FUNCTION AND REGULATION OF CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2 IN THE ATTENUATION OF PAIN IN MICE

CHEW WEE SIONG

(B. Sc. (Hons), NUS)

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Chew Wee Siong

22 January 2015

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Summary

Calcium-independent phospholipase A_2 ($iPLA_2$) is an 85 kDa enzyme that releases docosahexaenoic acid (DHA) from glycerophospholipids. Prefrontal cortical $iPLA_2$ has been shown to be important in hippocampo-prefrontal cortical LTP and for the antidepressive effect of the antidepressant, maprotiline. In the first part of the study, we investigated the role of prefrontal cortical $iPLA_2$ in the antinociceptive effect of maprotiline and another TCA, amitriptyline. Antidepressant treatment reduced pain behavioral responses indicating antinociceptive effect of maprotiline and amitriptyline treatment in a model of inflammatory orofacial pain. Injection of antisense oligonucleotide to $iPLA_2$ in the dorsolateral prefrontal cortex abolished the antinociceptive effect of maprotiline but not amitriptyline. In contrast, $iPLA_2$ antisense injection in the somatosensory cortex had no effect on maprotiline-induced antinociception. Real-time RT-PCR and Western blot results showed increased $iPLA_2$ mRNA and protein expression in the prefrontal cortex after maprotiline administration, thereby suggesting that prefrontal cortical $iPLA_2$ is involved in the antinociceptive effect of maprotiline. Lipidomic analysis showed decreased PC and increased LPC species in the prefrontal cortex after maprotiline treatment, indicating increased $iPLA_2$ enzymatic activity and endogenous release of DHA and EPA. These changes were blocked by intracortical $iPLA_2$ antisense injection. Together, our results indicate an important role of prefrontal cortical $iPLA_2$ in the antinociceptive effect of maprotiline, thereby suggesting a role of $iPLA_2$ not only in the antidepressive, but also antinociceptive effects of maprotiline and possibly other similar antidepressants.

In the second part of the study, we elucidated the potential cellular mechanisms involved in $iPLA_2$ expression induction, in particular the stimulation of adrenergic receptors. Treatment of SH-SY5Y neuroblastoma cells with maprotiline and another TCA with strong noradrenaline reuptake inhibition activity, nortriptyline, as well as the alpha-1 adrenergic receptor agonist, phenylephrine, resulted in increased $iPLA_2$ expression. This increase was blocked by inhibitors to the alpha-1 adrenergic receptors, MAPK/ERK, and sterol regulatory element binding protein (SREBP). Maprotiline and phenylephrine induced binding of SREBP-2 to the SRE region on the $iPLA_2$ gene, as determined by electrophoretic mobility shift assay (EMSA). Our results indicate that stimulation of adrenergic receptors increased $iPLA_2$ expression via MAPK/ERK and SREBP-2

Docosanoids such as resolvin D1 (RvD1) have been shown to be effective in treatment of inflammatory conditions and pain. RvD1 is metabolized from DHA by 15-lipoxygenase (15-LOX). In the last part of the study, we postulate that besides inducing $iPLA_2$ expression, antidepressants with strong noradrenaline reuptake inhibition activity will similarly induce an increase in 15-LOX expression. Real-time RT-PCR showed a significant increase in 15-LOX mRNA expression after maprotiline and nortriptyline treatment which was blocked by prazosin. This was supported by Western blot analysis which showed similar results. Overall, our findings suggest that treatment with antidepressants, especially those with strong noradrenaline reuptake inhibition activity, will induce $iPLA_2$ expression leading to increased DHA levels and subsequent production of resolvins via a concurrent increase in 15-LOX expression. The increase in DHA and its metabolites levels may

then contribute to the antidepressant-induced antinociception by facilitating activity or plasticity in the dorsolateral prefrontal cortex to stimulate the PAG and descending pain inhibitory pathway.

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List of Abbreviations

15-LOX	15-lipoxygenase
AA	Arachidonic acid
AC	Adenylate cyclase
Acyl-CoA	Acyl-Coenzyme A
ADHD	Attention-deficit hyperactivity disorder
ALA	α-linolenic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Cer	Ceramide
CFA	Complete Freund's adjuvant
CID	Collision-induced dissociation
CO_2	Carbon dioxide
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2

CT	Threshold cycle
CYP	Cytochrome P450
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBNA	Epstein-Barr Nuclear Antigen
EMSA	Electrophoretic mobility shift assay
EPA	Eicosapentaenoic acid
EPSCs	Excitatory postsynaptic currents
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GST	Glutathione S-transferase

i.c.	Intracortical
i.p.	Intraperitoneal
IASP	International Association for the Study of Pain
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-trisphosphate
$iPLA_2$	Calcium-independent phospholipase A_2
ISTD	Internal standard
K^+	Potassium
KCl	Potassium chloride
LA	Linoleic acid
LC-MS	Liquid chromatography-mass spectrometry
LOX	Lipoxygenase
LPC	Lysophosphatidylcholine
LTP	Long term potentiation
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitors
MAPK	Mitogen-activated protein kinase
MDD	Major Depressive Disorder
MRM	Multiple Reaction Monitoring
mRNA	Messenger ribonucleic acid

Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate – (reduced form)
NMDA	N-methyl-D-aspartate
NPD1	Neuroprotectin D1
NRIs	Noradrenaline reuptake inhibitors
NRM	Nucleus raphe magnus
PAF	Platelet activating factor
PAG	Periaqueductal gray
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PKA	Protein kinase A
PKC	Protein kinase C
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D

PlsEtn-selective	Plasmalogen-selective
PS	Phosphatidylserine
PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
RIMA	Reversible inhibitors of monoamine oxidase type A
rTMS	Repetitive transcranial magnetic stimulation
RvD1	Resolvin D1
RvE1	Resolvin E1
s.s.	Somatosensory cortex
S1P	Site-1 protease
S2P	Site-2 protease
SCAP	SREBP cleavage-activating protein
SDS	Sodium dodecyl sulfate
SM	Sphingomyelin
SNRIs	Serotonin-noradrenaline reuptake inhibitors
$sPLA_2$	Secretory phospholipase A_2
SRE	Sterol regulatory element
SREBP	Sterol regulatory element-binding protein
SSRIs	Selective serotonin reuptake inhibitors

TAG-1	Transient axonal glycoprotein-1
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TCAs	Tricyclic antidepressants
TESS	Transcription Element Search System
TMD	Temporomandibular disorders
UV	Ultraviolet

Publications

Various parts of this study have been published in international refereed journals:

1. **Chew WS**, Ong WY (2014). Regulation of calcium-independent phospholipase A_2 expression by adrenoceptors and sterol regulatory element binding protein - potential crosstalk between sterol and glycerophospholipid mediators. Molecular Neurobiology 2014 Dec 9 [Epub ahead of print].
2. Shalini SM, **Chew WS**, Rajkumar R, Dawe GS, Ong WY (2014). Role of constitutive calcium-independent phospholipase A_2 beta in hippocampo-prefrontal cortical long term potentiation and spatial working memory. Neurochemistry International 78C:96-104.

Chapter 1: Introduction

1. Glycerophospholipids in the brain

Glycerophospholipids are glycerol-based phospholipids which are amphipathic in nature with nonpolar and polar ends (Farooqui et al., 2000a). They are present in relatively high levels in brain tissue and consist of up to 20 to 25 % of the dry weight in the adult brain (Farooqui et al., 2000a). Together with cholesterol and glycolipids, glycerophospholipids encompass approximately 50 to 60 % of the whole membrane mass in the neural membrane (Farooqui et al., 2000a). There are four main categories of glycerophospholipids in the neural membrane. Three of these four categories contain a glycerol backbone with a normally unsaturated fatty acid at the carbon-2 position and a phosphobase at the carbon-3 position of the glycerol moiety which is made up of either ethanolamine, serine, inositol or choline (Farooqui et al., 2000a). They include 1-alkyl-2-acyl glycerophospholipid, 1,2-diacyl glycerophospholipid and 1-alk-1'-enyl-2-acyl glycerophospholipid or plasmalogen (Farooqui et al., 2000a). The last type of glycerophospholipid consists of sphingomyelin which contains phosphocholine-linked ceramide at the primary hydroxyl group (Farooqui et al., 2000a).

Glycerophospholipids play an important role in neural membrane fluidity, stability and permeability (Farooqui et al., 2000a). They are involved in membrane molecular packing, charge and reactivity and are essential for regulation of membrane-bound ion channel and enzyme activity (Crews, 1982; Farooqui et al., 2000a). Besides their role in neural membranes, glycerophospholipids are important for membrane anchoring (Farooqui et al., 2000a). Glycans, phosphoethanolamines and phosphatidylinositols (PIs) form glycosylphosphatidylinositol anchors that attach important proteins to

biomembranes (Low, 1989; Englund, 1993). The attached proteins include enzymes such as aminopeptidase P, alkaline phosphatase, acetylcholinesterase, 5'-nucleotidase and carboxypeptidase M which are present in all brain tissue and are essential for numerous metabolic activities (Hooper, 1997). Proteins such as axonin-1, transient axonal glycoprotein-1 (TAG-1) and the neural cell adhesion molecule are also linked to the glycosylphosphatidylinositol anchor and were found to activate axonal elongation and neurite outgrowth in PC12 rat cells (Doherty and Walsh, 1993). Endocytosis, fusion and secretory granule formation are examples of several membrane trafficking processes that also involve polyphosphoinositides (Martin, 1997).

In addition, glycerophospholipids are involved in regulation of enzymatic functions and they are needed for a number of enzymes to carry out their activity (Farooqui et al., 2000a). Some of these enzymes require specific glycerophospholipids and one such example is protein kinase C (PKC) which is activated in the presence of phosphatidylserine (PS) (Spector and Yorek, 1985; Yeagle, 1989; Farooqui et al., 2000a). PKC activation involves linkage with neural membranes via PS in the presence of calcium ions which will increase neural membrane surface pressure to help insert the protein domain of PKC into the membrane (Orr and Newton, 1992; Farooqui et al., 2000a). Once inserted into the membrane, PKC will then bind to diacylglycerol (DAG) to be fully functional (Farooqui et al., 1988; Farooqui et al., 2000a). Glycerophospholipids also act as precursors for DAG and it was suggested that DAG changes the membrane bilayer properties linked with lipid hexagonal-phase propensity in the activation of PKC (Senisterra and Epand, 1993). Moreover, DAG promotes membrane fusion which is associated with

the release of neurotransmitters (Nieva et al., 1989). By regulating PKC function, PS can likewise affect the binding activity of AMPA receptors with subsequent effects on synaptic plasticity (Gagne et al., 1996). PS also regulates the activities of acetylcholine receptor channel, Na^+/ K^+-ATPase, B-Raf protein kinase, dynamin GTPase and DAG kinase, while PC is needed for the activity of the inner mitochondria enzyme, β-Hydroxybutyrate dehydrogenase (Sunshine and McNamee, 1992; Farooqui et al., 2000a). Specific phospholipids are also needed by enzymes such as adenylate cyclase (AC) and Ca^{2+}-ATPase which are involved in sustaining regular ion homeostasis in glial cells and neurons (Farooqui and Horrocks, 1985; Spector and Yorek, 1985). Any disease-induced changes in the composition of glycerophospholipids can potentially affect ion permeability and fluidity of the membrane which will subsequently induce unregulated influx of calcium ions (Mecocci et al., 1996). This, in turn, will lead to oxidative stress and inflammatory responses in the brain (Farooqui and Horrocks, 1994).

Glycerophospholipids also act as a reservoir for the production of a number of bioactive mediators and lipid second messengers (Dennis et al., 1991; Exton, 1994; Farooqui et al., 1995; Farooqui et al., 1997b). Different second messengers are produced depending on the type and activity of the phospholipase involved (Dennis et al., 1991; Exton, 1994; Farooqui et al., 1995; Farooqui et al., 1997b). Phospholipases are a group of enzymes that hydrolyze glycerophospholipids and are classified according to their site of action (Fig. 1.1) (Farooqui et al., 2000a). The ester bond at the *sn*-1 position is acted on by phospholipase A_1 (PLA_1) to form a 2-acyl lysophospholipid and free fatty acid while phospholipase A_2 (PLA_2) hydrolyzes the fatty acid ester

bond at the *sn*-2 position to produce a 1-acyl lysophospholipid and free fatty acid (Farooqui et al., 2000a). The 1-acyl lysophospholipid produced by PLA_2 can then undergo subsequent acylation by acyl-Coenzyme A (acyl-CoA) in the presence of acyltransferase or it can be hydrolyzed by lysophospholipase to form a phosphobase and fatty acid (Farooqui et al., 2000a). The phosphodiester bond *sn*-3 position is cleaved by phospholipase C (PLC) to produce a phosphobase and DAG while phospholipase D (PLD) hydrolyzes glycerophospholipids to form a free base and phosphatidic acid (Farooqui et al., 2000a). The free fatty acids produced by phospholipase are active signaling molecules and their signaling actions are stopped by their conversion to fatty acyl-CoA (Horrobin, 2001). Acyl-CoA:lysophospholipid acyltransferase can then reacylate fatty acyl-CoA together with lysoglycerophospholipids to form glycerophospholipids (Lands, 1958). All four groups of phospholipases have several isoforms which are present in the brain and they have been purified and characterized from brain tissue (Hirashima et al., 1992; Rhee and Choi, 1992; Pete et al., 1994; Ross et al., 1995; Negre-Aminou et al., 1996; Exton, 1997, 1999; Farooqui et al., 2000a).

Phospholipase activity on glycerophospholipids to produce lipid second messengers are part of a signal transduction system which can potentially contribute to cross-talk between effector systems that are regulated by receptors and are important for regular glial cell and neuronal growth maintenance (Farooqui et al., 1992; Farooqui et al., 2000a). A common agonist was found to activate all four groups of phospholipases and the products of individual phospholipases were shown to stimulate other phospholipases, supporting the presence of a cross-talk between these

enzymes (Clark et al., 1995; Farooqui et al., 2000a). PLC activation produces DAG which translocates and stimulates PKC to activate both PLD and PLA_2 (Farooqui et al., 2000a). Besides producing DAG, PLC activity on PI 4,5-bisphosphate also results in the production of inositol 1,4,5-trisphosphate (IP_3) which is involved in intracellular calcium release and subsequent calcium signaling processes (Farooqui and Horrocks, 2007). PLC has been implicated in the maintenance of cell proliferation, secretion, contraction and phototransduction (Rhee and Choi, 1992).

PLD-generated second messengers include phosphatidic acid and choline which are hydrolyzed from PC in response to a number of extracellular stimuli (Klein et al., 1995; Exton, 1997, 1999). Phosphatidic acid acts as a precursor for lysophosphatidic acid which has autocrine or paracrine signaling effects and can activate the G protein-coupled receptor mechanism to trigger tyrosine kinase activation and subsequent Ras-Raf-MAPK stimulation (Moolenaar, 1995). Lysophosphatidic acid is present in high levels in the brain and the highest level of lysophosphatidic acid binding proteins and receptors are found in brain tissue (Das and Hajra, 1989). Lysophosphatidic acid causes retraction of neurites and rounding of neuronal cells in neuroblastoma cells and reduced uptake of glucose and glutamate in astrocytes (Tokumura, 1995; Keller et al., 1996). AC activity is also inhibited by phosphatidic acid and lysophosphatidic acid via a pertussis-toxin sensitive procedure which, in turn, causes a decrease in cAMP levels (Farooqui et al., 2000a). Besides acting as a precursor for lysophosphatidic acid, phosphatidic acid is involved in the activation of enzymes such as PLC, PKC, monoacylglycerol acyltransferase and PI 4-kinase (Farooqui et al., 2000a).

Moreover, phosphatidic acid was found to increase the GTP-bound form of Ras (Farooqui et al., 2000a). PLD also has a role in inflammation, cell proliferation, diabetes oncogenesis, secretion, mitogenesis and membrane trafficking (Exton, 1994; Liscovitch, 1996; Jones et al., 1999).

PLA_2 activity produces arachidonic acid (AA) which can be further metabolized into eicosanoids such as leukotrienes, prostaglandins and thromboxanes (Wolfe and Horrocks, 1994). AA is involved in pathological as well as physiological activities and it was shown to control ion channels and regulate DAG kinase, protein kinase A (PKA), PKC, Na^+/ K^+-ATPase and NADPH oxidase enzymatic activity (Dennis et al., 1991; Farooqui et al., 1997a; Farooqui et al., 2000a). AA was also shown to affect excitatory amino acid transporter-mediated glutamate uptake (Volterra et al., 1994). Additionally, AA and eicosanoids are known to be involved in the activation of PLD (Klein et al., 1995). Eicosanoids also act as intracellular second messengers which are essential for oxidative stress, inflammation and cell proliferation regulation (Farooqui, 2009a). In addition, they are involved in blood flow regulation, behavioral control and regulation of immune and neural activities (Chiu and Richardson, 1985; Wolfe and Horrocks, 1994; Katsuki and Okuda, 1995). Furthermore, PLA_2 is involved in the production of lysophospholipids which play important roles in membrane-membrane and membrane protein interactions (Fuller and Rand, 2001). Lysophospholipids also act as precursors for platelet activating factor (PAF) (Farooqui et al., 2000a; Fuller and Rand, 2001). One of the lysophospholipids, lysophosphatidylcholine (LPC), was found to activate alkaline phosphatase, PKC, phenylalanine hydroxylase, glycosyltransferase, sialyltransferase and

3,5-nucleotide phosphodiesterase while inhibiting lysophospholipase, AC, acyl-CoA:lysophosphatidylcholine acyltransferase and guanylate cyclase (Weltzien, 1979). PLA_2 is also involved in regeneration, apoptosis, neurodegeneration and neuritogenesis (Farooqui et al., 1997b).

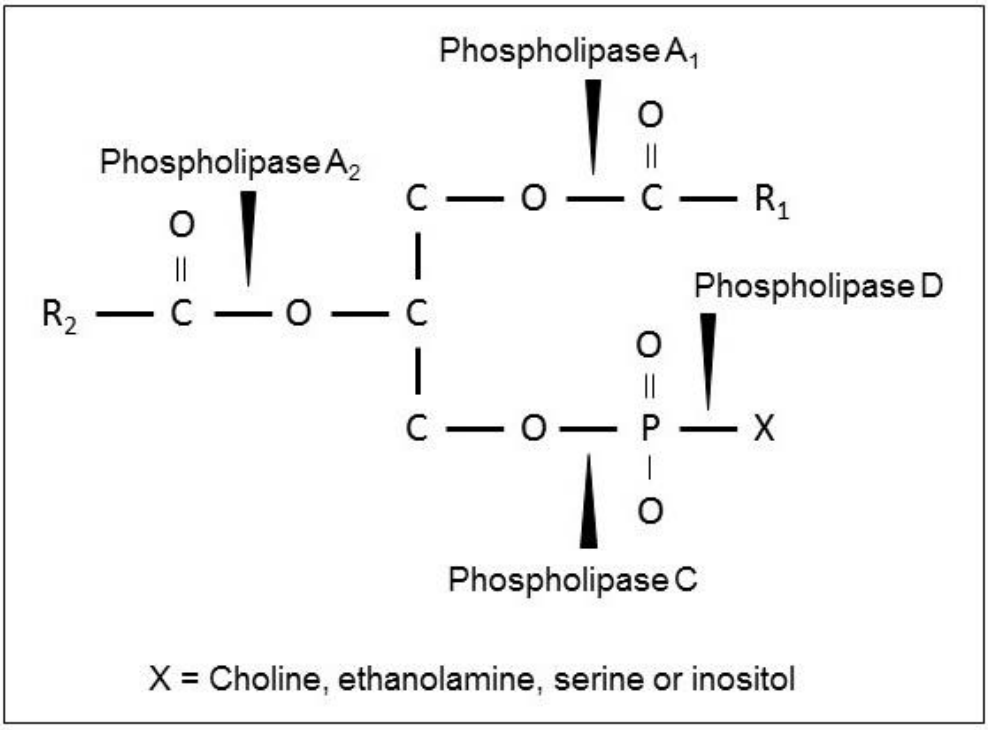


Fig. 1.1 Phospholipase enzymes and their site of action. Adapted from (Farooqui et al., 2000a)

1.1. Phospholipase A_2

Phospholipase A_2 (PLA_2, EC 3.1.1.4), as mentioned previously, comprises a group of enzymes that hydrolyze the acyl ester bond at the *sn*-2 position to produce a 1-acyl lysophospholipid and free fatty acid such as AA from glycerophospholipids (Dennis, 1994; Farooqui et al., 2000a). They are commonly found in mammalian tissue and can be further divided into several groups depending on their enzymatic reaction, structure, cellular location and

function (Farooqui et al., 1997b). PLA_2 enzymes include secretory phospholipase A_2, cytosolic phospholipase A_2, plasmalogen-selective phospholipase A_2 and calcium-independent phospholipase A_2 (Dennis, 1994; Farooqui et al., 1997b). There are different isozymes for each type of PLA_2 (Dennis, 1994; Farooqui et al., 1997b)

1.1.1. Secretory phospholipase A_2

Secretory phospholipase A_2 ($sPLA_2$) is produced intracellularly and is secreted to act on glycerophospholipids extracellularly (Farooqui and Horrocks, 2007). The $sPLA_2$ enzyme family consist of eleven isozymes which can be found in mammalian tissues and they are named in the order of their discovery: $sPLA_2$-IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, -XIIA and -XIIB (Kudo and Murakami, 2002). It was suggested that each $sPLA_2$ isozyme hydrolyzes specific phospholipid membrane moieties due to their distinct tissue expression patterns and diverse enzymatic activity (Murakami and Kudo, 2004).

$sPLA_2$ enzymes have low molecular mass (14-19 kDa), high content of disulfide bonds and are structurally related due to a common His-Asp catalytic dyad (Murakami and Kudo, 2004; Yang et al., 2009). Activation of $sPLA_2$ enzymatic activity requires Ca^{2+} in the millimolar range before they can act on the *sn*-2 ester bond without strict preference for any particular fatty acid side chain of the glycerophospholipids (Murakami and Kudo, 2002; Schaloske and Dennis, 2006; Farooqui and Horrocks, 2007; Burke and Dennis, 2009). $sPLA_2$ has low activity in the olfactory bulb and cerebellum, moderate activity in the

thalamus, hypothalamus and cerebral cortex, and has the highest levels of activity in the pons, hippocampus and medulla oblongata (Thwin et al., 2003).

Certain $sPLA_2$ isozymes, especially $sPLA_2$-IIA, have been suggested to be involved in inflammation, given that $sPLA_2$-IIA and $sPLA_2$-V were found to be highly expressed during inflammation resolution (Gilroy et al., 2004). In addition to acting on glycerophospholipids such as phosphatidylglycerol, $sPLA_2$-IIA is involved in the production of AA from cellular membranes, consequently enhancing the effects of AA on the inflammatory pathway (Hanasaki et al., 1999). $sPLA_2$-IIA sera concentrations are linked with the seriousness of inflammatory disorders, which is exemplified by the high catalytic $sPLA_2$-IIA level in the synovial fluids of rheumatoid arthritis and osteoarthritis patients (Nakano et al., 1990; Crowl et al., 1991; Oka and Arita, 1991; Pruzanski et al., 1991; Pruzanski et al., 1995). Inflammatory responses were also increased after $sPLA_2$-IIA injection into the hind paw of rats with adjuvant arthritis, further supporting the involvement of $sPLA_2$-IIA in inflammatory pain (Murakami et al., 1990; Koike et al., 1997).

1.1.2. Cytosolic phospholipase A_2

The enzymes in the cytosolic phospholipase A_2 ($cPLA_2$) family have high molecular weights (85-110 kDa) and consist of $cPLA_{2\alpha}$, $cPLA_{2\beta}$, $cPLA_{2\gamma}$, $cPLA_{2\delta}$, $cPLA_{2\varepsilon}$ and $cPLA_{2\zeta}$, where $cPLA_{2\alpha}$, $cPLA_{2\beta}$ and $cPLA_{2\gamma}$ are localized in brain tissue (Molloy et al., 1998; Pickard et al., 1999; Balboa et al., 2002). $cPLA_{2\alpha}$ is primarily expressed in gray matter astrocytes while maintaining very low levels in glial and neuronal cells (Owada et al., 1994; Farooqui et al.,

2000b; Ong et al., 2010). $cPLA_{2\beta}$ is predominantly present in the cerebellum while $cPLA_{2\gamma}$ is mainly found in the heart, skeletal muscle and brain (Pickard et al., 1999; Ong et al., 2010). All three isoforms were shown to be expressed in the amygdala, thalamus, corpus callosum, hippocampus, subthalamic nucleus and substantia nigra (Schaeffer et al., 2010). Although there are very low levels of $cPLA_2$ present in the liver, kidney, pancreas and heart, $cPLA_2$ is still found in most peripheral tissues (Molloy et al., 1998). $cPLA_2$ expression was also shown to be localized in dendritic spines or dendrites in the spinal cord ventral and dorsal horn (Sandhya et al., 1998; Ong et al., 1999a).

$cPLA_2$ catalytic activity does require need Ca^{2+} but submicromolar concentrations of Ca^{2+} are necessary for its translocation to internal membranes from the cytosol so that it can undergo phosphorylation for its enzymatic activity (Farooqui et al., 2000b; Murakami and Kudo, 2002). $cPLA_2$ preferentially acts on AA at the *sn*-2 position as compared to other unsaturated fatty acids in phospholipid substrates to produce lysophospholipids and AA (Diez et al., 1992; Clark et al., 1995; Balsinde et al., 2006). As previously mentioned, lysophospholipids, AA and its metabolite eicosanoids, are highly involved in physiological and pathological processes. Hence, regulation of $cPLA_2$ activity is necessary to maintain concentrations of lysophospholipids and AA for cellular homeostasis (Tanaka et al., 2012).

Due to their role in producing AA, $cPLA_2$ has been implicated in inflammatory processes (Leslie, 1997; Tanaka et al., 2012). Activation of $cPLA_2$ by proinflammatory factors will lead to increased $cPLA_2$ activity and higher levels of AA which can be further metabolized into eicosanoids that are involved in stimulation and maintenance of inflammatory responses (Farooqui

and Horrocks, 2007). Long term potentiation (LTP) induction in the dentate gyrus also leads to $cPLA_2$ activation and AA generation from glycerophospholipids, in particular PCs (Clements et al., 1991). Depending on the type of cell or tissue, $cPLA_2$ is involved in numerous other cellular processes such as mitogenesis, differentiation and cytotoxicity (Leslie, 1997). In addition, $cPLA_2$ was suggested to have an important role in the pain pathway due to its localization in the brainstem's facial motor nucleus and part of the ascending auditory pathway which includes the cochlear nuclei (Sandhya et al., 1998; Kishimoto et al., 1999; Ong et al., 1999b; Shirai and Ito, 2004). Intrathecal administration of $cPLA_2$ inhibitors significantly reduced $cPLA_2$ activity in spinal homogenates, which suggest the involvement of $cPLA_2$ in spinal processing of nociceptive inputs (Lucas et al., 2005). PLA_2 inhibitors similarly lessen the production of excitatory amino acids from the cortex after ischemia (Phillis and O'Regan, 1996). $cPLA_2$ activity was also found to be elevated in the dentate granule gyrus after brain ischemia which could induce higher neural membrane phospholipid metabolism and subsequent production of AA-derived lipid metabolites leading to oxidative stress, neurodegeneration, nociception and neuroinflammation (Koike et al., 1997; Ong et al., 2010). An increase in expression of both $cPLA_2$ mRNA and protein was demonstrated after transient forebrain ischemia or excitotoxicity injury followed by increased concentration of a toxic lipid peroxidation product, 4-hydroxynonenal (Owada et al., 1994; Clemens et al., 1996; Sandhya et al., 1998; Ong et al., 2003). 4-hydroxynonenal level was reduced after $cPLA_2$ inhibitor treatment which induced a neuroprotective influence on hippocampal neurons after excitotoxicity damage (Lu et al., 2001). $cPLA_2$

inhibitor treatment also increased functional recovery in spinal cord damage and protected hippocampal neurons against oxygen-glucose deficit (Arai et al., 2001; Huang et al., 2009).

1.1.3. Plasmalogen-selective phospholipase A_2

Plasmalogen-selective phospholipase A_2 (PlsEtn-selective PLA_2) has a molecular weight of 39 kDa and is found in the cytosol (Yang et al., 1996). PlsEtn-selective PLA_2 is involved in a receptor-mediated metabolism of plasmalogens in neural membranes and does not require Ca^{2+} for its enzymatic activity (Yang et al., 1996). PlsEtn-selective PLA_2 preferentially acts on AA and docosahexaenoic acid (DHA) at the *sn*-2 position of plasmalogens to generate free fatty acids and lysoplasmalogens (Farooqui and Horrocks, 2001a). The rate of release of DHA was found to be three to five times faster compared to AA (Ong et al., 2010). Plasmalogens contain a particularly high DHA content, where close to 70 % of plasmalogens in neuronal membranes possess DHA at the *sn*-2 position (Farooqui and Horrocks, 2001b). PlsEtn-selective PLA_2 is mainly linked with astrocytes due to its co-localization with glial fibrillary acidic protein (Farooqui and Horrocks, 2001a). Gangliosides, glycosaminoglycans and sialoglycoproteins were found to strongly inhibit PlsEtn-selective PLA_2 and the interaction between glycoconjugates and PlsEtn-selective PLA_2 is involved in the regulation of its enzymatic activity (Yang et al., 1996). PlsEtn-selective PLA_2 hydrolyzes plasmalogen to generate second messengers such as eicosanoids under physiological conditions. However, PlsEtn-selective PLA_2 was implicated to substantially release free

fatty acid under pathological settings which may lead to significant cellular and tissue injury (Yang et al., 1996).

PlsEtn-selective PLA_2 activity was also suggested to have a role in Alzheimer's disease, exemplified by the increased activity in the nucleus basalis and hippocampal areas in the brain of Alzheimer's disease patients (Farooqui, 2010). PlsEtn-selective PLA_2 was found to be activated by a sphingolipid metabolism lipid mediator, ceramide, in a dose-dependent manner (Latorre et al., 2003). It was then suggested that increased PlsEtn-selective PLA_2 activity in Alzheimer's disease patients may be attributed to ceramide build-up in the brain (Han et al., 2002; Han, 2005; Farooqui, 2010). Increased PlsEtn-selective PLA_2 activity may also lead to plasmalogen deficit and synapse loss in the brain of Alzheimer's disease patients (Wells et al., 1995; Ginsberg et al., 1998; Guan et al., 1999; Han et al., 2001; Pettegrew et al., 2001). Deficits in ethanolamine plasmalogen may induce destabilization of the neural membrane by affecting the core temperature needed for membrane lipid bilayer stability (Ginsberg et al., 1998; Farooqui, 2009b). Excessive PlsEtn-selective PLA_2 activity may similarly cause an increase in lysoplasmalogen production which may affect membrane permeability and fluidity as well as permit calcium influx, leading to neural membrane destabilization (Farooqui et al., 2008). Serum plasmalogen concentrations are also associated with severity of dementia and Alzheimer's disease neuropathology (Goodenowe et al., 2007). In addition to being associated with Alzheimer's disease, PlsEtn-selective PLA_2 activity was also found to be up-regulated in neuronal cell cultures undergoing kainate-induced toxicity and

ischemic damage in heart disease (Farooqui et al., 2001; Farooqui et al., 2008).

