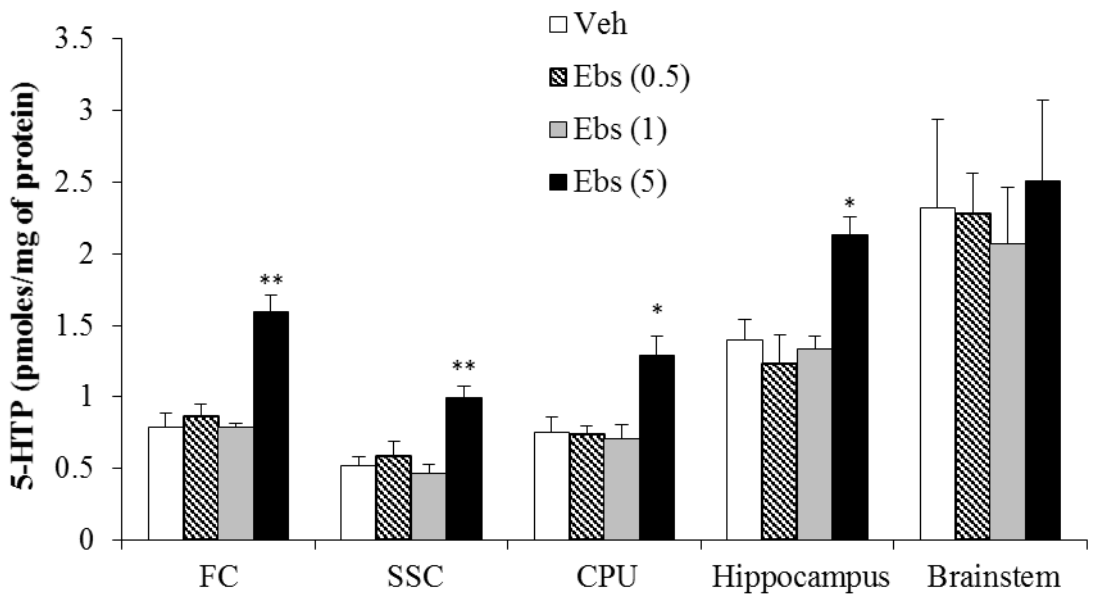

Figure 3: Effect of different doses of ebselen (0.5, 1 and 5 mg/kg) on 5-HTP in different mouse brain regions. *p < 0.005, **p < 0.001 treatment vs vehicle. Data shown are mean ± SEM (6 animals/group). One way ANOVA followed by LSD post-hoc. Abbreviations: FC: frontal cortex; SSC: somatosensory cortex; CPU: caudate putamen.

4.3.2 Effect of ebselen in combination with citalopram on extracellular 5-HT and 5-HIAA in ventral hippocampus *in vivo*

The effect of ebselen alone and on citalopram-induced increase in 5-HT in the ventral hippocampus was investigated *in vivo* by microdialysis. Ebselen alone (10 mg/kg) did not have an effect on extracellular 5-HT in the 60 min prior to citalopram administration compared to vehicle treated mice (Figure 4). Citalopram (5 mg/kg) induced a statistically significant increase in the levels of 5-HT of approximately 400% ($F(3, 24) = 35.655$, $p < 0.000$). Treatment with ebselen (10 mg/kg) 1 h prior to citalopram tended to augment the increase in 5-HT induced by citalopram but this effect was not statistically significant ($p = 0.182$) when analysed with repeated measures ANOVA. To further confirm this outcome

unpaired Student's *t*-test was performed at each time point following ebselen and citalopram injection was analysed separately. This analysis showed no significant difference between Ebs-Citalopram and Veh-Citalopram group (Figure 5).

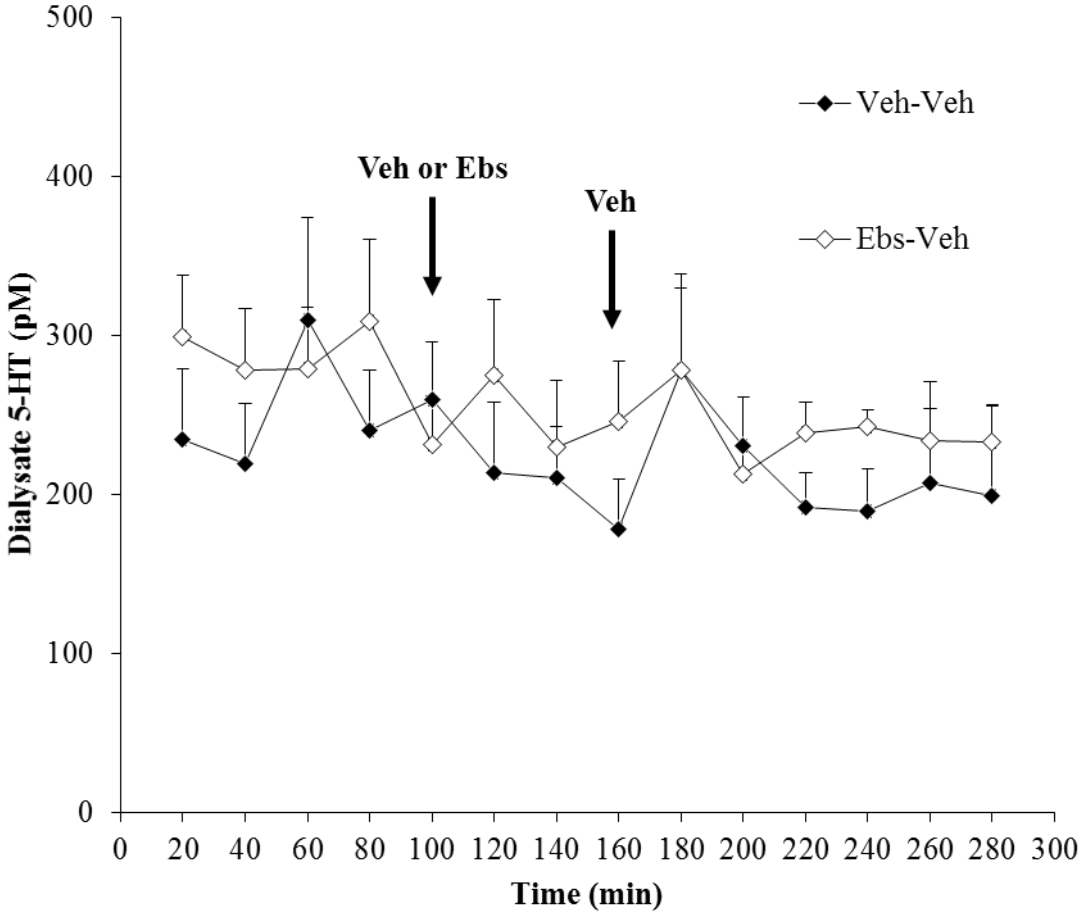

Figure 4 : Microdialysis data showing effect of ebselen (10 mg/kg) on 5-HT in the ventral hippocampus of the mouse. Data shown are mean ± SEM (4-7 animals/group). Repeated measures ANOVA.

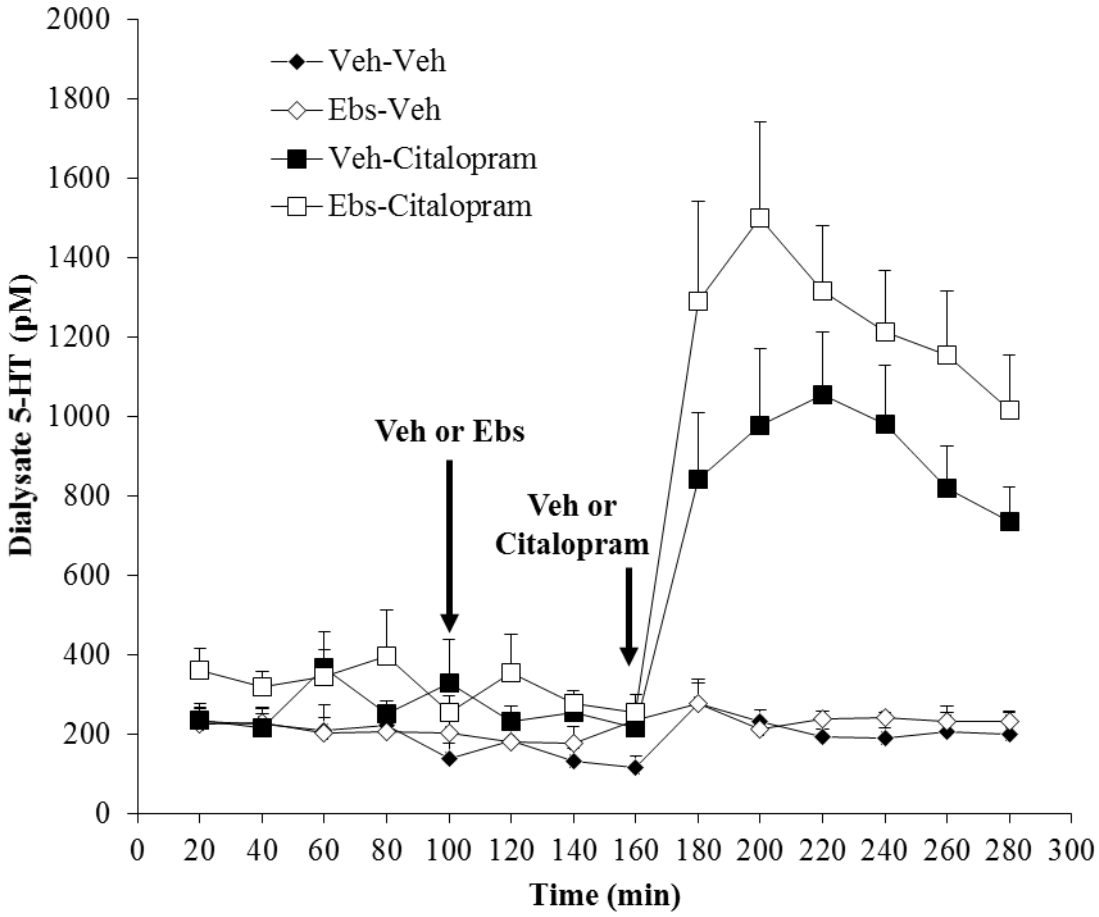

Figure 5: Microdialysis data showing effect of ebselen (10 mg/kg) and citalopram (5 mg/kg) alone or in combination on 5-HT in the ventral hippocampus of the mouse. Data shown are mean ± SEM (4-7 animals/group). Repeated measures ANOVA.

The effect of ebselen alone and in combination with citalopram on extracellular 5-HIAA levels was also investigated *in vivo* by microdialysis. Ebselen alone or in combination with citalopram did not alter the levels of 5-HIAA in the ventral hippocampus of the mouse compared to controls. Similarly, citalopram did not alter extracellular 5-HIAA levels (Figure 6).

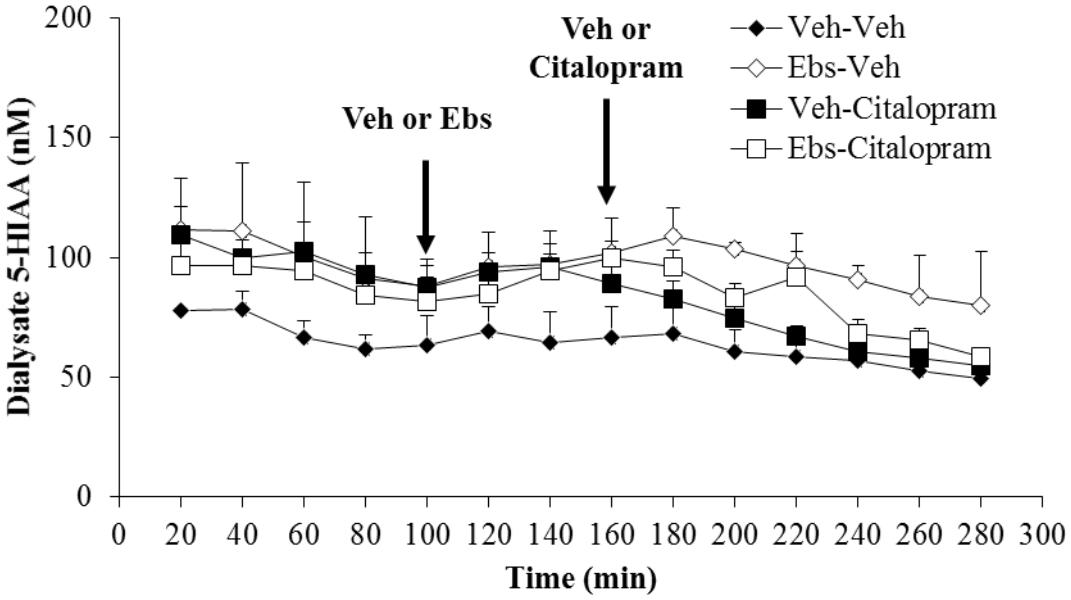

Figure 6: Microdialysis data showing effect of ebselen (10 mg/kg) and citalopram (5 mg/kg) alone or in combination on 5-HIAA in the ventral hippocampus of the mouse. Data shown are mean ± SEM (4-7 animals/group). Repeated measures ANOVA.

4.3.3 Effect of the combination of ebselen and citalopram on Arc mRNA abundance

Representative autoradiograms showing the distribution of Arc mRNA are shown in Figure 7.

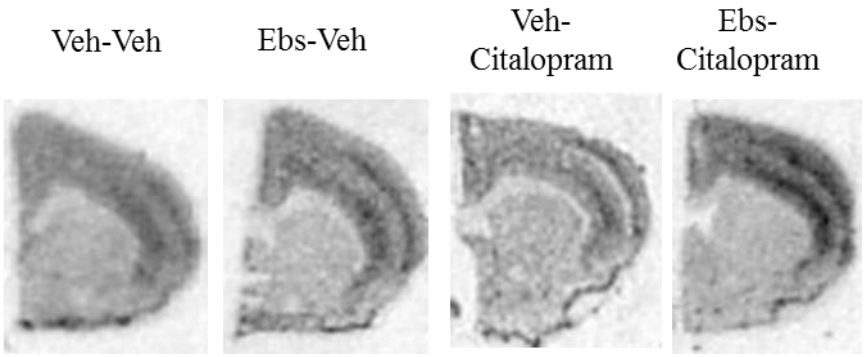

Figure 7: Representative autoradiograms showing distribution of Arc in the caudate putamen of mice treated with either vehicle or ebselen (10 mg/kg, i.p.) followed by citalopram (5 mg/kg, i.p.) or saline.

The effect of the combination of ebselen and citalopram on Arc mRNA abundance was investigated. Ebselen increased the abundance of Arc mRNA when administered in combination with citalopram, compared to vehicle-treated animals and animals that were treated only with citalopram. In particular, ebselen combined with citalopram elicited a statistically significant increase in Arc mRNA in the PrL ($F(3,20) = 1.621$, $p = 0.042$), SSCo ($F(3, 21) = 4.840$, $p = 0.025$), SSCi ($F(3, 21) = 2.971$, $p = 0.041$), Cg ($F(3, 21) = 5.464$, $p = 0.059$), Den ($F(3,21) = 4.591$, $p = 0.025$) and Pir ($F(3, 21) = 5.782$, $p = 0.023$). The increase of Arc mRNA induced by the combination of ebselen and citalopram was also statistically significant compared to citalopram treatment alone in the SSCo, SSCi, Cg, Den

and Pir (SSCo: $p = 0.002$; SSCi: $p = 0.040$; Cg: $p = 0.001$; Den: $p = 0.003$; Pir: $p = 0.001$). Citalopram treatment alone did not elicit a statistically significant change in Arc mRNA with the exception of Cg, where citalopram decreased Arc mRNA by around 15% ($p = 0.043$) compared to vehicle-controls. Ebselen treatment alone did not change Arc mRNA abundance in any of the regions investigated (Figure 8).