1.1.4. Calcium-independent phospholipase A_2

Calcium-independent phospholipase A_2 ($iPLA_2$) was originally purified and characterized from the murine $P388D_1$ macrophage-like cell line and has a molecular weight of 80-88 kDa (Ackermann et al., 1994; Murakami and Kudo, 2002; Farooqui and Horrocks, 2007). Besides its phospholipase A_2 activity, $iPLA_2$ has additional lysophospholipase activity and was demonstrated to have intricate interactions with interfaces (Lio and Dennis, 1998). $iPLA_2$ activity does not require Ca^{2+} but it utilizes a catalytic serine and can be affected by lipid coupled serine-reactive-type blockers (Ackermann et al., 1995; CondeFrieboes et al., 1996; Lio et al., 1996; Murakami and Kudo, 2002; Farooqui and Horrocks, 2007). Although $iPLA_2$ was suggested to not have a significant fatty acid selectivity, it has been shown to preferentially act on linoleic acid and DHA at the *sn*-2 position (Murakami and Kudo, 2002; Farooqui et al., 2006; Rapoport, 2013). $iPLA_2$ is strongly inhibited by the blocker, bromoenol lactone, while its activity is enhanced by ATP (Farooqui and Horrocks, 2007). $iPLA_2$ mRNA can be found in all tissues, with a higher expression in the central nervous system (CNS) and lower levels in the testes, pancreas and spleen at the periphery (Molloy et al., 1998; Lucas et al., 2005). Three $iPLA_2$ isoforms have been found in brain tissues and they include $iPLA_{2\alpha}$, $iPLA_{2\beta}$ and $iPLA_{2\gamma}$ (Molloy et al., 1998; Zanassi et al., 1998; Balboa et al., 2002). There is a significantly higher expression of $iPLA_2$ in the brain

compared to $cPLA_2$, evidenced by $iPLA_2$ immunohistochemical studies where dense staining of $iPLA_2$ was observed in the striatum, cortex and hippocampus (Farooqui et al., 1999; Ong et al., 2005; Ong et al., 2010). Neurons and astrocytes have been shown to express $iPLA_2$ in the brain (Ong et al., 2005; Sun et al., 2005)

$iPLA_2$ is an important "housekeeping" enzyme. Studies showed that knockdown of the $iPLA_2$ gene affected the incorporation of AA in phospholipids of the murine $P388D_1$ macrophage cells, implicating its role in the maintenance and remodeling of phospholipids via constitutive deacylation of phospholipids (Balsinde et al., 1995; Balsinde et al., 1997). Besides its role in phospholipid remodeling, $iPLA_2$ is also involved in the release of neurotransmitters, LTP, signal transduction, learning and memory (Winstead et al., 2000; Farooqui and Horrocks, 2007; Shalini et al., 2014). Bromoenol lactone treatment inhibited LTP induction in hippocampal slices and induced nuclear deterioration and neurite loss in cortical neurons (Wolf et al., 1995; Kurusu et al., 2008). Administration of bromoenol lactone at the hippocampus also affected short-term and long-term memory acquisition in inhibitory avoidance learning (Schaeffer and Gattaz, 2005). Similarly, $iPLA_2$ knockdown in the prefrontal cortex abolished hippocampo-prefrontal cortex LTP induction and inhibited spatial working memory (Shalini et al., 2014). $iPLA_2$ also has a possible role in the cortex-striatum-thalamus-cortex circuitry where inhibition of $iPLA_2$ induced vacuous chewing movements in a rat model for tardive dyskinesia (Lee et al., 2007). Moreover, intrastriatal knockdown of $iPLA_2$ induced deficits in prepulse inhibition of the auditory startle reflex in rats, similar to the prepulse inhibition deficits observed in schizophrenic patients

(Braff et al., 1978; Braff et al., 2001; Lee et al., 2009). iPLA$_2$ is also involved in the protection of mitochondrial processes from oxidative injury and in maintaining the structure of a mitochondrial membrane lipid component, cardiolipin, for the electron transport chain mechanism (Seleznev et al., 2006; Kinsey et al., 2008). In addition, prefrontal cortical iPLA$_2$ activity was decreased in a frontal variant of Alzheimer's disease (Talbot et al., 2000).

iPLA$_2$ has another significant role in its capacity to generate DHA from brain glycerophospholipids where iPLA$_2$ deficiency caused a decrease in brain DHA signaling and metabolism (Strokin et al., 2003, 2007; Green et al., 2008; Basselin et al., 2010). Inhibition of iPLA$_2$ using bromoenol lactone or small interfering RNA suppressed the release of DHA without affecting AA production in astrocytes after ATP stimulation (Strokin et al., 2003, 2007). DHA is metabolized into docosanoids such as resolvins and neuroprotectins which have anti-inflammatory actions and are involved in synaptic plasticity and cell survival signaling (Tassoni et al., 2008; Bazan, 2009; Xu et al., 2010; Park et al., 2011; Serhan and Petasis, 2011). They are also potentially involved in neuroprotective actions against ischemia-reperfusion injury (Marcheselli et al., 2003).

1.2. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are long-chain fatty acids that include omega-6 fatty acids and omega-3 fatty acids such as DHA (Fig. 1.2) (Farooqui, 2009b). They have more than one double bond in their backbone and are derived from the essential fatty acids, omega-3 α-linolenic acid (ALA;

18:3) and omega-6 linoleic acid (LA; 18:2) (Logan, 2003; Scorletti and Byrne, 2013). Plants are able to synthesize ALA via the Δ-15 desaturase enzyme which is absent in mammals (Scorletti and Byrne, 2013). Thus, the aforementioned essential fatty acids must be obtained from dietary sources such as walnuts and fish as these fatty acids cannot be produced by the human body (Logan, 2003; Scorletti and Byrne, 2013). Elongase and desaturase enzymes are involved in the conversion of ALA and LA to PUFAs (Fig. 1.3) (Hulbert et al., 2005). ALA is first converted to eicosapentaenoic acid (EPA; 20:5) which is subsequently converted to DHA (22:6) while LA is converted to AA (20:4), the primary omega-6 fatty acid which acts as a precursor for the proinflammatory series of eicosanoids (Kim, 2007; Farooqui, 2009b; Scorletti and Byrne, 2013).

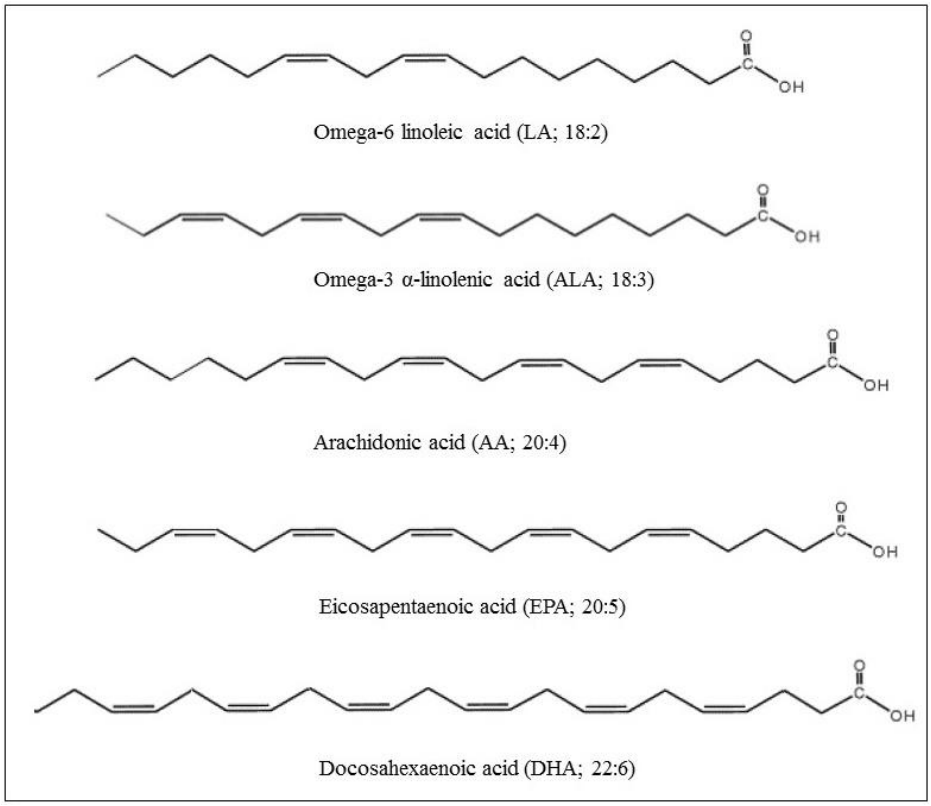


Fig. 1.2 Structure of several PUFAs. Adapted from (Farooqui, 2009b).

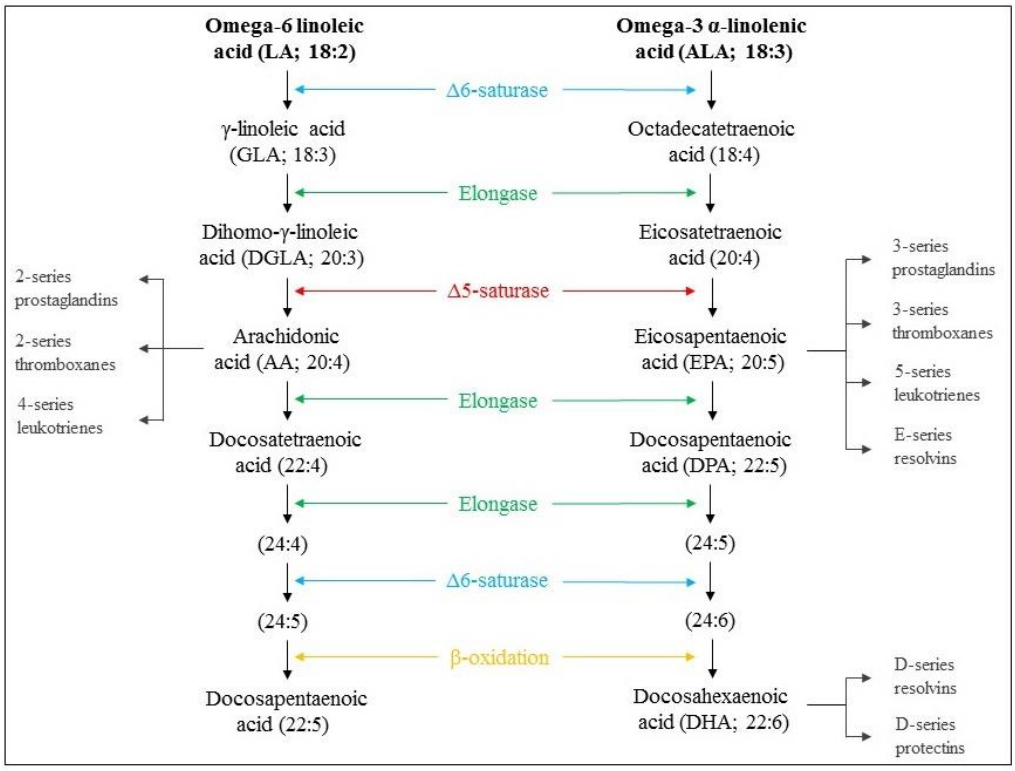


Fig. 1.3 Synthesis and metabolites of omega-3 and omega-6 fatty acids as well as the enzymes involved. Adapted from (Scorletti and Byrne, 2013).

1.2.1. DHA in the brain

Although there is a high level of DHA in the brain which is accumulated in neuronal membranes, DHA cannot be produced *de novo* in the brain as the neurons lack the necessary desaturase enzymes (Glomset, 2006; Kim, 2007; Nguyen et al., 2014). DHA in the body is transported by liver-formed plasma lipoproteins through the bloodstream, whereby some of the DHA is imported across the blood-brain barrier and esterified to the *sn*-2 position of the glycerol backbone of membrane glycerophospholipids (Glomset, 2006). A recent study found that the major transporter involved in the uptake of DHA into the brain is Mfsd2a which is a member of the major

facilitator superfamily and was previously an orphan transporter (Nguyen et al., 2014). DHA is found to be especially enriched in glycerophospholipids such as PCs, PSs, phosphatidylethanolamines (PEs) and ethanolamine plasmalogens, and comprises up to 50 % of the total quantity of PUFAs esterified at the *sn*-2 position of brain glycerophospholipids (Garcia et al., 1998; Kim, 2007; Farooqui, 2009b). DHA is produced from ethanolamine plasmalogens via the activity of PlsEtn-selective PLA_2 and can also be generated from glycerophospholipids via $iPLA_2$ activity as shown in several *in vitro* studies (Strokin et al., 2003, 2007; Farooqui, 2009b). As stated previously, brain imaging of $iPLA_2$-deficient mice also revealed reduced DHA signaling and metabolism, supporting the role of $iPLA_2$ in generating DHA in the brain (Basselin et al., 2010).

Both DHA and EPA have highly fluidizing characteristics and the presence of DHA at the *sn*-2 position of glycerophospholipids aids in the maintenance of membrane properties such as regulation of membrane fluidity (Salem et al., 1986). Administration of DHA/EPA increased brain phosphatides and synaptic protein concentrations while AA treatment did not induce any effects (Cansev and Wurtman, 2007). DHA was also shown to be important for initiation of LTP. Administration of DHA attenuated the inhibitory effects of $iPLA_2$ inhibitors on LTP induction in rat hippocampal slices, demonstrating the importance of DHA for LTP initiation (Fujita et al., 2001). In addition, as stated previously, our recent study showed that knockdown of $iPLA_2$ in the prefrontal cortex abolished hippocampo-prefrontal cortical LTP induction and inhibited spatial working memory (Shalini et al., 2014). Taken together, results from these studies suggest that $iPLA_2$ activation

and subsequent production of DHA are highly involved in synaptic plasticity, learning and memory. In a 2006 Framingham Heart Study, higher plasma PC DHA level was found to be significantly linked with a decreased risk of dementia development in the study subjects (Schaefer et al., 2006). Moreover, dietary intake of fatty fish and marine omega-3 fatty acids in middle-age subjects was associated with a decreased risk of impaired cognitive function in a cross-sectional population-based study, indicating an important role of DHA in learning and memory (Kalmijn et al., 2004).

Studies have also showed a possible role of PUFAs and DHA in pain. Patients with chronic pain were found to possess high levels of omega-6 fatty acids (Tokuyama and Nakamoto, 2011). Furthermore, patients with rheumatoid arthritis or joint pain caused by dysmenorrhea or inflammatory bowel disease reported reduced pain intensity after supplementation with omega-3 fatty acid such as DHA (Goldberg and Katz, 2007). Additionally, dose-dependent administration of DHA was shown to exert antinociceptive effects in animal pain models (Nakamoto et al., 2010). DHA, in turn, can be metabolized by the 15-lipoxygenase (15-LOX) enzyme to form docosanoids which comprise resolvins and neuroprotectins (Farooqui, 2011). 15-LOX is part of the LOX family of enzymes that is non-heme, contains iron, and has a molecular mass of approximately 75-78 kDa (Brash, 2001; Radmark and Samuelsson, 2009). Resolvins and neuroprotectins have anti-inflammatory as well as neuroprotective properties and are able to modulate the effects of AA-derived proinflammatory eicosanoids (Hong et al., 2003; Bazan, 2009). One of the neuroprotectins, neuroprotectin D1 (NPD1), has been shown to stimulate anti-apoptotic gene and protein expression coupled with a decrease in pro-

apoptotic proteins which may lead to suppression of amyloid-beta neurotoxicity in Alzheimer's disease (Mukherjee et al., 2004; Lukiw et al., 2005). Resolvins, including resolvin D1 (RvD1) and resolvin E1 (RvE1), were also reported to significantly reduce mice inflammatory pain behavior, whereas NPD1 was shown to effectively reduce neuropathic pain in mice, further highlighting the involvement of DHA and its metabolites in mediating neuroinflammation and pain (Yacoubian and Serhan, 2007; Nakamoto et al., 2010; Xu et al., 2013).

2. Pain

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Bonica, 1979; IASP, 1979; Merskey et al., 1994). Pain is very complex as well as subjective and each individual acquires the knowledge or application of the word through personal experiences related to damage or injury in early life (IASP, 1979). Pain also has a robust motivational aspect which could encourage withdrawal reflexes in addition to highly ordered avoidance and escape actions (McNeill and Dubner, 2001). This motivational component is essential for organisms to survive (McNeill and Dubner, 2001). Pain can be categorized as acute pain or chronic pain. Acute pain lasts a short time and resolves quickly, whereas chronic pain lasts longer and can also be defined as "pain that extends beyond the expected period of healing" (Turk and Okifuji, 2001). Chronic pain may also last for a period of several months

or longer, or occurs frequently for at least several months. Epidemiologic studies revealed that pain is one of the main reasons that patients look for medical attention and that the lifetime prevalence of pain indications such as back pain, chest pain, headache and joint pain is around 24 to 37 % (Regier et al., 1984; Komaroff, 1990; Kroenke, 2001; Bair et al., 2003). In Singapore, a large epidemiological survey on the prevalence and impact of pain revealed that 8.7 % of the population, which translates to more than 300000 adults, suffer from chronic pain (Yeo and Tay, 2009).

There are four components of the clinical occurrence of pain, namely nociception, pain, suffering and pain behavior (Loeser, 2006). Nociception is defined as the neural system of processing and encoding noxious or harmful stimuli (Merskey et al., 1994). Nociception does not equate pain as each can occur in the absence of the other (Loeser and Treede, 2008). Nociceptors are involved in nociception and they are sensory receptors which are particularly sensitive to noxious or harmful stimuli (Merskey et al., 1994). There are two main categories of nociceptors: A-delta fibers and C fibers. A-delta fibers are lightly myelinated and are involved in mediating fast, acute and sharp pain, whereas the unmyelinated C fibers facilitate slower, delayed, dull and more diffused pain (Julius and Basbaum, 2001). Threatened or actual injury to non-neural tissue will lead to nociceptor stimulation and subsequent nociceptive pain which can be either somatic or visceral (Table 1.1) (Merskey et al., 1994; Jann and Slade, 2007). Suffering is a negative affective reaction in the brain to pain or other affective conditions such as depression (Loeser, 2006). Pain behaviors are closely associated with suffering and include things a person says, does or does not do that are attributed to injury or tissue damage (Loeser,

2006). All pain behaviors are genuine and can be measured in a clinical situation (Loeser, 2006). Examples of pain behaviors include moaning, grimacing, and limping (Loeser, 2006).

Table 1.1 Differences between somatic and visceral pain. Adapted from (Bond and Simpson, 2006).

Somatic	Visceral
Well localized at site of pain	Poorly localized at site of pain
May follow somatic nerve distribution	Diffused radiation
Definite and sharp pain	Ambiguous and dull
Linked with external causes	Linked with internal causes
Pain is often constant though may be periodic sometimes	Pain is often periodic and leads to peaks though may be constant sometimes

2.1. Orofacial pain

Orofacial pain is defined as "pain within the structures of the oral cavity and face, usually of a diffuse pattern" (Zwemer, 1998). Orofacial pain includes pain disorders that are linked with the hard and soft tissues of the neck, head, face and all of the intraoral structures (American Academy of Orofacial Pain and Okeson, 1996). The trigeminal nerve forms the primary sensory supply to the orofacial area and has a big representation in the sensory cortex (Renton et al., 2012). Thus, any pain in the orofacial area can actually lead to substantial social and functional effects, where daily social activities such as eating, drinking or sleeping may be affected (Renton and Yilmaz, 2011; Renton et al., 2012). Besides common conditions such as headaches,

toothaches and temporomandibular disorders (TMD), orofacial pain also includes other rare orofacial pain disorders (Shephard et al., 2014). TMD is one of the most common orofacial pain disorder and has similar intensity, persistence and psychological impact to that of back pain (Von Korff et al., 1988; Manfredini et al., 2011).

2.2. Pain pathway

Pain pathways involve first-order, second-order and third-order neurons which convey nociceptive signals from the periphery to the brain (de Leeuw et al., 2005). Nociceptive input is transmitted to the spinal cord or brainstem via the spinal or trigeminal nerve and relay in the dorsal horn or spinal trigeminal nucleus, before onward transmission to the thalamus (Fig. 1.4). The ascending pain pathway involves two main systems, namely the lateral and medial pain system (Brooks and Tracey, 2005; de Leeuw et al., 2005). The lateral pain system transmits information to the ventral posterior lateral nucleus, ventral posterior medial nucleus, and ventral posterior inferior nucleus of the thalamus to the somatosensory cortex (de Leeuw et al., 2005). When stimuli travel through this lateral pain system, contralateral brain activation was observed (Treede et al., 1999; Rome and Rome, 2000). The medial pain system primarily involves medial thalamic structures which include the ventral part of the ventral medial nucleus, the ventrocaudal part of the medial dorsal nucleus, the intralaminar nucleus and the contralateral nucleus which eventually relays to the cingulate cortex (de Leeuw et al., 2005). A descending pain inhibitory pathway originating from the brainstem

also exists, which involves relays in the brainstem nuclei and affect all sensory feedbacks going up into the brainstem (Basbaum and Fields, 1984; Pertovaara and Almeida, 2006). The periaqueductal gray (PAG) and the nucleus raphe magnus (NRM) are two important components of the descending pain inhibitory pathway where the PAG projects to the raphe nuclei in the brainstem (Okeson, 2005). Serotoninergic neurons from the raphe nuclei project to the dorsal horn neurons in the spinal cord or the spinal trigeminal nucleus and act on opioid containing neurons to cause modulation of synaptic transmission between the first and second order sensory neurons in the pain pathway to modulate nociception (Pertovaara and Almeida, 2006). Besides these raphe neurons, noradrenergic neurons from the locus coeruleus also form a descending projection that inhibits pain transmission (Martin, 2003).

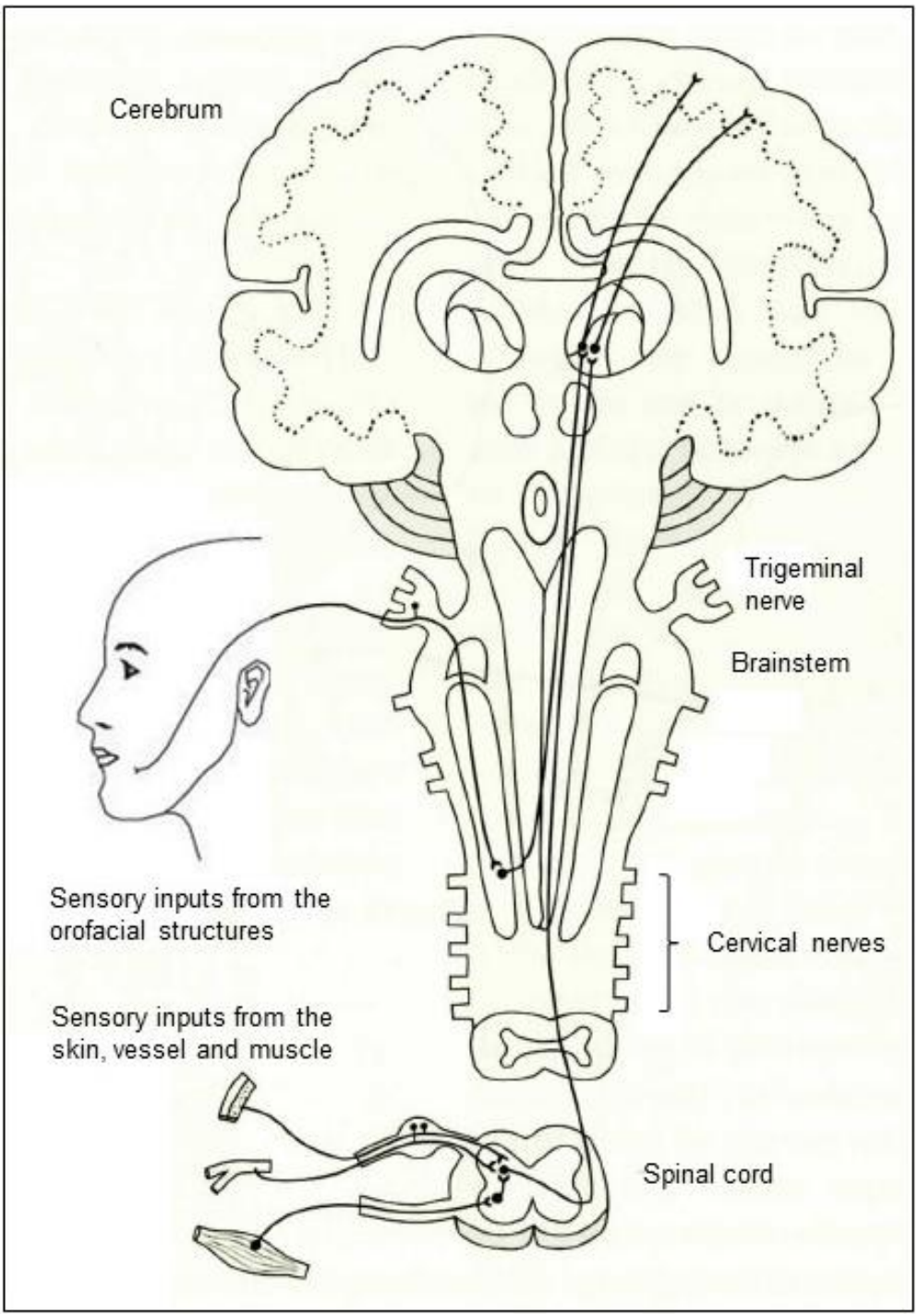


Fig. 1.4 Transmission of sensory inputs to the brain. Adapted from (Okeson, 2005).

2.3. Animal pain models

Scales and questionnaires in self-ratings of pain have been shown to be precise, dependable and versatile for both clinical and experimental pain

measurement in humans (Price et al., 1983; Mogil, 2009). However, unlike humans, animals cannot self-report and self-rate (Mogil, 2009). Nonetheless, their behavioral responses to noxious stimuli can be accurately and consistently scored although some behaviors such as licking an inflamed paw may appear to lack clinical face validity (Mogil, 2009). Some of the commonly used animal pain models include formalin, chronic constriction injury, carrageenan, spinal nerve ligation and complete Freund's adjuvant (CFA) (Mogil, 2009; Hsieh et al., 2010; Jaggi et al., 2011). Formalin, CFA and carrageenan (Fig. 1.5) are used in inflammatory pain models where they are injected subcutaneously into rodents to induce orofacial pain or hind paw edema (Poh et al., 2009; Hsieh et al., 2010). Studies have utilized these animal pain models to investigate nociceptive functions of molecules or potential antinociceptive and anti-inflammatory effects of drug treatment which could shed light on the mechanisms and pathways involved (Honore et al., 2002; Lucas et al., 2005; Oliveira et al., 2007; Poh et al., 2012).

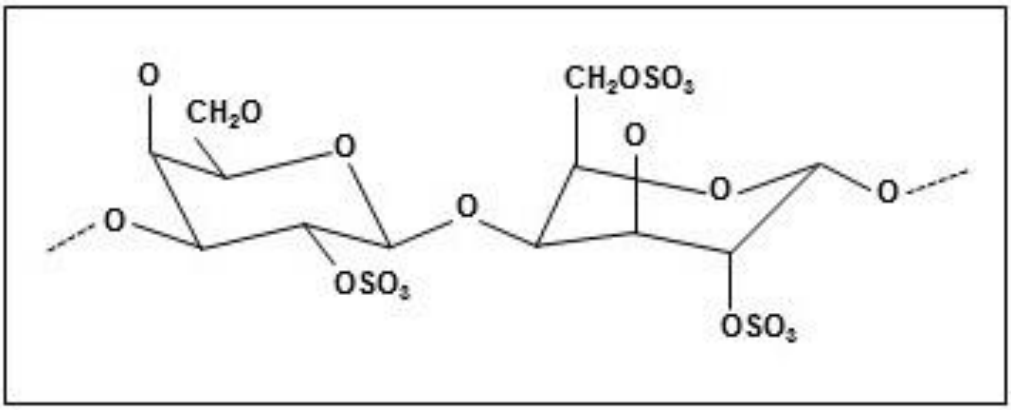


Fig. 1.5 Basic structure of carrageenan. Adapted from (O'Sullivan et al., 2010).

2.4. Prefrontal cortex in pain

The prefrontal cortex is the anterior portion of the frontal lobe and includes Brodmann areas 9, 10, 11, 12, 46 and 47 (Fuster, 2008). The main role of the prefrontal cortex involves executive functions such as working memory, planning and decision-making (Fuster, 2008). The prefrontal cortex receives information from different parts of the brain such as the hypothalamus, the midbrain and the limbic cortex (Fuster, 2008). In turn, the prefrontal cortex sends fibers back to these structures with the exception of the basal ganglia and pontine nuclei (Fuster, 2008). For these two structures, the prefrontal cortex sends projections to them without receiving any in return (Fuster, 2008). Like the brainstem and spinal cord, the prefrontal cortex is also richly innervated with aminergic fibers (Jordan et al., 1994; Carvalho et al., 2005).

Besides being involved in executive functions, there is also evidence suggesting a potential role of the prefrontal cortex in pain and antinociception. The Brodmann areas 9 and 46 make up the dorsolateral prefrontal cortex which is vital for conservation of information in short-term memory, maintaining competent performance control in the incidence of interfering stimuli and constant monitoring of the external world (MacDonald et al., 2000; Bunge et al., 2001; Sakai et al., 2002; Lorenz et al., 2003). Correlation analysis of functional neuroimaging studies suggest that the dorsolateral prefrontal cortex is involved in pain modulation (Iadarola et al., 1998; Baron et al., 1999; Bornhovd et al., 2002). Dorsolateral prefrontal cortex stimulation using the non-invasive repetitive transcranial magnetic stimulation (rTMS) was shown to increase tolerance to human experimental pain (Graff-Guerrero

et al., 2005). Other functional neuroimaging studies showed that there was increased activity in the prefrontal cortex during anticipation of analgesia with placebo treatment (Benedetti et al., 2005; Wager, 2005), and suggest that increased prefrontal cortical activity may modulate pain through activation of the descending pain inhibitory pathway (Valet et al., 2004; Ohara et al., 2005; Xie et al., 2009). In addition, electrical activation of prefrontal cortical fiber projections to the midbrain was found to induce antinociceptive effect in rodents (Hardy and Haigler, 1985; Zhang et al., 1998). Diffusion tensor imaging, a magnetic resonance imaging-based method to allow *in vivo* mapping of the anatomical connections in the human brain showed that tract paths could be defined between the PAG and the prefrontal cortex, amygdala, thalamus, hypothalamus and rostroventral medial medulla bilaterally (Hadjipavlou et al., 2006). In addition, retrograde tracing studies showed that the prefrontal cortex sends projections to the PAG mainly from the medial prefrontal cortical wall and a few other orbital/anterior insular prefrontal cortical regions, suggesting the possible involvement of the prefrontal cortex in a top-down modulation of pain through the PAG and subsequent activation of the descending pain inhibitory pathway (An et al., 1998; Floyd et al., 2000).

3. Depression and pain

Depression is defined as an ephemeral mood or emotional state which is experienced by almost everyone at some point in life (Fava and Kendler, 2000). Depression can also be described as a clinical or medical syndrome termed as Major Depressive Disorder (MDD) (Fava and Kendler, 2000). MDD

is a major health problem which affects mood, cognition, neurovegetative activities as well as psychomotor function, and it also causes one of the highest levels of non-fatal burden and disability (Fava and Kendler, 2000; Chua et al., 2012; Ho et al., 2013). Epidemiological studies show a high prevalence of depression worldwide, with 9 % to 20 % of the population possibly being affected during their lifetime (Licinio and Wong, 1999; Chua et al., 2012). In Singapore, nationwide epidemiological studies in 2003/2004 showed a 4.9 % prevalence of depression in adults and 3.1 % prevalence in elderly populations, while the Singapore National Mental Health Survey in 2010 revealed that there is a 6.3 % lifetime prevalence of depression in adults (Chua et al., 2012).

The monoamine hypothesis of depression was first suggested in 1965 and states that depression is caused by a lack of monoamine neurotransmitters and function in the brain (Schildkraut, 1965). This theory was supported by the mechanism of action of antidepressants which involves increasing the levels of monoamine neurotransmitters, predominantly noradrenaline and serotonin, and improving depressive symptoms (Delgado, 2000; Hirschfeld, 2000). However, studies on patients with depression suggest that this hypothesis may not be completely accurate and is insufficient to fully explain the actions of antidepressants (Delgado, 2000; Hirschfeld, 2000). An additional fact is that antidepressant drugs induce their direct biochemical effects rather rapidly but their antidepressant effects take weeks to develop (Rang et al., 2007). This led to the suggestion that instead of the primary drug effects on monoamines in the brain, the clinical improvement of depressive symptoms are a result of secondary adaptive responses induced by long-term

antidepressant treatment (Rang et al., 2007). Nonetheless, although the monoamine hypothesis is inadequate to fully explain depression, drugs affecting monoamine levels and functions remain the most effective therapeutic method to treat depression.

Depression is also associated with pain, given that both depression and pain symptoms are often found together and are mutually interacting (Bair et al., 2003; Williams et al., 2006). Studies showed a higher pain prevalence in patients with depression and a higher prevalence of depression in patients experiencing pain as compared to individual evaluation of depression and pain conditions (Bair et al., 2003). Depression induced a greater number of pain grievances and impairment in patients with pain while moderate to severe pain is linked to additional symptoms of depression and worse depressive outcomes such as reduced work functions (Bair et al., 2003). There are overlying neurobiology, biological pathways, therapy and phenomenology linking depression and pain (Bair et al., 2003; Williams et al., 2006). This interaction has significant implications on treatment and outcome of both depression and pain (Bair et al., 2003; Williams et al., 2006).

3.1. Antidepressants

In patients with moderate to severe depression, antidepressants are generally recommended as the first line of treatment based on recent clinical practice guidelines (Chua et al., 2012). Most antidepressants increase the levels of monoamine neurotransmitters either by interacting with their receptors or affecting their metabolism (Fig. 1.6) (Owens et al., 1997; Coyle

and Duman, 2003). Antidepressants generally can be separated into monoamine oxidase inhibitors, monoamine reuptake inhibitors and other miscellaneous 'atypical' antidepressants which have non-selective receptor-blocking effects and poorly understood mechanisms (Frazer, 1997a; Rang et al., 2007).

Monoamine oxidases (MAOs) are a family of enzymes that are involved in the oxidation of monoamines and MAO inhibitors (MAOIs) are the first drugs that are used clinically as antidepressants (Edmondson et al., 2004; Rang et al., 2007). MAO is present in nearly all tissues and there are two subtypes of MAO: MAO-A and MAO-B (Pletscher, 1991; Rang et al., 2007). The main target for MAOIs is the subtype MAO-A which is involved in the oxidation of serotonin, noradrenaline and dopamine (Rang et al., 2007). MAOIs such as phenelzine and iproniazid generally work by binding irreversibly and indiscriminately to both MAO subtypes to inhibit their functions (Rang et al., 2007). MAOI were found to have a number of undesirable drug and food interactions which could lead to problems such as the 'cheese reaction' and subsequent acute hypertension (Pletscher, 1991; Rang et al., 2007). These unwanted side effects and interactions led to a decline in the clinical usage of MAOIs to treat depression. A newer class of reversible MAOIs such as moclobemide however, was developed and was shown to have fewer interactions and higher specificity for MAO-A compared to the older MAOIs (Lotufo-Neto et al., 1999). These new reversible inhibitors of monoamine oxidase type A (RIMAs) were suggested to potentially have a useful albeit limited role in the differential treatment of depressive disorders (Lotufo-Neto et al., 1999).

Monoamine reuptake inhibitors act by inhibiting the actions of monoamine transporters such as the serotonin transporter. This will lead to an increase in extracellular monoamine neurotransmitter levels and subsequent monoaminergic transmission which in turn causes neurochemical changes that eventually induce the desired therapeutic effect in the CNS (Richelson, 2003; Walter, 2005). Monoamine reuptake inhibitors include selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs), noradrenaline reuptake inhibitors (NRIs) and tricyclic antidepressants (TCAs).

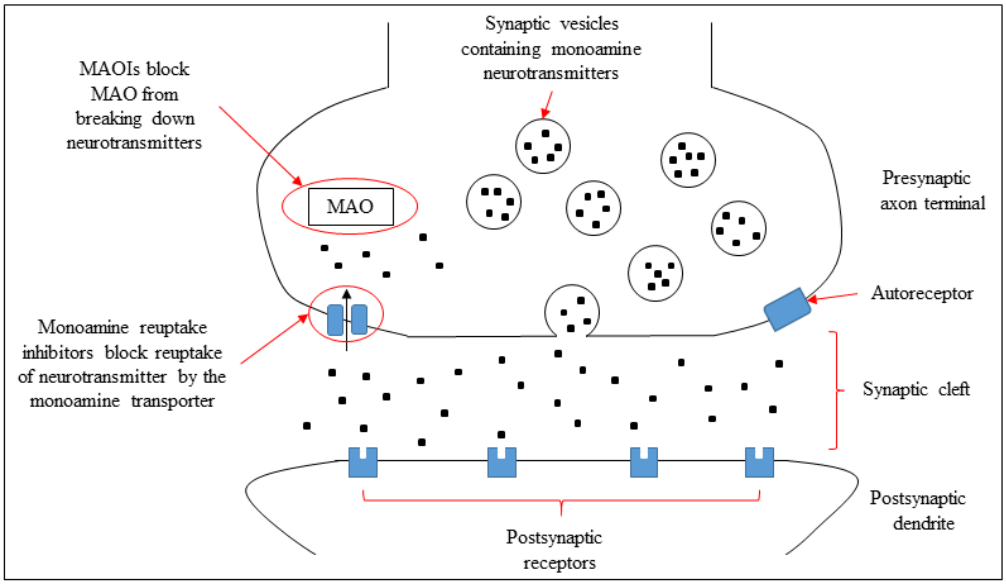


Fig. 1.6 Sites of action of antidepressants.

3.2. Pain and antidepressant treatment

Inhibitors of cyclooxygenase (COX) enzymes or opioids are used in pain conditions such as chronic inflammatory pain. However, long-term usage of these drugs is not without problems. Nonselective COX inhibitors may

induce gastrointestinal bleeding and kidney damage, while more selective COX-2 inhibitors increase cardiovascular disease risk (Mukherjee et al., 2001). Even though opioids are effective and strong analgesics for acute pain treatment, long-term usage may require increasing doses leading to adverse effects such as sedation, cognitive disruptions, nausea and constipation (Noble et al., 2010). In addition to these serious side effects, major concerns surrounding opioid use include the risk of addiction, abuse, misuse and diversion.

On the other hand, there are other drugs such as antidepressants and anticonvulsants which have attracted attention as alternative medication for pain (Lynch and Watson, 2006). Antidepressants have been observed to induce a distinct effect on pain. The dose to modulate pain are mostly found to be lower than the dose needed to treat depression and the onset of activity has also been shown to be faster (Ryder and Stannard, 2005). In addition, antidepressants are analgesic even in non-depressed patients with chronic pain and they are also shown to be effective in experimental and acute pain (Ryder and Stannard, 2005; Mico et al., 2006). Clinical studies on antidepressants found them to be effective in chronic pain conditions such as chronic lower back pain (Fishbain, 2000; Salerno et al., 2002; Staiger et al., 2003). Neuropathic pain was found to be most responsive to the analgesic and antinociceptive effects of antidepressants, whereas TCAs were shown to have the greatest analgesic efficacy in several different studies (Finnerup et al., 2005; Mico et al., 2006).

Antidepressant-induced antinociception is thought to be linked to the central inhibition of monoamine neurotransmitters reuptake, in particular

noradrenaline and serotonin, in the CNS (Jasmin et al., 2003; Ryder and Stannard, 2005). Besides inducing monoaminergic effects in other parts of the CNS, monoamine reuptake inhibition may also lead to increased monoamine levels and activity in the synapse which may stimulate descending inhibitory action in the spinal cord and affect nociceptive processing. (Jasmin et al., 2003; Ryder and Stannard, 2005). Formalin tests in rats treated with antidepressants and monoamine receptors antagonists indicate that alpha-1 adrenergic receptors and serotonin receptors in the brain have an important role in antidepressant-induced antinociception (Yokogawa et al., 2002). It was also suggested that functional interactions between serotonergic and noradrenergic neurons in the brain are involved in the antinociceptive effects of antidepressants (Yokogawa et al., 2002).

Nevertheless, there is still a large gap in the knowledge of the actual mechanism in which antidepressants exert their antinociceptive effect. It has been shown that blockade of monoamine reuptake and subsequent increase in monoamine levels may not be the only mechanism involved as the antinociceptive effect of the antidepressant, fluvoxamine, was reliant on ATP-dependent potassium channels activation (Hajhashemi and Amin, 2011) Thus, it is possible that the therapeutic effects of antidepressants not only involve the inhibition of monoamine neurotransmitters such as serotonin or noradrenaline, but also include other mechanisms and potential adaptive cellular changes caused by antidepressant treatment.

3.3. Tricyclic antidepressants

Tricyclic antidepressants (TCAs) are a group of heterocyclic chemical compounds that are used as antidepressants. TCAs contain three rings of atoms in their molecular structure which they are named after. A closely-related group of antidepressant compounds are the tetracyclic antidepressants which contain four rings of atoms. Psychopharmacological characterization of a series of structural analogs of phenothiazines that were being developed as potential antihistamines, sedatives, and analgesic drugs led to the initial development of the TCAs (Hollister, 1981; Baldessarini, 2006).

One of the first few TCAs is a dibenzazepine analog, imipramine, which was shown to be ineffective in schizophrenia but had a notable effect in patients with depression (Kuhn, 1958). Subsequent findings on the effectiveness of imipramine in treating major depression led to the synthesis of other chemically related compounds such as desipramine and clomipramine (Thase and Perel, 1982; Potter et al., 1998; Baldessarini, 2006). Desipramine is the main active metabolite and secondary-amine congener of imipramine while clomipramine is its 3-chloro derivative (Baldessarini, 2006). In addition, there were also other similar compounds developed which include dibenzocycloheptadienes such as amitriptyline and its N-demethylated metabolite nortriptyline, along with the dibenzoxepine, doxepin and the dibenzocycloheptatriene protriptyline (Baldessarini, 2006). Other analogs include the tetracyclic antidepressant, maprotiline, which has an extra ethylene bridge across the central six-carbon ring, and amoxapine which is a piperazinyldibenzoxazepine with combined neuroleptic and antidepressant effects (Baldessarini, 2006). All these analogs possess the three-ring molecular

core and most have similar clinical and pharmacological properties (Baldessarini, 2006). They are thus collectively termed as "tricyclic antidepressants".

Antidepressants generally have a relatively good absorption rate after oral administration and the serum levels of most TCAs peak within a few hours (Baldessarini, 2006; Rang et al., 2007). TCAs are extensively distributed in the body once they are absorbed as they are rather lipophilic and can bind strongly to plasma albumin (Baldessarini, 2006; Rang et al., 2007). Most TCAs are bound up to 90-95 % at therapeutic plasma levels and they bind to extravascular tissue which contribute to their low elimination rate and high distribution capacity (Rang et al., 2007). However, TCAs and their relatively cardiotoxic metabolites tend to accumulate in the cardiac tissue of the heart which increase their cardiotoxic risks (Pollock and Perel, 1989; Prouty and Anderson, 1990; Wilens et al., 1992). Serum levels of TCAs higher than 500 ng/ml can cause toxic effects while concentrations higher than 1 µg/ml can be lethal (van Harten, 1993; Catterson et al., 1997; Preskorn, 1998). TCAs are metabolized via oxidation by microsomal cytochrome P450 (CYP) enzymes in the liver followed by conjugation with glucuronic acid (Baldessarini, 2006). There are several preferred CYP enzymes for the metabolism of TCAs which include CYP1A2, CYP2D6, CYP2C19 and CYP3A3/4 (Table 1.2) (Baldessarini, 2006; Mays, 2006). CYP1A2 and CYP2D6 are generally involved in aromatic hydroxylation while CYP3A3/4 are essential for the N-oxidation and N-dealkylation reactions (Baldessarini, 2006). Preferential oxidation of TCAs occurs at different positions depending on the antidepressant involved (Baldessarini, 2006). For example, oxidation of

imipramine and desipramine takes place at the 2-position while oxidation of amitriptyline and nortriptyline occurs at the 10-position (Baldessarini, 2006). The 10-hydroxy metabolites of amitriptyline and nortriptyline were found to be less cardiotoxic than the 2-hydroxy metabolites of imipramine and desipramine (Pollock and Perel, 1989; Baldessarini, 2006). Any residual biological activity of these metabolites are generally quenched by conjugation with glucuronic acid and subsequently excreted in the urine (Baldessarini, 2006; Rang et al., 2007). It is noted that the N-methylated derivatives of a few TCAs still possess pharmacological activity and could potentially affect their pharmacodynamics if they accumulate to levels close to or more than the parent drug (Baldessarini, 2006). Metabolism and excretion generally take place over several days and most TCAs are almost entirely eliminated within a week (Baldessarini, 2006). However, there are a few exceptions in which the elimination half-life of the N-methylated metabolites of SSRIs and most secondary-amine TCAs are around twice that of the parent drugs (van Harten, 1993; Baldessarini, 2006).

As mentioned earlier, the main mechanism of action of TCAs is to block the reuptake of monoamine neurotransmitters at the nerve terminals (Rang et al., 2007). They do so by competitive inhibition of the binding site of monoamine neurotransmitter transporter protein (Rang et al., 2007). TCAs generally do not directly affect the synthesis, storage and release of monoamine transmitters but some TCAs were found to block presynaptic alpha-2 adrenergic receptors and indirectly increase the release of noradrenaline (Rang et al., 2007). Most TCAs inhibit the noradrenaline and serotonin transporters while having much fewer effect on the dopamine

transporters (Richelson, 1996; Frazer, 1997b; Owens et al., 1997; Leonard and Richelson, 2000). It was postulated that the TCA-induced increase in serotoninergic transmission improves emotional indications while the increase in noradrenergic transmission modulates the biological symptoms of depression (Rang et al., 2007). Nonetheless, elucidation of the actual effects of TCAs continue to be challenging as the pharmacological activities of the main derivatives of TCAs are frequently different from the parent drug (Rang et al., 2007). Besides affecting the monoamine neurotransmitter transporter, most TCAs also interact with other receptors such as histamine receptors and muscarinic acetylcholine receptors which also contribute to their various adverse effects (Frazer, 1997b; Leonard and Richelson, 2000).

Antidepressants commonly have significant side effects and TCAs predominantly induce adverse autonomic responses due to their relatively potent anti-muscarinic properties (Baldessarini, 2006; Rang et al., 2007). Atropine-like effects of TCAs include blurred vision, dry mouth, epigastric distress, constipation, palpitations, urinary retention and dizziness whereas cardiovascular effects include sinus tachycardia and orthostatic hypotension (Baldessarini, 2006; Rang et al., 2007). Other unwanted effects include postural hypertension, weakness, fatigue and sedation (Baldessarini, 2006; Rang et al., 2007). Interaction with other drugs also increases the probability of TCA-induced adverse effects. TCAs were found to interact with a number of antihypertensive drugs and potentiate the effects of alcohol (Rang et al., 2007). In addition, drugs competing to bind to plasma proteins such as aspirin will increase the effects of TCA (Rang et al., 2007).

TCAs were the main drugs used in depression treatment until the later development of SSRIs (Baldessarini, 2006). Besides treating depression, TCAs have been used in treatment of pain. TCAs, together with anticonvulsants, are considered to be first-line drugs for the treatment of neuropathic pain (Mico et al., 2006). However, as stated previously, the exact mechanisms involved in the antinociceptive effects of TCAs especially in nociceptive pain remain unclear.

3.3.1. Amitriptyline

Amitriptyline is one of the first 'reference' TCAs and is sold under brand names such as Elavil and Enovil (Fig. 1.7) (Mays, 2006). Even though it has been more than 50 years since amitriptyline was synthesized and approved in 1961, it was found to have at least equal or better efficacy against depression as compared to the newer classes of antidepressants such as SSRIs and is still commonly used (Barbui and Hotopf, 2001; Fangmann et al., 2008). Amitriptyline is predominantly a SNRI with strong effects on the serotonin transporter and weaker effects on the noradrenaline transporter (Table 1.2) (Frazer, 1997b; Owens et al., 1997; Tatsumi et al., 1997; Leonard and Richelson, 2000). It has very weak effects on the dopamine transporter and, thus, does not affect the reuptake of dopamine (Frazer, 1997b; Tatsumi et al., 1997).

Besides treating depression, amitriptyline is used for conditions such as attention-deficit hyperactivity disorder (ADHD), tension headache and post-traumatic stress disorder (Lance and Curran, 1964; Davidson et al., 1990;

Banaschewski et al., 2004). Amitriptyline is also effective in modulating pain and symptoms in conditions such as post-herpetic neuralgia, diabetic neuropathy pain and ankylosing spondylitis (Watson et al., 1982; Max et al., 1987; Koh et al., 1997). In addition, amitriptyline was shown to have analgesic activity in a mouse model of acute pain and a dose-response analgesic effect in chronic pain in human patients (McQuay et al., 1993; Paudel et al., 2007). Amitriptyline is also commonly used for treatment of chronic neuropathic pain and fibromyalgia (Moore et al., 2012).

Patients taking amitriptyline have an increased risk for suicide and they need to be observed and monitored for clinical deterioration and suicidal tendencies (Murinson, 2013). Other side effects of amitriptyline include dry mouth and sedation (Baldessarini, 2006). Sedation caused by amitriptyline, however, can be used to provide greater relief and better sleep especially in patients who experience higher pain severity in the evening or at night (Nishishinya et al., 2008; Murinson, 2013).

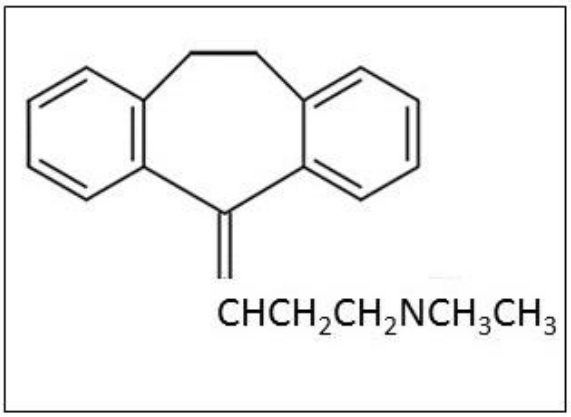


Fig. 1.7 Amitriptyline structure. Adapted from (Baldessarini, 2006)

3.3.2. Nortriptyline

Nortriptyline is a second generation TCA sold under brand names such as Aventyl and Pamelor (Fig. 1.8) (Mays, 2006). Nortriptyline is the active N-demethylated metabolite of amitriptyline and is similar to amitriptyline (Baldessarini, 2006). Nortriptyline functions as a SNRI with weak effects on the dopamine transporter (Baldessarini, 2006). However, nortriptyline has much stronger effects on the noradrenaline transporter and weaker effects on the serotonin transporter as compared to amitriptyline (Table 1.2) (Frazer, 1997b; Owens et al., 1997; Leonard and Richelson, 2000). Nortriptyline is also more selective for the inhibition of noradrenaline reuptake compared to amitriptyline (Fig. 1.10) (Baldessarini, 2006; Rang et al., 2007).

Nortriptyline is FDA-approved for the treatment of symptoms of MDD and besides treating depression, nortriptyline is also used in treatment of ADHD and irritable bowel syndrome (Wilens et al., 1993; Clouse, 2003; Murinson, 2013). In addition, nortriptyline has been shown to be effective in management for pain conditions such as chronic low back pain and is commonly used by pain specialists (Atkinson et al., 1998; Murinson, 2013). Nortriptyline showed comparable efficacy to morphine in post-herpetic neuralgia and has been found to have at least equal efficacy as gabapentin in treatment of diabetic polyneuropathy (Raja et al., 2002; Gilron et al., 2009). Furthermore, treatment with both nortriptyline and gabapentin led to better pain relief in patients (Gilron et al., 2009).

Similar to amitriptyline, patients treated with nortriptyline need to be monitored for clinical worsening and suicidal inclinations (Murinson, 2013).

However, the incidence of side effects associated with nortriptyline is lower compared to amitriptyline (Baldessarini, 2006; Mays, 2006). Nortriptyline also has lower sedating effects than amitriptyline (Murinson, 2013).

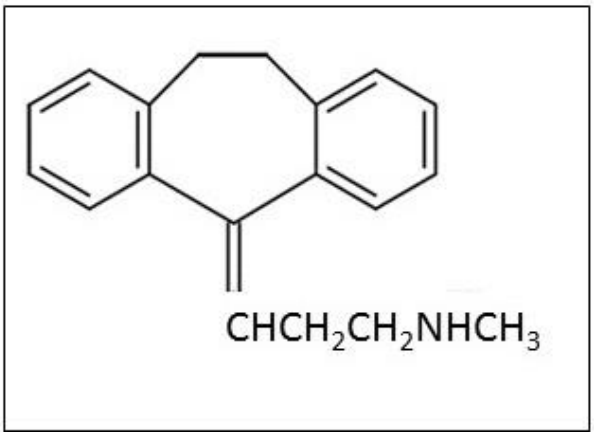


Fig. 1.8 Nortriptyline structure. Adapted from (Baldessarini, 2006)

3.3.3. Maprotiline

Maprotiline is a tetracyclic antidepressant with an extra ethylene bridge across the central six-carbon ring (Fig. 1.9). It is pharmacologically related to the TCAs and is, thus, also termed as a "tricyclic antidepressant". .It is sold under brand names such as Ludiomil and Psymion (Kessell and Holt, 1975; Baldessarini, 2006). Maprotiline primarily functions as a strong inhibitor of noradrenaline reuptake with robust effects on the noradrenaline transporter and much weaker effects on the serotonin and dopamine transporter (Table 1.2) (Baldessarini, 2006; Rang et al., 2007). Maprotiline has a higher selectivity for noradrenaline reuptake inhibition compared to both amitriptyline and nortriptyline (Fig. 1.10) (Rang et al., 2007).

Maprotiline is effective in treating different forms and severity of depression such as depressive neurosis (Kessell and Holt, 1975; Pinder et al., 1977). Studies also showed that maprotiline is effective in the treatment of chronic pain, chronic tension headache, painful polyneuropathy and chronic lower back pain (Fogelholm and Murros, 1985; Lindsay and Olsen, 1985; Vrethem et al., 1997; Atkinson et al., 1999). In addition, long-term combined treatment of maprotiline and diazepam significantly reduced the symptoms and signs of TMD (Ivkovic et al., 2008). Maprotiline was shown to be effective in post-herpetic neuralgia as well (Watson et al., 1992).

Maprotiline has comparable side effects as the other TCAs which include dry mouth, dizziness and constipation (d'Elia et al., 1981; Vaz-Serra et al., 1994; Baldessarini, 2006). Similar to amitriptyline and nortriptyline, maprotiline treatment was found to increase the risk of suicidal tendencies (Pinder et al., 1977). Adverse skin reactions occurred more frequently with maprotiline treatment (Pinder et al., 1977). However, results from controlled trials showed that there are very few differences between maprotiline and amitriptyline in the general incidence and severity of adverse effects (Pinder et al., 1977). Nonetheless, maprotiline has been reported to have a higher risk of causing seizures than the other TCAs (Skowron and Stimmel, 1992; Baldessarini, 2006).

The antidepressive effect of maprotiline was found to involve the phospholipase A_2 enzyme, $iPLA_2$, in the prefrontal cortex (Lee et al., 2012). Maprotiline treatment caused a significant increase in lysophosphatidylcholine levels coupled with a decrease in phosphatidylcholine levels, signifying increased PLA_2 activity and endogenous release of long chain polyunsaturated

fatty acids (Lee et al., 2012). Inhibition of $iPLA_2$ in the prefrontal cortex eliminated the change in lipid profile and abolished the antidepressive effect of maprotiline in a mouse forced swim test model of depression (Lee et al., 2012). The results suggest that prefrontal cortical $iPLA_2$ has an essential role in the antidepressive effect of maprotiline, potentially via the release and activity of long chain PUFAs (Lee et al., 2012). Thus, it is possible that the expression and activity of $iPLA_2$ in the prefrontal may also have a role in the antinociceptive effect of maprotiline.

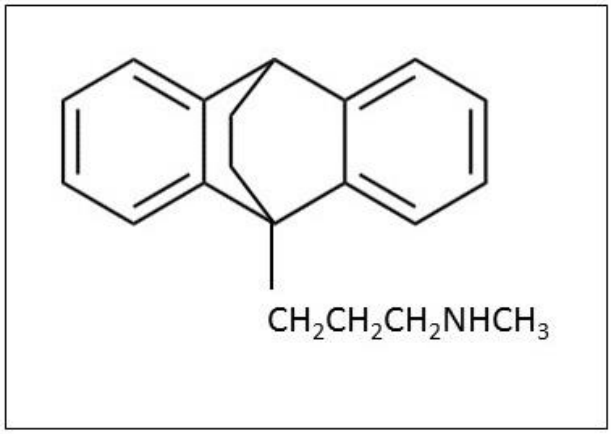


Fig. 1.9 Maprotiline structure. Adapted from (Baldessarini, 2006)

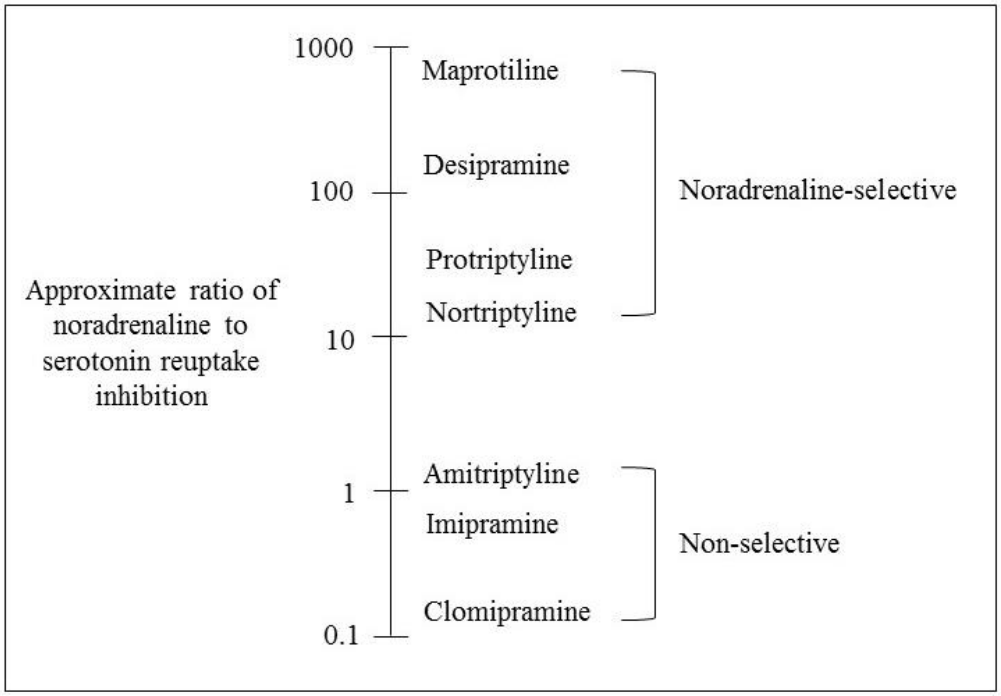


Fig. 1.10 Selectivity for inhibition of noradrenaline reuptake by several TCAs. Adapted from (Rang et al., 2007)

Table 1.2 Potencies and elimination profile of amitriptyline, nortriptyline and maprotiline based on radioactive ligand transport competition assays. Adapted from (Frazer, 1997b; Owens et al., 1997; Leonard and Richelson, 2000; Baldessarini, 2006).

Drug	**Potencies at monoamine neurotransmitter transporter[1]**			**Elimination profile**			
	Noradrenaline transporter	**Serotonin transporter**	**Dopamine transporter**	**Elimination half-life for parent drug (hours)**	**Elimination half-life for active metabolite (hours)**	**Usual serum concentrations (ng/ml)**	**Preferred CYP isozymes**
Amitriptyline	34.5	4.33	3200	16	30	100 – 250	2D6, 2C19, 3A3/4
Nortriptyline	4.35	18.5	1140	30	-	60 – 150	2D6, 2C19, 3A3/4
Maprotiline	11.1	5900	1000	48	-	200 – 400	2D6, 2C19, 3A3/4

1 Potency is expressed as inhibition constant (K_i) in nanomoles.

Chapter 2: Aims of Study

Aims of Study

Clinical studies found that certain classes of antidepressants are effective in the management of pain conditions including lower back pain, migraine, tension headache, atypical facial pain and chronic orofacial pain (Lynch and Watson, 2006). However, the biological substrates through which antidepressants exert their effects are largely unknown. Studies found that antidepressants that are effective in antinociception are most commonly those that act on the noradrenergic system, or noradrenergic and serotoninergic system, whereas those that act solely on the serotoninergic system are generally less effective (Sansone and Sansone, 2008; Verdu et al., 2008).

At the supraspinal level, the prefrontal cortex has been shown to be involved in antinociception and may also be affected by antidepressant treatment. The frontal cortex is richly innervated by aminergic fibers (Jordan et al., 1994; Carvalho et al., 2005), and is activated in acute and chronic pain (Peyron et al., 2000; Maihofner et al., 2004; Maihofner and Handwerker, 2005). Antinociception in rodents was found to involve electrical stimulation of prefrontal cortical fiber connections to the midbrain (Hardy and Haigler, 1985; Zhang et al., 1998).

Our recent study revealed an important role of prefrontal cortical $iPLA_2$ in the effectiveness of maprotiline to treat depression (Lee et al., 2012). Besides treating depression, maprotiline was also shown to induce an antinociceptive effect in pain conditions, including chronic lower back pain and TMD (Atkinson et al., 1999; Ivkovic et al., 2008). Thus, the present study was carried out with three main aims in mind. The first main aim is to study a

possible role of prefrontal cortical $iPLA_2$ in the antinociceptive effect of maprotiline and another TCA, amitriptyline. Secondly, we would like to investigate the effect of antidepressant treatment on $iPLA_2$ expression and determine the possible mechanisms involved in $iPLA_2$ expression. The third and final aim of this study is to examine the effect of antidepressant treatment on 15-LOX which metabolizes DHA to generate docosanoids such as resolvins (Farooqui, 2011). It is hoped that our results could provide insights into the brain regions involved in supraspinal antinociception as well as possible cellular mechanisms involved in $iPLA_2$ expression and the antinociceptive effect of antidepressants.

Chapter 3: Role of Prefrontal Cortical iPLA$_2$ in Antidepressant-Induced Antinociception

1. Introduction

The prefrontal cortex is part of the cerebral cortex which encompasses the anterior portion of the frontal lobe and includes Brodmann areas 9, 10, 11, 12, 46 and 47 (Fuster, 2008). In addition to playing an important role in executive functions, the prefrontal cortex has been shown to be involved in antinociception by correlation analysis of functional neuroimaging studies (Iadarola et al., 1998; Baron et al., 1999; Bornhovd et al., 2002). Activation of the dorsolateral prefrontal cortex by rTMS activation also increased pain tolerance in human experimental pain studies (Graff-Guerrero et al., 2005). Studies suggest that increased prefrontal cortical activity may modulate pain through projections to the midbrain to stimulate PAG and subsequent activation of the descending pain inhibitory pathway (Hardy and Haigler, 1985; Valet et al., 2004; Ohara et al., 2005; Xie et al., 2009). Similar to the brainstem and spinal cord, there are high levels of aminergic neurons and fibers in the prefrontal cortex (Jordan et al., 1994; Carvalho et al., 2005). Thus, the prefrontal cortex may also be affected by changes in the monoamine neurotransmitter levels after antidepressant treatment.

Antidepressants such as TCAs have been shown to be effective in the management of pain (Lynch and Watson, 2006). However, the biological substrates and the mechanisms involved in antidepressant-induced antinociception remain largely unknown. Our recent study showed an important role of prefrontal cortical iPLA$_2$ in the hippocampo-prefrontal cortical LTP and spatial working memory as well as in the antidepressive effect of maprotiline (Lee et al., 2012; Shalini et al., 2014). Besides its

antidepressive effects, maprotiline was also shown to possess antinociceptive properties in pain conditions such as TMD (Atkinson et al., 1999; Ivkovic et al., 2008). Thus, it is possible that prefrontal cortical iPLA$_2$ may similarly have an essential role in the antinociceptive effect of maprotiline and other similar antidepressants. In this part of the study, we investigated a possible role of prefrontal cortical iPLA$_2$ in the antinociceptive effect of maprotiline and another TCA, amitriptyline, using a carrageenan mouse model of inflammatory orofacial pain (Yeo et al., 2004; Poh et al., 2012). We also studied the effects of maprotiline treatment on prefrontal cortical iPLA$_2$ expression and the lipid profile of the prefrontal cortex.

Pain behavioral assay was first carried out to examine the potential antinociceptive effect of antidepressant treatment on pain responses and whether knockdown of prefrontal cortical iPLA$_2$ will affect the observed pain responses. The antinociceptive effect of maprotiline treatment was shown to be modulated by prefrontal cortical iPLA$_2$ knockdown. Another set of pain behavioral assay was then carried out after iPLA$_2$ knockdown at the somatosensory cortex to examine whether the effects of iPLA$_2$ knockdown on maprotiline treatment is specific to the prefrontal cortex. Real-time RT-PCR and Western blot analyses were performed to investigate the effect of maprotiline treatment on iPLA$_2$, specifically iPLA$_{2\beta}$, which is involved in brain DHA signaling and metabolism (Basselin et al., 2010). Western blot analysis was also carried out to validate the efficacy of iPLA$_2$ antisense oligonucleotide and this was followed by lipidomic analysis to study the effects of maprotiline treatment and iPLA$_2$ knockdown on the lipid profile of the prefrontal cortex.

2. Materials and methods

2.1. Experimental animals

A total of 80 male C57BL mice weighing between 20 – 30 g each and around 6 – 8 weeks old were used throughout this study. They were obtained from NUS Comparative Medicine Animal Facility and were housed under defined conditions (65 % relative humidity, 22 °C room temperature and 12 hours of lighting daily) with unrestricted access to water and food. All mice were randomized to treatment and all animal procedures were carried out in accordance to the approval and guidelines of the Institutional Animal Care and Use Committee of NUS.