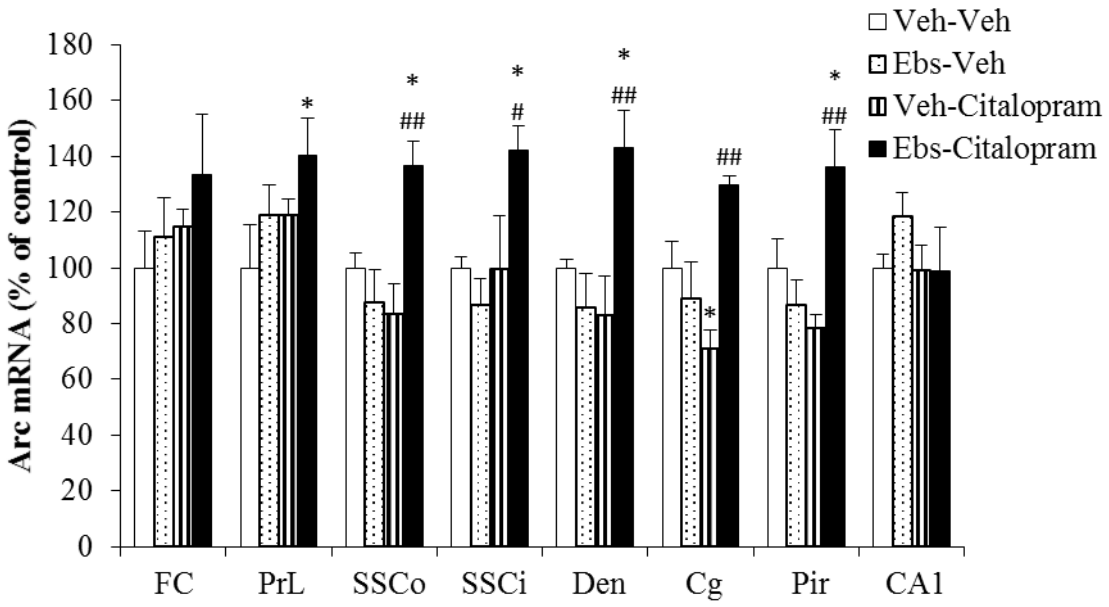

Figure 8: Effect of the combination of acute ebselen (10 mg/kg) and citalopram (5 mg/kg) on Arc mRNA abundance. Data shown are mean ± SEM percentage of Veh-Veh (6 animals/group). $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, treatment vs Veh-Citalopram, $^{*}p < 0.05$ treatment vs Veh-Veh. One-way ANOVA followed by LSD post-hoc. Abbreviations as in methods section.

4.4 Discussion

The aim of the experiments described in this chapter was to investigate the effect of ebselen on 5-HT synthesis and also explore possible SSRI augmentation properties of ebselen. Previous experiments in our lab showed that ebselen increased the intracellular levels of 5-HT in the mouse brain (see Table 1 in Introduction). In the experiments described in this Chapter it was found that ebselen increased the synthesis of 5-HT in various brain regions, as shown by the increased accumulation of 5-HTP. In microdialysis experiments, ebselen had no effect on basal extracellular 5-HT but tended to increase 5-HT when combined with citalopram although this effect was not statistically significant. Combination of ebselen with citalopram was found to elevate Arc mRNA abundance compared to vehicle injected animals across a variety of brain regions, while neither ebselen nor citalopram alone had an effect on Arc mRNA. Lastly, acute ebselen (10 mg/kg) administration prior to citalopram (5 mg/kg) did not induce a statistically significant increase in 5-HT in the ventral hippocampus of mice.

In previous experiments in our lab ebselen (0.5, 1 and 5 mg/kg) increased the tissue levels of 5-HT in mouse brain regions. Here, ebselen (5 mg/kg) increased regional brain 5-HT synthesis in the frontal cortex, caudate putamen, somatosensory cortex and hippocampus of mice, but not in the brainstem. These findings suggest that the increase in tissue 5-HT induced by ebselen results from an increase in the synthesis of 5-HT. As with ebselen, lithium has been reported to increase the 5-HT synthesis when given to rats {Perez-Cruet, 1971 #187; Poitou, 1974 #197}. It is not known whether the effect of ebselen or lithium on 5-HT synthesis is mediated through the blockade of IMPase. Therefore, it would be

interesting to determine the effect of a selective IMPase inhibitor on 5-HT synthesis. It should be noted that ebselen may increase the levels of 5-HT through an independent of IMPase mechanism. In particular, ebselen has been reported to inhibit indoleamine 2,3 deoxygenase (IDO) in *in vitro* experiments with an IC_{50} = 90 nM, similar to that for IMPase (IC_{50} = 1.5 μM). Since IDO breaks down the precursor of 5-HT, tryptophan to kynurenine, IDO inhibition would likely result in increased tryptophan levels and elevation of 5-HT synthesis. In previous experiments, no increase in the levels of tryptophan was found, which would indicate that IDO inhibition is not the mechanism behind the increase in 5-HT synthesis (Fowler *et al.*, unpublished data).

Ebselen (10 mg/kg) when combined with citalopram increased Arc mRNA abundance in a variety of brain regions, while neither ebselen nor citalopram increased Arc mRNA when administered alone. The IEG Arc is commonly used as a molecular neuronal marker of antidepressant action as it was found to increase following repeated administration of antidepressants (Pei *et al.*, 2003b; Serres *et al.*, 2012). The functional relevance of Arc to the adaptive changes of antidepressants at the level of the synapse arises from studies showing that Arc is a marker of increased neuronal activation, while disruption of Arc leads to behavioural deficits in memory consolidation (Steward *et al.*, 1998; Guzowski *et al.*, 2000). Additionally, in an animal paradigm of depression, that of social defeat, Arc mRNA reduction has been associated to vulnerability in depressive behaviour under stressful situations (Covington *et al.*, 2010). Different classes of antidepressants such as the selective serotonin re-uptake inhibitor paroxetine, the serotonin and norepinephrine inhibitor venlafaxine and the tricyclic antidepressant desipramine when administered acutely were found to have no effect on Arc mRNA abundance in rats (Pei *et al.*, 2003b). However, when the same antidepressants were administered repeatedly for 14 days to rats, they elicited a

clear increase in Arc mRNA in a variety of brain regions (Pei *et al.*, 2003b). In agreement with these findings, acute citalopram alone was not found to alter Arc mRNA in this study. However, co-administration of ebselen and citalopram elicited an acute increase in Arc mRNA. The exact mechanism through which ebselen might accelerate the onset of increase of Arc mRNA by citalopram is not known but the increase in 5-HT synthesis by ebselen is hypothesised to contribute to the findings on Arc. Nevertheless, augmentattion of SSRI-induced Arc mRNA abundance in a way similar observed for ebselen in the present study has been reported to depend on 5-HT. The 5-HT synthesis inhibitor *p*-chlorophenylalanine, inhibited the augmentation effects of co-administration of 5-HT_{1A} antagonists and paroxetine on Arc mRNA, highlighting that the 5-HT system is involved (Castro *et al.*, 2003).

Another mechanism that could contribute to the enhancement of 5-HT function when ebselen is co-administered with citalopram and could further result in the increase in Arc mRNA is the attenuation of 5-HT_{2A} and 5-HT_{2C} receptor function by ebselen, as demonstrated in the previous two Chapters. 5-HT_{2A} and 5-HT_{2C} receptors are involved in a negative feedback control of 5-HT neurons. In particular, 5-$HT_{2A/2C}$ agonists administered systemically inhibit the firing of midbrain 5-HT neurons (Boothman *et al.*, 2003). Activation of 5-HT_{2A} and 5-HT_{2C} might contribute to the delay of onset of effects of SSRIs, as it would counteract the increase in 5-HT following blockade of the 5-HT transporter. In fact, microdialysis studies revealed that the combination of either 5-HT_{2A} or 5-HT_{2C} antagonists with SSRIs enhanced the acute effects of SSRIs on 5-HT release in the hippocampus and frontal cortex of rats (Cremers *et al.*, 2004; Boothman *et al.*, 2006b). Preliminary findings suggest that co-administration of either 5-HT_{2A} or 5-HT_{2C} antagonists with citalopram increased Arc mRNA, although neither citalopram nor antagonists had an effect on Arc

when administered alone (Serres *et al.*, unpublished data). Similar findings on IEG expression have been reported for 5-HT_{1A} autoreceptor antagonists. The 5-HT_{1A} autoreceptors are also involved in the negative feedback control of 5-HT neurons in the dorsal raphe nucleus and acute blockade of these receptors by the antagonists WAY 100635, NAD-299, *p*-MPPI and LY 426965 prior to acute paroxetine has been shown to elicit an acute increase in Arc mRNA, while administration of paroxetine alone has no acute effect on Arc mRNA (Castro *et al.*, 2003; Tordera *et al.*, 2003).

In order to further investigate whether the augmentation of the effects of ebselen on citalopram administration at the molecular level (IEG expression), correlated to an augmentation of citalopram-induced increase of extracellular 5-HT, ebselen was co-administered with citalopram and the levels of 5-HT in the ventral hippocampus were monitored *in vivo*, in freely moving mice, using microdialysis. Ebselen alone did not have an effect on 5-HT release in the ventral hippocampus. Ebselen showed a clear tendency to augment the effect of citalopram on extracellular 5-HT, but this effect was not statistically significant. It is likely that the present data lack statistical power, due to the use of small number of subjects per group (n = 4-7). Therefore the effect of ebselen should be further investigated by increasing the size of the treatment groups of the present study. The enhancement of 5-HT release by ebselen in combination with citalopram, could be related to the increased 5-HT levels and synthesis, elicited by ebselen in a similar way that it has been suggested for lithium. Lithium is shown to increase brain tissue 5-HT levels and synthesis but not extracellular baseline 5-HT and these effects are believed to contribute to augmentation of antidepressants (discussed in more detail below) (Berggren, 1985; Berggren, 1987; Friedman *et al.*, 1988; Sharp *et al.*, 1991). Additionally, as discussed above,

attenuation of the 5-HT_{2A} and 5-HT_{2C} receptors by ebselen, could enhance the increase in 5-HT by citalopram as well.

Lithium has been reported to augment the increase in 5-HT induced by SSRIs. Thus, repeated lithium administration to rats, enhanced the 5-HT increase induced by citalopram and milnacipran in the medial prefrontal cortex of the rat (Muraki *et al.*, 2001; Kitaichi *et al.*, 2005). In the ventral hippocampus, however, two studies reported contradictory findings. One study showed that 7 days lithium enhanced citalopram induced increase in 5-HT but this was not confirmed in a second study (Wegener *et al.*, 2000; Wegener *et al.*, 2003). It would therefore be interesting to investigate the effect of ebselen on citalopram-induced increase in 5-HT in the frontal cortex of the mouse as well, as it is possible that regional specificity in the augmentation effects of ebselen might occur. In the present study, ebselen was administered acutely prior to citalopram, since acute ebselen both reduced 5-HT_{2A} and 5-HT_{2C} function and increased 5-HT levels and 5-HT synthesis occurred. However, it could be investigated whether repeated treatment with ebselen would increase 5-HT when combined with citalopram as it has been reported for repeated lithium.

Another point of consideration is the dose of citalopram used in the present study. It has been reported that the magnitude of the increase in 5-HT induced by co-administration of augmentation agents with SSRIs is proportional to the dose of SSRI used (Sharp *et al.*, 1997). In fact, in the previous studies using lithium in combination with citalopram, a dose of 20 or 30 mg/kg of citalopram was administered, while in the present study a much lower dose of 5 mg/kg of citalopram was used (Muraki *et al.*, 2001; Wegener *et al.*, 2003). It cannot therefore be excluded that a higher dose of citalopram in combination with ebselen would produce a greater enhancement in extracellular 5-HT.

In summary, experiments described in this chapter provide evidence that ebselen increased presynaptic 5-HT function by increasing the synthesis of 5-HT. Lithium and 5-HT_2 antagonists are also reported to augment the effect of an SSRI on extracellular 5-HT, and in the current study ebselen had a tendency to produce a similar effect. Further evidence for an SSRI enhancing action of ebselen was found in experiments measuring Arc expression. Collectively, these data suggest that ebselen augments the effects of SSRI administration, as it has been shown for lithium. Overall, ebselen was found to bear similarities to lithium in terms of 5-HT neurochemistry and SSRI-augmentation. Experiments in the next chapter explore whether ebselen has properties similar to lithium in another model: expression of markers of neuronal plasticity.

Chapter 5

Investigation of the effects of ebselen on markers of neuronal plasticity

5 Investigation of the effects of ebselen on markers of neuronal plasticity

5.1 Introduction

In Chapter 4 it was found that ebselen increased the rate of 5-HT synthesis and augmented the effects of citalopram on gene expression. As with ebselen, lithium has been shown to increase 5-HT synthesis while it is proven to be effective in augmenting the effects of antidepressants in clinic. In light of the possible antidepressant properties of ebselen and the neuropharmacological similarities it bears with lithium as shown in Chapters 2, 3 and 4, the experiments of the present chapter aimed to explore the effects of ebselen on markers of neuronal plasticity and compare to lithium.

Neuronal plasticity is a term used to describe the ability of the brain to adapt to internal and external stimuli (e.g. environment, injury, behaviour, learning) at the cellular and molecular level (Soeiro-de-Souza *et al.,* 2012). Current mood disorder theories converge on the hypothesis that enhancement of neuronal plasticity is a key mediator of therapeutic effect in mood disorder. These theories offer an explanation for the delay in the onset of therapeutic effects seen in clinic, that does not correlate with the acute pharmacological effects of antidepressants such as the inhibition of monoamine transporters, which occurs rapidly. Antidepressant drugs including lithium are hypothesised to repair structural and molecular abnormalities in the course of repeated treatment, by regulating gene expression which leads

to enhanced neuronal plasticity, exhibited as increased synaptogenesis and synaptic strength or neuronal survival and neurotrophic support (Sharp, 2013).

A good example of neuronal plasticity alterations by antidepressants is brain-derived neurotrophic factor (BDNF) which is increased by repeated but not acute antidepressant administration in rodents. Moreover, BDNF is necessary for antidepressant activity as BDNF knockout mice do not respond to antidepressant treatment (Vaisanen *et al.*, 2003; Monteggia *et al.*, 2004). Increased BDNF has functional significance in terms of enhanced dendritic growth (McAllister *et al.*, 1997), synaptic efficacy (Levine *et al.*, 1995) and monoaminergic activity (Siuciak *et al.*, 1996). Additionally, other genes have been reported to increase following antidepressant administration in rodents, including Arc (Castro *et al.*, 2003; Pei *et al.*, 2003b; Serres *et al.*, 2012), shank (Serres *et al.*, 2012), homer (Serres *et al.*, 2012) and the vesicular glutamate transporter 1 (VGluT1) (Moutsimilli *et al.*, 2005; Tordera *et al.*, 2005; Serres *et al.*, 2012).

Lithium has been shown to increase markers of neuronal plasticity at the molecular level including BDNF. Thus, lithium has been found to increase BDNF mRNA and protein in both cell cultures and whole animals. For instance, in rat cortical neuronal cultures, lithium activated the promoter IV of BDNF (Yasuda *et al.*, 2009). Systemic administration of lithium for 3 weeks elevated BDNF mRNA and protein levels in cortical and hippocampal regions of rats (Jacobsen *et al.*, 2004). These effects of lithium were dependent on the duration of treatment as 1 or 7 days lithium treatment did not increase BDNF in hippocampal and cortical regions, while 14 or 28 days lithium resulted in a significant increase (Fukumoto *et al.*, 2001).

Lithium increased the serum levels of BDNF in patients with unipolar depression who were refractory to antidepressant treatment (Ricken *et al.*, 2013), and also in patients with bipolar disorder (de Sousa *et al.*, 2011). Another study demonstrated that bipolar disorder patients have reduced BDNF serum levels compared to healthy volunteers, and that repeated lithium treatment increased BDNF to control levels (Tramontina *et al.*, 2009). Interestingly, a subgroup of lithium-treated patients who showed complete remission of symptoms, displayed elevated BDNF in the serum compared to non-responding patients (Rybakowski *et al.*, 2010).

In addition to BDNF, repeated lithium treatment has been reported to alter the expression of other markers of neuronal plasticity. For example, treatment of mice for 19 days with lithium, increased the mRNA and protein of VGluT1 mRNA in cortical and hippocampal regions (Moutsimilli *et al.*, 2005). On the other hand, the mRNA of the post-synaptic density protein homer and the IP3 receptor have been reported to be down-regulated by treatment of rats for 4 weeks with lithium (de Bartolomeis *et al.*, 2012). Lithium also increased the levels of the adhesion molecule neurocan (NCAN) in a human neuroblastoma cell line (Italia *et al.*, 2011). NCAN is a gene that has recently been associated with bipolar disorder. NCAN knockout mice show a manic behavioural phenotype which is rescued by lithium (Miro *et al.*, 2012).

Adaptive changes to mRNA abundance of genes related to the PI cycle have also been reported following lithium treatment in both rodents and humans. In particular, lithium administration for 10 or 28 days has been shown to increase the activity, mRNA and protein levels of IMPase in the brain of rats and mice (Parthasarathy *et al.*, 2003; Shamir *et al.*,

2003). Increased IMPase mRNA in neutrophils of patients with bipolar disorder has also been detected in response to lithium treatment (Nemanov *et al.*, 1999). On the other hand, mRNA of the sodium myo-inositol transporter (SMIT) has been reported to be decreased after lithium treatment in neutrophils of bipolar disorder patients (Willmroth *et al.*, 2007).