2.2. Pain behavioral studies

2.2.1 Effect of antidepressant and oligonucleotide treatment on pain behavioral responses

Pain behavioral studies were first carried out to study the effect of antidepressant treatment and iPLA$_2$ knockdown on pain behavioral responses in a mouse model of inflammatory orofacial pain. There were two parts for the pain behavioral studies where the first part involved intracortical oligonucleotide injections at the dorsolateral prefrontal cortex while the second part involved oligonucleotide injections at the somatosensory cortex.

For the first part of the pain behavioral studies involving intracortical (i.c.) injections at the dorsolateral prefrontal cortex, mice were divided into

seven equal groups (six mice per group) and given daily intraperitoneal (i.p.) injections of maprotiline (two groups), amitriptyline (two groups) or saline (three groups) throughout the duration of the study (Fig. 3.1). For maprotiline treatment, the chosen dose of 10 mg/kg was based on previous studies which showed both behavioral and neurochemical changes in animal models after maprotiline administration (Parra et al., 2000; Tan et al., 2006; Lee et al., 2012). A dose of 10 mg/kg was likewise chosen for amitriptyline based on previous pain studies (Paudel et al., 2007). For the second part of the study involving somatosensory cortex (s.s.) injection, three groups of mice (six mice per group) were given daily i.p. maprotiline (two groups) or saline (one group) injections throughout the duration of the study (Fig. 3.1). Only one saline treatment group was used in the second part of the study as a baseline pain reference for comparison to the maprotiline treated groups after oligonucleotide injections at the somatosensory cortex and also to reduce the number of necessary animals used as accordance to the Institutional Animal Care and Use Committee of NUS guidelines. Our previous study had shown that the number of six mice per group was sufficient to display significant differences in nociceptive behavior between different treatment groups (Poh et al., 2011).

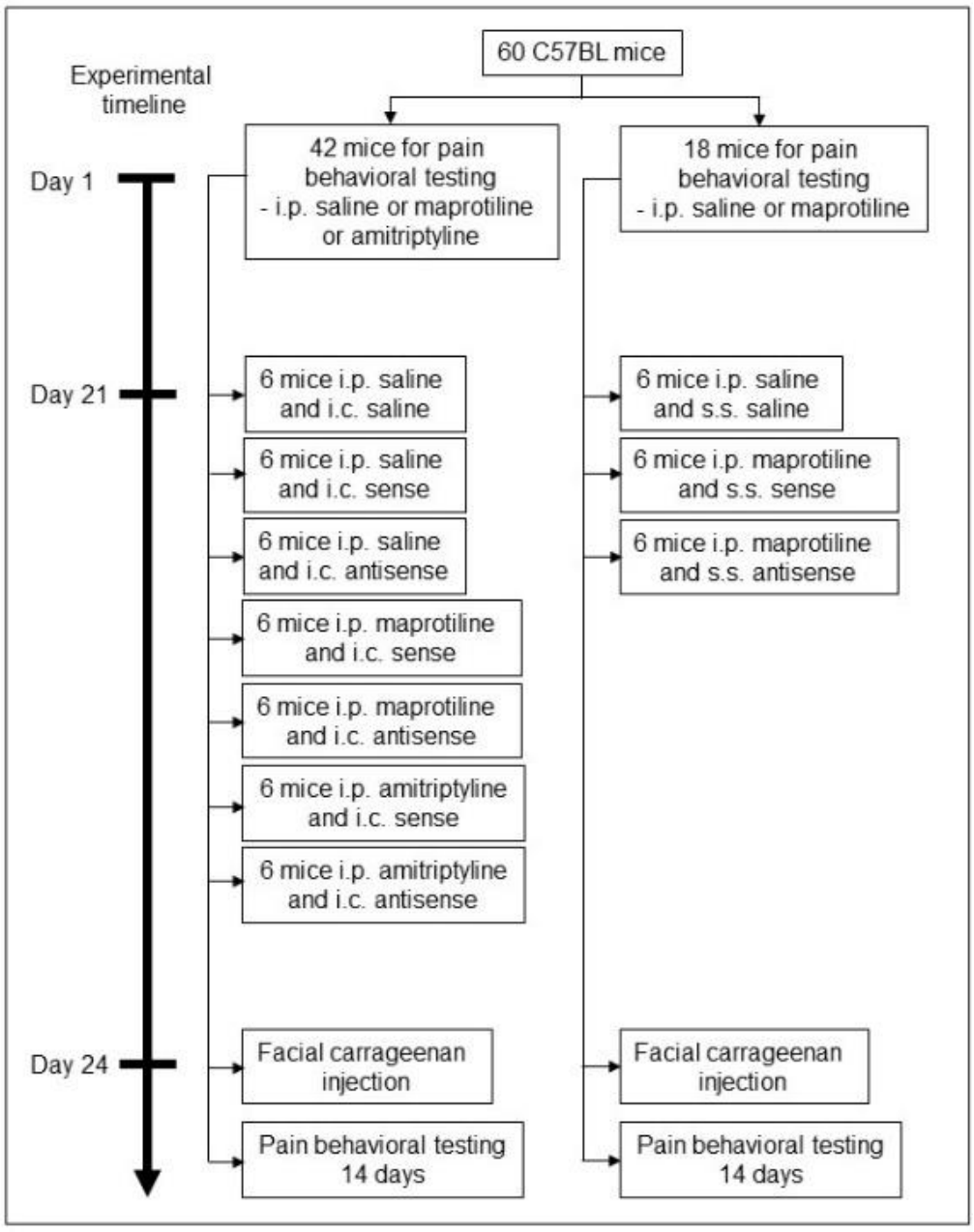


Fig. 3.1 Schematic flowchart of the experimental outline and animal grouping for the pain behavioral studies.

2.2.2 Dorsolateral prefrontal cortex intracortical (i.c.) oligonucleotide injection

On day 21 of maprotiline, amitriptyline or saline treatment, mice were anesthetized by i.p. injection of ketamine/medetomidine mixture and

positioned on a stereotaxic device (Stoelting, USA). Dorsolateral prefrontal cortex intracortical (i.c.) oligonucleotide injections were carried out one hour after maprotiline, amitriptyline or saline treatment. Antisense oligonucleotide (3 µg/µl), sense oligonucleotide (3 µg/µl) or sterile saline was stereotaxically administered into the right and left sides of the dorsolateral prefrontal cortex through small craniotomies on the skull (coordinates: 2.5 mm rostral to bregma, 1.5 mm lateral to midline and 1.5 mm from surface of cortex). A total volume of 1 µl was injected over 5 minutes on each side and the scalp was sutured after the injection was done. The antisense oligonucleotide used was a 20-base oligonucleotide which corresponds to nucleotides 59–78 of the murine iPLA$_2$ sequence (5'-CTCCTTCACCCGGAATGGGT-3') and was shown to reduce iPLA$_2$ expression in mouse P388D1 macrophage-like cells (Balsinde et al., 1997). Our previous study also showed the effectiveness of the iPLA$_2$ antisense oligonucleotide in decreasing iPLA$_2$ expression in the mouse prefrontal cortex (Lee et al., 2012). Scrambled sense oligonucleotide was used as a control (5'-ACCCATTCCGGGTAAAGGAG-3'). Both antisense and sense oligonucleotides contained phosphorothioate linkages to prevent nuclease degradation.

2.2.3. Somatosensory cortex (s.s.) oligonucleotide injection

The procedures and timeline for somatosensory cortex oligonucleotide injections were similar to that of the prefrontal cortex injections, except that maprotiline or saline treated groups received sense or antisense oligonucleotides or saline in the somatosensory cortex at coordinates: 1.5 mm

caudal to bregma, 3 mm lateral to the midline and 1.5 mm from the surface of the cerebral cortex.

2.2.4. Facial carrageenan injection and pain behavioral assay

On day 24 of antidepressant or saline treatment, mice were first anesthetized and subsequently injected with 50 µL carrageenan (2 mg/50 µL of lambda carrageenan) in the subcutaneous tissue over the left maxillary, ophthalmic and mandibular region. Carrageenan administration resulted in a constant swelling of approximately 4 mm in diameter and induced allodynia in the days following the injection as shown in our previous study (Poh et al., 2012). Mice were tested for pain behavioral responses before carrageenan injection, and once daily after carrageenan injection at one hour after daily antidepressant or saline i.p. treatment. Testing of pain behavioral responses was performed in a blinded manner and was carried out individually in a deep rectangular tank, sized (L x W x H) 60 × 40 × 25 cm (Vos et al., 1994; Poh et al., 2009). A von Frey hair filament (Touch-Test Sensory Evaluator, North Coast Medical, Morgan Hill, USA) delivering 1.4 g force (4.17 log units = 13.725 mN) was used to test the pain behavioral responses. The von Frey hair was first reached into the tank for 5 – 10 minutes to ensure that the mice were familiarized with the reaching movements before testing. Mice were observed to confirm that they were able to move without restrictions during this period of time. Test stimuli using the von Frey hair was administered when the mice were neither freezing nor moving and with all four paws placed on the ground while displaying sniffing behavior. To acquire an adequate number of

responses for the reduction of variability among individual mice in each treatment group, the carrageenan-injected area of the face was probed 20 times with the von Frey hair. Each new stimulus with the von Frey hair was administered at least 30 seconds after the previous administration. Directed facial grooming (a continuous series of facial wash strokes directed to the stimulated facial area by the mice) was utilized as an indicator of unilateral facial pain in freely moving mice. The number of immediate asymmetric facial grooming/scratching strokes was then obtained to calculate the total number of facial strokes displayed by each animal after 20 stimulations using the von Frey hair filament. The mean and standard error of total facial strokes were calculated and possible significant differences studied using two-way mixed ANOVA to analyze the interactions between treatment and time on the mean pain behavioral responses, followed by two-tailed Student's t-tests to compare the possible significant differences in the mean pain behavioral responses between the different treatment groups at each time point (IBM SPSS Statistics, Version 20.0, New York, USA). $P < 0.05$ was considered significant.

2.3. Effect of maprotiline treatment on iPLA$_2$ mRNA and protein expression in the prefrontal cortex

This part of the study was then carried out to investigate the effect of maprotiline treatment on prefrontal cortical iPLA$_2$ mRNA and protein expression. Two groups of mice (four mice per group) were given daily i.p. injections of either maprotiline (Sigma, St. Louis, USA) or saline for 28 days.

After 28 days, mice were sacrificed by i.p. injection of ketamine/medetomidine cocktail and decapitated, and the prefrontal cortex dissected out for subsequent Real-time RT-PCR and Western blot analyses (Fig. 3.2).

2.4. Effect of iPLA$_2$ knockdown on iPLA$_2$ protein expression and lipid profile

The antisense oligonucleotide to iPLA$_2$ being used in this study was previously shown to successfully knockdown iPLA$_2$ expression in the normal mouse prefrontal cortex (Lee et al., 2012). Thus, this part of the study was carried out to further validate the efficacy of this antisense oligonucleotide to similarly reduce iPLA$_2$ levels in maprotiline-treated mice using Western blot analysis. Lipidomic analysis was also performed to study the effects of maprotiline treatment and iPLA$_2$ knockdown on the lipid profile of the prefrontal cortex (Fig. 3.2). Three groups of mice (four mice per group) were given daily i.p. injections of either maprotiline (two groups) or saline (one group). On day 21 of i.p. injection, saline-treated mice received intracortical injection of normal saline to the prefrontal cortex, while one group of maprotiline-treated mice was intracortically injected with iPLA$_2$ antisense oligonucleotide, and the other with scrambled sense oligonucleotide. Facial carrageenan injection was then administered to the above mice on day 24 of i.p. injection. Pain behavioral response testing was carried out daily until the time of sacrifice on 4 days after carrageenan injection. The prefrontal cortex was harvested for Western blot analysis to verify the efficacy of antisense oligonucleotide to iPLA$_2$ and for lipidomic analysis.

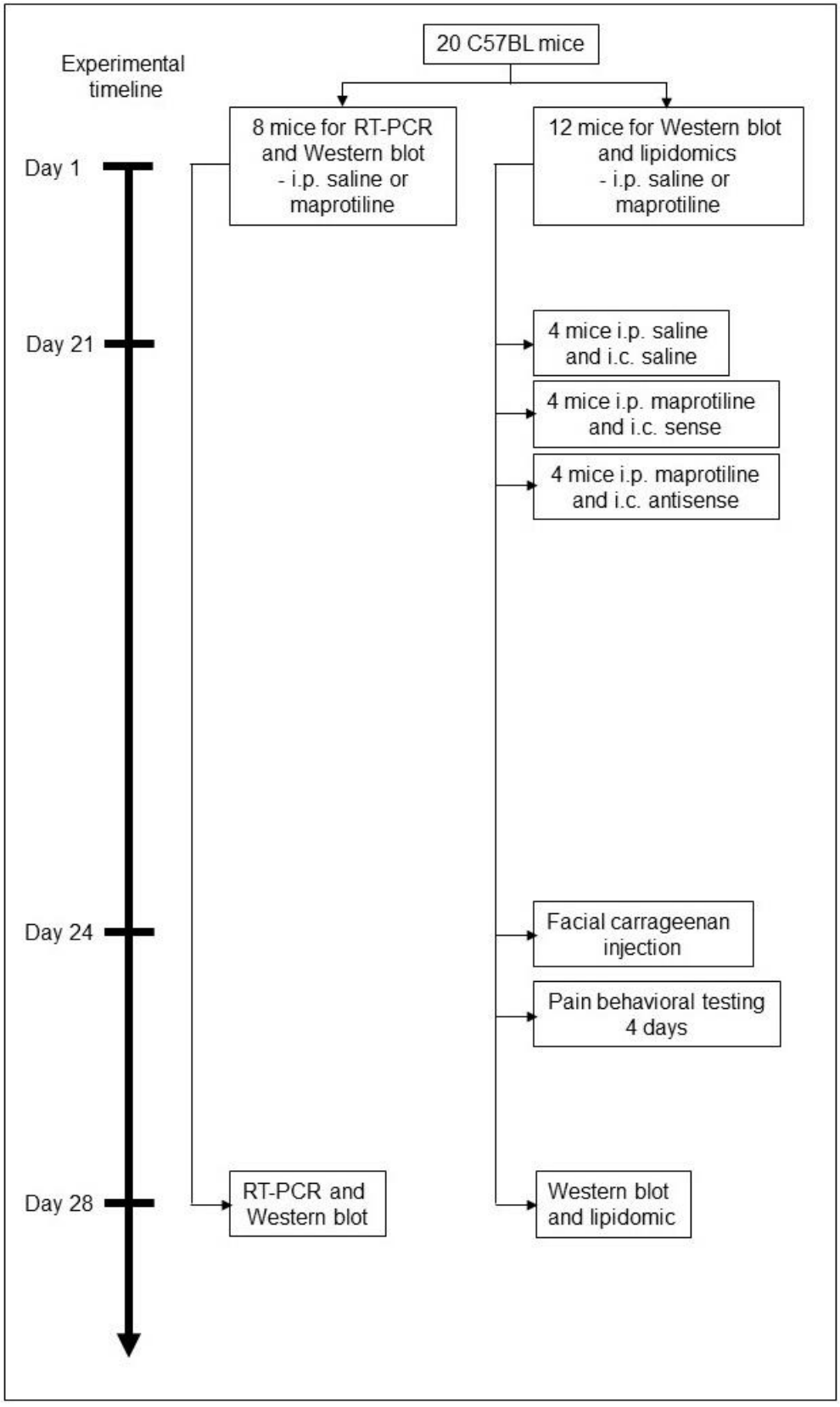


Fig. 3.2 Schematic flowchart of the experimental outline and animal grouping for the real-time RT-PCR, Western blot and lipidomic analyses.

2.5. Real-time RT-PCR

RNA was extracted from the prefrontal cortex and reverse transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, CA, USA). The conditions for the reverse transcription reactions were 25 °C for 10 minutes followed by 37 °C for 120 minutes and 85 °C for 5 minutes. Quantitative real-time PCR amplification was then performed in the 7500 Real-time PCR system (Applied Biosystems, CA, USA) using the converted cDNA together with TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA) and TaqMan® probes for mouse iPLA$_{2\beta}$ and beta-actin (Mm01299491_m1 and #4331182 respectively, synthesized by Applied Biosystems). The conditions for real-time PCR amplification were initial incubation at 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All amplification reactions were carried out in triplicates. The number of reaction cycles at which the reporter fluorescence emission exceeds the preset threshold level is the threshold cycle, CT. There is an inverse correlation between the CT value and the levels of target mRNA. The amplified transcripts were then quantified using the comparative CT method with the formula for relative fold change = $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The mean and standard error for each treatment group were calculated and possible significant differences analyzed using two-tailed Student's t-test. $P < 0.05$ was considered significant.

2.6 Western blot analysis

Proteins were first extracted from the prefrontal cortex using T-PER® Tissue Protein Extraction solution containing 1 % Halt^{TM} protease inhibitor and 1 % EDTA solution (Thermo Fischer Scientific, Rockford, IL, USA). Concentrations of the protein obtained were measured using the Bio-Rad protein assay kit. Protein samples (30 µg) were then mixed with a loading dye consisting of SDS and DTT before undergoing denaturation at 95 – 100 °C for 10 minutes and resolved in 10 % SDS-polyacrylamide gels under reducing conditions. A protein ladder containing molecular weight of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa was also used to monitor the electrophoresis run and to check for protein size as well as protein transfer efficacy (Precision Plus Protein Dual Color Standards, Bio-Rad Laboratories, CA, USA). The resolved proteins were then electrotransferred to a polyvinylidene difluoride (PVDF) membrane and non-specific binding sites were blocked by incubation for 1 hour with 5 % non-fat milk in tris-buffered saline containing 0.1 % Tween-20 (TBST). After blocking, the PVDF membrane was incubated overnight with iPLA$_2$ antibody (#sc-14463, Santa Cruz Biotechnology, CA, USA; diluted 1:2000 in 5 % non-fat milk in TBST) at 4 °C. The PVDF membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated anti-goat IgG (Thermo Fisher Scientific, Rockford, IL, USA; diluted 1:2000 in 5 % non-fat milk in TBST) for 1 hour at room temperature. The protein bands were visualized with an enhanced chemiluminescence kit (Supersignal West Pico, Thermo Fisher Scientific,

Rockford, IL, USA). For the loading controls, the membrane was incubated with a stripping buffer for 10 minutes at room temperature (Restore Western Blot Stripping Buffer, Thermo Fisher Scientific, Rockford, IL, USA). After stripping, the membrane was again blocked with 5 % non-fat milk in TBST before incubating with a mouse monoclonal antibody to beta-actin (Sigma-Aldrich, St. Louis, MO, USA; diluted 1:10000 in 5 % non-fat milk in TBST) for 1 hour at room temperature. The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL, USA; diluted 1:10000 in 5 % non-fat milk in TBST) for 1 hour. Visualization of the protein bands were then carried out as mentioned earlier. Visualized films containing the protein bands were scanned and their densities were measured using the Gel-Pro Analyzer 3.1 program (Media Cybernetics, Silver Spring, MD, USA). The densities of the target bands were normalized against those of beta-actin. The mean and standard error were then calculated and possible significant differences analyzed using two-tailed Student's t-test or one-way ANOVA with Bonferroni multiple comparison post-hoc test. $P < 0.05$ was considered significant.

2.7. Lipidomic analysis

Prefrontal cortex tissue samples were homogenized using a sonicator for 30 minutes at 4 ºC in 750 µL of chloroform/methanol, 1:2 (v/v) and 5 µL of an internal standards solution, containing N-lauroyl-D-erythro-sphingosylphosphorycholine, N-heptadecanoyl-D-erythro-sphingosine and 1,2-dimyristoyl-sn-glycero-3-phosphocholine, all from Avanti Polar Lipids

(Alabaster, AL, USA). Samples were then mixed with 250 µL chloroform and 450 µL 0.88 % potassium chloride (KCl), vortexed and centrifuged at 9000 g at 4 °C for 2 minutes. The aqueous phase was re-extracted with 250 µl of chloroform and the extracted organic fractions pooled. Lipids were isolated from the organic phase, vacuum-dried (Thermo Savant SpeedVac, USA), and resuspended for analyses. An Agilent 1290 UPLC system connected with an Agilent 6460 Triple Quadrupole mass spectrometer (Santa Clara, CA, USA) was used for quantification of individual polar lipids. The column used was a Kinetex HILIC (150 X 2.10 mm) packed with 2.6 µM core-shell particles from Phenomenex (Torrance, CA, USA). Solvents used for the HILIC LC: 50 % acetonitrile in water containing 25 mM ammonium formate pH 4.6 (solvent A) and 95 % acetonitrile containing 25 mM ammonium formate H 4.6 (solvent B). Analytes were eluted with the following gradient: 0.1 % solvent A and 99.9 % solvent B from 0 to 6 minutes, 75 % solvent A and 25 % solvent B from 6 to 7 minutes, 90 % solvent A and 10 % solvent B from 7 to 7.1 minutes, and 0.1 % solvent A and 99.9 % solvent B from 7.1 to 10.1 minutes with a constant flow rate of 0.5 mL/minute. MS source parameters: gas temperature 300 ºC with a flow of 5 L/minute and nebulizer at 45 psi. Sheath gas temperature 250 ºC with a gas flow rate of 11 L/minute. The Agilent 6460 triple quadrupole was operated in positive mode for Multiple Reaction Monitoring (MRM). In the positive ion MRM mode, product ions at 184 m/z were monitored after CID of the lipid precursors for both cholines and sphingomyelins. Product ions at 262, 264 and 266 m/z were monitored for d18 ceramides. Quantification was performed according to the internal standard method, comparing peak areas of the sample to the ISTD. All samples were

resuspended in solvent B before injecting the sample for LC-MS analyses. Data were extracted and analyzed using Agilent MassHunter Qualitative and Agilent MassHunter Quantitative software (Santa Clara, CA, USA). The mean and standard error for each lipid species were calculated and then analyzed using one way ANOVA with Bonferroni multiple comparison post-hoc test to check for possible significant differences. $P < 0.05$ was considered significant.

3. Results

3.1. Pain behavioral studies

3.1.1. Antidepressant and prefrontal cortex oligonucleotide treatment groups

A higher number of facial strokes indicate more pain and a higher pain behavioral response. Responses of the saline + iPLA$_2$ antisense oligonucleotide, saline + scrambled sense oligonucleotide and saline + saline groups showed a similar trend and all three peaked at day 4 after carrageenan injection. Responses of the amitriptyline + antisense and amitriptyline + sense treatment groups also showed a similar trend and peaked at day 4 after carrageenan injection. Responses of the maprotiline + antisense oligonucleotide treatment peaked at day 5 while the maprotiline + sense oligonucleotide treatment peaked at day 3 after carrageenan injection. The maprotiline + antisense treatment group had a higher trend in pain behavior as compared to the maprotiline + sense treatment group. This trend by the maprotiline + antisense group was similar to that of the saline treatment groups (Fig. 3.3).

Two-way ANOVA indicated that the within subject effects were significant, $F(14, 490) = 304.688$, $P < 0.001$. This showed that there was a significant interaction between treatment and time on the pain behavioral responses. Further two-tailed Student's t-test carried out to compare between the treatment groups showed no significant differences between the saline + saline and saline + antisense groups, indicating that antisense $iPLA_2$ treatment to the prefrontal cortex in the absence antidepressant treatment did not have any effect on pain behavior (Fig. 3.3).

Similar to that of saline-treated mice, there were no significant differences between the two amitriptyline treatment groups throughout the duration of the study, which indicate that antisense treatment to the prefrontal cortex did not have any effect on the pain behavior of amitriptyline-treated mice. However, there were significant differences between both amitriptyline treatment groups and all three saline treatment groups from days 4 to 14 after carrageenan injection, highlighting the antinociceptive effect of amitriptyline treatment ($P < 0.05$) (Fig. 3.3).

The maprotiline + sense treatment group had significantly lower pain behavioral responses from days 4 to 14 after carrageenan injection as compared to all three saline treatment groups (Fig. 3.3). Interestingly, the number of responses of maprotiline + antisense treatment group was similar to that of the saline treatment groups and had significantly greater responses compared to the maprotiline + sense treatment group from days 4 to 12 after carrageenan injection ($P < 0.05$) (Fig. 3.3). Overall, the maprotiline + sense treatment group showed a lower trend in the number of facial strokes as

compared to the other groups. The results indicate that maprotiline exerts an antinociceptive effect in the facial carrageenan pain model. Antisense oligonucleotide to iPLA$_2$ treatment was found to abolish this antinociceptive effect and return the facial stroke values similar to that of saline treatment groups. The maprotiline + sense treatment group also showed significantly lower number of facial strokes as compared to both amitriptyline treatment groups on days 4, 5, 7, 9 and 12 after carrageenan injection which suggest that maprotiline treatment has a relatively stronger antinociceptive effect in this carrageenan mouse model of inflammatory orofacial pain.

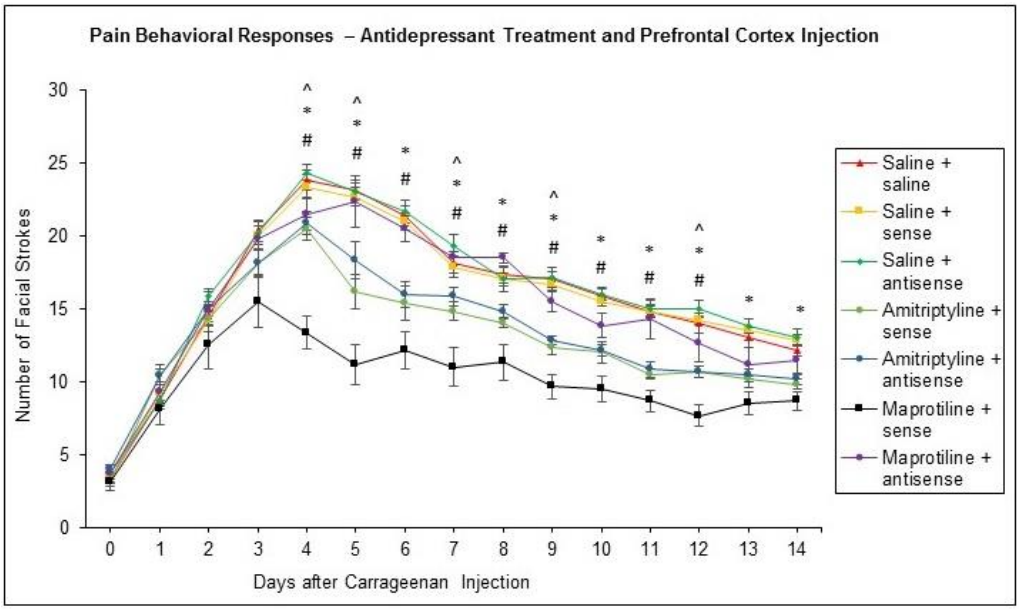


Fig. 3.3 Pain behavioral responses – antidepressant treatment and prefrontal cortex injection. Responses to von Frey hair stimulation after prefrontal cortex injection and facial swelling induced by carrageenan administration in addition to daily intraperitoneal injection of maprotiline (10 mg/kg), amitriptyline (10mg/kg) or saline. The Y-axis represents number of responses to von Frey hair stimulation of the carrageenan-injected areas of the face. Day 0 refers to the day before carrageenan injection was carried out while Day 1 to Day 14 refers to 1 day to 14 days after injection. Asterisks (*) indicate significant differences at $P < 0.05$ between the two amitriptyline treatment groups and all three saline treatment groups as well as significant differences at $P < 0.05$ between the maprotiline + sense treatment group and all three

saline treatment groups. Symbol (#) indicate significant differences at $P < 0.05$ between maprotiline + sense treatment group and maprotiline + antisense treatment group. Symbol (^) indicate significant differences at $P < 0.05$ between maprotiline + sense treatment group and both amitriptyline treatment groups. Data represent the mean and standard error of six mice per treatment group.

3.1.2. Maprotiline and somatosensory cortex oligonucleotide treatment groups

Responses of the saline + saline treatment group peaked at day 4 after carrageenan injection. Responses of both maprotiline treatment groups showed a similar trend and peaked at day 4 after carrageenan injection. Both maprotiline treatment groups also showed a lower trend as compared to the saline + saline treatment group. Two way ANOVA indicated that the within subject effects were significant, $F(14, 210) = 110.830$, $P < 0.001$. This showed that there was a significant interaction between treatment and time on the pain behavioral responses. Further two-tailed Student's t-test carried out to compare the treatment groups at each time point showed significant differences between the two maprotiline treated groups and the saline + saline group from days 4 to 14 after carrageenan injection, indicating antinociceptive effect of maprotiline treatment ($P < 0.05$). However, in contrast to $iPLA_2$ antisense oligonucleotide injection in the prefrontal cortex, no significant difference was detected between the maprotiline + antisense and maprotiline + sense treatment group, after oligonucleotide injection to the somatosensory cortex (Fig. 3.4). The results suggest that unlike the prefrontal cortex, administration of $iPLA_2$ antisense oligonucleotide to the somatosensory cortex did not affect the antinociceptive effect of maprotiline.

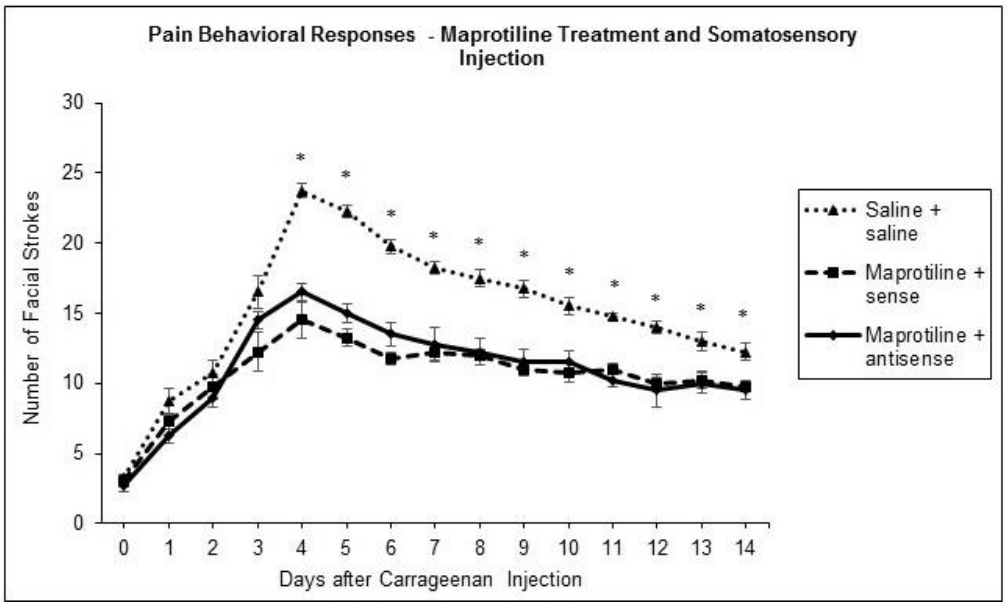


Fig. 3.4 Pain behavioral responses - maprotiline treatment and somatosensory injection. Responses to von Frey hair stimulation after somatosensory cortex oligonucleotide injections and facial swelling induced by carrageenan administration in addition to daily intraperitoneal injection of maprotiline (10 mg/kg) or saline. Asterisks (*) indicate significant differences at $P < 0.05$ between the saline + saline group and both maprotiline treatment groups. Data represent the mean and standard error of six mice per treatment group.

3.2. Effect of maprotiline treatment on prefrontal cortical iPLA$_2$ expression

3.2.1. Real-time RT-PCR

Quantitative real-time RT-PCR using the iPLA$_{2\beta}$ probe showed a significant 1.59 fold increase in iPLA$_2$ mRNA expression in the mouse prefrontal cortex after maprotiline treatment for 28 days as compared to saline treatment ($P < 0.05$) (Fig. 3.5). The results indicate that maprotiline treatment increased iPLA$_2$ mRNA expression in the prefrontal cortex.

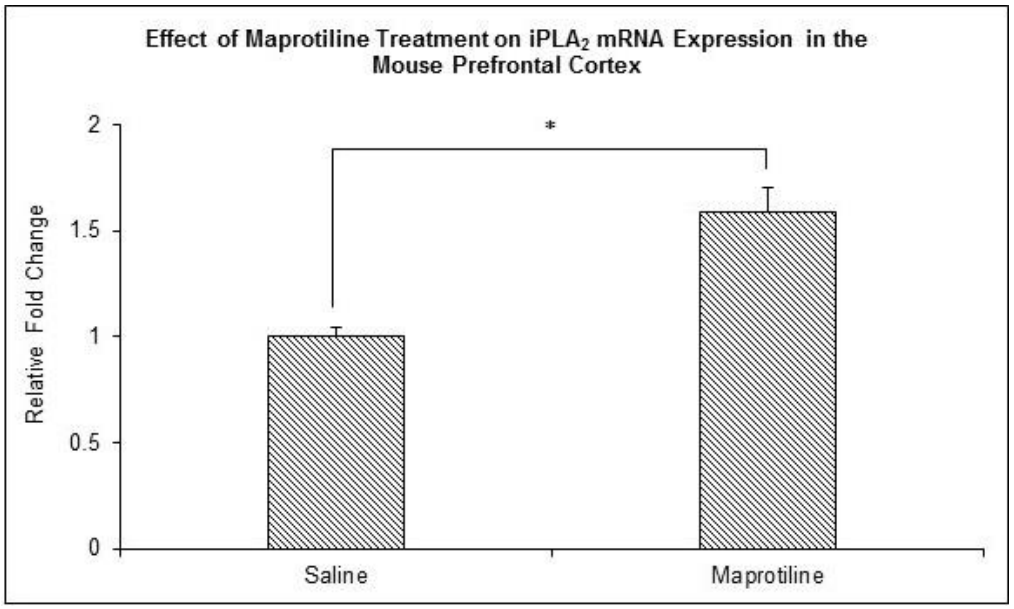


Fig. 3.5 Effect of maprotiline treatment on iPLA$_2$ mRNA expression in the mouse prefrontal cortex. Real-time RT-PCR analysis on iPLA$_2$ expression in the mouse prefrontal cortex after maprotiline treatment. Data represent the mean and standard error of four mice per treatment group. Asterisks (*) indicate significant differences at $P < 0.05$ when compared to saline control.

3.2.2. Western blot analysis

Western blot analysis of the mouse prefrontal cortex showed the presence of a band at around 85 kDa which corresponds to the band size of the iPLA$_2$ protein. Densitometric analyses of the protein blots showed a significant 4.2 fold increase in iPLA$_2$ protein expression in the mouse prefrontal cortex after maprotiline treatment for 28 days as compared to saline treatment ($P < 0.05$) (Fig. 3.6). The increase in iPLA$_2$ protein expression is consistent with the increase in iPLA$_2$ mRNA expression in the prefrontal cortex after maprotiline treatment.

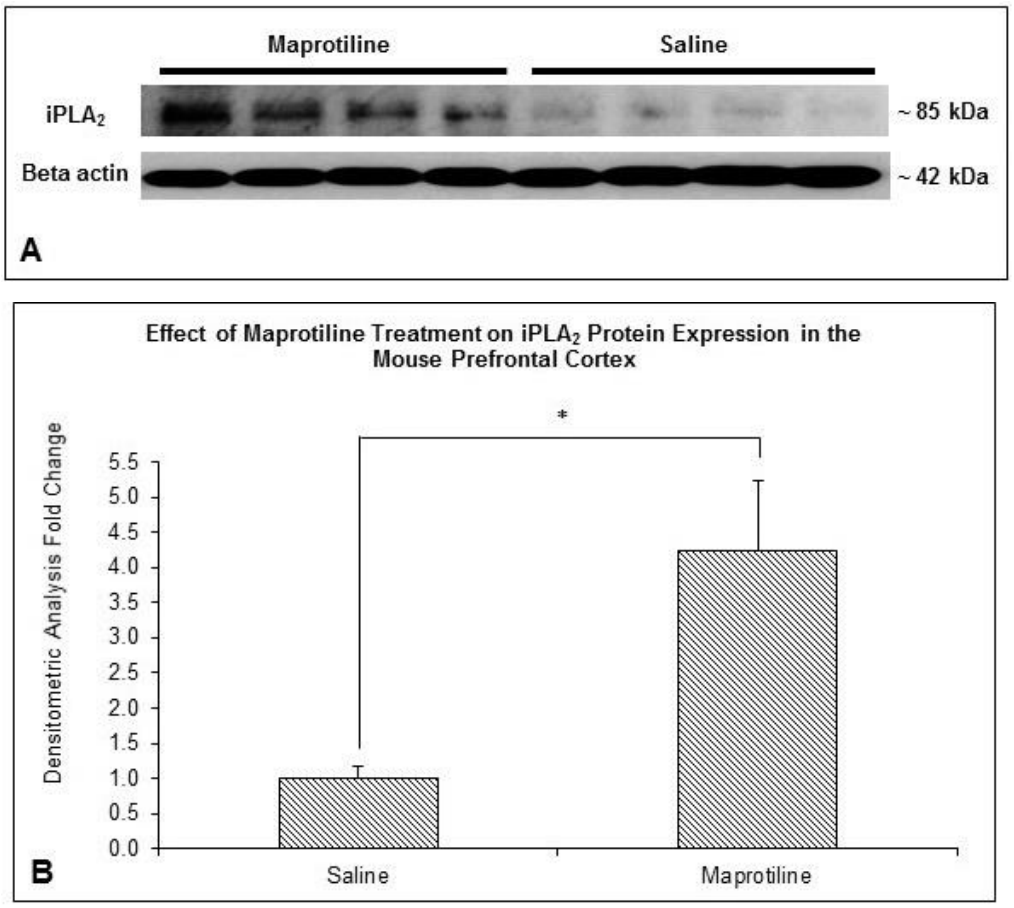


Fig. 3.6 Effect of maprotiline treatment on iPLA$_2$ protein expression in the mouse prefrontal cortex. (A) Western blot and (B) densitometric analysis of iPLA$_2$ protein expression in the mice prefrontal cortex after antidepressant treatment. Asterisks (*) indicate significant differences at $P < 0.05$ when compared to saline control.

3.3. Effects of maprotiline treatment and prefrontal cortical iPLA$_2$ knockdown on iPLA$_2$ protein expression and lipid profile

3.3.1. Pain behavioral responses

Responses of the maprotiline + antisense group and the saline + saline group showed a similar trend up to day 4 after carrageenan injection. Responses of the maprotiline + sense group showed a peak at day 3 after

carrageenan injection similar to the responses in Fig. 3.3 and the responses at day 4 was lower compared to the maprotiline + antisense group and the saline + saline group. Two way ANOVA indicated that the within subject effects were significant, $F(4, 36) = 546.781$, $P < 0.001$. This showed that there was a significant interaction between treatment and time on the pain behavioral responses. Further two-tailed Student's t-test carried out to compare between the treatment groups at each time point showed a significant difference between the maprotiline + antisense treatment group and saline + saline treatment group and also between the maprotiline + antisense treatment group and the maprotiline + sense treatment group on day 4 (Fig. 3.7) after carrageenan injection similar to the results in Fig. 3.3 ($P < 0.05$). The results in Fig 3.7 thus importantly showed the reproducibility of the pain behavioral responses as seen in Fig 3.3.

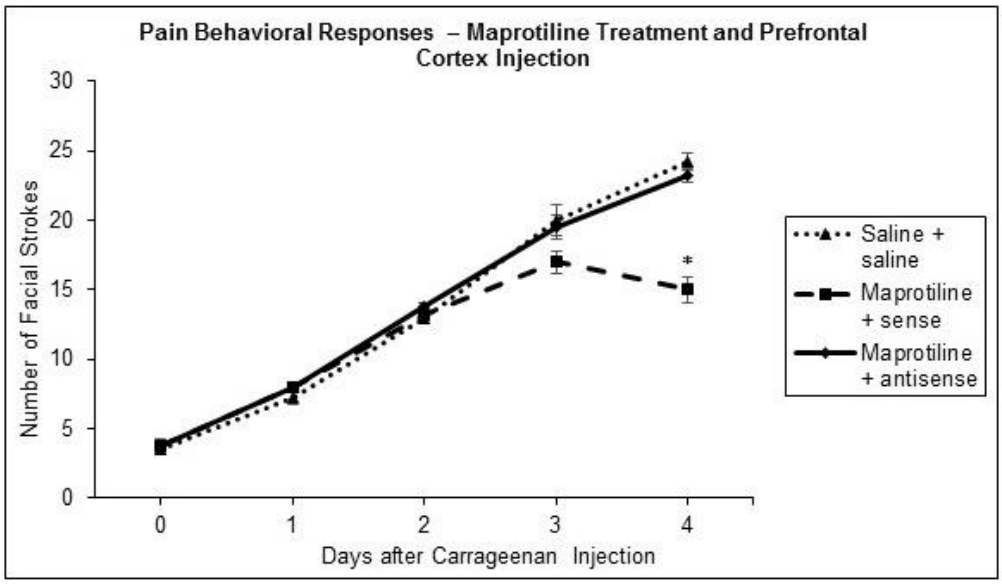


Fig. 3.7 Pain behavioral responses – maprotiline treatment and prefrontal cortex injection. Responses to von Frey hair stimulation after prefrontal cortex injection and facial swelling induced by carrageenan administration at up to day 4 after carrageenan injection and after daily intraperitoneal injection of maprotiline (10 mg/kg). Data represent the mean and standard error of four mice per treatment group. Asterisks (*) indicate significant differences at $P < 0.05$ when comparing maprotiline + sense treatment group to both saline + saline and maprotiline + antisense treatment groups.

3.3.2. Western blot analysis

Western blot analysis of the mouse prefrontal cortex similarly showed the presence of a band at around 85 kDa which corresponds to the band size of the iPLA$_2$ protein. Densitometric analyses of the protein blots showed a significant 3.0 fold increase in iPLA$_2$ protein expression in the mouse prefrontal cortex after maprotiline + sense treatment as compared to the saline + saline treatment ($P < 0.05$) (Fig. 3.8). This result is consistent with the increase in iPLA$_2$ mRNA and protein after maprotiline treatment observed earlier. Administration of antisense oligonucleotide to iPLA$_2$ to maprotiline treated mice, however, significantly abolished the increase in iPLA$_2$ protein

expression. Results confirmed the effectiveness of antisense oligonucleotide to iPLA$_2$ in reducing iPLA$_2$ protein expression in maprotiline treated mice even at day 4 after carrageenan injection.

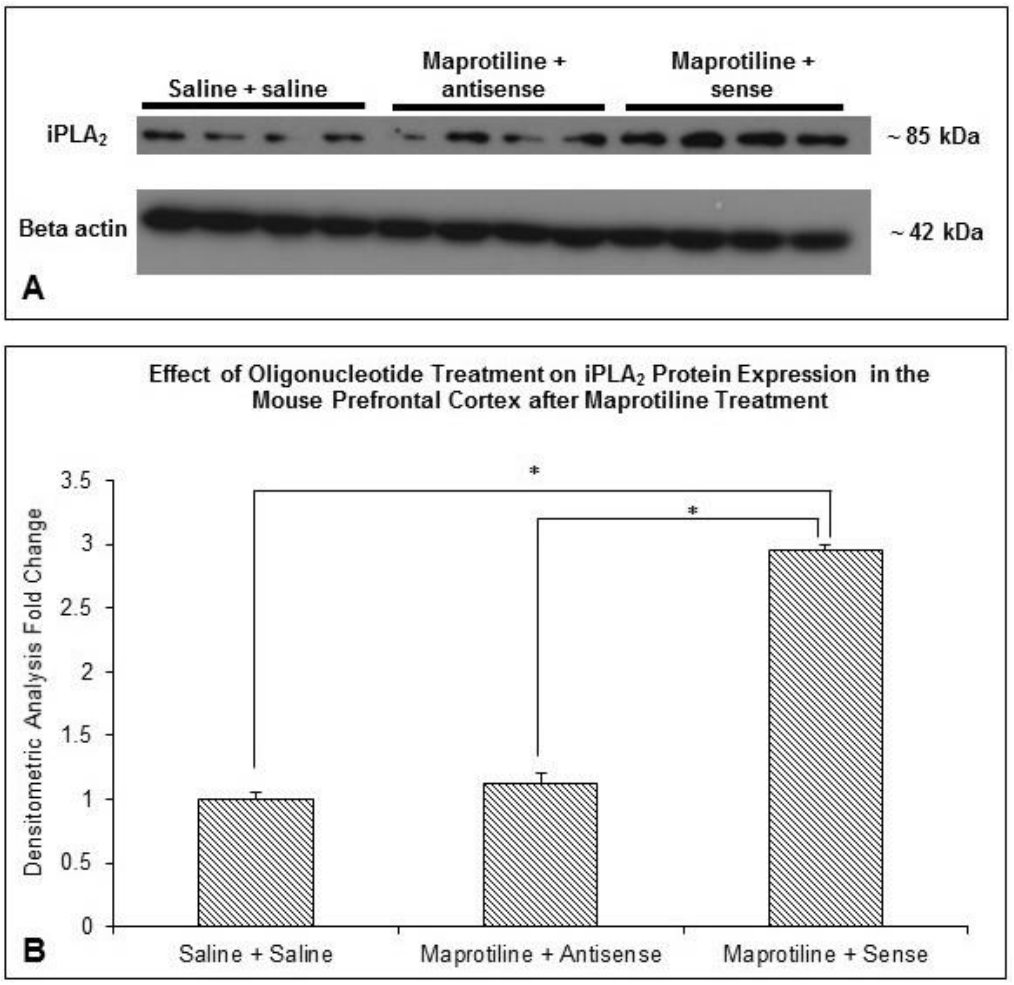


Fig. 3.8 Effect of oligonucleotide treatment on iPLA$_2$ protein expression in the mouse prefrontal cortex after maprotiline treatment. (A) Western blot and (B) densitometric analysis on the effect of oligonucleotide treatment on iPLA$_2$ protein expression in the mouse prefrontal cortex after maprotiline treatment. Asterisks (*) indicate significant differences at $P < 0.05$.

3.3.3. Lipidomic analysis

Lipidomic analysis results showed that maprotiline treatment had significant effects on several different lipid species which was abolished by antisense oligonucleotide to iPLA$_2$ treatment. There was a significant increase in the PC 32:0 lipid species after maprotiline + sense treatment as compared to the other treatment groups, but concentrations of PC 36:1, PC 36:3, PC 38:3, PC 38:5, PC 40:4, PC 40:5 and PC 40:6 were significantly reduced after maprotiline + sense treatment ($P < 0.05$). Treatment with maprotiline + antisense abolished the changes in phosphatidylcholine lipid species (Fig. 3.9). Concurrently, maprotiline + sense treatment caused a significant increase in the LPC 16:0, LPC 18:0, 18:1 and LPC 20:4 species, and these were similarly abolished after treatment with antisense oligonucleotide ($P < 0.05$) (Fig. 3.10). The general decrease in PC species levels and the increase in LPC levels indicate an increased iPLA$_2$ enzymatic activity as iPLA$_2$ hydrolyzes PCs to form LPCs and fatty acids. The fatty acids produced was found to include DHA as shown by the decrease in PC 40:6 and the increase in LPC 18:0 to release a 22:6 fatty acid which is DHA. Similarly, EPA (20:5) was also one of the possible fatty-acid side chain released, based on the decrease of PC 38:5 and the increase of LPC 18:0. In addition, maprotiline + sense treatment induced a significant increase in several ceramide species, including Cer d18:1/C20:0, Cer d18:1/C22:0, Cer d18:1/C24:0 and Cer d18:1/C24:1, and these were repressed by antisense treatment ($P < 0.05$) (Fig. 3.11). Maprotiline + sense treatment also increased SM 18/16:0 species as well as decreased SM 18/20:0 and SM 18/24:1 species levels and these changes were abolished by antisense treatment ($P < 0.05$) (Fig. 3.12). The changes in the ceramide and

sphingomyelin levels indicate an increased sphingomyelinase activity after maprotiline treatment.

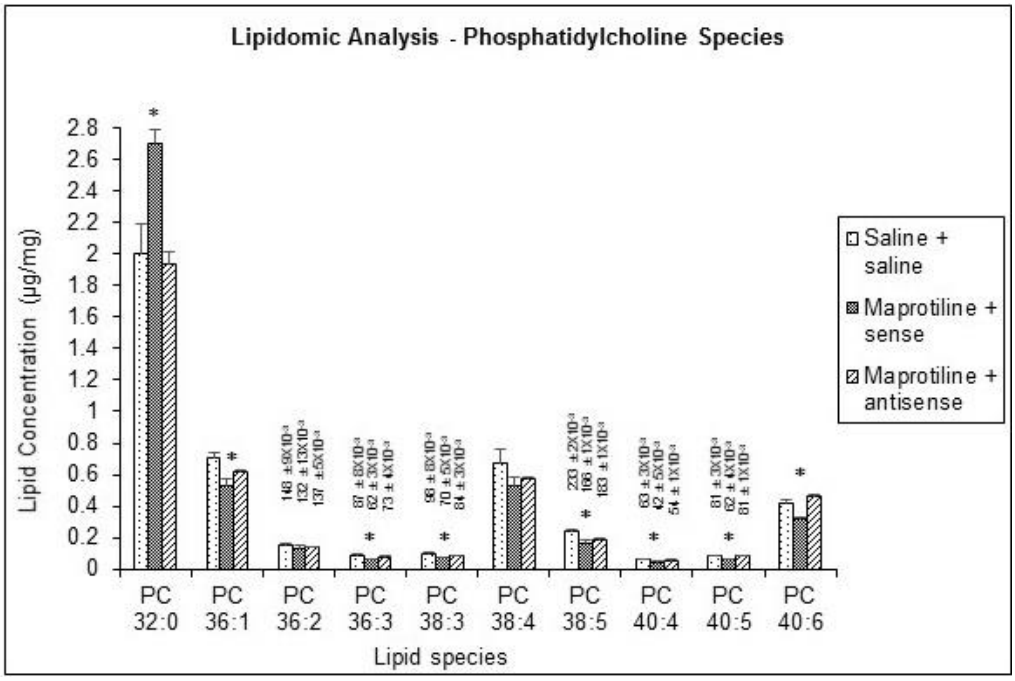


Fig. 3.9 Lipidomic analysis - phosphatidylcholine species. Lipidomic analysis of PC species in the mice prefrontal cortex after maprotiline and oligonucleotide treatment. Data represent the mean and standard error of four mice per treatment group. Asterisks (*) indicate significant differences at $P < 0.05$ in the lipid concentration of maprotiline + sense compared to all other treatment groups.

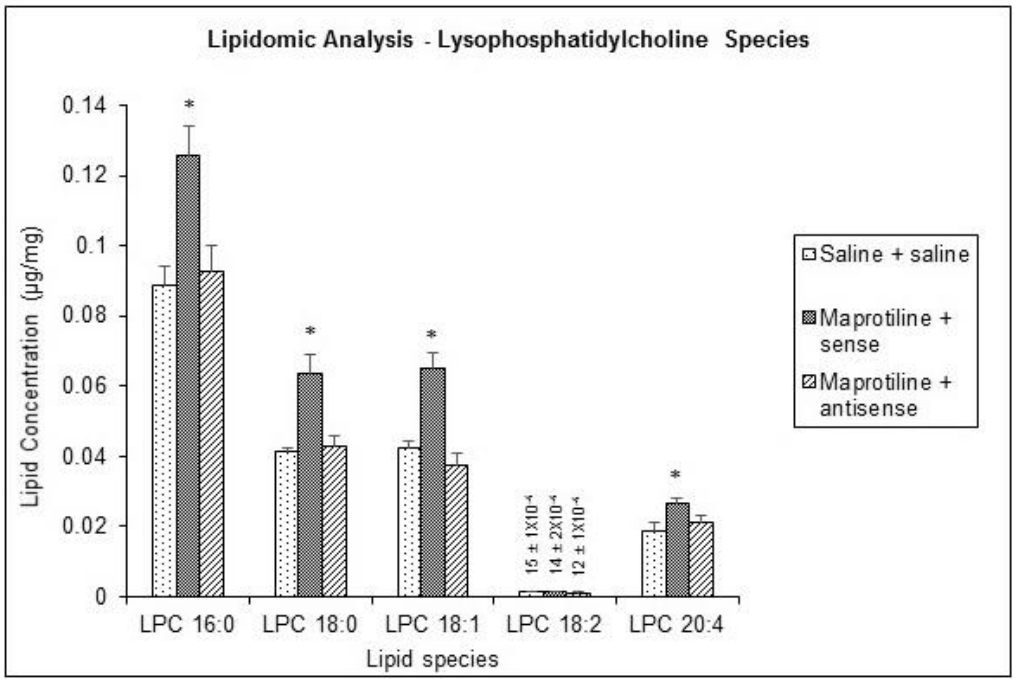


Fig. 3.10 Lipidomic analysis - lysophosphatidylcholine species. Lipidomic analyses of LPC species in the mice prefrontal cortex after maprotiline and oligonucleotide treatment. Asterisks (*) indicate significant differences at $P < 0.05$ in the lipid concentration of maprotiline + sense compared to all other treatment groups.

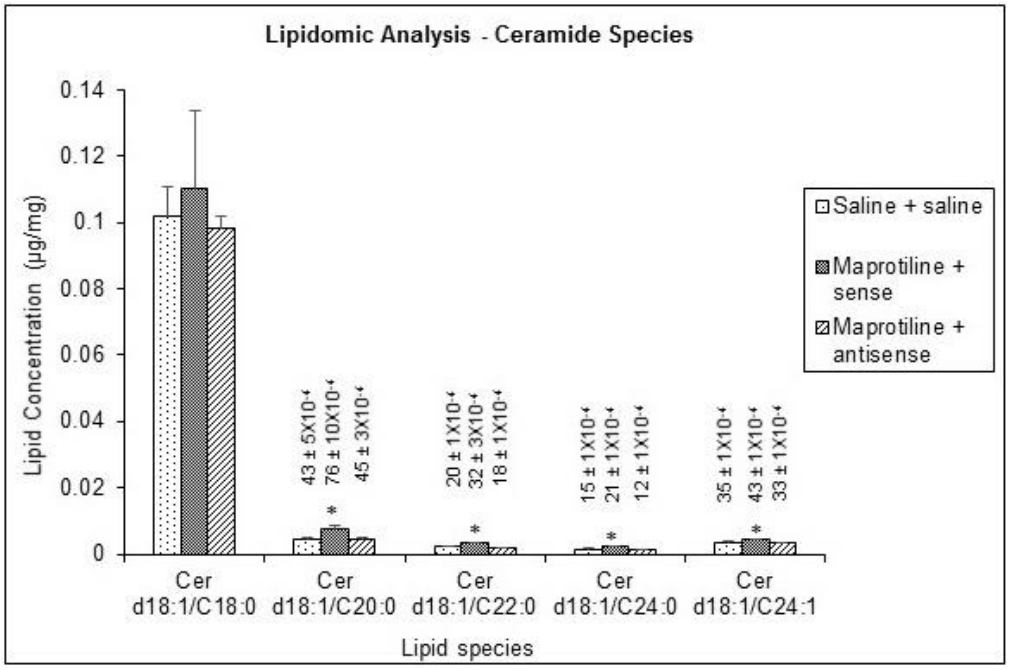


Fig. 3.11 Lipidomic analyses - ceramide species. Lipidomic analyses of Cer species in the mice prefrontal cortex after maprotiline and oligonucleotide treatment. Data represent the mean and standard error of four mice per treatment group. Asterisks (*) indicate significant differences at $P < 0.05$ in the lipid concentration of maprotiline + sense compared to all other treatment groups.

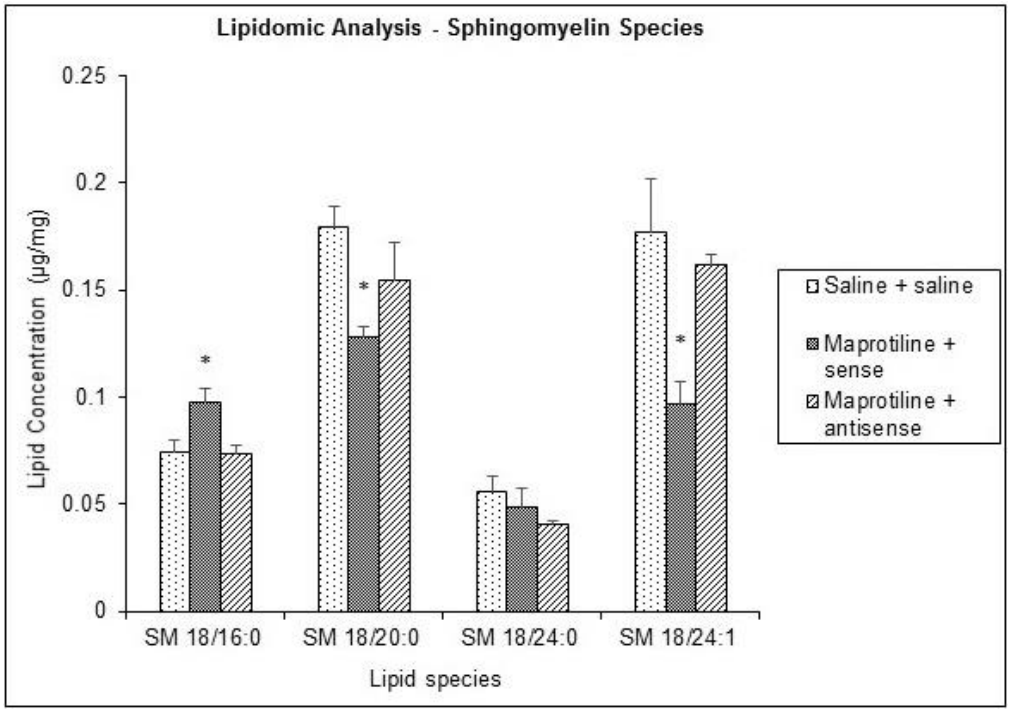


Fig. 3.12 Lipidomic analyses - sphingomyelin species. Lipidomic analyses of SM species in the mice prefrontal cortex after maprotiline and oligonucleotide treatment. Asterisks (*) indicate significant differences at $P < 0.05$ in the lipid concentration of maprotiline + sense compared to all other treatment groups.

4. Discussion

The present study was carried out to investigate the role of prefrontal cortical iPLA$_2$ in the antinociceptive effect of maprotiline and amitriptyline as well as to examine the effect of maprotiline treatment on prefrontal cortical iPLA$_2$ expression and lipid profile. Pain behavioral studies were first carried out to determine the effect of antidepressant treatment on nociception in a carrageenan mouse model of inflammatory orofacial pain. Behavioral analysis of mice that received daily intraperitoneal saline injection and facial carrageenan injection showed no difference in the number of facial strokes after intracortical injections of saline, sense oligonucleotide, or antisense

oligonucleotide to iPLA$_2$ at the prefrontal cortex. A higher number of facial strokes indicate more pain and a higher pain behavioral response. Our results showed that non-specific effect of iPLA$_2$ knockdown on cortical neurons is unlikely, and that inhibition of constitutive prefrontal cortex iPLA$_2$ did not have a significant effect on pain behavior. This is consistent with our previous findings where inhibition of iPLA$_2$ levels in the prefrontal cortex in the absence of antidepressant treatment did not affect despair behavior during the forced swim test (Lee et al., 2012).

Both amitriptyline treatment groups showed significantly lower number of facial strokes as compared to the saline treatment groups, highlighting the antinociceptive activity of amitriptyline. The observed effect of amitriptyline on nociception is consistent with its clinical usage in managing pain conditions (Fishbain, 2000; Salerno et al., 2002; Staiger et al., 2003). Similar to the saline treatment groups, administration of antisense oligonucleotide to iPLA$_2$ did not affect the pain behavioral responses of the amitriptyline treatment groups. The maprotiline + sense treatment group also exhibited antinociceptive activity as demonstrated by the reduced number of facial wash strokes after von Frey hair stimulation of the carrageenan-injected area as compared to the saline treatment groups. In addition, the maprotiline + sense treatment group was found to have significantly lower pain responses as compared to the amitriptyline groups at several time points of the study which seem to suggest that maprotiline treatment is marginally more effective than amitriptyline in modulating nociception in the carrageenan mouse model of inflammatory orofacial pain. However, the reduction in facial wash strokes induced by maprotiline treatment was abolished when the prefrontal cortex

was intracortically injected with antisense oligonucleotide to iPLA$_2$, suggesting that the loss of this enzyme could impact maprotiline-induced antinociception. Interestingly, our results showed that knockdown of prefrontal cortical iPLA$_2$ only abolished the antinociceptive effects of maprotiline but not amitriptyline, suggesting that unlike amitriptyline, maprotiline-induced antinociception is highly dependent on prefrontal cortical iPLA$_2$. Both maprotiline and amitriptyline are classified as TCAs (Baldessarini, 2006). However, amitriptyline has stronger effects on the serotonin transporter and weaker effects on the noradrenaline transporter as compared to maprotiline (Owens et al., 1997). Maprotiline also has a much higher selectivity for inhibition of noradrenaline reuptake as compared to amitriptyline (Rang et al., 2007). Thus, it is possible that the antinociceptive activity of other TCAs with similarly strong effects on the noradrenaline transporter may also involve prefrontal cortical iPLA$_2$. Additional work, however, is needed to further investigate this possibility.

To investigate whether the role of iPLA$_2$ in antidepressant-induced antinociceptive effect is specific to the prefrontal cortex, iPLA$_2$ antisense was injected into the somatosensory cortex of maprotiline-treated mice followed by pain behavioral analysis. Treatment with maprotiline significantly reduced pain behavior in mice compared to saline treatment, but in contrast to the prefrontal cortex, no significant differences in pain behavior was detected between the maprotiline + antisense and the maprotiline + sense groups after somatosensory cortex antisense injection. Results indicate that the effect of iPLA$_2$ in antinociception is specific to the prefrontal cortex. These findings are consistent with the view that the prefrontal cortex and PAG, rather than the

somatosensory cortex, is a source of supraspinal antinociception (Sessle et al., 1976; Christie et al., 1986; Floyd et al., 2000).

We then studied the effect of maprotiline treatment to confirm that maprotiline does indeed affect iPLA$_2$ expression in the prefrontal cortex. Real-time RT-PCR showed a significant increase in iPLA$_2$ mRNA expression in the mouse prefrontal cortex after maprotiline treatment and a similar significant increase in iPLA$_2$ protein expression was shown by Western blot analysis. This increase in iPLA$_2$ expression is consistent with the up-regulation in phospholipase A$_2$ activity in the prefrontal cortex after maprotiline treatment as shown in our previous study, suggesting a potentially important role of iPLA$_2$ in mediating the pharmacological effects of maprotiline (Lee et al., 2012). Western blot analysis confirmed the efficacy of antisense oligonucleotide to iPLA$_2$ in reducing prefrontal iPLA$_2$ protein expression, as shown in our previous study on mice without carrageenan injection (Lee et al., 2012). In that study, we also demonstrated that administration of iPLA$_2$ antisense oligonucleotide to the prefrontal cortex did not induce apoptosis in the prefrontal cortex (Lee et al., 2012).

Increased iPLA$_2$ expression in the brain may lead to endogenous release of DHA and EPA as shown in our previous study (Lee et al., 2012). Similar to our previous study on mice without carrageenan injection (Lee et al., 2012), lipidomic analysis of the prefrontal cortex in mice that received maprotiline and facial carrageenan injection showed significant increases in LPC lipid species and decreases in PC lipid species, suggesting increased iPLA$_2$ activity. The fatty-acid side chain released by iPLA$_2$ activity was found

to include DHA (22:6), based on the decrease of PC 40:6 and the increase of LPC 18:0. Similarly, EPA (20:5) was also one of the possible fatty-acid side chain released, based on the decrease of PC 38:5 and the increase of LPC 18:0. DHA is metabolized to D-series resolvins and neuroprotectins, while EPA is metabolized to E-series resolvins (Serhan et al., 2004). These metabolites have anti-inflammatory properties and effects on neural plasticity (Hasturk et al., 2006; Sun et al., 2007; Xu et al., 2010; Cortina et al., 2013; Erdinest et al., 2014). Resolvins and neuroprotectins also facilitate cell survival signaling and synaptic plasticity (Tassoni et al., 2008; Bazan, 2009; Park et al., 2011; Serhan and Petasis, 2011). It is possible that plasticity changes associated with increased resolvins and neuroprotectins in the dorsolateral prefrontal cortex may mediate the antidepressive and antinociceptive effects of TCAs, particularly those with strong noradrenaline reuptake inhibition activity. It is postulated that the antinociceptive effect of maprotiline involves the induction of iPLA$_2$ activity and subsequent DHA and EPA release. Increased production of DHA, EPA and their metabolites may then facilitate activity or plasticity in the dorsolateral prefrontal cortex leading to activation of the PAG and subsequent descending pain inhibitory pathway. Thus, inhibition of prefrontal cortical iPLA$_2$ may prevent activation of the prefrontal cortex and abolish PAG stimulation leading to decreased antinociceptive activity of antidepressants as shown by maprotiline in our study.