There are not studies investigating the involvement of IMPase on neuronal plasticity in the mammalian brain. Indirect evidence that inhibition of IMPase might be involved in the neuroplastic effects of lithium, come from studies in cell cultures and animals including drosophila or *C. elegans.* In rat dorsal root ganglia cultures, lithium has been shown to increase growth cone area, decrease growth cone collapse and increase axon branches in rat dorsal root ganglia cultures, and these effects are reversed by inositol supplementation suggesting that lithium might have neurotrophic effects linked to inositol depletion (Williams *et al.*, 2002). Furthermore, local application of lithium increased the number of functional synapses in hippocampal neuronal cultures which was reversed by inositol supplementation while GSK-3 inhibition did not have an effect (Kim *et al.*, 2009). In addition, IMPase itself has been reported to be important for synaptic function. *C. elegans* with IMPase gene mutations, show severely affected thermotactic behaviour and defects in the localisation of synaptic vesicle proteins in the neural circuit encoding this behaviour, effect also reversed by inositol supplementation (Tanizawa *et al.*, 2006). Lastly, drosophila with mutations in the IPP gene, which is also involved in the recycling of inositol were unable to sustain long tetanic stimulation. In the same report, lithium mimicked the effects of the gene mutation (Acharya *et al.*, 1998). These data add to the hypothesis that the PI pathway is fundamental for proper adaptive neural network function.

The effect of ebselen on markers of neuronal plasticity has not been investigated. Indirect evidence that ebselen might have an effect on neuronal plasticity comes from a human study, in which ebselen was found to improve recovery after stroke (Yamaguchi *et al.*, 1998). Ebselen also has neuroprotective effects in animal models of stroke with the drug being found to increase neuronal viability under toxic conditions of excess glutamate concentrations (Dawson *et al.*, 1995; Porciuncula *et al.*, 2001; Moretto *et al.*, 2005).

Given the similarities between ebselen and lithium reported in this thesis the aim of the present study was to investigate the effect of ebselen and lithium treatment on neuronal plasticity related genes and genes involved in inositol recycling. A number of candidate genes were selected as markers of neuronal plasticity, in particular specific genes reported to be regulated by lithium and/or antidepressants were investigated. These included Arc, BDNF, Shank1B, VGluT1, homer 1b/c, IP3R and NCAN. In addition, IMPase and the SMIT mRNA levels were also studied, as it has been reported that lithium treatment modulates their expression (Nemanov *et al.*, 1999; Vaden *et al.*, 2001; Parthasarathy *et al.*, 2003; Shamir *et al.*, 2003; Willmroth *et al.*, 2007).

5.2 Materials and methods

5.2.1 Experimental animals

Adult, male C57BL/6 (7-9 weeks old) (Harlan, Bicester, UK) mice were housed in groups of 4 under controlled conditions of lighting (12 h light-dark cycle) and temperature (21±1 ^{o}C). Food and water was available *ad libitum*. One week acclimatisation in the animal facility was allowed before the experiments were carried out. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and Home Office Guidelines.

5.2.2 Drug treatments

For the ebselen study, 2 groups of mice (*n*=8 per group) were treated with ebselen (5 mg/kg) twice daily or vehicle (4% (w/v) (2-hydroxypropyl)-β-cyclodextrin, 0.0003% (v/v) DMSO) for 14 days. For the lithium study, 2 groups of mice (*n*=8 per group) were treated with lithium (10 mmol/kg on day 1 and 3 mmol/kg twice daily on days 2-14) or saline for 14 days. 16 h post last injection brains were dissected, snap frozen in ice-cold isopentane and stored in -80 ^{0}C until used.

5.2.3 Protocol for *in situ* hybridization

Coronal sections (12 µm) were collected onto gelatinised slides at the level of the caudate putamen (Figure 23 in (Paxinos *et al.*, 2007). Sections were stored in -80 ^{0}C until pre-treatment. Sections were pre-treated using standards protocols, as it was described in Chapter 2. Briefly, slides were defrosted, fixed in paraformaldehyde, acetylated in acetic anhydride in triethanolamine hydrochloride, dehydrated in increasing concentrations of alcohols, delipidated in chloroform and rehydrated in increasing concentration of alcohols. Sections were then left to air dry and stored in -20 ^{0}C.

Oligonucleotide probes complementary to Arc (5'-CTCGGTTGCCCATCCTCACCTGGCCCCCAAGACTGATATTGCTGA-3'), VGluT1 (5'-GCACTGGGAACAAGGGAGGACTTGCATCTT-3'), BDNF (5'-GGTCTCGTAGAAATATTGCTTCAGTTGGCCTTT-3'), Shank1B (5'-GTACCACATCCTGTTCCCGATGGTTACGAATCAGTT-3'), IP3R (5'-TGACATCAAAATTCACCCGGGAGATGACACTGACTGGTCA-3'), homer 1b/c (5'-GGTACCCTGTCTTCTGTGAAAACTCTGTAGGCCTGTGGT-3'), NCAN (5'-CTGATGCAGTCTGAACCTTAGTCCACTTGATCCGAGGAA-3'), SMIT (5'-TCTACTGGTGTTTCTGCCTCTGAATGACCCATGGAAGCC-3'), IMPase1 (5'-AGTTCACCATTACACAACAGTACACAGGTCAGCACCAGG-3') were 3'-tailed labelled with [^{35}S]-dATP (see Chapter 2 for the detailed protocol). Briefly, 2 µM of each oligonucleotide probe was incubated with [^{35}-S]-dATP and terminal

deoxynucleotidyl transerase (TdT) at 37 ^{0}C for 35 min. The unincorporated oligonucleotide probe was then separated from the labelled using illustra NICK column.

Sections from selected brain regions were defrosted and incubated overnight at 34 ^{0}C with the labelled probe in hybridization buffer ($2.4x10^{6}$ cpm/section for all except for SMIT where 3.6 cpm/section were applied) containing 50 mM DTT (see Chapter 2 for detailed protocol). Sections were washed 16 h post incubation, in 1 x SSC for 3 x 20 min at 55 ^{0}C followed by 2 x 60 min at room temperature. Sections were allowed to dry overnight and exposed for 7 days to BioMax MR film (except VGluT and Shank1B for which 3 days exposure was sufficient, and SMIT for which 10 days exposure was required). Films were developed using an automatic X-ray film processor.

Image analysis of autoradiograms

The mRNA abundance was calculated by densitometric quantification of selected brain regions (see Figure 1) on the autoradiograms, using MCID software as described in Chapter 2. In particular, the selected regions of interest were frontal cortex (FC), somatosensory cortex outer layer (SSCo), somatosensory cortex inner layer (SSCi), cingulate cortex (Cg), piriform cortex (Pir), endopiriform nucleus (Den), caudate putamen (CPU), dentage gyrus (DG), habenula (Hb), posterior thalamic nuclear group (Po), basolateral amygdaloid nucleus (BLA) and zona incerta (ZI). The abundance of mRNA was determined by measuring the optical density in selected brain regions using MCID software. The optical density readings were converted in nCi/g of tissue by

calibration with the use of [^{14}C] microscales, which were co-exposed with the slides. Measurements of regions of interest were taken bilaterally from 3 sections per slide and the values for each region of interest were averaged.

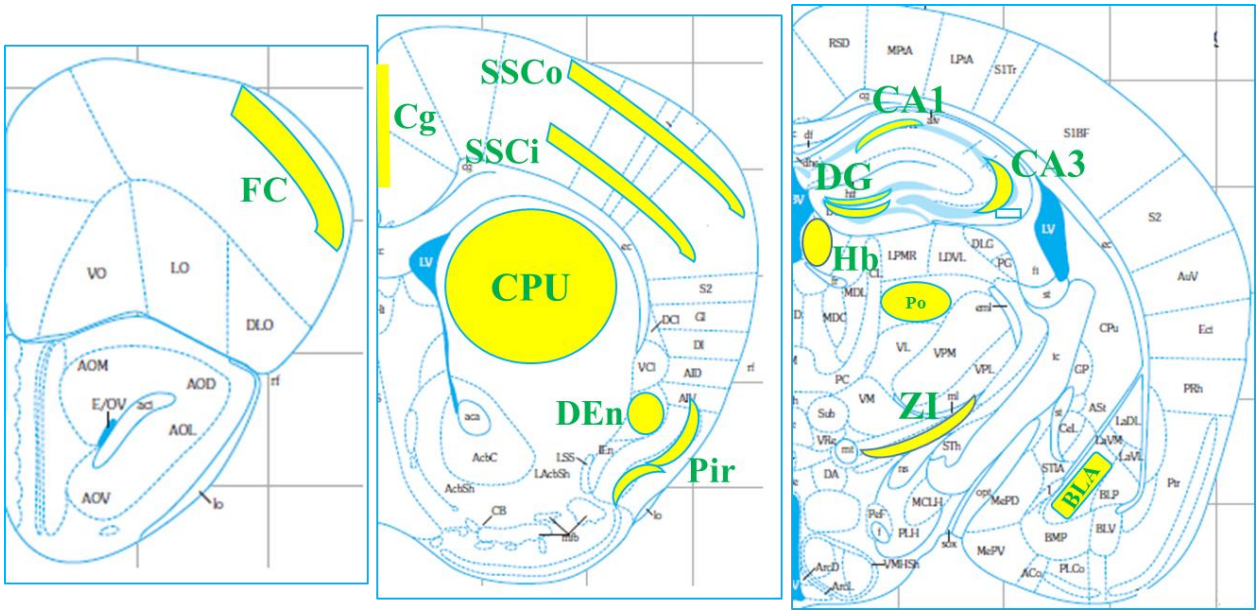

Figure 1: Pictures from coronal sections of the mouse brain at three different levels (A) frontal cortex, (B) caudate putamen and (C) hippocampus. Image modified from (Paxinos *et al.*, 2007).

5.2.4 Data analysis

All data were analysed using IBM SPSS Statistics software, version 20. Data were expressed as percentage of controls and presented as mean ± SEM values. Effects were considered statistically significant when $p < 0.05$. The effect of each treatment was evaluated by using one-way ANOVA followed by LSD *post-hoc* test. Each region was analysed separately.

5.3 Results

5.3.1 Effect of ebselen and lithium on Arc mRNA abundance

Autoradiograms showing the distribution of Arc mRNA in vehicle and ebselen or lithium treated animals are illustrated in Figure 2. The distribution of Arc mRNA was found to be similar to studies presented in Chapters 2, 3 and 4.

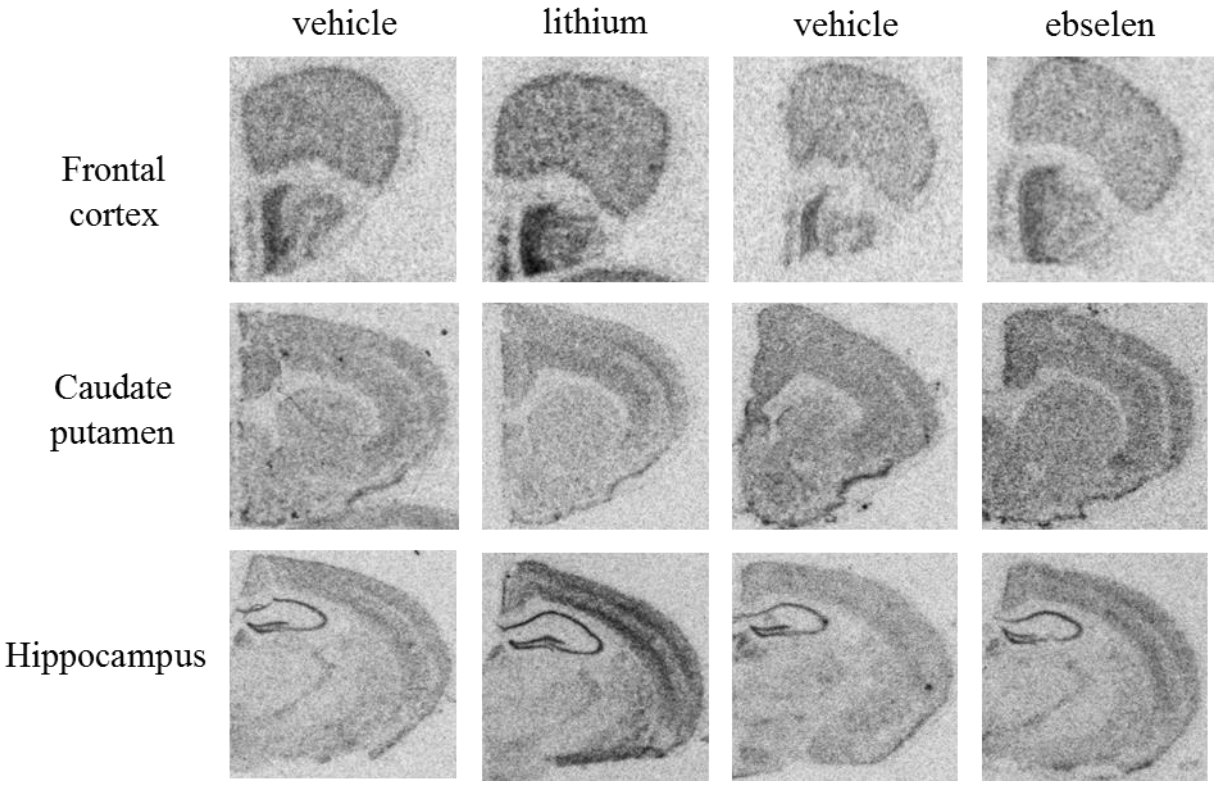

Figure 2: Representative autoradiograms showing distribution of Arc mRNA at the level of frontal cortex, caudate putamen and hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Ebselen treatment for 14 days increased Arc mRNA abundance in both cortical and hippocampal regions. In particular, compared to vehicle controls ebselen increased Arc mRNA in the SSC (t (11) = 3.407, p = 0.006), Pir (t (12) = 3.982, p =0.002) and the CA3 region of the hippocampus (t (14) = 2.953, p = 0.010) (Figure 3A).

Compared to vehicle controls lithium treatment for 14 days also increased Arc mRNA abundance and this effect was statistically significant in the CA3 region of the hippocampus (t (11) = 4.523, p = 0.001) with only upward trends in other regions (Figure 3B).

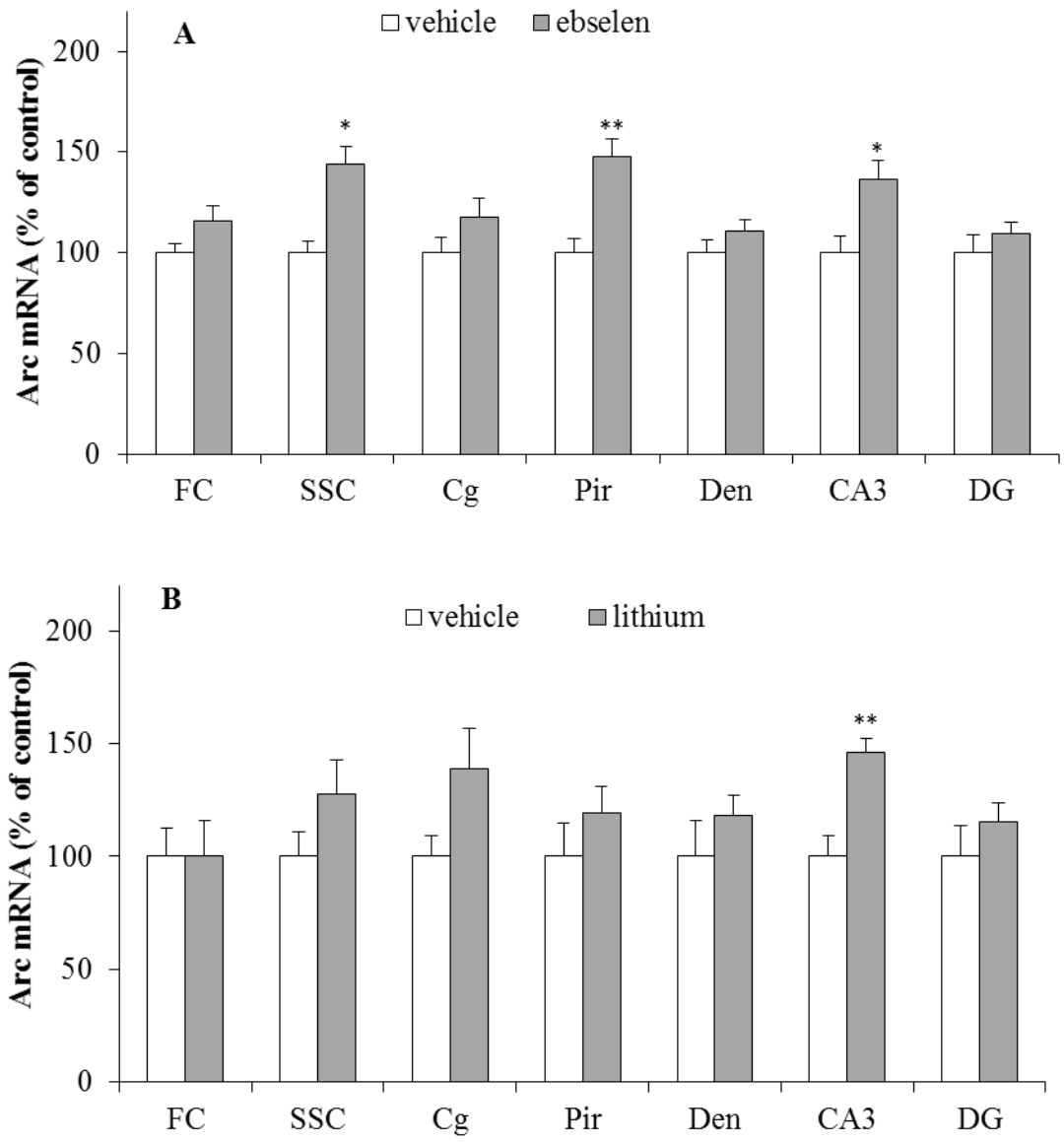

Figure 3: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of Arc mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (5-8 animals/group). *$p < 0.05$, **$p < 0.005$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.2 Effect of ebselen and lithium on BDNF mRNA abundance

Representative autoradiograms of BDNF mRNA distribution in control and ebselen or lithium treated animals are shown in Figure 4. BDNF mRNA abundance was found to be highest in the hippocampus and in particular in the CA3 region and the DG of the hippocampus with lowest levels in the SSC and the Cg.

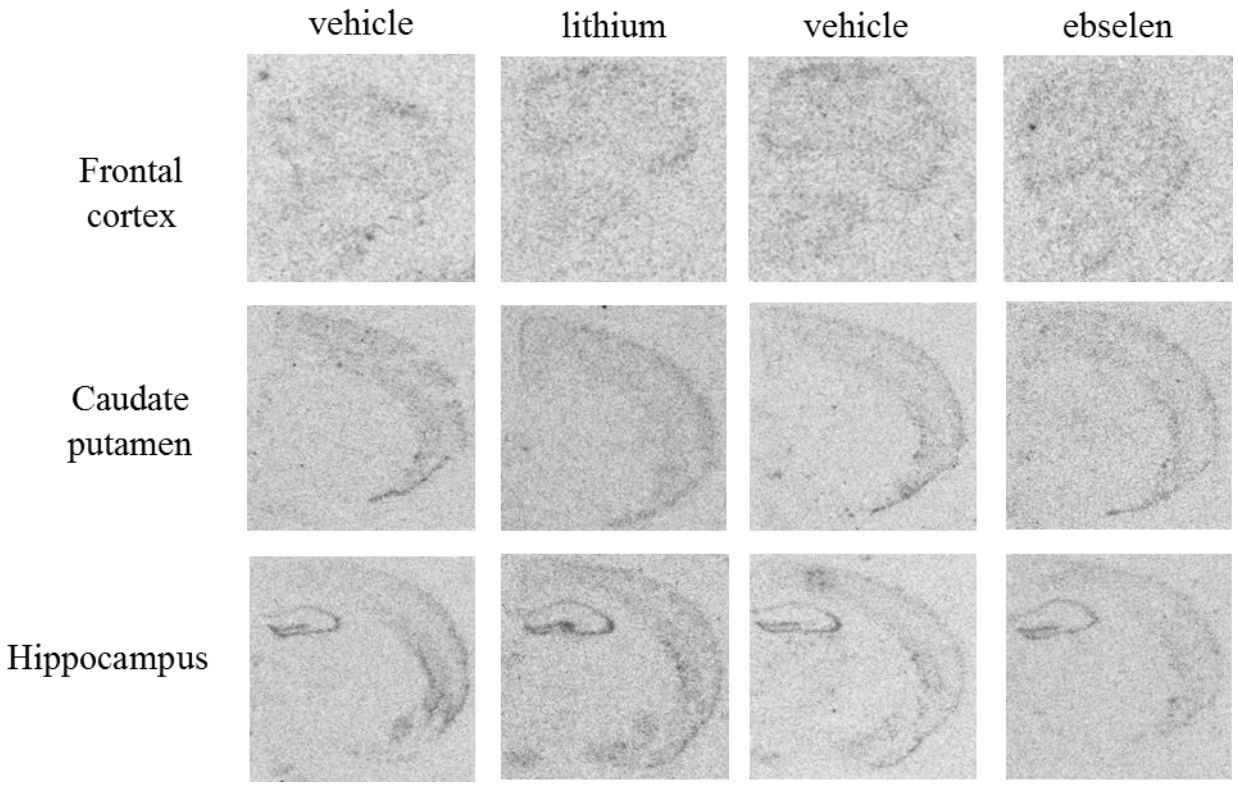

Figure 4: Representative autoradiograms showing distribution of BDNF mRNA at the level of the frontal cortex, caudate putamen and hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Compared to vehicle-injected controls ebselen administration for 14 days increased BDNF mRNA in the Cg (t (12) = 2.482, p = 0.020), Pir (t (12) = 5.281, p = 0.000) and Den (t (12) = 2.434, p = 0.032), DG (t (14) = 2.157, p = 0.049) and the SSC (t (12) = 2.978, p = 0.012) (Figure 5A).

Compared to controls, lithium treatment for 14 days also increased BDNF mRNA compared to controls and this effect was statistically significant in the FC (t (18) = 3.563, p = 0.002), Cg (t (17) = 3.462, p = 0.008), Pir (t (17) = 5.435, p = 0.000), DEn (t (17) = 3.399, p =0.003) and DG (t (18) = 2.705, p = 0.014) (Figure 5B).

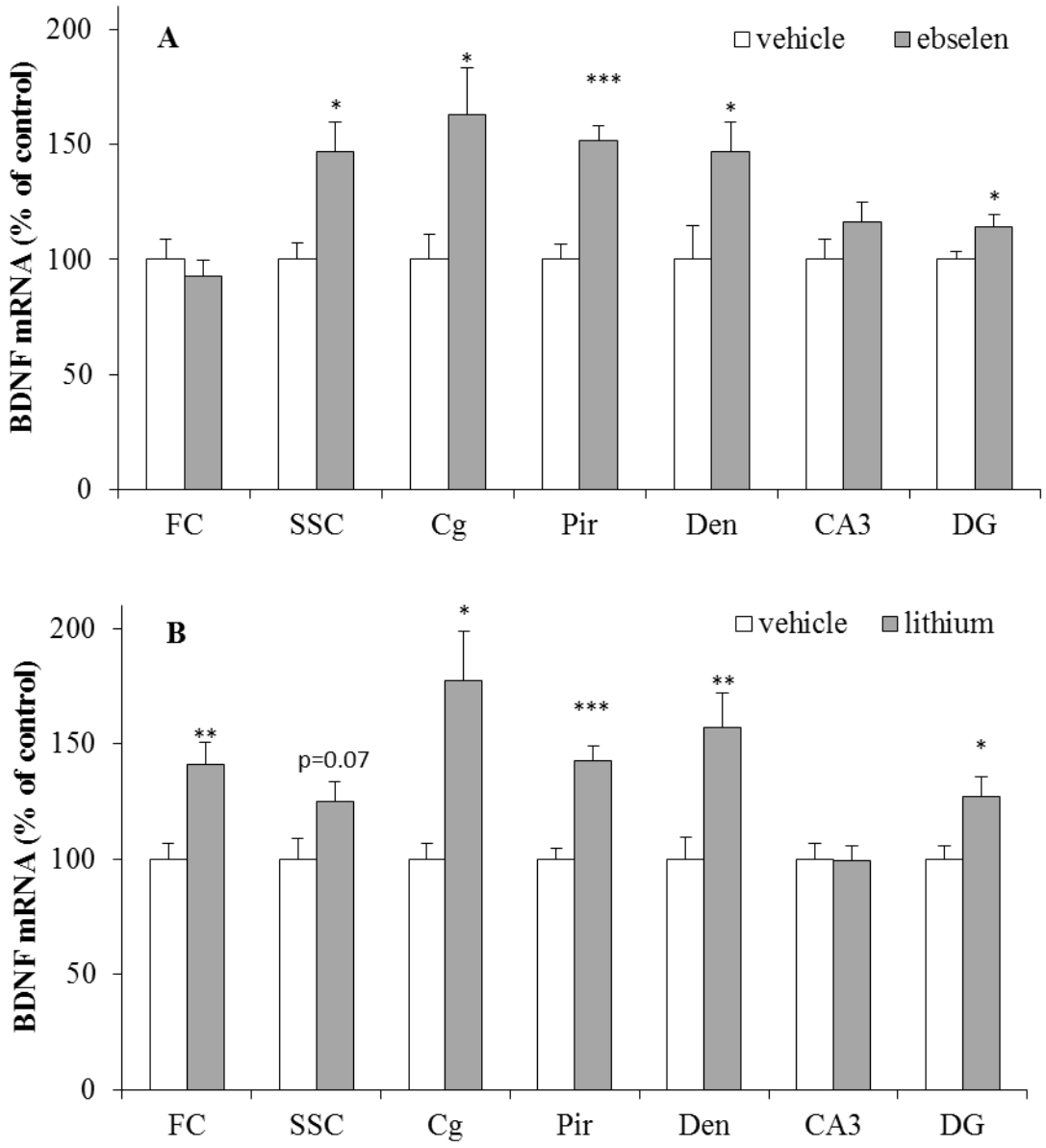

Figure 5: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of BDNF mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). * $p < 0.05$, **$p < 0.005$, ***$p < 0.001$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.3 Effect of ebselen and lithium on VGluT1 mRNA abundance

Representative autoradiograms of VGluT1 mRNA distribution in vehicle and ebselen or lithium treated animals are shown in Figure 6. The abundance of VGluT1 mRNA was found to be highest in the CA3 region of the hippocampus with lowest levels in the SSC.

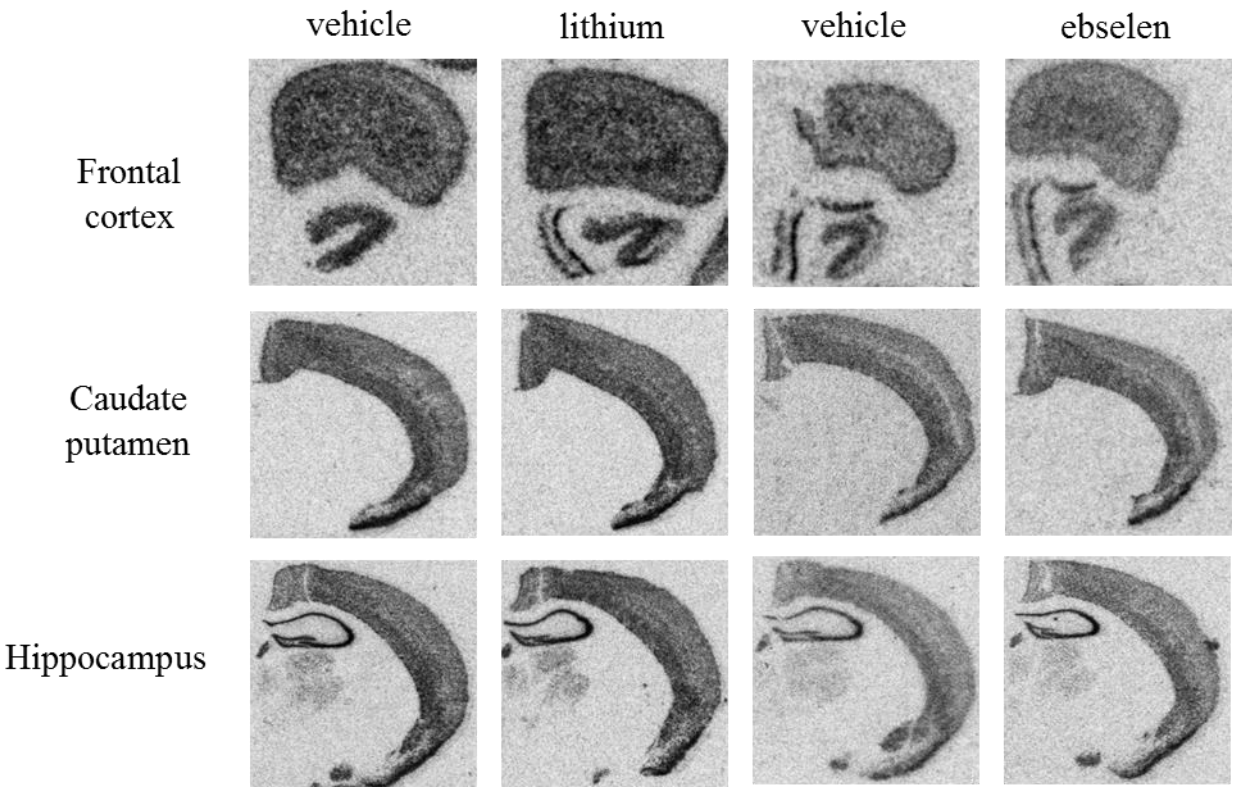

Figure 6: Representative autoradiograms showing distribution of VGluT1 mRNA at the level of the frontal cortex, caudate putamen and hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Repeated treatment of mice with ebselen increased VGluT1 mRNA compared to controls across a range of regions, specifically in the Pir (t (13) = 3.287, p = 0.006), Den (t (12) = 2.555, p = 0.025), CA3 (t (11) = 2.406, p = 0.035) and DG (t (11) = 2.583, p = 0.025) (Figure 7A).

Unlike Arc and BDNF mRNA that were increased across a range of regions following lithium treatment VGluT1 mRNA was only increased in the FC from repeated lithium (t (11) = 2.727, p = 0.020) (Figure 7B).

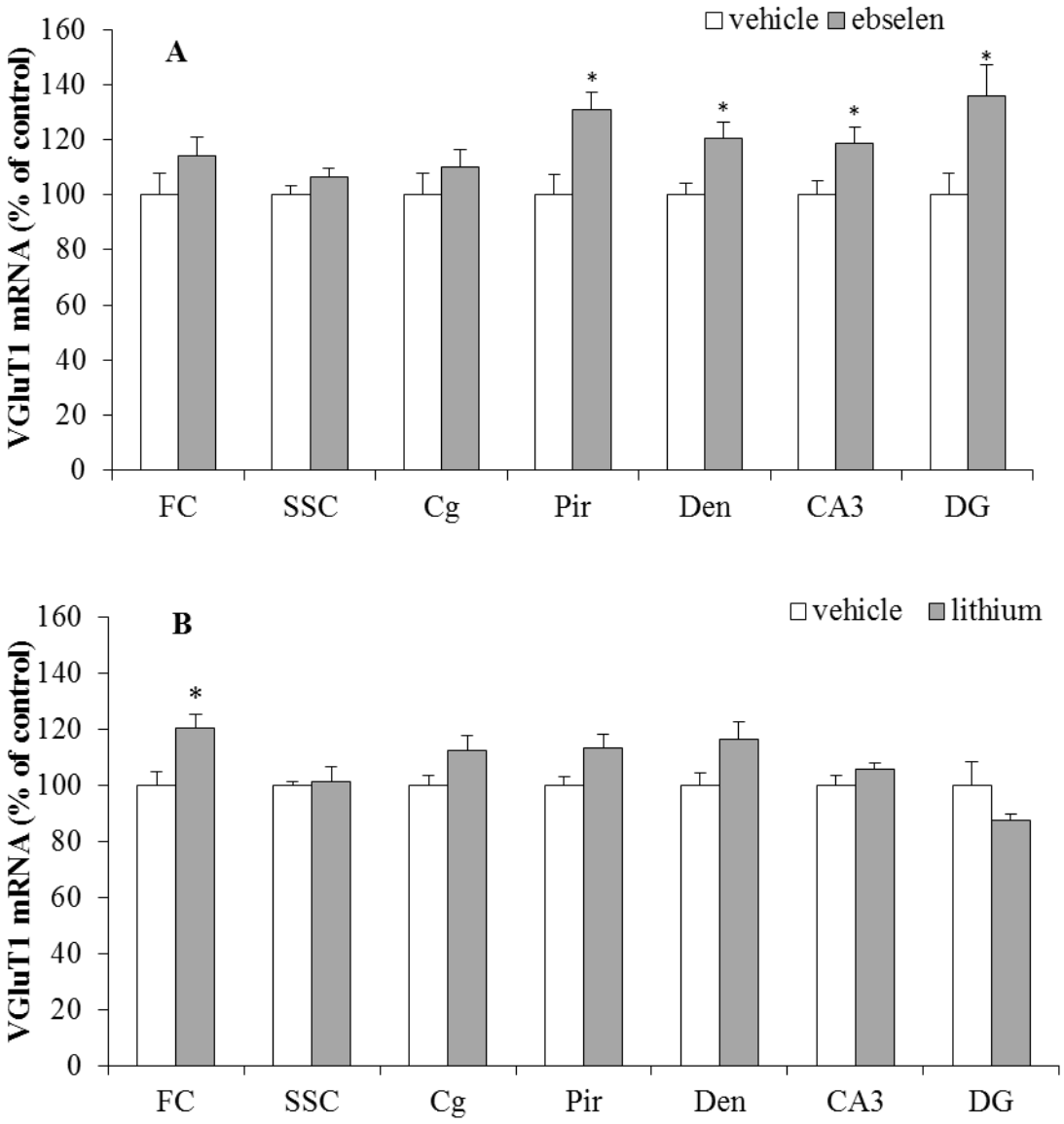

Figure 7: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of VGluT1 mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (5-8 animals/group). *$p < 0.05$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.4 Effect of ebselen and lithium on NCAN mRNA abundance

Representative autoradiograms of NCAN mRNA in vehicle and ebselen or lithium treated animals are shown in Figure 8. NCAN mRNA was found to be highly abundant in the hippocampus and in particular in the CA3 and DG regions. In the FC there was also great abundance of NCAN mRNA, although lower than the hippocampus. The lowest levels of NCAN mRNA were detected in the SSC.