Chapter 4: Regulation of iPLA$_2$ Induction by Adrenergic Receptors, MAPK/ERK and SREBP Pathways

1. Introduction

Induction of prefrontal cortical iPLA$_2$ was previously revealed to be essential for the antidepressive effect of maprotiline, and iPLA$_2$ activity is correlated with endogenous release of DHA (Lee et al., 2012). We have also shown earlier in the first part of our study that the antinociceptive activity of maprotiline also involves prefrontal cortical iPLA$_2$ activity and expression, coupled with the production of DHA and EPA. Little, however, is known about the possible mechanisms and pathways involved in iPLA$_2$ expression. Since maprotiline acts by inhibiting noradrenaline reuptake and increasing the level of noradrenaline, it is highly likely that induction of iPLA$_2$ involves activation of adrenergic receptors. It is also possible that regulation of iPLA$_2$ expression induction involves the MAPK/extracellular signal-regulated kinase (MAPK/ERK) or cAMP-dependent PKA (cAMP/PKA) signaling pathways downstream from the adrenergic receptors.

The second messenger cAMP/PKA cascade is linked with the pathophysiology and treatment of depression (Liu et al., 2012). AC-cAMP-PKA signaling cascade dysfunction as well as reduced G-protein and cAMP levels were shown in patients with depression (Cowburn et al., 1994; Shelton et al., 1996; Dowlatshahi et al., 1999). Studies also revealed that chronic antidepressant treatment increases AC and GTP expression, induces the level and accumulation of forskolin-stimulated cAMP as well as augments PKA activity, leading to up-regulation of the cAMP/PKA signaling pathway (Nestler et al., 1989; Perez et al., 1989; Ozawa and Rasenick, 1991; Jensen et al., 2000).

In addition to the cAMP/PKA cascade, there is also evidence suggesting a possible role of the MAPK/ERK signaling pathway in the maprotiline-induced $iPLA_2$ expression. The MAPK 1 and 3 (ERK1/2) activation pathway is one of the most researched alpha-1 adrenergic receptors-stimulated pathways (Garcia-Sainz et al., 1999; Hague et al., 2002). Inhibition of MAPK signaling blocked the behavioral actions of antidepressants, resulting in a depressive-like phenotype (Duman et al., 2007). In addition, antidepressant efficacy is linked with altered limbic phosphorylated ERK1/2 in an animal model of chronic depression (Gourley et al., 2008). Researchers also showed that the induction of PLA_2 protein expression involves the MAPK/ERK cascade (Anfuso et al., 2007).

Besides the cAMP/PKA cascade and MAPK/ERK signaling pathway, the sterol regulatory element-binding proteins (SREBPs), in particular SREBP-2, may also be implicated in the regulation of $iPLA_2$ induction. SREBPs are transcription factors that bind to the SRE DNA sequence TCACNCCAC to regulate cellular fatty acid and cholesterol biosynthesis (Raeder et al., 2006). There exists two homologous SREBP proteins: SREBP-1 and SREBP-2 (Raeder et al., 2006). SREBP-1 has two different isoforms, SREBP-1a and SREBP-1c, and is mainly involved in regulating genes required for fatty acid synthesis while SREBP-2 primarily controls the genes involved in cholesterol biosynthesis (Raeder et al., 2006). SREBPs are initially generated as inactive precursor proteins located in the membrane of the endoplasmic reticulum (ER) (Brown and Goldstein, 1997). Activation of the SREBP pathway will induce SREBP translocation to the Golgi apparatus where it will be proteolytically cleaved by site-1 protease (S1P) and site-2

protease (S2P) to release the cytoplasmic portion of SREBP, which then travels to the nucleus to activate transcription of target genes with the SRE sequence (Brown and Goldstein, 1997). Antidepressants such as imipramine were found to activate the SREBP system, leading to increased expression of downstream genes (Raeder et al., 2006). The iPLA$_2$ gene was revealed to possess a putative SRE sequence which can bind to SREBP-2 (Seashols et al., 2004). It was therefore suggested that induction of iPLA$_2$ involves stimulation of adrenergic receptors followed by activation of either the cAMP/PKA cascade or MAPK/ERK signaling pathway to initiate the SREBP pathway and subsequent SREBP-2 binding to the iPLA$_2$ gene. In this part of the study, we investigated the role of adrenergic receptors and the possible mechanisms and pathways involved in regulation of iPLA$_2$ expression induction.

Real-time RT-PCR and Western blot analyses were carried out to investigate the effect of antidepressant treatment and selective pathway inhibitors on SREBP-2 and iPLA$_2$ expression. Electrophoretic mobility shift assay (EMSA) was also performed to investigate the effect of maprotiline, alpha-1 adrenergic receptor agonist and inhibitor treatment on the binding of SREBP-2 to the SRE region of the iPLA$_2$ gene. Lastly, immunocytochemistry was carried out to study the effect of maprotiline and inhibitor treatment on iPLA$_2$ cellular staining and expression.

2. Materials and methods

2.1. Cells and treatment

2.1.1. Cell culture

SH-SY5Y neuroblastoma cells obtained from ATCC (Manassas, VA, U.S.A.) were used for this part of the study. SH-SY5Y cells are derived from the parental SK-N-SH line and they are relatively easy to manipulate and work with (Kovalevich and Langford, 2013). Human neuroblastoma cells such as LA-N-1 cells and SK-N-SH cells have also been successfully used in previous studies that showed novel functions of iPLA$_2$ (Sun et al., 2004). In addition, SH-SY5Y cells are shown to express alpha adrenergic receptors and are able to synthesize, store and release catecholamines such as noradrenaline (Atcheson et al., 1994; Ou et al., 1998; Parsley et al., 1999; Perez, 2005; Mathieu et al., 2010; Korecka et al., 2013). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % penicillin-streptomycin solution and 10 % fetal bovine serum (FBS) at 37 °C with 5 % CO_2 aeration. They were cultured on 100 mm culture plates until 80 % confluency and used for subsequent treatments.

2.1.2. Treatment with antidepressants

Treatment with different antidepressants was first carried out to investigate their effect on iPLA$_2$ expression. Besides maprotiline and amitriptyline, another TCA with strong noradrenaline reuptake inhibiting properties, nortriptyline, was used in this part of the study. SH-SY5Y cells were separated into 4 groups: 1) Treatment with vehicle, dimethyl sulfoxide (DMSO) 2) Treatment with 25 µM maprotiline 3) Treatment with 25 µM amitriptyline 4) Treatment with 25 µM nortriptyline. Each group consisted of five 100 mm culture plates (n = 5). The i*n vitro* concentrations of

antidepressants used are based on other studies (Liao et al., 2010; Hisaoka et al., 2011). Incubation was carried out for 24 hours, after which the cells were washed in phosphate-buffered saline (PBS) and harvested for real time RT-PCR analysis. A 24 hour time point was carried out to emulate the chronic treatment of antidepressants in the *in vivo* part of our study.

2.1.3. Treatment with maprotiline and alpha-1 adrenergic receptor blocker

Treatment with maprotiline and alpha-1 adrenergic receptor blocker was carried out to examine their effect on SREBP-2 and iPLA$_2$ expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM alpha-1 adrenergic receptor blocker, prazosin 3) Treatment with 25 µM maprotiline 4) Treatment with 25 µM maprotiline and 10 µM prazosin. The concentration of prazosin is based on previous cell studies (Segura et al., 2013). Prazosin pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR and Western blot analyses.

2.1.4. Treatment with maprotiline and alpha-2 adrenergic receptor blocker

Treatment with maprotiline and alpha-2 adrenergic receptor blocker was performed to study their effect on iPLA$_2$ expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM alpha-2 adrenergic receptor blocker, idazoxan 3) Treatment with 25 µM

maprotiline 4) Treatment with 10 µM idazoxan and 25 µM maprotiline. The concentration of idazoxan is similar to that used in previous studies (Hu et al., 1996). Idazoxan pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.5. Treatment with maprotiline and non-selective beta adrenergic receptor blocker

Treatment with maprotiline and non-selective beta adrenergic receptor blocker was carried out to examine their effect on iPLA$_2$ expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM non-selective beta adrenergic receptor blocker, nadolol 3) Treatment with 25 µM maprotiline 4) Treatment with 10 µM nadolol and 25 µM maprotiline. The concentration of nadolol used is based on previous studies (Cawley et al., 2011). Nadolol pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.6. Treatment with maprotiline and serotonin receptor antagonist

Treatment with maprotiline and serotonin receptor antagonist was performed to investigate their effect on iPLA$_2$ expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 100 nM selective serotonin receptor antagonist, WAY100635 3)

Treatment with 25 µM maprotiline 4) Treatment with 100 nM WAY100635 and 25 µM maprotiline. The concentration of WAY100635 is similar to that used in other cell studies (Jordan et al., 2002). WAY100635 pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.7. Treatment with nortriptyline and alpha-1 adrenergic receptor blocker

Treatment with nortriptyline and alpha-1 adrenergic receptor blocker was carried out to study their effect on iPLA$_2$ expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 25 µM nortriptyline 4) Treatment with 10 µM prazosin and 25 µM nortriptyline. Prazosin pre-treatment was carried out for 1 hour, followed by nortriptyline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.8. Treatment with nortriptyline and serotonin receptor antagonist

Treatment with nortriptyline and serotonin receptor antagonist was performed to determine their effect on iPLA$_2$ expression, SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle 2) Treatment with 100 nM WAY100635 3) Treatment with 25 µM nortriptyline 4) Treatment with 100 nM WAY100635 and 25 µM nortriptyline. WAY100635 pre-treatment was carried out for 1 hour, followed by nortriptyline or vehicle incubation for

another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.9. Treatment with maprotiline, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors

For this part of the study, PKA inhibitors were used to block the cAMP/PKA signaling cascade. Treatment with maprotiline, PKA inhibitors and MAPK/ERK signaling inhibitors was carried out to investigate their effect on SREBP-2 and iPLA$_2$ expression. SH-SY5Y cells were divided into 6 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 100 nM PKA inhibitor, H-89 3) Treatment with 25 µM maprotiline 4) Treatment with 25 µM maprotiline and 100 nM H-89 5) Treatment with 10 µM of the selective ERK inhibitor, FR180204 6)Treatment with 25 µM maprotiline and 10 µM FR180204. PKA and MAPK/ERK inhibitor pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis. The experiments were repeated using 10 nM of another PKA inhibitor, PKI, and 20 µM of another MAPK/ERK pathway inhibitor, PD98059, to validate their effect on SREBP-2 and iPLA$_2$ expression. The concentrations of PKA inhibitors used are based on previous studies (Brigino et al., 1997; Collas et al., 1999; Harmon et al., 2005; Queen et al., 2006). The concentrations of MAPK/ERK inhibitors used are also similar to those used in previous studies (Singh et al., 2009; Volpi et al., 2011; Zeng et al., 2013).

2.1.10. Treatment with alpha-1 adrenergic receptor agonist and blocker

Both maprotiline and nortriptyline have strong noradrenaline reuptake inhibition activity which will lead to increasing noradrenaline level and subsequent adrenergic receptor activation. Thus, to validate the involvement of adrenergic receptors, in particular the alpha-1 adrenergic receptor, in SREBP-2 and iPLA$_2$ expression, treatment with alpha-1 adrenergic receptor agonist and blocker was carried out. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 50 µM alpha-1 adrenergic receptor agonist, phenylephrine 4) Treatment with 50 µM phenylephrine and 10 µM prazosin. The concentration of phenylephrine used is similar to other cell studies (Endoh and Blinks, 1988; Capogrossi et al., 1991). Prazosin pre-treatment was carried out for 1 hour, followed by phenylephrine or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.11. Treatment with alpha-1 adrenergic receptor agonist, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors

Treatment with alpha-1 adrenergic receptor agonist, cAMP/PKA and MAPK/ERK pathway inhibitors was performed to study their effect on SREBP-2 and iPLA$_2$ expression. SH-SY5Y cells were divided into 6 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 100 nM PKA inhibitor, H-89 3) Treatment with 10 µM of the ERK selective inhibitor, FR180204 4) Treatment with 50 µM phenylephrine 5) Treatment with 50 µM phenylephrine and 10 µM H-89 6) Treatment with 50 µM of phenylephrine and 10 µM

FR180204. Inhibitor pre-treatment was carried out for 1 hour, followed by phenylephrine or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.12. Treatment with maprotiline and SREBP pathway inhibitors

Treatment with maprotiline and SREBP pathway inhibitors was carried out to examine their effect on iPLA$_2$ expression. The SREBP pathway inhibitors used were betulin and the SREBP S1P enzyme inhibitor, PF-429242. SH-SY5Y cells were divided into 6 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM betulin 3) Treatment with 10 µM PF-429242 4) Treatment with 25 µM maprotiline 5) Treatment with 25 µM maprotiline and 10 µM betulin 6) Treatment with 25 µM maprotiline and 10 µM PF-429242. The concentrations of SREBP pathway inhibitors used are similar to previous cell studies (Hawkins et al., 2008; Tang et al., 2011). Inhibitor pre-treatment was carried out for 1 hour, followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.1.13. Treatment with alpha-1 adrenergic receptor agonist and SREBP pathway inhibitors

Treatment with alpha-1 adrenergic receptor agonist and SREBP pathway inhibitors was performed to investigate their effects on iPLA$_2$ expression. SH-SY5Y cells were divided into 6 groups: 1) Treatment with

vehicle, DMSO 2) Treatment with 10 µM betulin 3) Treatment with 10 µM PF-429242 4) Treatment with 50 µM phenylephrine 5) Treatment with 50 µM phenylephrine and 10 µM betulin 6) Treatment with 50 µM phenylephrine and 10 µM PF-429242. Inhibitor pre-treatment was carried out for 1 hour, followed by phenylephrine or vehicle incubation for another 24 hours, after which the cells were harvested for real time RT-PCR analysis.

2.2. Real time RT-PCR

SH-SY5Y RNA samples were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as described previously. Quantitative real-time PCR amplification was then carried out in the 7500 Real-time PCR system (Applied Biosystems, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA) and Applied Biosystem TaqMan® probes for human SREBP-2, iPLA$_2$ and beta-actin (Hs01081784_m1, Hs00185926_m1 and #4326315E respectively). All reactions were carried out in triplicates and the PCR conditions were as described previously.

2.3. Electrophoretic mobility shift assay

SREBP-2 is a transcription factor that can bind to the SRE sequence present on the iPLA$_2$ gene to induce iPLA$_2$ expression (Seashols et al., 2004). Thus, electrophoretic mobility shift assay (EMSA) was performed to investigate the effect of maprotiline, alpha-1 adrenergic receptor agonist and

blocker treatment on the binding of SREBP-2 to the SRE region of the $iPLA_2$ gene. Two sets of cell treatment experiments were carried out. For the first set of cell treatment, SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 50 µM phenylephrine 4) Treatment with 50 µM phenylephrine and 10 µM prazosin. For the second set, SH-SY5Y cells were divided into 4 groups: 1) Treatment with DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 25 µM maprotiline 4) Treatment with 25 µM maprotiline and 10 µM prazosin. Prazosin pre-treatment for both sets of cell treatment was carried out for 1 hour, followed by maprotiline, phenylephrine or vehicle incubation for another 24 hours, after which protein was extracted from the cells for subsequent EMSA analysis. $iPLA_2$ oligonucleotides EMSA probe with the following sequences were then synthesized for EMSA analysis: 5′ ACG TCG GTG GTC AGG CCA TCA CGT GGC CCG AGG C 3′ and 5′ ACGT TGC CTC GGG CCA CGT GAT GGC CTG ACC ACC G 3′. They represent nucleotides -106 to -77 pf the 5’ flanking region of the $iPLA_2$ gene which contains a SRE binding site as shown in a previous study (Seashols et al., 2004). The LightShift Chemiluminescent EMSA kit (Thermo Fischer Scientific, IL, USA) for gel-shift assays without digoxigenin or radioisotopes was used for EMSA analysis. $iPLA_2$ oligonucleotides were first tagged with biotin and subsequently annealed. Non-isotopic biotin labeling of the $iPLA_2$ oligonucleotides EMSA probe was carried out using the DNA 3' End Biotinylation Kit (Thermo Fischer Scientific, IL, USA). Binding reactions containing the biotin end-labeled $iPLA_2$ oligonucleotides, protein extracts and other binding buffer solutions as according to the LightShift

Chemiluminescent EMSA kit were then incubated for 20 minutes at room temperature. After incubation, the binding reactions were mixed with 5X loading buffer, loaded onto a polyacrylamide gel and electrophoresed. The binding reactions were then transferred to a nylon membrane, UV cross-linked, and the binding between biotin-labeled iPLA$_2$ oligonucleotides EMSA probe and target SREBP-2 protein was then detected by chemiluminescence. Visualized films containing the EMSA blots were scanned and densities of the bands measured, using Gel-Pro Analyzer 3.1 program (Media Cybernetics, Silver Spring, MD, USA). To check the binding specificity of the iPLA$_2$ oligonucleotides EMSA probe containing the SRE region of the iPLA$_2$ gene, excess unlabeled iPLA$_2$ oligonucleotide was incubated together with the labeled iPLA$_2$ oligonucleotide and protein followed by EMSA analysis. Binding reactions for control Epstein-Barr Nuclear Antigen (EBNA) system were also carried out to ensure that the kit components and overall EMSA procedure was working properly.

2.4. Western blot analysis

Proteins were extracted from SH-SY5Y cells and their concentrations were measured as stated previously. Subsequent western blot analysis was carried out as described earlier using 30 µg of protein and rabbit polyclonal antibody to SREBP-2 (ab30682, Abcam, New Territories, HK; 4 µg/mL).

2.5. Immunocytochemistry

Treatment with maprotiline, alpha-1 adrenergic receptor blocker and SREBP pathway inhibitor was carried to examine their effect on iPLA$_2$ expression and immunolabeling. SH-SY5Y cells were cultured on poly-L-lysine coated coverslips in 24-well plates and maintained in DMEM supplemented with 10 % fetal bovine serum, 1 % penicillin-streptomycin solution at 37 °C with 5 % CO_2 aeration until reaching 80 % confluency. Cells were then separated into 6 treatment groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 25 µM maprotiline 4) Treatment with 25 µM maprotiline and 10 µM prazosin 5) Treatment with 10 µM betulin 6) Treatment with 25 µM maprotiline and 10 µM betulin. Prazosin and betulin pre-treatment were carried out for 1 hour followed by maprotiline or vehicle incubation for another 24 hours. Cells were then washed with PBS and fixed with 2 % paraformaldehyde. After fixing, cells were washed with PBS and reacted with 70 % formic acid for 20 minutes before undergoing permeabilization by TBS-Triton X for 15 minutes. Cells were then blocked in 1 % bovine serum albumin (BSA) in PBS for 1 hour and incubated with goat polyclonal anti-iPLA$_2$ (sc-14463, Santa Cruz Biotechnology, CA, USA; diluted 1:100 in 1 % BSA) for 1 hour. Cells were subsequently washed with PBS and reacted with Alexa Fluor® 488 Donkey Anti-Goat IgG (H+L) Antibody (Applied Biosystems, CA, USA; diluted 1:200 in 1 % BSA) for 1 hour at room temperature. ProLong Gold anti-fade reagent with DAPI (Invitrogen, USA) was used as a nuclear counterstaining and

mounting agent. Cells were mounted on to glass slides and examined using an Olympus FluoView FV1000 confocal microscope. The total cell fluorescence corrected for background was then calculated using ImageJ software.

2.6. Statistical analyses

The mean and standard errors of the values were calculated for each experimental group, and possible significant differences between the means were detected using one-way ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Real time RT-PCR

3.1.1. Effect of antidepressant treatment on iPLA$_2$ expression

There were significant increases in iPLA$_2$ mRNA expression in SH-SY5Y cells after treatment with maprotiline (2.36 fold change) and nortriptyline (1.42 fold change) for 24 hours. In contrast, no significant increase in iPLA$_2$ expression was detected after treatment with amitriptyline (1.26 fold change) (Fig. 4.1).

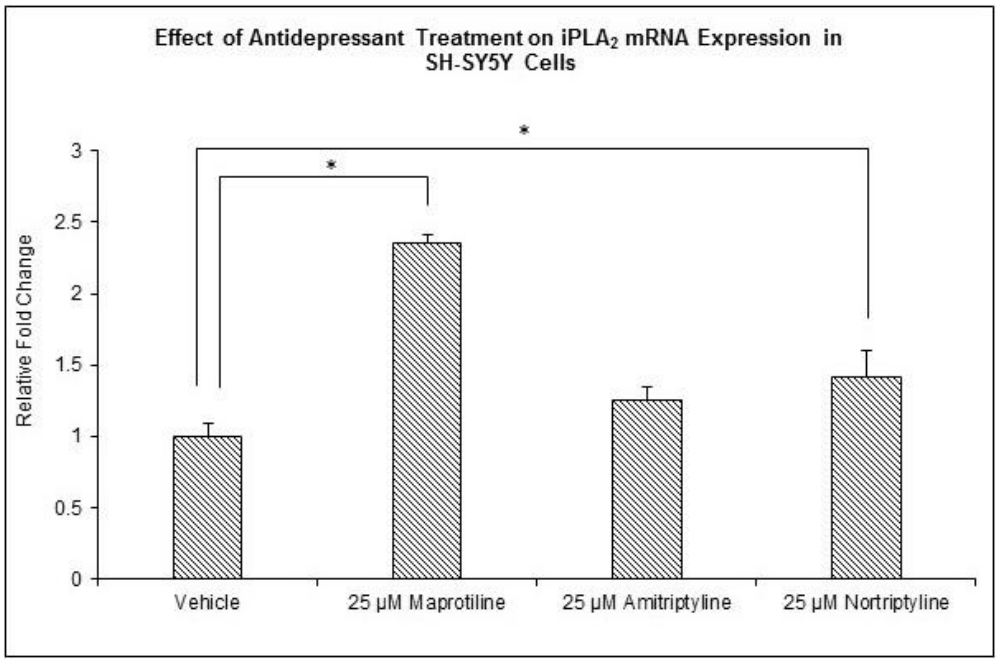


Fig. 4.1 Real time RT-PCR results. Effect of antidepressant treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.2. Effect of maprotiline and alpha-1 adrenergic receptor blocker on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 25 µM maprotiline (2.15 fold change), consistent with the increase in iPLA$_2$ expression after maprotiline treatment as shown previously. Co-treatment of maprotiline with the alpha-1 adrenergic receptor blocker, prazosin, significantly reduced the increase in iPLA$_2$ expression (1.55 fold change) (Fig. 4.2). The results suggest the involvement of alpha-1 adrenergic receptors in maprotiline-induced iPLA$_2$ expression.

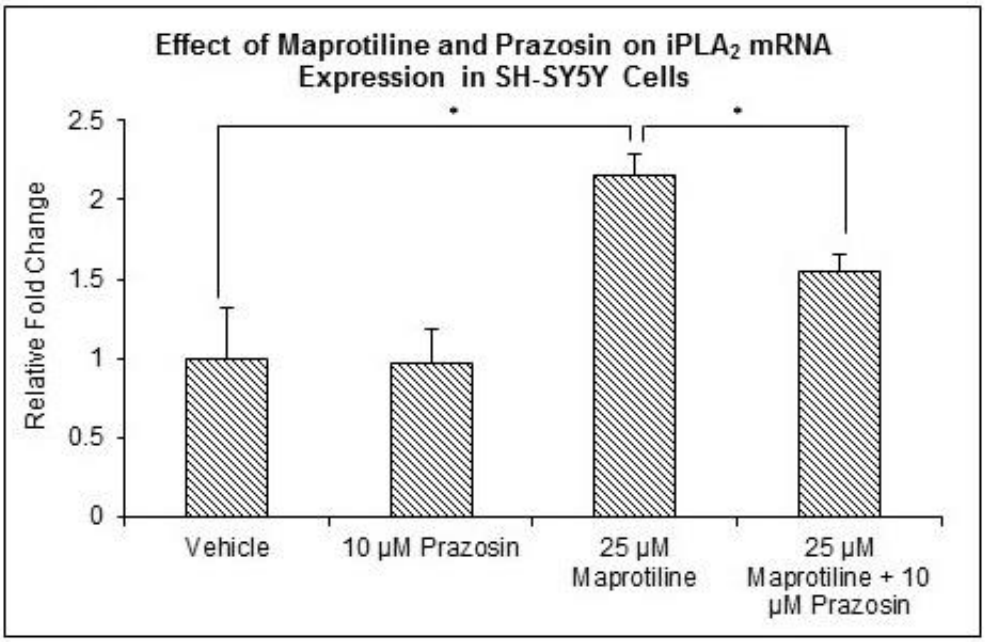


Fig. 4.2 Real time RT-PCR results. Effect of maprotiline and alpha-1 adrenergic receptor blocker, prazosin, on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.3. Effect of maprotiline and alpha-2 adrenergic receptor blocker on iPLA$_2$ expression

There was a significant 1.8 fold increase in iPLA$_2$ expression after treatment with 25 µM maprotiline (Fig. 4.3). Co-treatment with the alpha-2 adrenergic receptor blocker, idazoxan, did not significantly affect the maprotiline-induced increase in iPLA$_2$ expression (1.7 fold change).

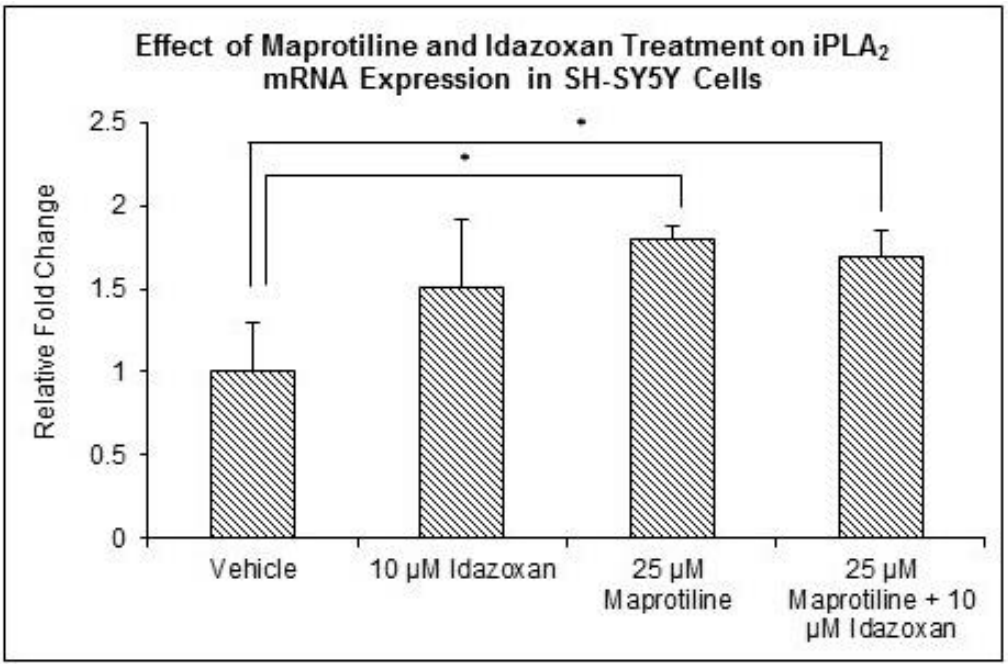


Fig. 4.3 Real time RT-PCR results. Effect of maprotiline and alpha-2 adrenergic receptor blocker, idazoxan, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.4. Effect of maprotiline and non-selective beta adrenergic receptor blocker on iPLA$_2$ expression

There was a significant 1.9 fold increase in iPLA$_2$ expression after treatment with 25 µM maprotiline (Fig. 4.4). Co-treatment of maprotiline with the non-selective beta adrenergic receptor blocker, nadolol, led to modulation of the maprotiline-induced iPLA$_2$ expression although there was still an increasing trend at 1.48 fold change.

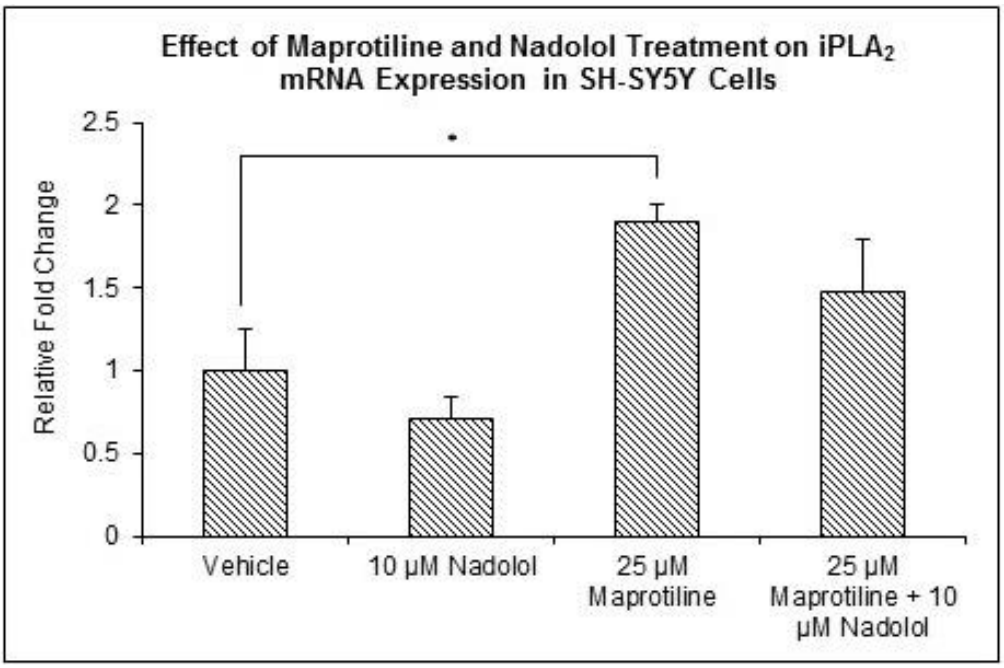


Fig. 4.4 Real time RT-PCR results. Effect of maprotiline and beta adrenergic receptor blocker, nadolol, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.5. Effect of maprotiline with serotonin receptor antagonist on iPLA$_2$ expression

There was a significant 2.05 fold increase in iPLA$_2$ expression after treatment with 25 µM maprotiline (Fig. 4.5). Co-treatment with the serotonin receptor antagonist, WAY100635, did not affect the maprotiline-induced increase in iPLA$_2$ expression (1.95 fold change).

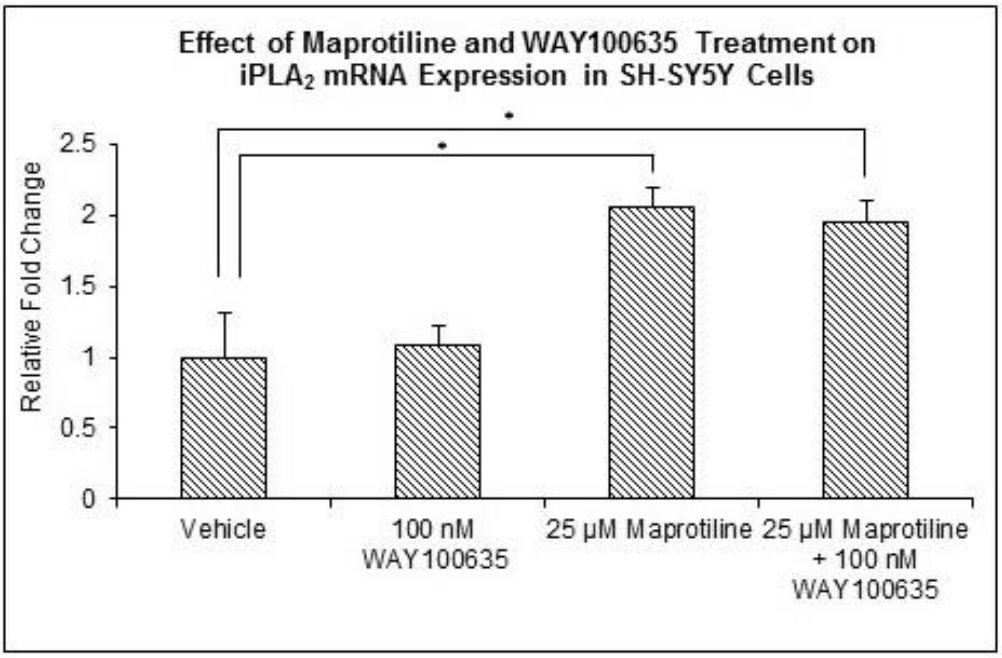


Fig. 4.5 Real time RT-PCR results. Effect of maprotiline and serotonin receptor antagonist, WAY100635, treatment on iPLA$_2$ mRNA expression in SY-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.6. Effect of nortriptyline with alpha-1 adrenergic receptor blocker on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression after treatment with 25 µM nortriptyline (1.87 fold change), consistent with the increase in iPLA$_2$ expression after nortriptyline treatment as shown previously. Co-treatment of nortriptyline with prazosin significantly reduced the increase in iPLA$_2$ expression (1.44 fold change) (Fig. 4.6). Our results suggest that besides being involved in maprotiline-induced iPLA$_2$ expression, the alpha-1 adrenergic receptors also play a role in nortriptyline-induced iPLA$_2$ expression.

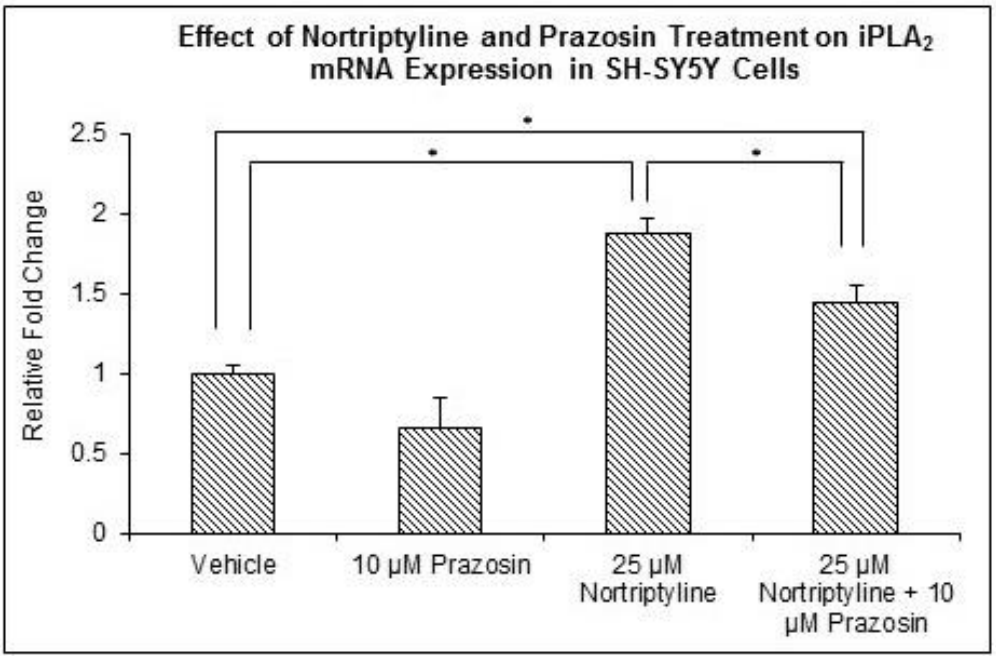


Fig. 4.6 Real time RT-PCR results. Effect of nortriptyline and alpha-1 adrenergic receptor blocker, prazosin, on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.7. Effect of nortriptyline and serotonin receptor antagonist on iPLA$_2$ expression

There was a significant 1.91 fold increase in iPLA$_2$ expression after treatment with 25 µM nortriptyline (Fig. 4.7). Co-treatment of nortriptyline with the serotonin receptor antagonist, WAY100635, did not affect the nortriptyline-induced increase in iPLA$_2$ expression (1.46 fold change).

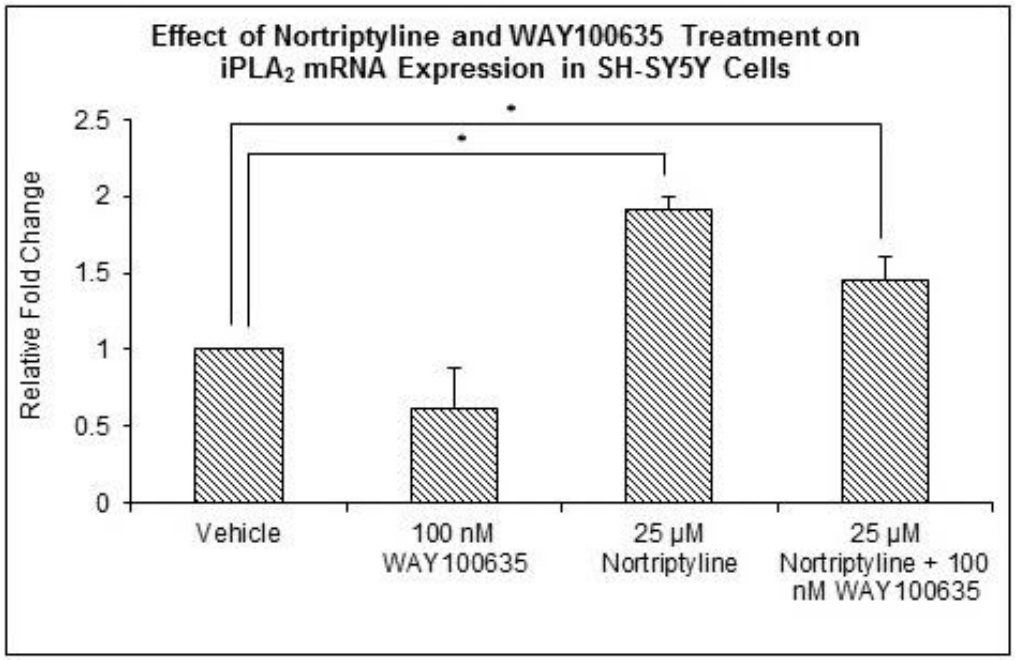


Fig. 4.7 Real time RT-PCR results. Effect of nortriptyline and serotonin receptor antagonist, WAY100635, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.8. Effect of maprotiline, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 25 µM maprotiline (1.59 fold change). Co-treatment of maprotiline with the PKA inhibitor, H-89, did not affect the maprotiline-induced increase in iPLA$_2$ expression (Fig. 4.8). However, co-treatment of maprotiline with the ERK inhibitor, FR180204, significantly reduced the increase in iPLA$_2$ expression (Fig. 4.8).

In the second set of experiments, there was a significant 2.3 fold increase in iPLA$_2$ expression after 25 µM maprotiline treatment. Co-treatment

with another PKA inhibitor, PKI, modulated the maprotiline-induced iPLA$_2$ expression although there was still an increasing trend in iPLA$_2$ expression (1.45 fold change) (Fig. 4.9). Co-treatment with another MAPK/ERK pathway inhibitor, PD98059, significantly reduced the increase in iPLA$_2$ expression (1.56 fold change) (Fig. 4.9). Together, results suggest that there is a stronger role for the MAPK/ERK signaling pathway than the cAMP/PKA cascade in regulating iPLA$_2$ expression induction.

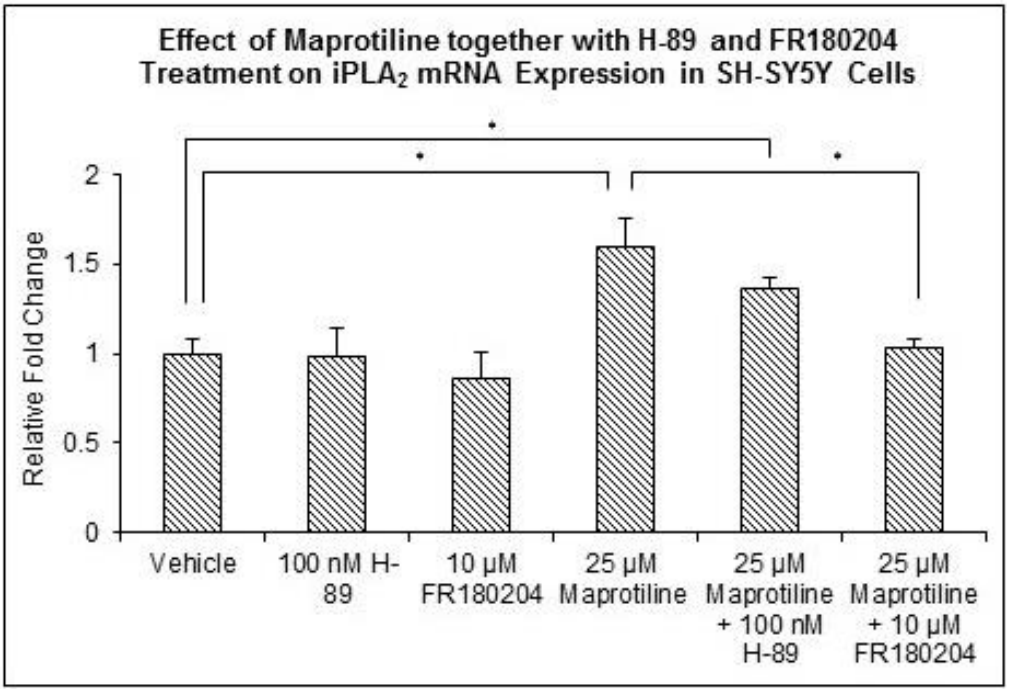


Fig. 4.8 Real time RT-PCR results. Effect of maprotiline together with PKA inhibitor, H-89, and ERK inhibitor, FR180204, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

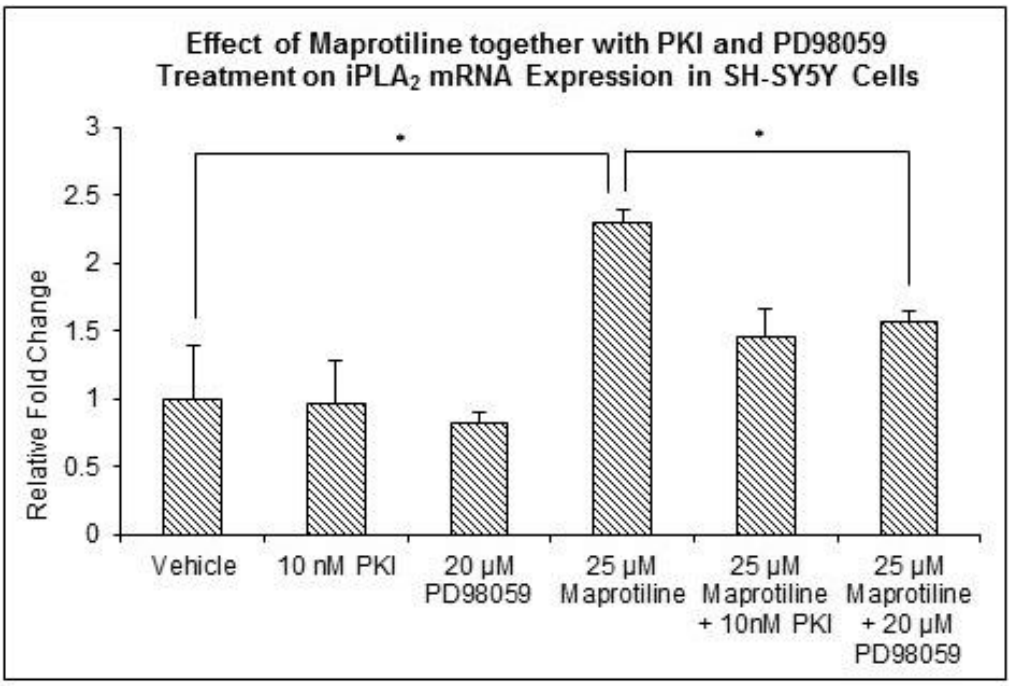


Fig. 4.9 Real time RT-PCR results. Effect of maprotiline together with PKA inhibitor, PKI, and MAPK/ERK pathway inhibitor, PD98059, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.9. Effect of alpha-1 adrenergic receptor agonist and alpha-1 adrenergic receptor blocker on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 50 µM alpha-1 adrenergic receptor agonist, phenylephrine (1.67 fold change). Co-treatment of phenylephrine with the alpha-1 adrenergic receptor blocker, prazosin, significantly reduced the increase in iPLA$_2$ expression (1.14 fold) (Fig. 4.10). Results support the involvement of alpha-1 adrenergic receptors in iPLA$_2$ expression induction.

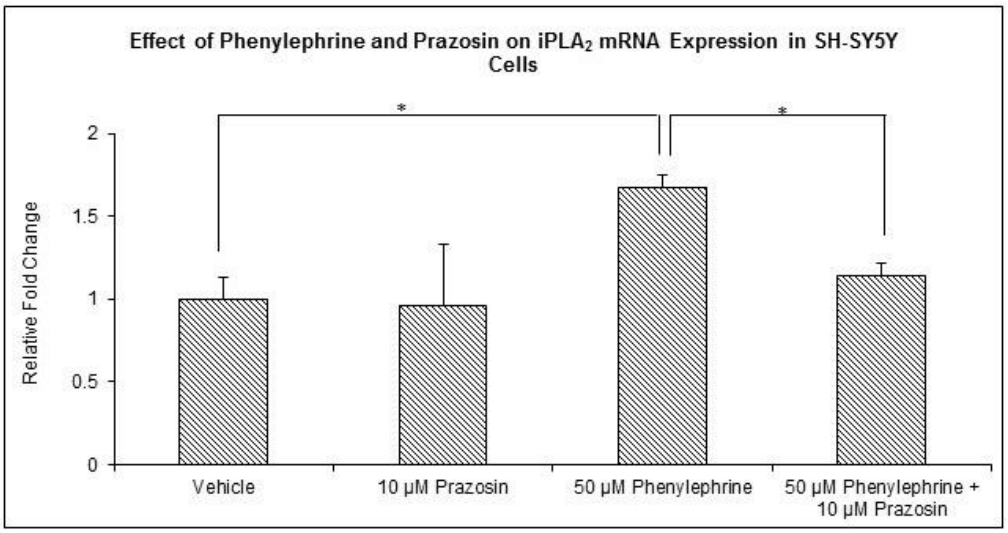


Fig. 4.10 Real time RT-PCR results. Effect of alpha-1 agonist, phenylephrine, and alpha-1 adrenergic receptor blocker, prazosin, on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.10. Effect of alpha-1 adrenergic receptor agonist, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 50µM alpha-1 adrenergic receptor agonist, phenylephrine (1.5 fold change). Co-treatment of phenylephrine with the selective ERK inhibitor, FR180204, abolished the phenylephrine-induced iPLA$_2$ expression (1.04 fold change) while co-treatment of phenylephrine with the PKA inhibitor, H-89, also modulated the phenylephrine-induced iPLA$_2$ expression although there was a higher trend present (1.29 fold change) (Fig. 4.11).

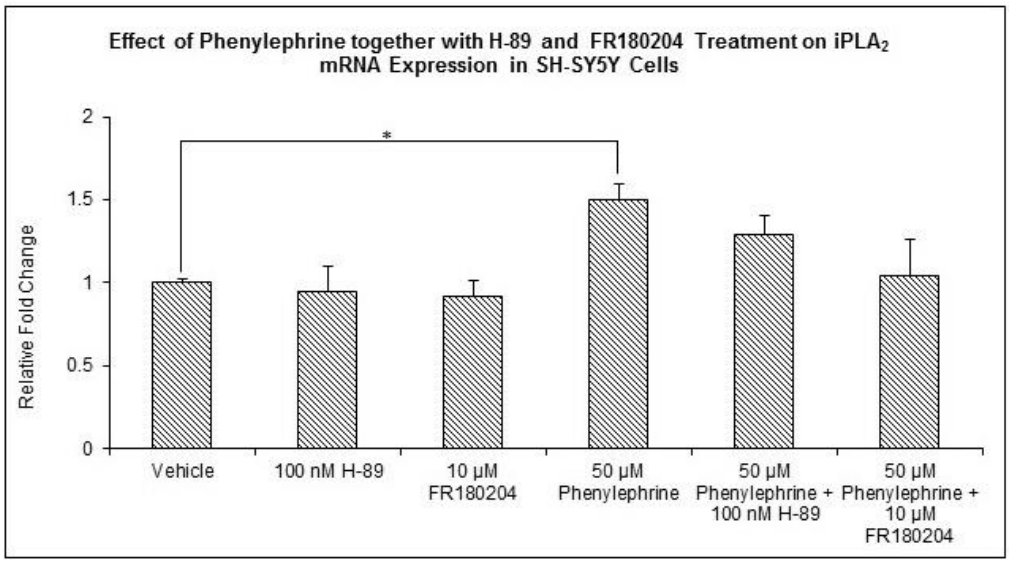


Fig. 4.11 Real time RT-PCR results. Effect of alpha-1 agonist, phenylephrine, and PKA inhibitor, H-89, and ERK inhibitor, FR180204, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.11. Effect of maprotiline and alpha-1 adrenergic receptor blocker on SREBP-2 expression

There was a significant increase in SREBP-2 expression in SH-SY5Y cells after treatment with 25 µM maprotiline (4.07 fold change). Co-treatment of maprotiline with the alpha-1 adrenergic receptor blocker, prazosin, significantly reduced the increase in SREBP-2 expression (Fig. 4.12). Results suggest that alpha-1 adrenergic receptors also play a role in maprotiline-induced SREBP-2 expression.

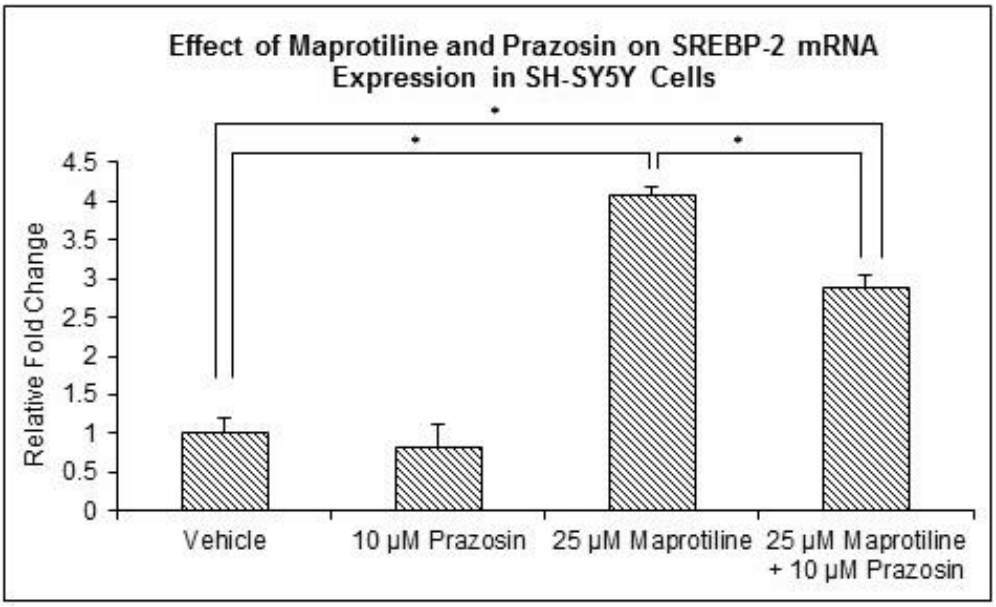


Fig. 4.12 Real time RT-PCR results. Effect of maprotiline and alpha-1 adrenergic receptor blocker, prazosin, on SREBP-2 mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.12. Effect of maprotiline, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors on SREBP-2 expression

There was a significant increase in SREBP-2 expression in SH-SY5Y cells after treatment with 25 µM maprotiline (3.06 fold change). Co-treatment of maprotiline with the PKA inhibitor, H-89, did not affect the increase in SREBP-2 expression (2.99 fold change) (Fig. 4.13). However, co-treatment of maprotiline with the selective ERK inhibitor, FR180204, significantly reduced the increase in SREBP-2 expression (Fig. 4.13).

In a second set of experiments, co-treatment with another PKA inhibitor, PKI, modulated the increase in SREBP-2 expression (Fig. 4.14). Co-treatment with another MAPK/ERK pathway inhibitor, PD98059, also

significantly reduced the maprotiline-induced SREBP-2 expression (2.65 fold change) (Fig. 4.14). Together, results suggest a stronger involvement of the MAPK/ERK signaling pathway as compared to the cAMP/PKA cascade in maprotiline-induced SREBP-2 expression.

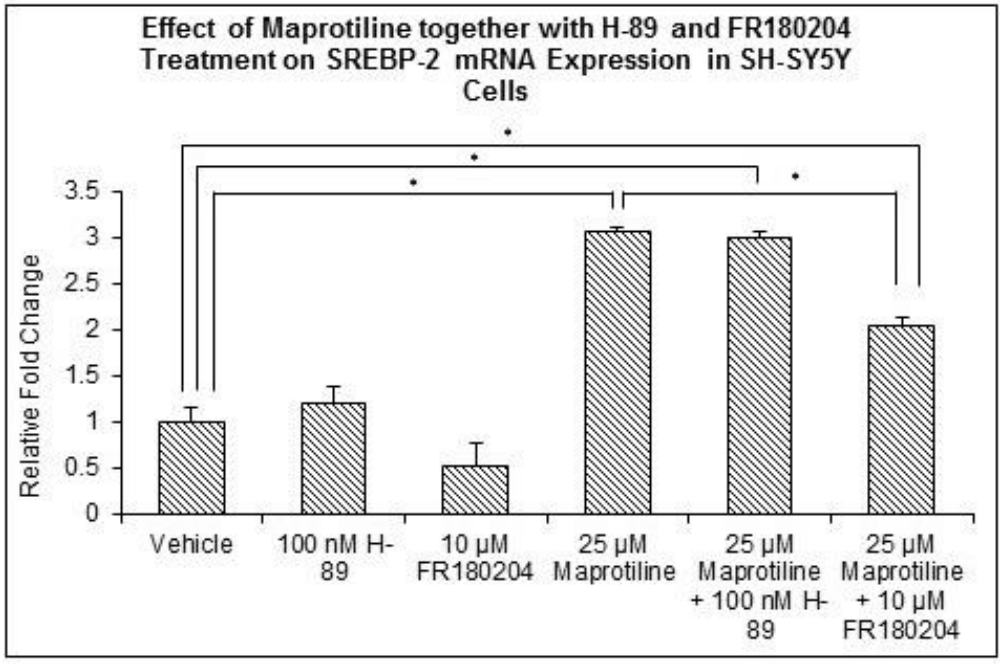


Fig. 4.13 Real time RT-PCR results. Effect of maprotiline together with PKA inhibitor, H-89, and ERK inhibitor, FR180204, treatment on SREBP-2 mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

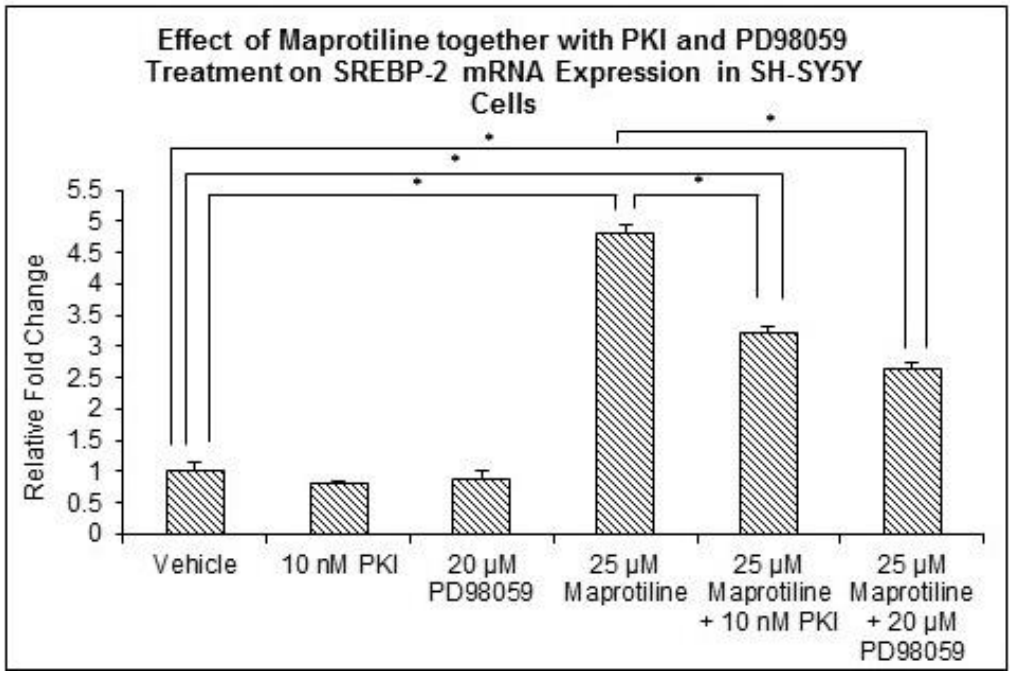


Fig. 4.14 Real time RT-PCR results. Effect of maprotiline together with PKA inhibitor, PKI, and MAPK/ERK pathway inhibitor, PD98059, treatment on SREBP-2 mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.13. Effect of alpha-1 adrenergic receptor agonist and blocker on SREBP-2 expression

There was a significant increase in SREBP-2 expression in SH-SY5Y cells after treatment with 50 µM alpha-1 agonist, phenylephrine (1.67 fold change). Co-treatment of phenylephrine with the alpha-1 adrenergic receptor blocker, prazosin, significantly abolished the increase in SREBP-2 expression (0.99 fold change) (Fig. 4.15).

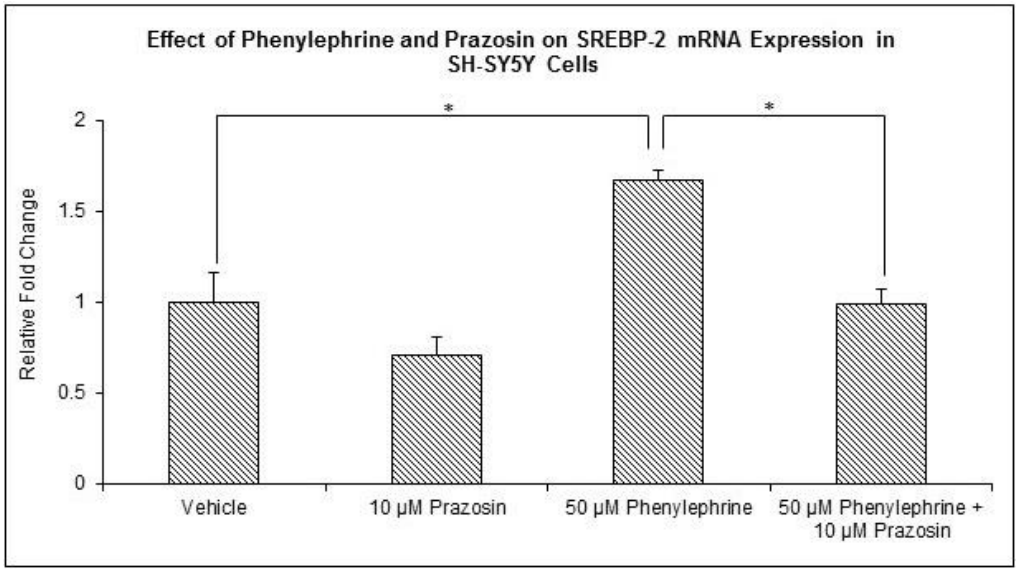


Fig. 4.15 Real time RT-PCR results. Effect of alpha-1 agonist, phenylephrine, and alpha-1 adrenergic receptor blocker, prazosin, on SREBP-2 mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.14. Effect of alpha-1 adrenergic receptor agonist, cAMP/PKA cascade inhibitors and MAPK/ERK signaling pathway inhibitors on SREBP-2 expression

There was a significant increase in SREBP-2 expression in SH-SY5Y cells after treatment with 50 µM agonist, phenylephrine (1.43 fold change). Co-treatment of phenylephrine with the ERK inhibitor, FR180204, abolished the increase in SREBP-2 expression (0.97 fold change), whereas co-treatment with the PKA inhibitor, H-89, had no effect on SREBP-2 expression (2.05 fold change) (Fig. 4.16). Results further support the role of the MAPK/ERK signaling pathway in SREBP-2 expression.

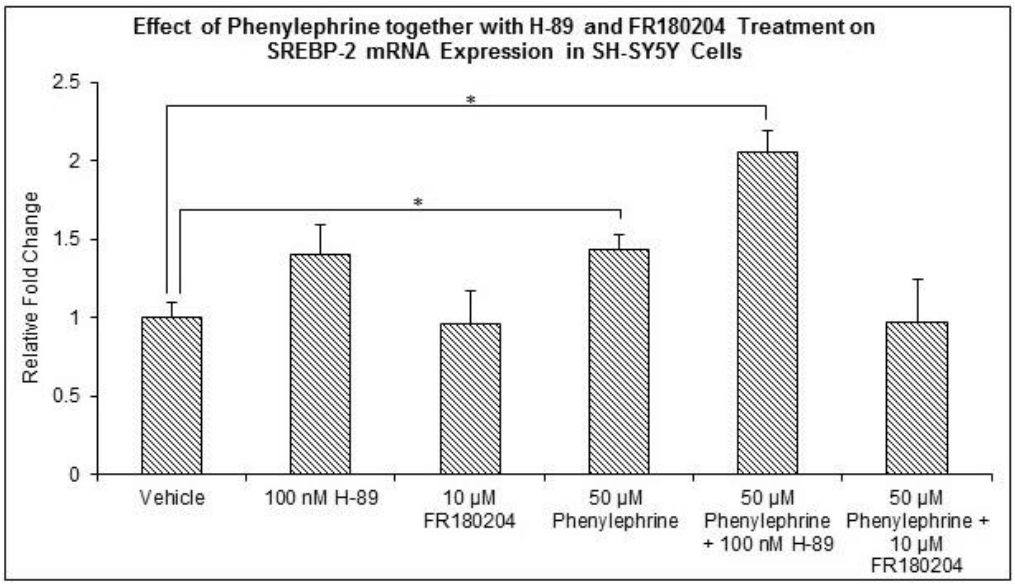


Fig. 4.16 Real time RT-PCR results. Effect of alpha-1 agonist, phenylephrine, and PKA inhibitor, H-89, and ERK inhibitor, FR180204, treatment on SREBP-2 mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.15. Effect of maprotiline and SREBP pathway inhibitors on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 25 µM maprotiline (2.25 fold change). Co-treatment of maprotiline with the SREBP pathway inhibitors, betulin and PF-429242, significantly abolished the increase in iPLA$_2$ expression (0.97 fold change and 1.25 fold change respectively) (Fig. 4.17). Results highlight the importance of the SREBP pathway in regulating iPLA$_2$ expression induction.

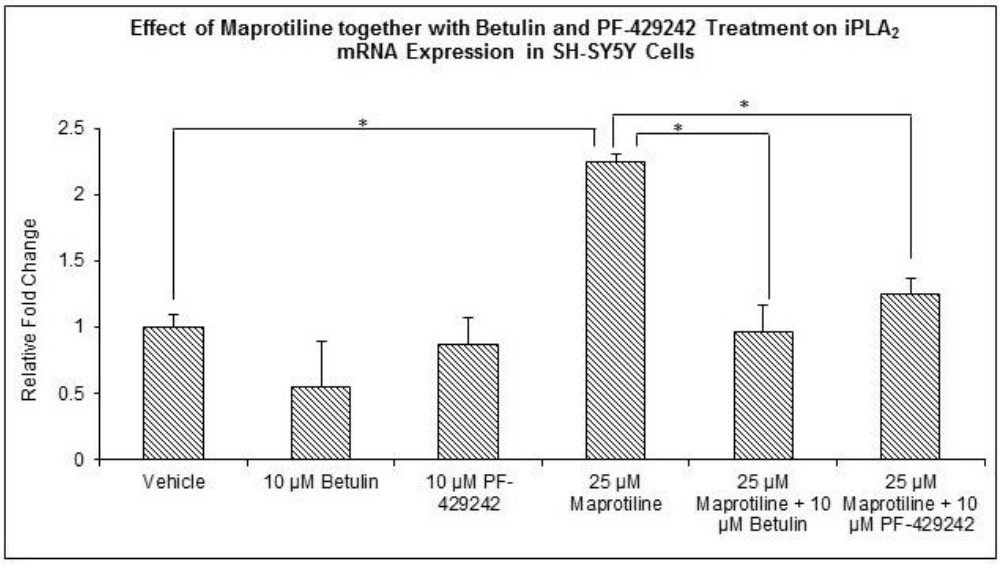


Fig. 4.17 Real time RT-PCR results. Effect of maprotiline and SREBP pathway inhibitors, betulin and PF-429242, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.16. Treatment with alpha-1 adrenergic receptor agonist and SREBP pathway inhibitors on iPLA$_2$ expression

There was a significant increase in iPLA$_2$ expression in SH-SY5Y cells after treatment with 50 µM alpha-1 receptor agonist, phenylephrine (1.41 fold change). Co-treatment of phenylephrine with the SREBP pathway inhibitor, betulin significantly reduced the phenylephrine-induced iPLA$_2$ expression (1.05 fold change). Co-treatment of phenylephrine with PF-429242 also abolished the phenylephrine-induced iPLA$_2$ expression (0.91 fold change) (Fig. 4.18). Results further support that the SREBP pathway plays a role in iPLA$_2$ expression induction.