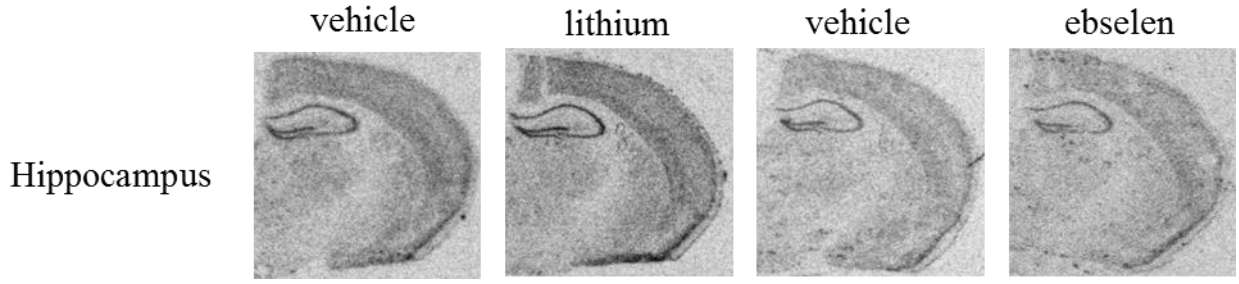

Figure 8: Representative autoradiograms showing distribution of NCAN mRNA in the level of hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Mice treated with ebselen repeatedly for 14 days were found to have increased NCAN mRNA abundance compared to vehicle controls in the hilus (t (9) = 2.181, p = 0.057) but this effect was borderline statistically significant. On the other hand, ebselen decreased NCAN mRNA in the CA3 (t (12) = 2.322, p = 0.039) region of the hippocampus (Figure 9A).

In comparison, lithium increased NCAN mRNA across a range or regions; the hilus of the hippocampus (t (16) = 2.733, p = 0.015), FC (t (18) = 2.265, p = 0.036), SSC (t (19) = 3.486, p = 0.002), Cg (t (19) = 2.186, p = 0.043) and Pir (t (19) = 4.495, p = 0.000) (Figure 9B).

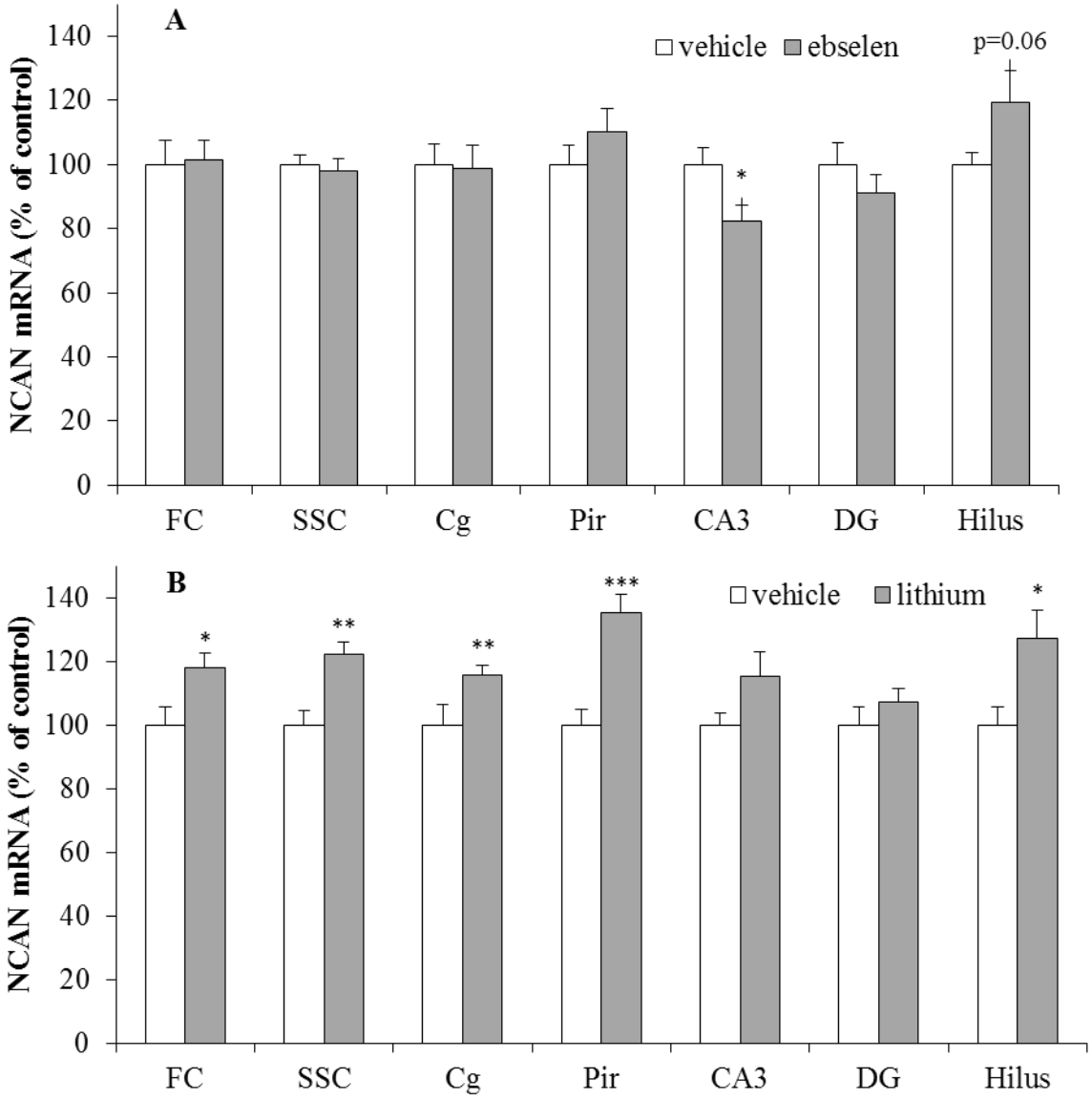

Figure 9: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of NCAN mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *p < 0.05, **p < 0.005, ***p < 0.001 treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.5 Effect of ebselen and lithium on Shank1B mRNA abundance

Representative autoradiograms of Shank1B mRNA distribution in vehicle and ebselen or lithium treatment is shown in Figure 10. Shank1B mRNA showed a similar distribution across all the cortical regions investigated, specifically the SSC, Cg and Pir, while in the CPU Shank1B mRNA was found to have lowest abundance.

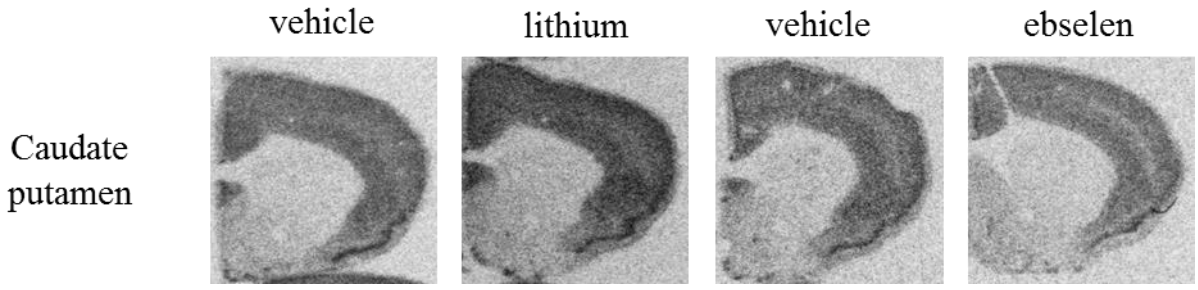

Figure 10: Representative autoradiograms showing distribution of Shank1B mRNA at the level of the caudate putamen of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Treatment of mice with ebselen for 14 days increased Shank1B mRNA abundance in the CPU (t (10) = 2.375, p = 0.039) (Figure 11A).

Treatment with lithium for 14 days also increased Shank1B mRNA compared to controls in all the brain regions investigated (SSC (t (15) = 6.041, p = 0.000); Cg (t (17) = 4.498, p = 0.000); CPU (t (15) = 5.521, p = 0.000); Pir (t (15) = 6.214, p = 0.000) (Figure 11B).

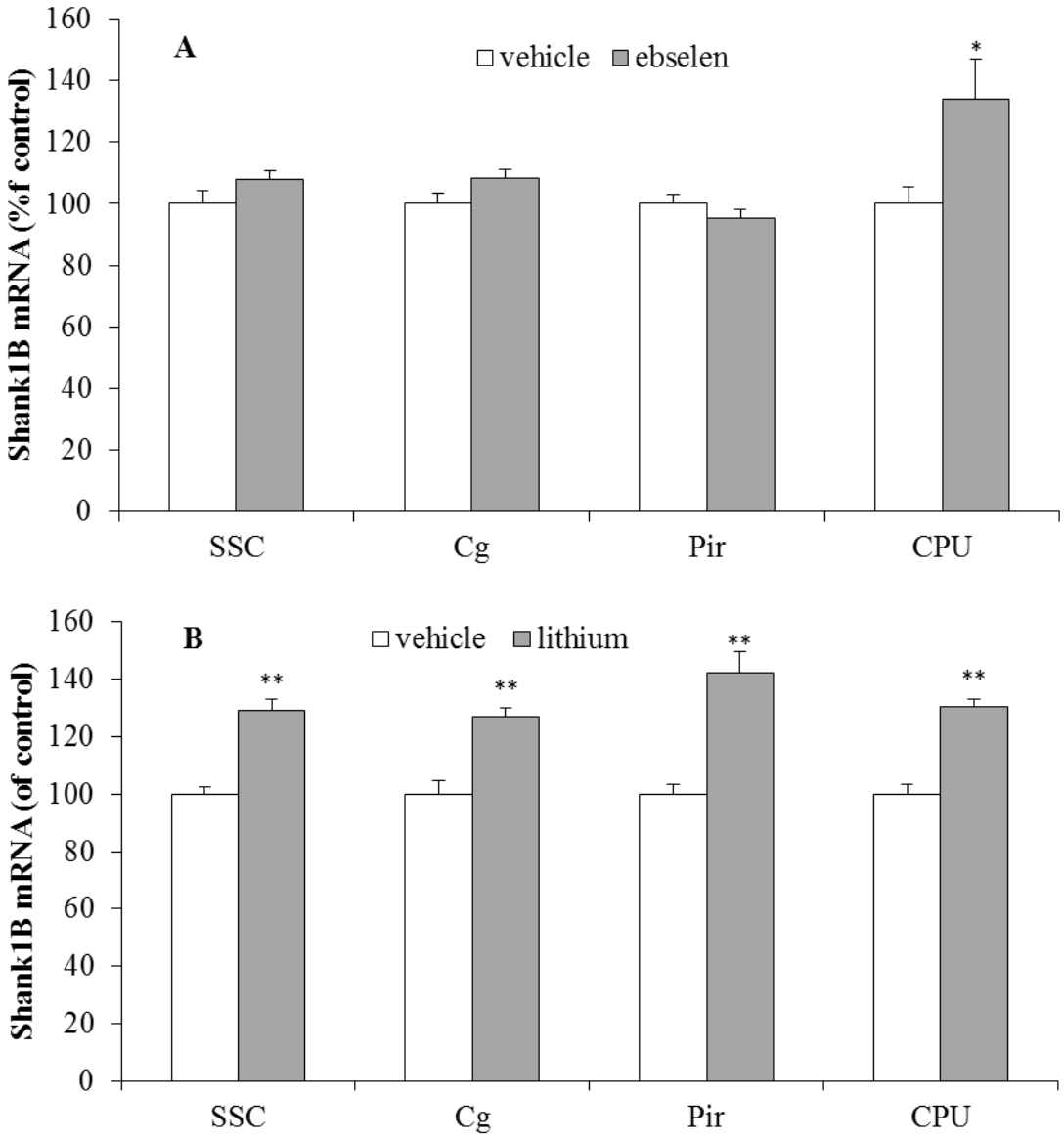

Figure 11: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of Shank1B mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *$p < 0.05$, **$p < 0.001$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.6 Effect of ebselen and lithium on homer1b/c mRNA abundance

Representative autoradiograms of homer1b/c distribution in vehicle and ebselen or lithium treated animals are shown in Figure 12. Homer1b/c mRNA showed a similar pattern of expression across all brain regions investigated.

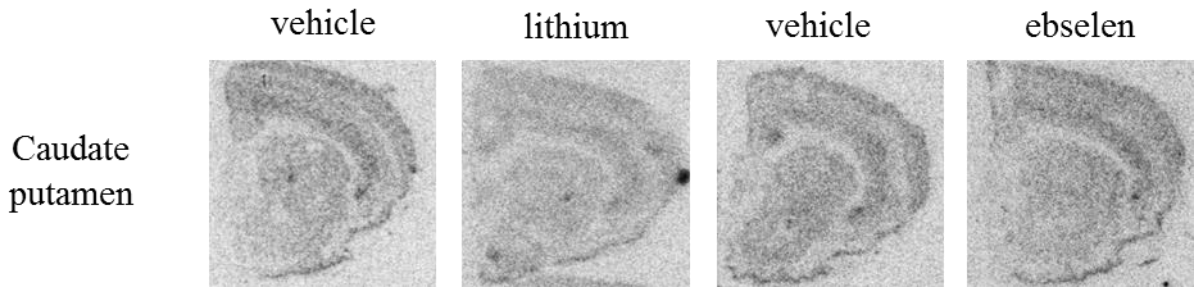

Figure 12: Representative autoradiograms showing distribution of homer1b/c mRNA at the level of caudate putamen of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

In contrast to Arc, BDNF, VGluT1, NCAN and Shank1B mRNA where lithium and ebselen were shown to have similar effects, homer1b/c mRNA was increased by ebselen and decreased by lithium. Repeated ebselen increased homer1b/c mRNA compared to controls in the SSC (t (12) = 3.475, p = 0.005) and Pir (t (12) = 3.567, p = 0.004) (Figure 13).

On the contrary, repeated lithium treatment decreased homer1b/c mRNA abundance compared to vehicle controls in the SSC (t (14) = 2.437, $p = 0.029$) and CPU (t (14) = 3.268, $p = 0.006$) (Figure 13).

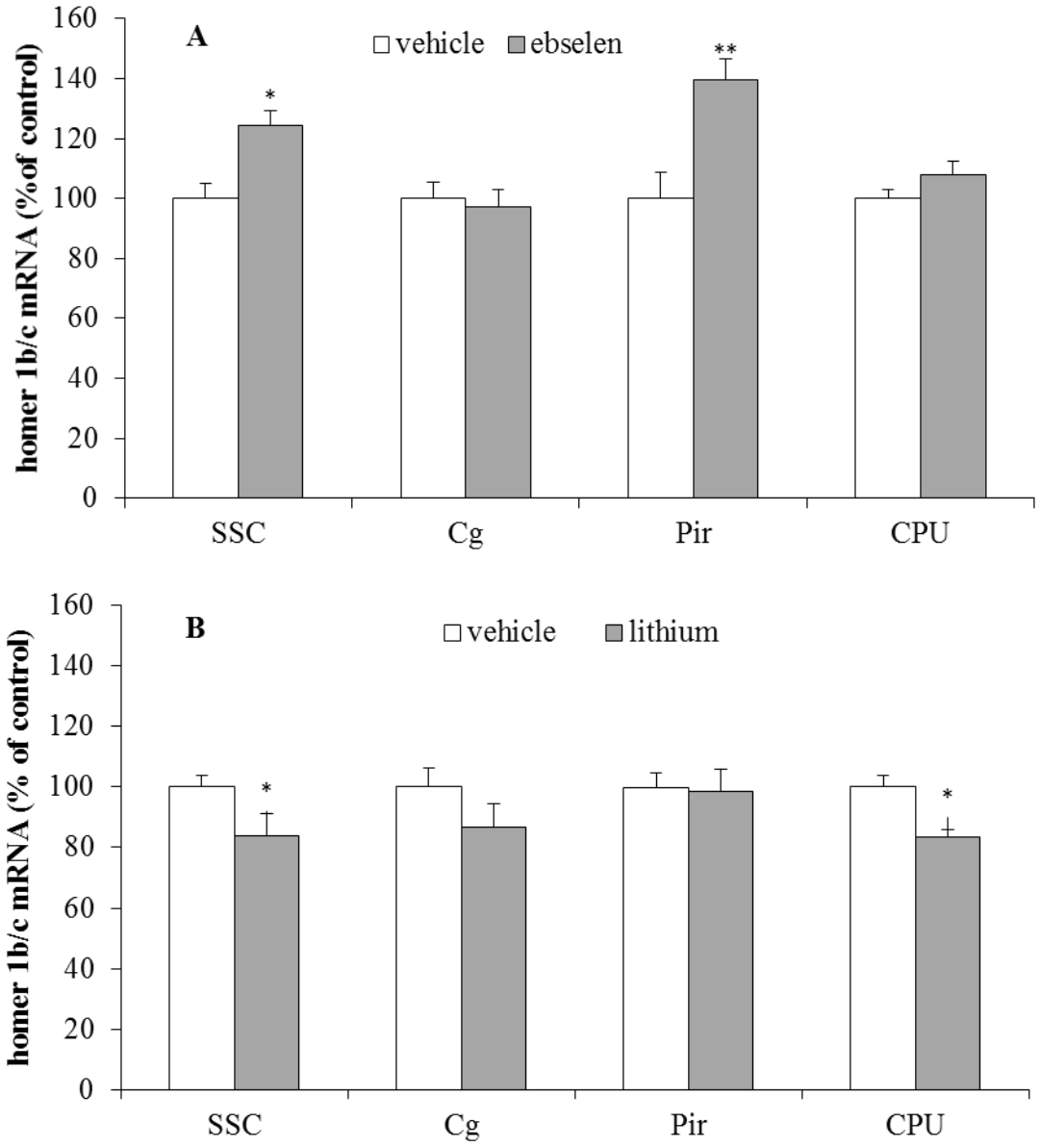

Figure 13: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of homer1b/c mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *p < 0.05, **p < 0.005 treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.7 Effect of ebselen and lithium on IP3R mRNA abundance

Representative autoradiograms showing the distribution of IP3R mRNA in vehicle and ebselen or lithium treated animals are shown in Figure 14. IP3R mRNA abundance was found to be similar across all regions investigated.

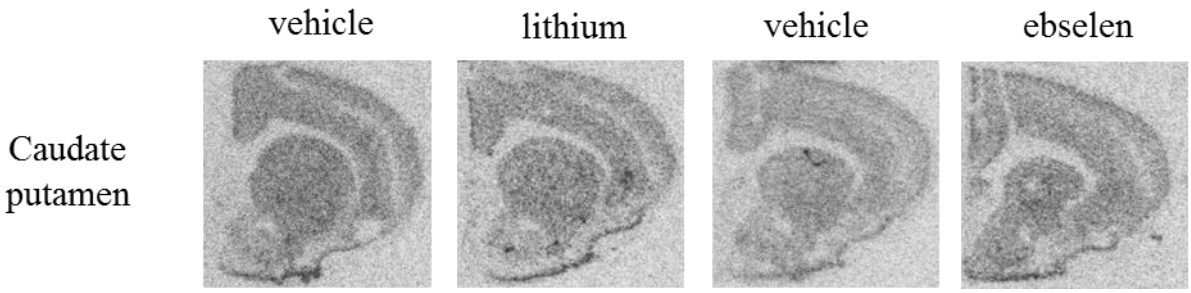

Figure 14: Representative autoradiograms showing distribution of IP3R mRNA at the level of the caudate putamen of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Repeated ebselen increased IP3R mRNA abundance compared to vehicle controls in the Pir (t (14) = 3.546, p = 0.003) (Figure 15A).

As with ebselen, repeated lithium increased the levels of IP3R mRNA compared to controls in the Cg (t (19) = 2.618, p = 0.017) and Pir (t (18) = 2.718, p = 0.014) (Figure 15B).

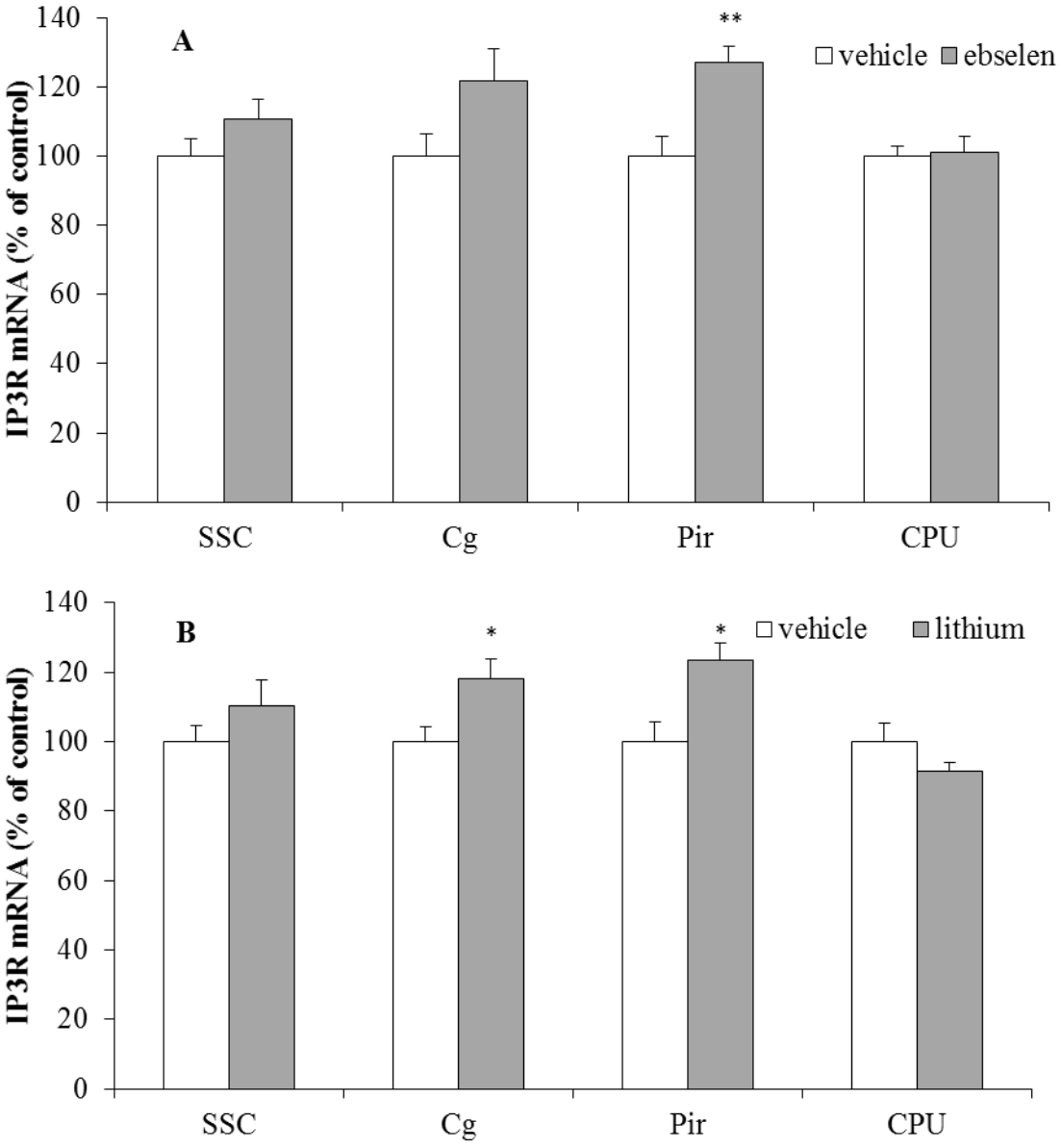

Figure 15: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of IP3R mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *$p < 0.05$, **$p < 0.005$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.8 Effect of ebselen and lithium on IMPase1 mRNA abundance

Representative autoradiograms showing the distribution of IMPase1 mRNA in the mouse brain of vehicle, ebselen and lithium treated animals are shown in Figure 16. The abundance of IMPase1 mRNA was found to be highest in the CA1, CA3 and DG of the hippocampus and the Hb. Relatively, moderate levels of IMPase1 mRNA were found in the Pir, FC and the SSCo. The lowest levels of IMPase1 mRNA were detected in the Cg, Po and CPU.

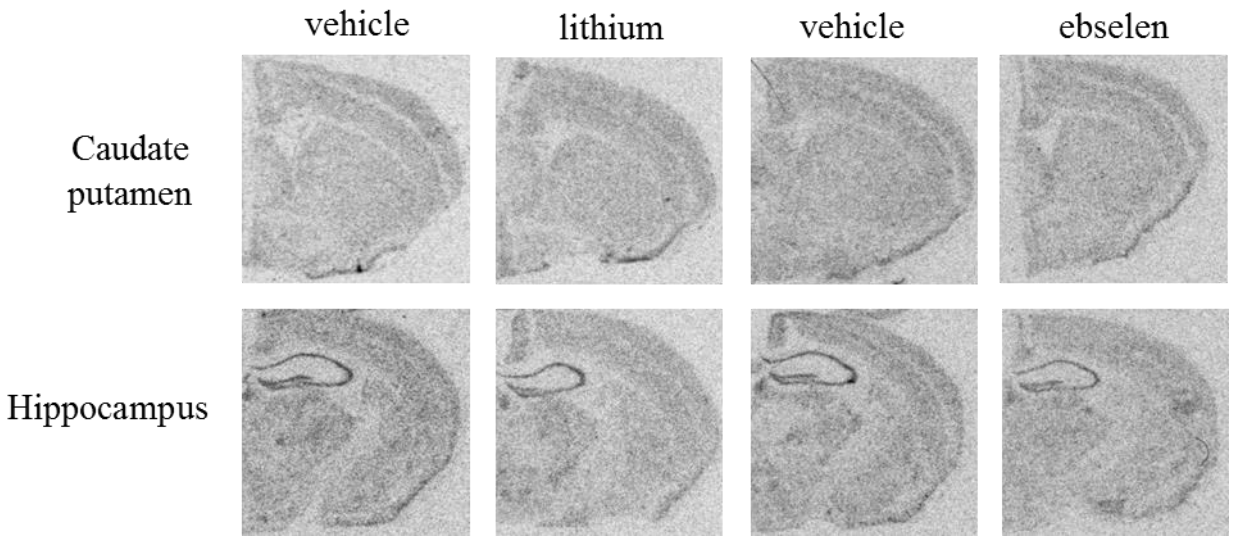

Figure 16: Representative autoradiograms showing distribution of IMPase1 mRNA at the level of the caudate putamen and hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Ebselen treatment for 14 days increased IMPase1 mRNA abundance compared to controls in the Pir (t (12) = 2.435, p = 0.031) and Po (t (12) = 3.027, p = 0.011) (Figure 17A).

In comparison, repeated lithium also increased IMPase1 mRNA in the SSC (t (17) = 2.483, p = 0.024), Cg (t (17) = 3.058, p = 0.007), Pir (t (17) = 5.006, p = 0.000) and CPU (t (17) = 2.810, p = 0.012) (Figure 17B).

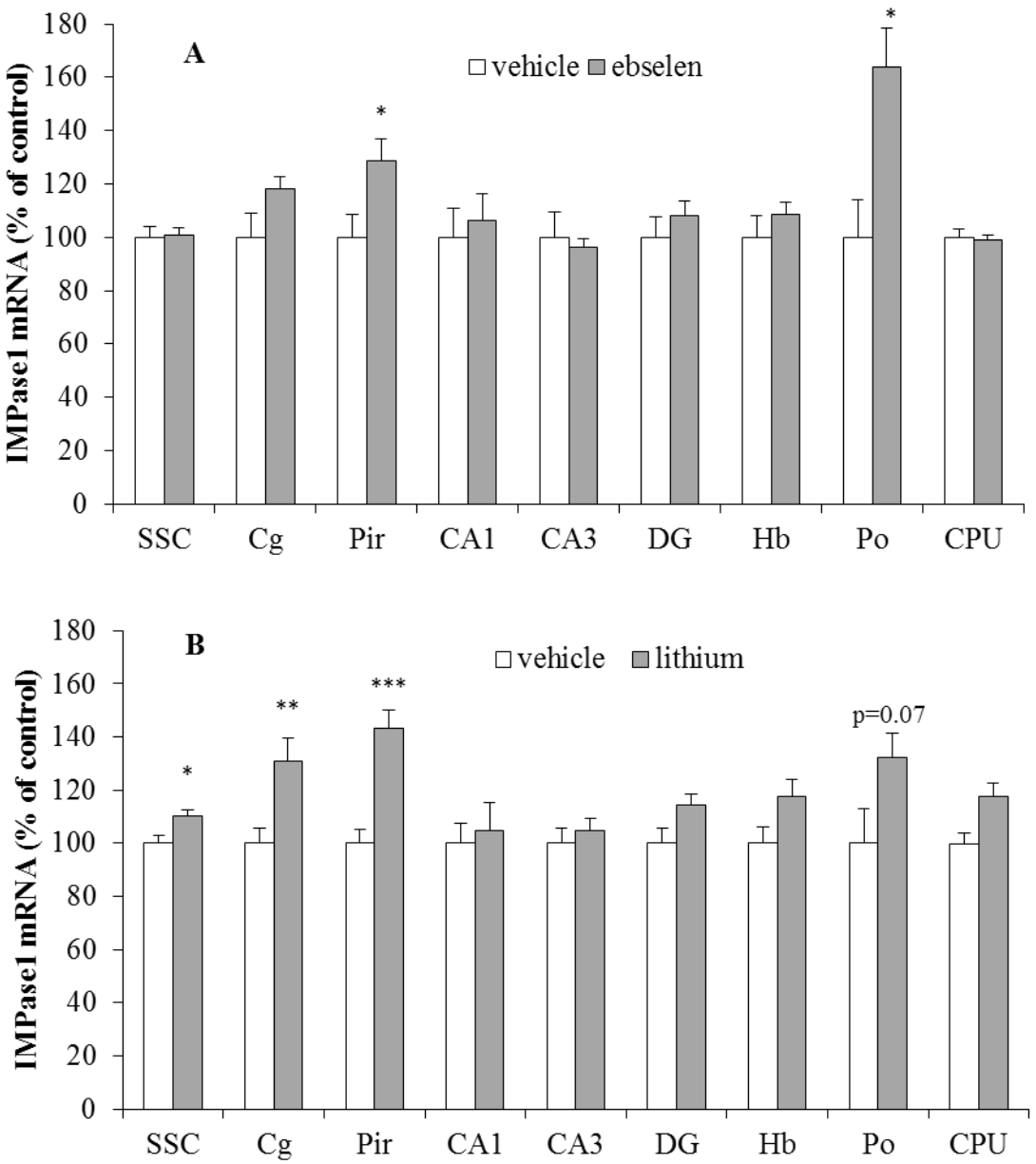

Figure 17: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of IMPase1 mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.3.9 Effect of ebselen and lithium on SMIT mRNA abundance

Representative autoradiograms of SMIT mRNA distribution or vehicle and lithium or ebselen treated animals is shown in Figure 18. Interestingly, SMIT mRNA showed a similar regional distribution compared to IMPase1 mRNA. The highest levels of SMIT mRNA were found in the CA1, CA3 and DG of the hippocampus, Hb and Po. Comparatively moderate levels were detected in the Pir, SSCo, SSCi and Cg. SMIT mRNA levels were lowest in the CPU.

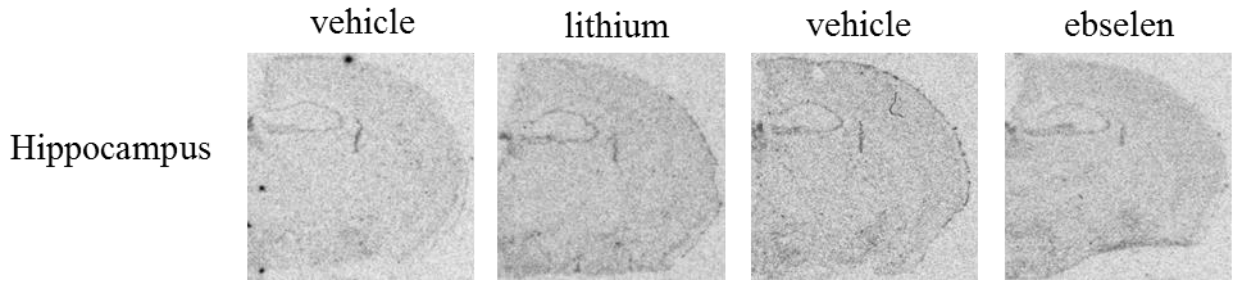

Figure 18: Representative autoradiograms showing distribution of SMIT mRNA at the level of the hippocampus of mice treated with either vehicle or lithium (10 mmol/kg on day 1, 3 mmol/kg twice daily on days 2-14 i.p.) or with vehicle or ebselen (5 mg/kg twice daily i.p.).

Repeated ebselen treatment increased SMIT mRNA abundance in the Cg (t (9) = 2.820, p = 0.020) and SSC (t (10) = 2.158, p = 0.056) (Figure 19).

In comparison, lithium treatment increased SMIT mRNA in the Cg (t (12) = 2.839, p =0.015), DG (t (18) = 4.786, p = 0.000), Hb (t (18) = 2.696, p = 0.015) and Po (t (18) =

2.508, $p = 0.022$). There was also a trend for increase in the CA1 (t (17) = 2.020, $p = 0.059$) (Figure 19B).

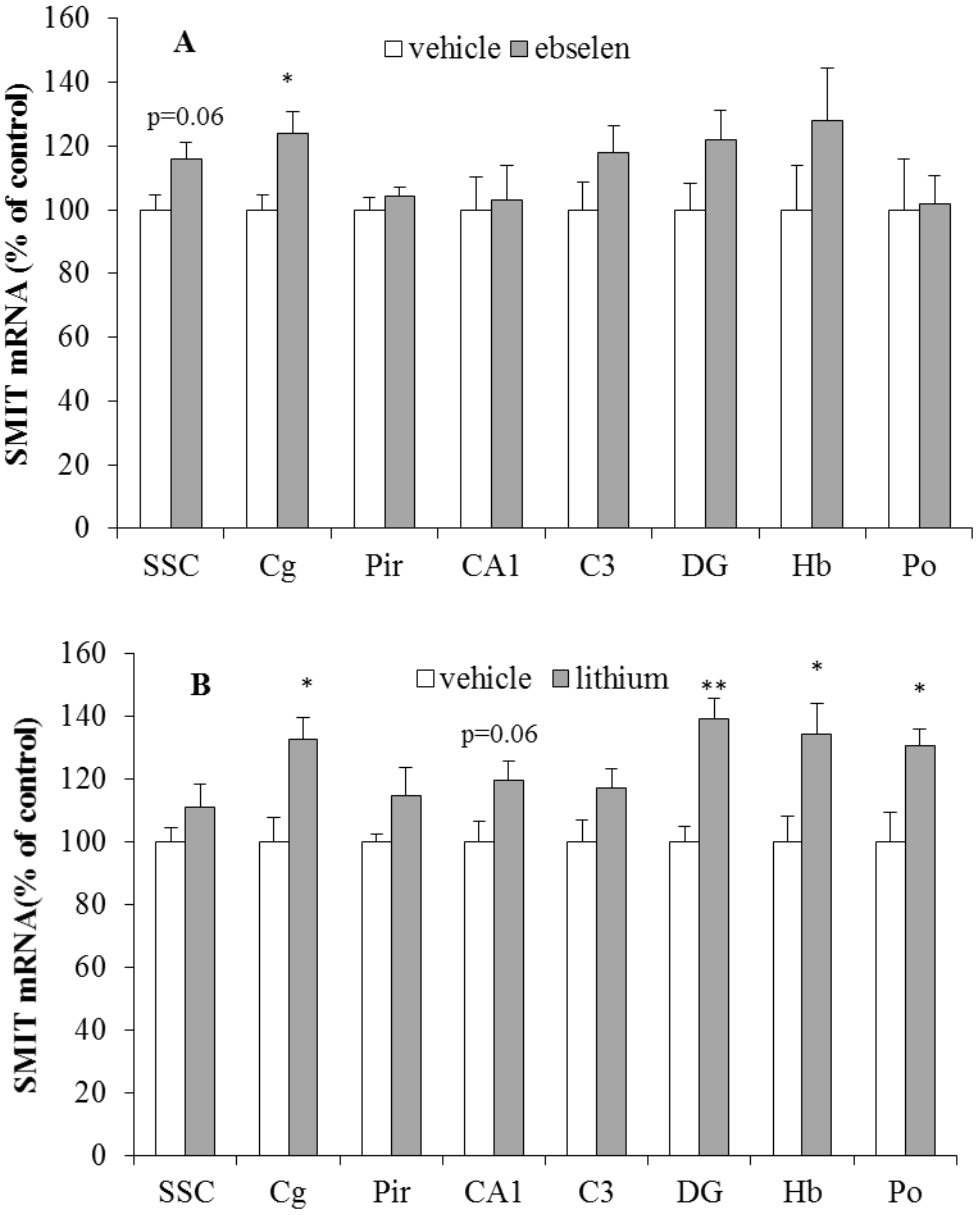

Figure 19: Effect of (A) ebselen (5 mg/kg, twice daily, i.p.) or (B) lithium (10 mmol/kg on day 1, 3 mmol/kg, twice daily on days 2-14, i.p.) on the abundance of SMIT mRNA in the mouse brain as measured by in situ hybridization. Data shown are mean ± SEM value (8-13 animals/group). *$p < 0.05$, **$p < 0.001$ treatment vs vehicle controls (Student's unpaired t-test). Abbreviations as in methods section.

5.4 Discussion

The aim of the experiments described in this chapter was to investigate the effect of ebselen and lithium on markers of neuronal plasticity and explore possible compensatory regulation of genes involved in phosphoinositide signalling. Overall, the present data demonstrated that repeated ebselen triggered the activation of a range of genes involved in neuronal plasticity and that this effect was accompanied by changes in PI signalling genes. Repeated lithium evoked similar changes in many of the same genes. In detail, compared to vehicle injected mice, both ebselen and lithium treatment increased Arc, BDNF, VGluT1, NCAN, Shank1B, IP3R, IMPase1 and SMIT mRNA abundance in specific forebrain regions. Ebselen and lithium had opposite effects on homer1b/c mRNA, where ebselen increased it and lithium decreased it.

Arc, BDNF and VGluT1 mRNA were increased by ebselen in a variety of cortical and hippocampal regions and lithium had a similar effect. The effect of lithium on BDNF mRNA confirms previous studies, showing that repeated lithium treatment increased BDNF in both cortical and hippocampal regions in rodents (Fukumoto *et al.*, 2001; Jacobsen *et al.*, 2004). Lithium has also been reported to increase VGluT1 mRNA in the hippocampus (Moutsimilli *et al.*, 2005) although in this study lithium was found to increase VGluT1 mRNA in the FC. This is the first time that the effect of lithium on Arc mRNA is investigated. The enhancement of these 3 markers of neuronal plasticity (Arc, BDNF, VGLUT1) by ebselen and the similarities observed with lithium, are of particular importance, given the functional relevance of these markers in pathological mood conditions in animal models but also in the effects of psychotropic drugs in mood. In particular, Arc, BDNF and VGluT1 mRNA have

been found to decrease in animal models of stress and depression, while repeated and not acute antidepressant treatment increased their expression (Nibuya *et al.*, 1995; Castro *et al.*, 2003; Pei *et al.*, 2003a; Tordera *et al.*, 2005; Covington *et al.*, 2010; Elizalde *et al.*, 2010; Serres *et al.*, 2012). It is also important to stress that functionally, these markers have fundamental role in psychotropic drug action. For example, BDNF knockout and heterozygous mice, do not respond to antidepressant drug treatment, illustrating that BDNF is a crucial component for antidepressant effects to take place (Saarelainen *et al.*, 2003; Monteggia *et al.*, 2004). Clinically, Arc and BDNF have also been shown to be relevant in mood disorders and their treatment, as Arc is found to decrease in post-mortem tissue of depressed patients (Covington *et al.*, 2010), while recent meta-analysis studies suggest that BDNF is consistently found to be decreased in patients undergoing manic or depressive episodes and is increased following antidepressant treatment of patients (Fernandes *et al.*, 2011; Teche *et al.*, 2013).

Repeated ebselen and lithium treatment increased Shank1B and IP3R mRNA but ebselen increased Homer1b/c, while lithium decreased it. Shank1B, homer and the IP3R have been reported to interact at the post-synaptic density with Shank1B to recruit IP3R and Homer1b/c proteins in new synapses (Sala *et al.*, 2001). The present findings on the effect of lithium on Shank1B and IP3R mRNA are not in agreement with a previous report where lithium was shown to have no effect on Shank1B mRNA and to decrease IP3R. In the same study, it was found that lithium decreased homer1b/c mRNA, in a way similar to the present (de Bartolomeis *et al.*, 2012). However, the above study was performed in rats and lithium was administered in diet, which leads to crucial differences in the circulating levels of the drug, which can subsequently have a major effect on gene expression. Shank1B is a protein

involved in synaptic function, spine density and memory retention, IP3R is a receptor that undergoes highly dynamic modulation of expression to regulate Ca^{+2} levels and homer1b/c is a post-synaptic density protein involved in LTP (Roussignol *et al.*, 2005; Kammermeier, 2006; Hung *et al.*, 2008; Gerstein *et al.*, 2012). Shank1B mRNA was found to be decreased in depressed rats, in the social defeat model of depression (Serres *et al.*, unpublished data) while repeated antidepressant treatment increased the abundance of Shank1B mRNA in the rat (Serres *et al.*, 2012). The effect of psychotropic drugs on homer1b/c and IP3R mRNA has not been explored with the exception of the study about lithium mentioned above, which was the basis for the investigation of the effect of ebselen on these genes here. The increase of the mRNA of IP3R by ebselen and lithium could emerge as a compensatory mechanism to the decreased levels of IP3, arising from the depletion of inositol by ebselen and lithium, through IMPase inhibition, but this needs further investigation. Homer1b/c was regulated in different ways by lithium and ebselen. This finding suggests that ebselen and lithium might be acting through additional pharmacological targets, which as a result can lead to contrasting effects. Homer1b/c is involved in regulating the distribution of mGluRs while it also plays an important role in maintaining LTP (Kammermeier, 2006; Gerstein *et al.*, 2012). Increase of homer1b/c by ebselen suggests, that the drug might be enhancing glutamate signalling, which is also supported by the increase it induced in the VGluT1.

Ebselen showed a trend to increase NCAN mRNA in the hilus of the hippocampus. Lithium increased NCAN abundance in the hilus of the hippocampus but also in the majority of the rest of the cortical regions investigated. NCAN is part of the extracellular matrix that surrounds neurons and plays a role in the differentiation of progenitor cells into neurons. Its expression peaks during precursor migration and differentiation (Kuhn *et al.*, 1996). The

increase of NCAN mRNA by ebselen and lithium in the hilus of the hippocampus which includes the subgranular zone, the brain region that neurogenesis occurs might represent the induction of increased neurogenesis by the two agents. Indeed, NCAN is considered to be a marker of recently generated synapses (Sandi, 2004). For lithium, it has been reported in the past that it increased hippocampal neurogenesis in the mouse (Chen *et al.*, 2000). Lastly, NCAN, has been implicated in the genetic predisposition to bipolar disorder, while NCAN knockout mice show a manic behavioural phenotype rescued by lithium in behavioural models of mania (Miro *et al.*, 2012).

Ebselen and lithium increased IMPase and SMIT mRNA, suggesting that compensatory mechanisms are activated in order to overcome the inositol depletion induced by IMPase inhibition. IMPase is involved in the recycling of inositol in the intracellular environment and SMIT regulates the transport of inositol from the extracellular environment inside the cell (see General Introduction). Previous data suggest that changes in inositol are associated with altered expression of genes that regulate intracellular inositol levels. For example, in the yeast extracellular inositol depletion leads to an increase in the mRNA of genes that regulate its *de novo* synthesis (Vaden *et al.*, 2001). Our findings on the effect of lithium on IMPase are in agreement with reports that repeated administration of lithium to rodents has been shown to increase the activity, the mRNA and protein levels of IMPase (Parthasarathy *et al.*, 2003; Shamir *et al.*, 2003). Increased IMPase mRNA following lithium treatment has been also detected in neutrophils of patients with bipolar disorder (Nemanov *et al.*, 1999). The effect of lithium on SMIT mRNA has not been explored in animal models. The present findings suggest that ebselen and lithium had the greatest effect on IMPase mRNA in regions with the lowest abundance. It is possible that in regions with higher IMPase

expression such as hippocampus IMPase mRNA levels are sufficiently high to ameliorate IMPase inhibition by ebselen or lithium. Regions in which ebselen or lithium increased IMPase mRNA were different from those that demonstrated increased SMIT mRNA. This observation might suggest that in specific brain regions, different mechanisms of inositol regulation are more dominant than others. In fact, in rat primary neuronal cultures SMIT was found to intake inositol at higher rates in discrete regions such as the cortex and the hippocampus compared to the cerebellum (Lubrich *et al.*, 2000).

Although both ebselen and lithium altered expression of a range of genes, regional differences were detected. Several factors could account for these differences. Firstly, the two agents do not have the same pharmacodynamic and pharmacokinetic properties and that at the moment of tissue collection the blood levels of lithium and ebselen could significantly differ. In fact, the half-life of elimination of ebselen in rats is 2.1 h, while for lithium, it is 6.07 h (Wood *et al.*, 1986; Masumoto *et al.*, 1997). In addition, ebselen has been shown to elicit a strong inhibition of IMPase1 in the brain 4.5 h post administration, while the peak of inositol reduction by lithium is 12 h following administration (Allison *et al.*, 1971; Singh *et al.*, 2013). Therefore, at the moment of tissue collection in the present study the magnitude of IMPase inhibition by ebselen and lithium could greatly differ. Since IMPase1 might be the target through which lithium and ebselen exert their effects on gene expression, this difference could account for the regional differences observed. Moreover, the genes investigated show highly dynamic expression patterns. Previous studies report that repeated administration of certain antidepressants increased Shank1B mRNA when the last injection was administered 16 h before tissue collection and not when administered 2 h before, while Arc showed the opposite (Serres *et al.*, 2012). Therefore, the present study might have only

captured a snapshot of the pattern of genes activated by ebselen and lithium. A final point, besides IMPase both lithium and ebselen have additional pharmacological targets (see below) which could contribute to the regional differences.

Although interactions between IMPase inhibition and neuronal plasticity are little investigated, evidence from studies of lithium using simple biological model systems suggests an important link. Thus, inositol depletion would appear to have fundamental role in mediating neuronal plasticity effects of lithium. Specifically, lithium has been shown to increase axonal sprouting and synapse formation in neuronal cultures and these effects were reversed by inositol supplementation (Williams *et al.*, 2002; Kim *et al.*, 2009). It is entirely plausible that these effects of lithium are liked to IMPase inhibition, and even altered expression of some of the genes measured herein. It is uncertain whether IMPase inhibition is the mechanism underlying the effects of ebselen. However, the similarities between ebselen and lithium in altering the expression of a range of genes suggests IMPase as a possible mechanism. Recent reports have proposed that IMPase is crucial for the neurotrophic effects mediated by NGF. In fact, IMPase was shown to be abundantly expressed in neuronal dendrites but also to be a fundamental factor for preventing the fragmentation of neuronal axons. NGF-mediated neurotrophic effects were shown to be dependent on IMPase function (Andreassi *et al.*, 2010). Therefore, the neurotrophic factor BDNF increase elicited by ebselen and lithium in the present study, could involve the inhibition of IMPase, but this assumption needs further investigation, and comparison with selective IMPase inhibitors. Nevertheless, the exact pathway through which IMPase inhibition and subsequently inositol depletion would lead to modulation of gene expression resulting in enhanced neuronal plasticity and neurotrophic support is not known. It has been

speculated though that PKC, which is downstream the PI pathway and is a protein known to influence the transcription of other proteins could be a link (Lenox *et al.*, 2003).

GSK-3 inhibition could also account for some of the effects of ebselen and lithium on neuronal plasticity. Ebselen has been shown to interact with GSK-3 (IC_{50}=30 μM) (Singh *et al.*, 2013). However, it has 20 times lower potency for GSK-3 compared to IMPase (IC_{50}=1.5 μM) which makes GSK-3 a less likely mediator of the effects of ebselen. On the other hand, GSK-3 inhibition has been found to increase neurogenesis (Ahn *et al.*, 2014) and activate BDNF promoter IV (Yasuda *et al.*, 2009). However, GSK-3 inhibition does not reproduce the effects of lithium on synaptic strengthening. In particular, although inositol supplementation has been shown to reverse the increase of axonal sprouting and synapse formation induced by lithium, GSK-3 inhibition does not mimic these effects of lithium (Williams *et al.*, 2002; Tanizawa *et al.*, 2006; Kim *et al.*, 2009). Lastly, in contrast to inositol, there are not any studies showing the involvement of GSK-3 inhibition in the enhancement of gene expression induced by lithium, making it a less relevant target.

In summary, the present study employed the expression of markers of neuronal plasticity in selected anatomical structures, as a molecular model to explore possible facilitation of neuroadaptive changes by ebselen and compare with lithium. It also evaluated whether repeated ebselen and lithium evoked a compensatory increase on the expression of genes that regulate PI cycle. Overall, it was shown that ebselen increased all markers of neuronal plasticity investigated and in comparison to ebselen, lithium had a similar effect. The most robust increase induced by ebselen and lithium was on BDNF and Arc while ebselen also had a large effect on VGluT1 mRNA. Lastly, both lithium and ebselen increased

IMPase and SMIT mRNA, suggesting that both agents regulate genes involved in the PI cycle. These findings demonstrate that ebselen elicited neuroplastic effects and suggest that ebselen and lithium share clear neuropharmacological similarities.

Chapter 6

General Discussion

6 General Discussion

The main aim of this thesis was to investigate the neuropharmacological effects of the putative lithium-mimetic ebselen, at the molecular, behavioural and neurochemical level in the mouse. Ebselen is a repurposed drug recently found to inhibit IMPase. IMPase is a key molecular target of lithium and suggested to underlie the mood stabilising properties of the drug. IMPase is involved in the recycling of inositol which is the precursor of PIP2, and the later the source of the second messengers IP3 and DAG. Therefore inhibition of IMPase is hypothesized to attenuate PI-linked receptor signalling. Here, the effect of ebselen was tested in models of 5-HT_{2A} and 5-HT_{2C} receptor function both of which are PI linked 5-HT receptors and also implicated in the actions of lithium. It was found that ebselen attenuated the function of 5-HT_{2A} and 5-HT_{2C} receptors as assessed in behavioural and molecular models. In comparison, lithium also attenuated 5-HT_{2A} and 5-HT_{2C} receptor function. Since antagonists at 5-HT_{2A} and 5-HT_{2C} receptors are reported to have SSRI augmentation effects, and lithium is clinically used as an augmentation agent in treatment resistant depression, the SSRI potentiating augmentation effect of ebselen was investigated in molecular and neurochemical models. Ebselen was found to increase 5-HT synthesis and also augment the effect of the SSRI citalopram. The effect of ebselen on markers of neuronal plasticity, which are reported to be upregulated by lithium and other antidepressant agents, was also investigated. Ebselen was found to increase abundance of various markers of neuronal plasticity in forebrain regions and lithium had similar effects but not always the same. Overall, the experiments of this thesis demonstrate that ebselen attenuates 5-HT_2 receptor function, increases 5-HT synthesis and elevates markers of neuronal plasticity. These effects of ebselen bear many

similarities to those observed with lithium and strongly support the contention that ebselen has a lithium-like neuropharmacological action.

6.1 Ebselen attenuated 5-HT$_{2A}$ receptor function in behavioural and molecular models

As demonstrated in Chapter 2, ebselen dose-dependently attenuated 5-HT$_{2A}$ function at the behavioural level, as evident through decreased HTR and ESR evoked by the 5-HT$_{2A}$ agonist DOI. This finding was further confirmed using a second 5-HT$_{2A}$ agonist, psilocin. This effect of ebselen on 5-HT$_{2A}$ function was maintained with repeated administration. Lithium also attenuated DOI-evoked HTR and ESR after repeated administration but not when given acutely. The IMPase inhibitor, L-690,330 also attenuated the HTR and ESR to DOI, but the GSK-3 inhibitor AR-A014418 did not. The behavioural findings were complemented with molecular studies. Thus, ebselen (acute and repeated) was found to decrease DOI-evoked IEG expression and lithium (repeated) had a similar effect.

Although ebselen and lithium clearly attenuated 5-HT$_{2A}$ function, the decrease in agonist-evoked IEG expression by ebselen and lithium did not always occur in the same brain regions. This result questions whether the two agents are acting by the same mechanism. One cannot exclude the possibility that additional pharmacological effects of ebselen and lithium might contribute to these differences. Ebselen is an anti-oxidant, as it mimics glutathione perodixase. However, at the dose administered here (10 mg/kg) ebselen has been shown to be ineffective in exerting an anti-oxidant effect *in vivo* in rats (Dawson *et*

al., 1995). Theoretically, lithium would also decrease the IEG response to DOI through inhibition of GSK-3. This however seems unlikely given that at the dose administered in the present study (10 mmol/kg on 1 day, 3 mmol/kg twice daily on days 2-7), lithium has been shown to reach levels in the rat brain which are unlikely to significantly inhibit GSK-3; lithium reaches a tissue concentration of approximately 0.8 mM and the IC_{50} of GSK-3 inhibition is 2 mM (Hillert *et al.*, 2012). Additionally, attenuation of 5-HT_{2A} agonist-evoked HTR and ESR was not attenuated by the GSK-3 inhibitor AR-A017748 as discussed above. Hence, at least in the case of those behavioural responses, GSK-3 is not expected to mediate the effect of ebselen or lithium. Overall, the most plausible account of the inhibition of 5-HT_{2A} receptor function by ebselen and lithium is inhibition of IMPase. This is in keeping with the fact that the 5-HT_{2A} receptor signals via the PI pathways and that IMPase is critical for this signalling.

6.2 Ebselen attenuated 5-HT_{2C} receptor function at the molecular level

The evidence that ebselen attenuated 5-HT_{2A} receptor function was followed up by experiments described in Chapter 3 which investigated the effect of ebselen on the function of the 5-HT_{2C} receptor, which also signals via the PI cycle. Ebselen attenuated the increase in IEG expression to 5-HT_{2C} receptor agonist (Ro 60-0175) and this effect was also observed with lithium. However, in a behavioural model of 5-HT_{2C} function, Ro 60-0175 induced hypolocomotion, neither ebselen nor lithium were effective. These findings were complicated by decrease in locomotion produced by ebselen alone but this was not the case for lithium. The difference in the effect of ebselen and lithium in the molecular versus the behavioural model of 5-HT_{2C} function raises the question of whether

the models have the same underpinning mechanism. In this regard, emerging evidence suggests that G-protein coupled receptors signal through more than one signalling cascade, depending on regulatory proteins. For example, both 5-HT_4 and 5-HT_7 receptors are members of the G_s family of proteins which are coupled to cAMP signalling. However, both receptors have been reported to activate PLC and PLA2 upon stimulation (for review (Woehler *et al.*, 2009). Similarly, evidence suggests that the signalling of the 5-HT_{2C} receptors may be regulated by effector proteins that lead to activation of non PI-linked signalling pathways. For instance, evidence suggests that 5-HT_{2C} receptor activation leads to stimulation of both PLC and PLA2 pathways (Berg *et al.*, 1998). Therefore, the hypolocomotion effects of 5-HT_{2C} agonists might not necessarily involve PI cycle stimulation, which could explain why ebselen or lithium were not effective in this model.

6.3 Ebselen increased 5-HT synthesis and augmented the effect of citalopram

Experiments described in Chapter 4 demonstrate that ebselen increases 5-HT synthesis in forebrain regions of the mouse. In microdialysis experiments although ebselen did not increase basal extracellular levels of 5-HT, the drug tended to augment the increase in 5-HT evoked by the SSRI citalopram. However, an overall increase in 5-HT function elicited by ebselen in combination with citalopram was evident in experiments showing an increase in Arc mRNA in a variety of brain regions when ebselen was combined with citalopram (but not when the drugs were administered alone). Previous studies have found that the combination of SSRI with 5-HT_{1A} antagonists elicits an increase in Arc

expression which is mediated by increased extracellular 5-HT (Pei *et al.*, 2003b). This idea of increased 5-HT concords with the increase in 5-HT synthesis induced by ebselen but also by the attenuation of 5-HT_2 receptor function. Thus, previous experiments show that 5-HT_2 antagonists augment the effect of SSRIs on extracellular 5-HT (Boothman *et al.*, 2006b) and also elevate Arc expression (Serres *et. al.*, unpublished data).

6.4 Ebselen increased markers of neuronal plasticity in discrete forebrain regions

Experiments described in Chapter 5 show that repeated ebselen increased markers of neuronal plasticity, as evident through an increase in BDNF, Arc, VGluT1, Shank1B, Homer1b/c, IP3R, NCAN mRNA. Lithium treatment also increased some of the same markers, although not always in the same regions. BDNF, Arc, VGluT1 and Shank1B are also increased by antidepressants including SSRIs (Serres *et al.*, 2012) (Castro *et al.*, 2003) (Pei *et al.*, 2003a; Tordera *et al.*, 2005). Hence, at least at the molecular level, ebselen has antidepressant properties which is in accord with the increase in 5-HT synthesis as discussed above. The increase in IMPase and SMIT mRNA is supposed to result as a compensatory regulation due to the decrease in inositol, following IMPase inhibition by ebselen.

6.5 Limitations of the current thesis

A limitation of the present project is that proof of a role of IMPase inhibition in the various neuropharmacological effects of ebselen is somewhat limited in that it often relies on lithium having analogous effects. This limitation is in part due to the lack of IMPase inhibitors with good *in vivo* potency as well as viable IMPase knockout mice. Although the selective IMPase inhibitor L-690,330 was helpful in experiments described in Chapter 2, it suffers low bioavailability and consequently has to be administered in a very high dose (240 mg/kg) to achieve inhibition of IMPase in the mouse brain (Atack *et al.*, 1993). Attempts were made to overcome this limitation, by administering L-690,330 directly in the mouse brain by icv injection at a dose (10 mM) required to inhibit IMPase and increase IP1 levels (Atack *et al.*, 1993) but this dose proved toxic. The toxic effect of L-690,330 following icv injection has recently been reported by other groups (Shtein *et al.*, 2013). IMPase knockout mice were not employed in this project whilst such a model seems attractive, as these mice die in uterus unless inositol is supplemented during pregnancy and even then show weak reductions in inositol levels in a variety of brain regions (Agam *et al.*, 2009). The future development of conditional IMPase knockout mice would help circumvent these problems.

On the other hand, IMPase inhibition seems a likely mediator of the effects of ebselen observed here. In particular as noted above, lithium had comparable effects to ebselen in many of the studies. Lithium was not included as a comparator in the study of 5-HT synthesis and SSRI augmentation but the action of lithium in these models had been established in previous studies. At the doses administered in mice here (10 mg/kg),

ebselen has been shown to inhibit central IMPase *ex vivo* (Singh *et al.*, 2013). Lithium was administered at doses (10 mmol/kg on day 1, 3mmol/kg, twice daily on days 2-7) that are reported to reach brain concentrations of approximately 0.8 mM which is the IC_{50} of IMPase inhibition (Hillert *et al.*, 2012). Therefore, both ebselen and lithium are likely to inhibit IMPase in the doses used while the similarities between ebselen and lithium in most experiments suggest IMPase as the putative mechanism. Notably this conclusion is also supported by the similarities between ebselen and L-690,330 in the behavioural model of 5-HT_{2A} receptor function. Furthermore, in accord with the hypothesis that inhibition of IMPase would result in dampening of PI signalling, ebselen attenuated the function of two Gq receptors coupled to PI signalling (5-HT_{2A} and 5-HT_{2C}) and this was mimicked by lithium. Finally, it is also worth noting that repeated ebselen increased both IMPase and SMIT mRNA which is evidence of an interaction with the PI cycle and could reflect the activation of a compensatory mechanisms triggered by inositol depletion, but further studies need to confirm that.

Future studies to overcome the lack of pharmacological and genetic tools to investigate the effect of inhibition of IMPase could include the use of techniques that allow the silencing of expression of certain genes with the use of short interfering RNAs (RNAi) delivered through lentiviruses (for reviews see (Dykxhoorn *et al.*, 2003; Rubinson *et al.*, 2003; Tiscornia *et al.*, 2003; Van den Haute *et al.*, 2003). Lentiviruses carrying RNAi which targets IMPase could be directly injected in the mouse brain and used to silence IMPase expression in selected brain regions. Based on the experiments of this thesis, it would be interesting to investigate the effect of IMPase silencing in the frontal cortex on 5-HT_{2A} receptor function. Thus 5-HT_{2A} receptor mediated HTR have been shown to be

mediated through 5-HT_{2A} receptors located in the frontal cortex (Willins *et al.*, 1997) (Gonzalez-Maeso *et al.*, 2007).

6.6 Future studies with ebselen

Ebselen has been tested in approximately 500 patients to date and evidence suggests that it has good tolerability and safety. Clearly, it would be very interesting to test ebselen in psychopharmacological studies in humans. Evidence from the present project suggests that ebselen would attenuate the function of central 5-HT_2 receptors in humans. In humans, inhibition of 5-HT_2 function has been linked to increased slow wave sleep as it has been shown for lithium (Friston *et al.*, 1989) (Sharpley *et al.*, 1994). This is a non-invasive model that could be easily employed for the investigation of the effect of ebselen in human volunteers. Evidence of decreased 5-HT_2 function in humans by ebselen would provide an important finding in further pursuing the testing of ebselen as a novel psychotropic agent, given the broad use of 5-HT_2 antagonists in several psychiatric disorders including bipolar disorder, anxiety, psychosis and depression (Jensen *et al.*, 2010) (Celada *et al.*, 2004).

6.7 Conclusions

Collectively, the experiments described in this thesis demonstrate that ebselen has many neuropharmacological effects in common with lithium. Like lithium, ebselen attenuated the function 5-HT_{2A} and 5-HT_{2C} receptors, a pharmacological effect that has been linked to the therapeutic effect of not only lithium but other important classes of psychotropic

drugs. Ebselen also augmented the effect of an SSRI and enhanced neuronal plasticity as observed previously for other antidepressant agents. IMPase inhibition is suggested to underlie the effects of ebselen in these experiments. Given that ebselen is a repurposed drug with known clinical safety and tolerability, evidence from this thesis supports the investigation of the drug at the clinical level.

7 Appendix: Effect of ebselen in the elevated plus maze

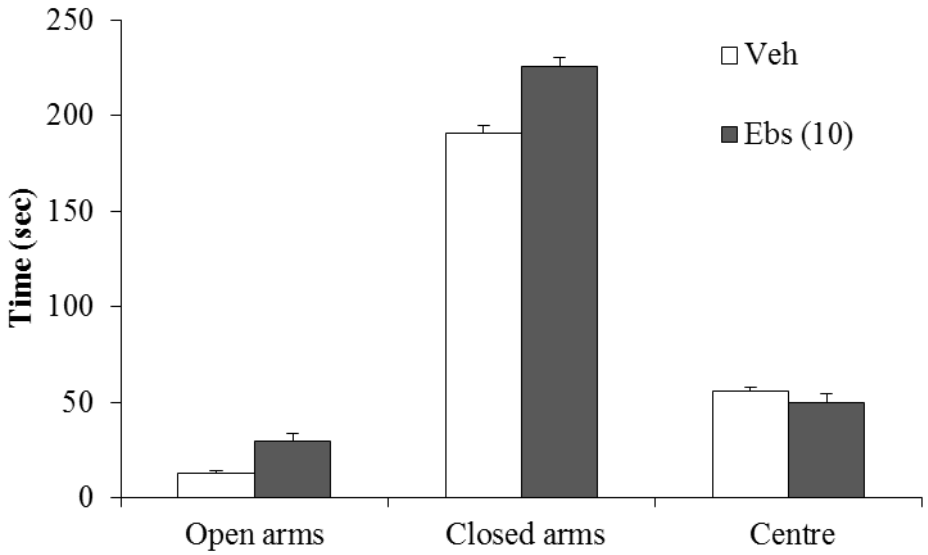

Figure 1: Effect of acute ebselen (10 mg/kg) in the elevated plus maze. Three parameters were tested; time spent in open arms, closed arms and centre of the maze. Data shown are mean ± SEM (*n*=8 animals/group). Student's unpaired t-test.

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