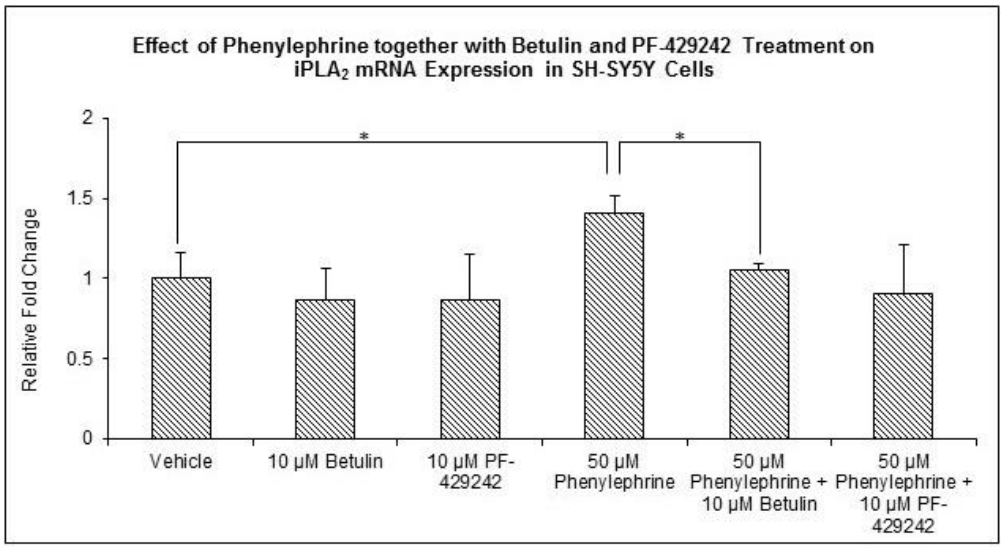


Fig. 4.18 Real time RT-PCR results. Effect of alpha-1 adrenergic receptor agonist, phenylephrine, and SREBP pathway inhibitors, betulin and PF-429242, treatment on iPLA$_2$ mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.2. Electrophoretic mobility shift assay

3.2.1. Effectiveness and binding specificity of iPLA$_2$ oligonucleotides EMSA probe to SREBP-2

To check the effectiveness and binding specificity of the iPLA$_2$ oligonucleotides EMSA probe containing the SRE region of the iPLA$_2$ gene, labeled iPLA$_2$ oligonucleotides EMSA probe and protein were incubated together with and without excess unlabeled iPLA$_2$ oligonucleotides followed by EMSA analysis. There was a shift present after labeled iPLA$_2$ oligonucleotides EMSA probe and protein incubation indicating binding of SREBP-2 to the SRE region of the iPLA$_2$ oligonucleotides, consistent with the EMSA results in a previous study which showed binding of SREBP-2 to the

EMSA probe (Seashols et al., 2004). Competition with excess unlabeled iPLA$_2$ oligonucleotide blocked this shift indicating binding specificity of iPLA$_2$ oligonucleotides EMSA probe to SREBP-2 (Fig. 4.19).

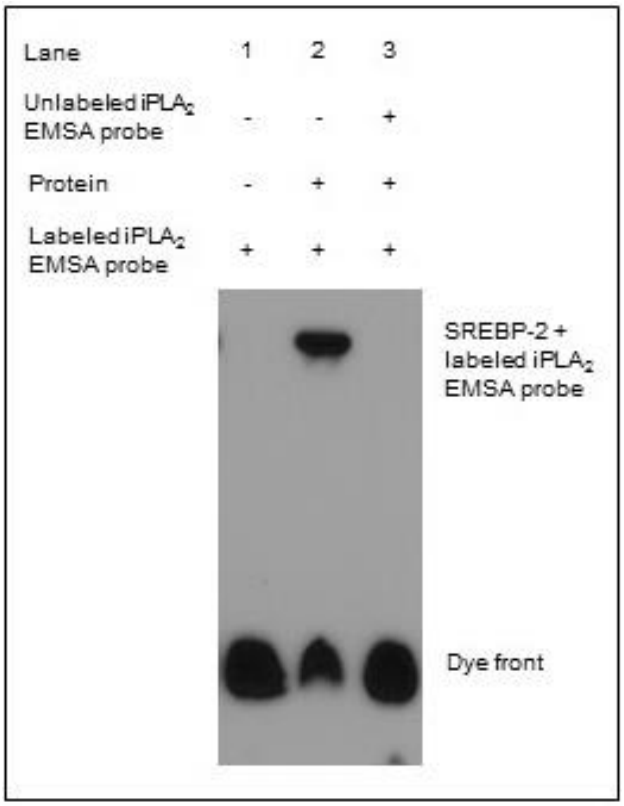
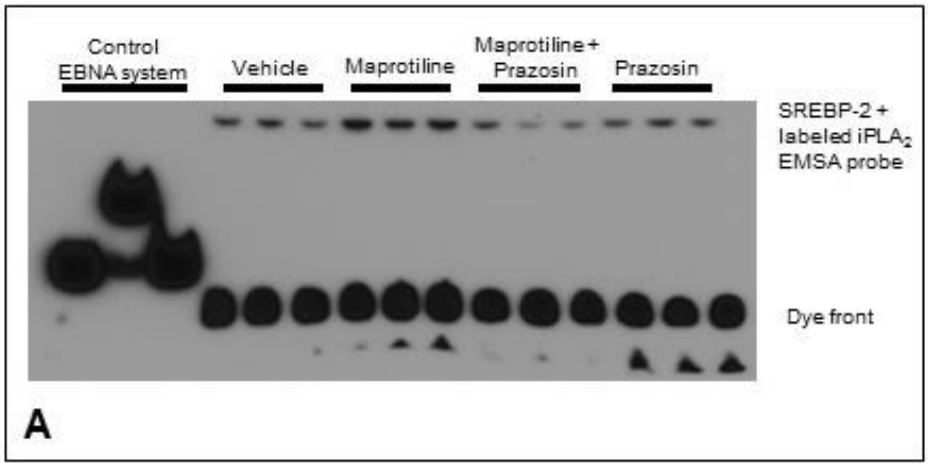


Fig. 4.19 EMSA blot showing the effectiveness and binding specificity of iPLA$_2$ oligonucleotides EMSA probe to SREBP-2. In Lane 1, there is no protein for the iPLA$_2$ EMSA probe to bind, thus no shift was observed. In Lane 2, there is protein present to effect the binding and shift of SREBP-2 and labeled iPLA$_2$ EMSA probe. A shift was thus detected in Lane 2 as compared to Lane 1. In Lane 3, the signal shift observed in Lane 2 was blocked by competition with excess unlabeled iPLA$_2$ EMSA probe indicating that the shift in Lane 2 is due to specific binding interactions between the target SREBP-2 protein and labeled iPLA$_2$ EMSA probe.

3.2.2. Effect of maprotiline and alpha-1 adrenergic receptor blocker treatment on the binding of SREBP-2 to the SRE region of the iPLA$_2$ gene

EMSA blot showed binding between SREBP-2 and labeled $iPLA_2$ oligonucleotides EMSA probe, similar to the EMSA blot presented earlier and consistent with a previous study (Seashols et al., 2004). Densitometric analysis found that there was a significant increase in SREBP-2 binding to the $iPLA_2$ oligonucleotides EMSA probe containing the SRE region of the $iPLA_2$ gene after maprotiline treatment (Fig. 4.20). Pre-treatment with the alpha-1 adrenergic receptor blocker, prazosin, significantly abolished this increase in SREBP-2 binding. Control EBNA system showed that the overall EMSA procedure was working properly.



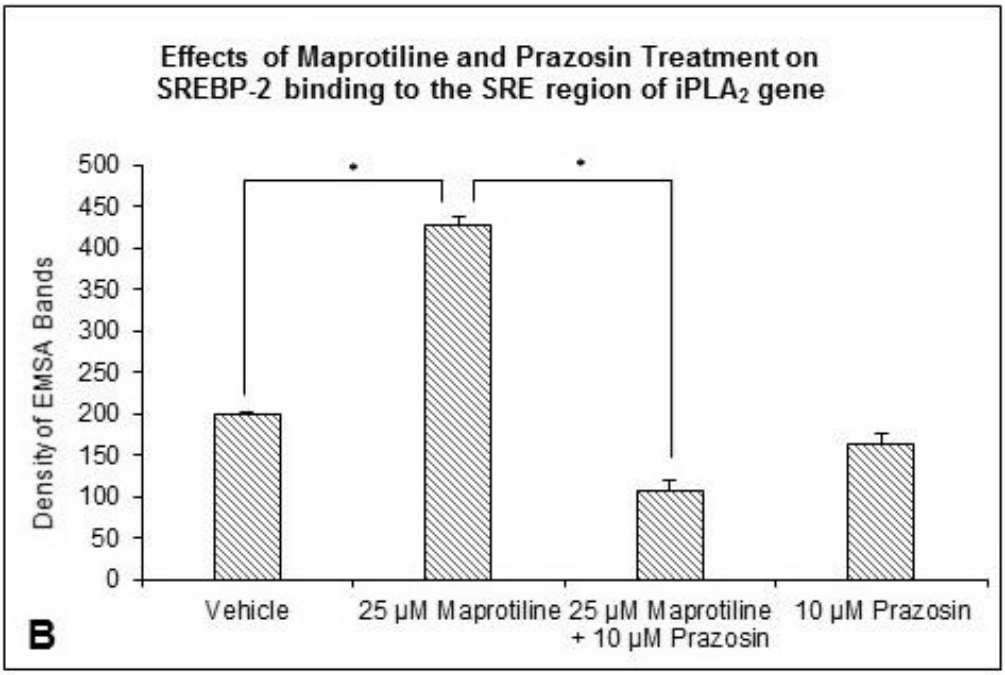


Fig. 4.20 EMSA blot densitometric analysis results. (A) EMSA blot and (B) densitometric analysis on the effects of maprotiline treatment and alpha-1 adrenergic receptor blocker, prazosin, on SREBP-2 binding to the SRE region of iPLA$_2$ gene. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$.

3.2.3. Effect of alpha-1 adrenergic receptor agonist and alpha-1 adrenergic receptor blocker treatment on the binding of SREBP-2 to the SRE region of the iPLA$_2$ gene

EMSA blot showed binding between SREBP-2 and labeled iPLA$_2$ oligonucleotides EMSA probe, similar to the EMSA blot presented earlier and consistent with a previous study (Seashols et al., 2004). Densitometric analysis revealed that there was a significant increase in SREBP-2 binding to the iPLA$_2$ oligonucleotides containing the SRE region of the iPLA$_2$ gene after treatment with alpha-1 adrenergic receptor agonist, phenylephrine (Fig. 4.21). Pre-treatment with the alpha-1 receptor blocker, prazosin abolished the increase in

SREBP2 binding. Control EBNA system showed that the overall EMSA procedure was working properly.

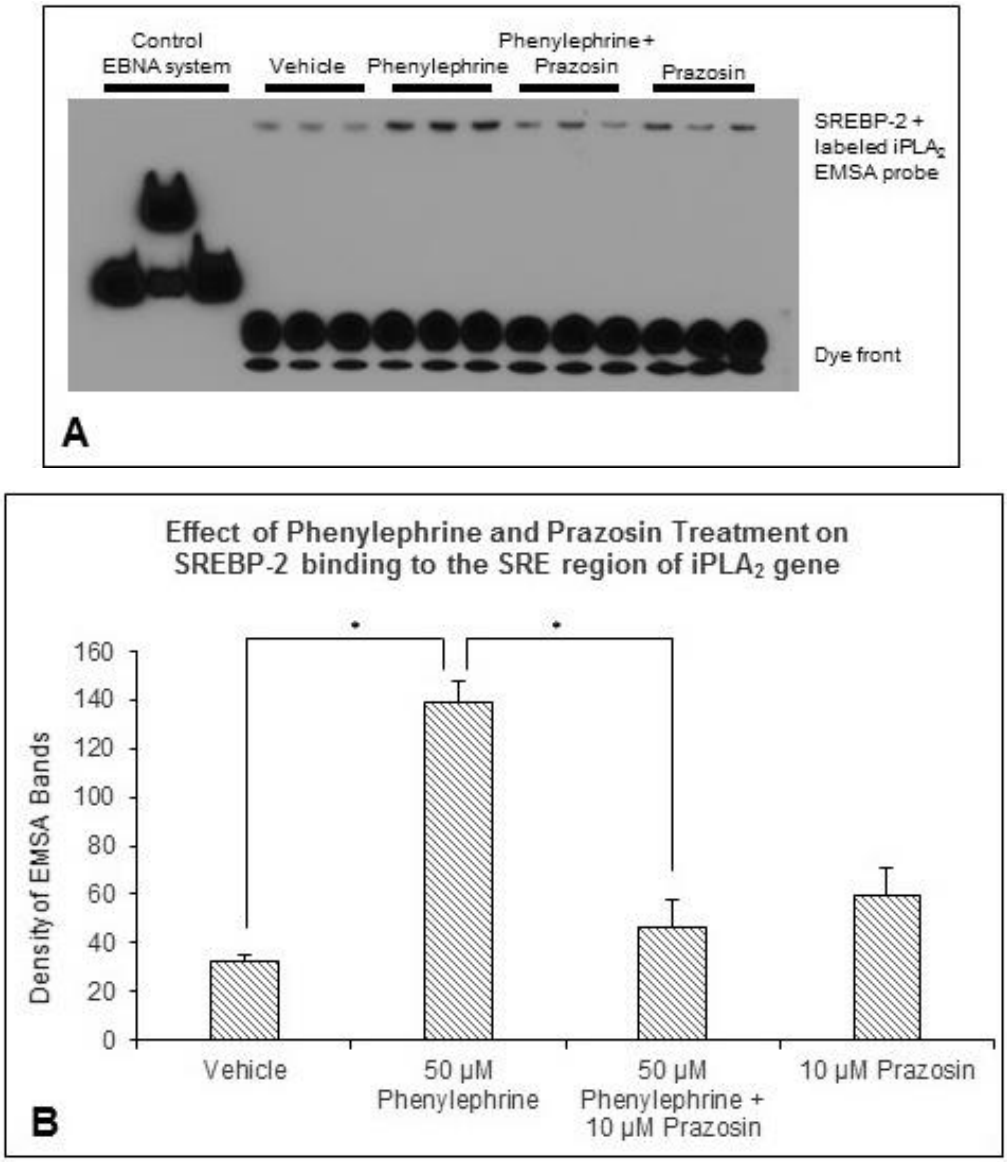
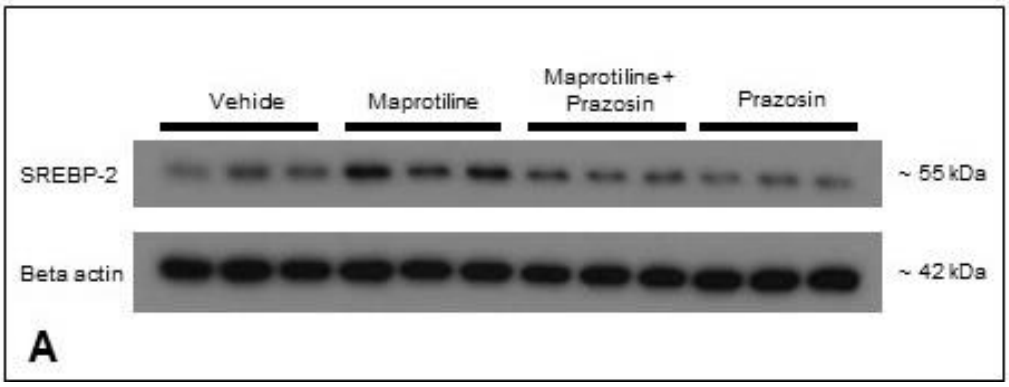


Fig. 4.21 EMSA blot densitometric analysis results. (A) EMSA blot and (B) densitometric analysis on the effect of alpha-1 adrenergic receptor agonist, phenylephrine, and alpha-1 adrenergic receptor blocker, prazosin, treatment on SREBP-2 binding to the SRE region of iPLA$_2$ gene. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$.

3.3. Western blot analysis

Western blot analysis showed the presence of a band at approximately 55 kDa which corresponds to the band size of activated SREBP-2 protein. Densitometric analysis of the protein blots showed a significant 2.17 fold increase in SREBP-2 protein expression in the SH-SY5Y cells after maprotiline treatment, compared to vehicle controls. This increase in SREBP-2 protein expression was significantly abolished after treatment with the alpha-1 adrenergic receptor blocker, prazosin (1.11 fold change) (Fig. 4.22).



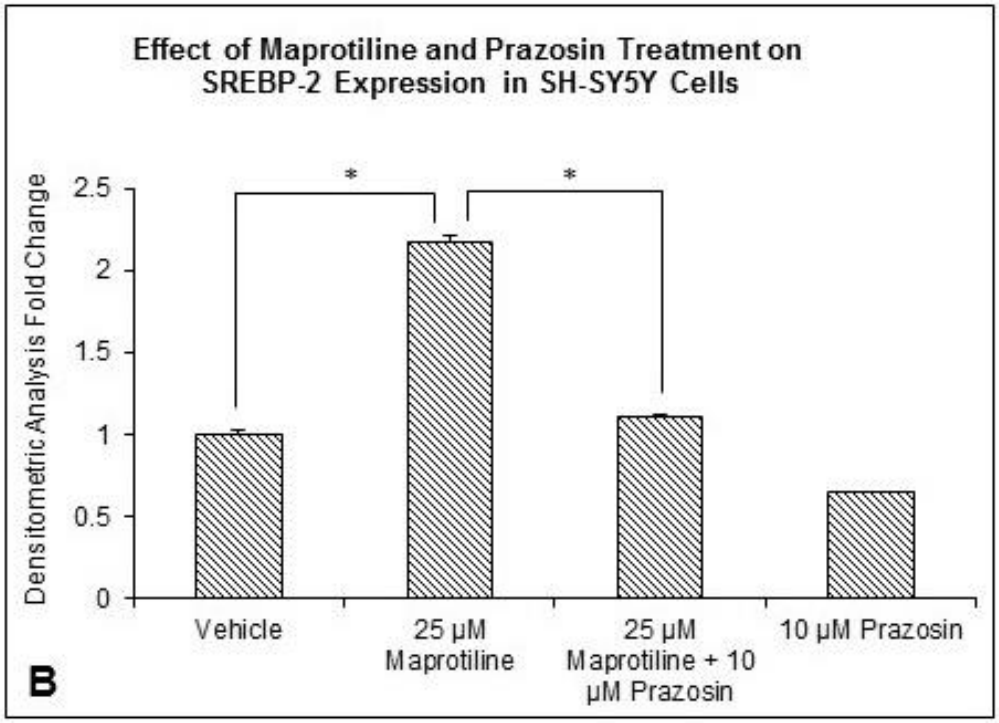


Fig. 4.22 Western blot densitometric analysis results. (A) Western blot and (B) densitometric analysis on the effect of maprotiline and alpha-1 adrenergic receptor blocker, prazosin, treatment on SREBP-2 expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$.

3.4. Immunocytochemistry

iPLA$_2$ immunofluorescence labeling showed localization of iPLA$_2$ protein in the cytoplasm (Fig. 4.23). A significant increase in fluorescence intensity was observed after maprotiline treatment, compared to vehicle control (Fig. 4.24). Blockade of alpha-1 adrenergic receptors by prazosin, or SREBP pathway by betulin, significantly abolished the maprotiline-induced increase in immunofluorescence.

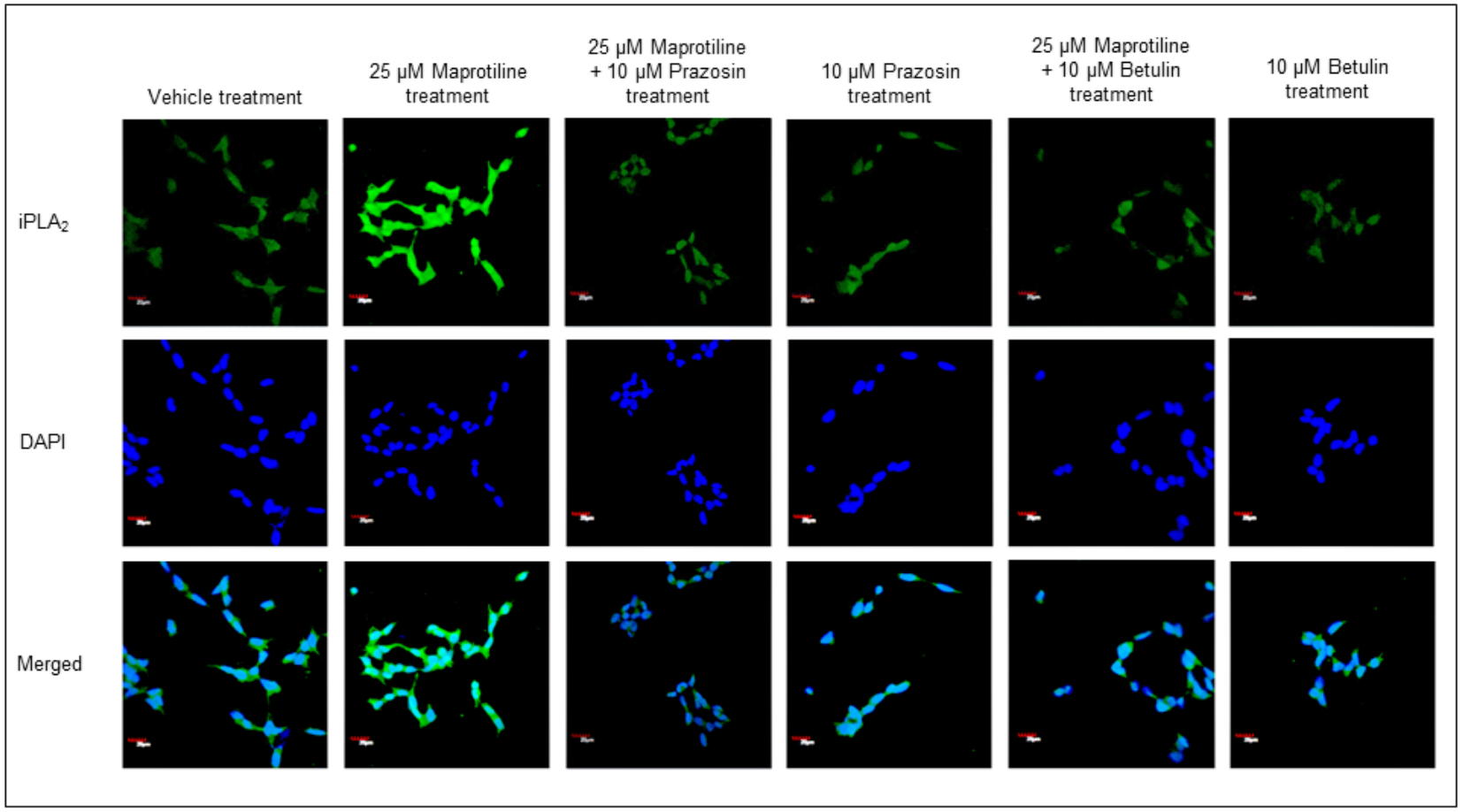


Fig. 4.23 Immunocytochemistry photos of iPLA$_2$ expression in SH-SY5Y cells after maprotiline, alpha-1 adrenergic receptor blocker, prazosin, and SREBP pathway inhibitor, betulin, treatment.

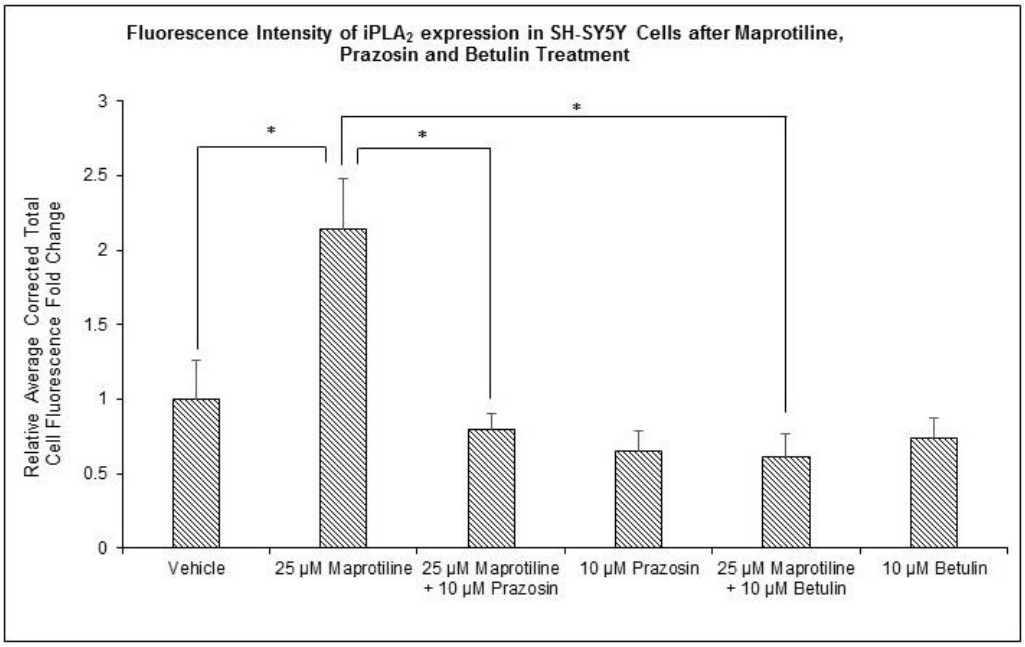


Fig. 4.24 Fluorescence intensity of iPLA$_2$ expression in SH-SY5Y cells after maprotiline, alpha-1 adrenergic receptor blocker, prazosin, and SREBP pathway inhibitor, betulin treatment. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$.

4. Discussion

This part of the study was carried out to investigate the role of adrenergic receptors and the possible mechanisms and pathways involved in regulation of iPLA$_2$ expression induction. Our previous study showed that maprotiline caused a significant increase in iPLA$_2$ expression in the mouse prefrontal cortex (Lee et al., 2012), and this was supported by the first part of this study. Similarly, real-time RT-PCR results showed that iPLA$_2$ expression in SH-SY5Y cells was significantly increased after maprotiline treatment. There was also a significant increase in iPLA$_2$ expression after nortriptyline but not amitriptyline treatment. Nortriptyline is a TCA with serotonin and

noradrenaline reuptake inhibition activity but with stronger selectivity for the inhibition of noradrenaline reuptake (Baldessarini, 2006; Rang et al., 2007). Thus, it is possible that the increase in iPLA$_2$ expression after maprotiline and nortriptyline treatment primarily involves inhibition of noradrenaline reuptake, leading to higher noradrenaline level and subsequent activation of the adrenergic receptors.

The involvement of adrenergic receptors in the antidepressant-induced iPLA$_2$ expression was further investigated, using adrenergic and serotonin receptor blockers. Treatment with the alpha-1 adrenergic receptor blocker, prazosin, significantly abrogated the maprotiline-induced increase in iPLA$_2$ expression. In addition, prazosin also significantly abolished the nortriptyline-induced iPLA$_2$ expression, suggesting that alpha-1 adrenergic receptors are essential for iPLA$_2$ expression induction. Alpha-1 adrenergic receptors are mainly located on the postsynaptic membrane (Starke et al., 1989; Rang et al., 2007). Neurotransmission facilitation by the alpha-1 adrenergic receptor has been found to be involved in the effect of antidepressant therapy (Vetulani, 1984; Vetulani et al., 1984). Studies also revealed that chronic antidepressant treatment caused an eventual up-regulation of alpha-1 adrenergic receptors coupled with a down-regulation of alpha-2 and beta adrenergic receptors in the rat cerebral cortex (Vetulani, 1984; Vetulani et al., 1984). The importance of alpha-1 adrenergic receptors in depression and antidepressant therapy was further highlighted by a study showing that a reduction in alpha-1 noradrenergic transmission resulted in depressive behavior (Stone and Quartermain, 1999). In comparison, treatment with idazoxan, a selective alpha-2 adrenergic receptor blocker, did not inhibit the increase in iPLA$_2$

expression induced by maprotiline treatment in SH-SY5Y cells. We also investigated a potential role of serotonin receptors in the antidepressant-induced iPLA$_2$ expression using a serotonin receptor blocker, WAY100635 (Fletcher et al., 1996). Treatment with WAY100635 did not significantly affect the increase in iPLA$_2$ expression after maprotiline or nortriptyline treatment. Together, our results suggest that the alpha-1 adrenergic receptor has an important role in regulation of iPLA$_2$ expression induction. This was further supported by our findings that treatment with the alpha-1 adrenergic receptor agonist, phenylephrine, also caused a significant albeit lower increase in iPLA$_2$ expression as compared to antidepressant treatment. In addition, this increase in iPLA$_2$ expression after phenylephrine treatment was significantly abolished by co-treatment with prazosin, further supporting the role of alpha-1 adrenergic receptors in iPLA$_2$ expression.

Regulation of iPLA$_2$ expression may also involve the MAPK/ERK or cAMP/PKA signaling pathways downstream from the adrenergic receptors. We then studied the potential roles of these signaling pathways in mediating iPLA$_2$ expression induction using specific signaling pathway inhibitors. Treatment with MAPK/ERK pathway inhibitors significantly abolished the maprotiline-induced expression of iPLA$_2$, indicating involvement of the MAPK/ERK signaling pathway in iPLA$_2$ expression induction. As stated earlier, one of the most studied alpha-1 adrenergic receptors-stimulated pathways is the ERK1/2 activation signaling pathway (Garcia-Sainz et al., 1999; Hague et al., 2002). Alpha-1 adrenergic receptors are G protein-coupled receptors and are activated by adrenaline and noradrenaline (Garcia-Sainz et al., 1999). Besides ERK1/2, alpha-1 adrenergic receptors are involved in

activation of PKC which was also shown to mediate iPLA$_2$ expression and activity (Garcia-Sainz et al., 1999; Hu et al., 1999; Rohde et al., 2000; Steer et al., 2002; Meyer et al., 2005). Moreover, induction of PLA$_2$ protein expression in endothelial cell-pericyte co-cultures was found to involve activation of PKC and the MAPK/ERK cascade, further supporting the involvement of the MAPK/ERK signaling pathway in mediating iPLA$_2$ expression induction (Anfuso et al., 2007). Besides the MAPK/ERK signaling pathway, the cAMP-dependent cascade which involves PKA activation could potentially have a role in the up-regulation of iPLA$_2$ expression. However, treatment with PKA inhibitors failed to significantly abolish the increase in iPLA$_2$ expression, suggesting that the cAMP/PKA pathway does not play a key role in iPLA$_2$ expression. Together, our results support a role of MAPK/ERK signaling pathway rather than the cAMP/PKA cascade in iPLA$_2$ expression induction.

SREBP-2 is a transcription factor and was previously shown to be involved in iPLA$_2$ expression (Seashols et al., 2004). The effect of antidepressant and alpha-1 adrenergic receptor agonist treatment on SREBP-2 expression was thus further elucidated. Treatment with maprotiline significantly increased SREBP-2 mRNA expression and this increase was abrogated by blockade of the alpha-1 adrenergic receptors with prazosin. Similarly, phenylephrine significantly increased SREBP-2 mRNA expression and this increase was abolished when the alpha-1 adrenergic receptors were blocked with prazosin, highlighting the involvement of alpha-1 adrenergic receptors in both iPLA$_2$ and SREBP-2 expression. MAPK/ERK pathway inhibitors also significantly abolished the increase SREBP-2 expression, indicating the involvement of the MAPK/ERK signaling cascade in SREBP-2

as well as iPLA$_2$ expression. This was supported by a previous study that showed an association between SREBP-2 and the MAPK/ERK signaling cascade (Kotzka et al., 2000). In that study, a direct link was found between MAPK activity and SREBPs as ERK1 and ERK2 were shown to phosphorylate GST-fusion SREBP-2 protein *in vitro*, suggesting that SREBP-2 is a direct substrate of ERK1 and ERK2. SREBP pathway inhibitors were then used to study the effect of SREBP inhibition on iPLA$_2$ expression induction. Our results showed that treatment with SREBP pathway inhibitors, betulin and PF-429242, significantly abolished the maprotiline-induced increase in iPLA$_2$ expression. SREBP pathway inhibitors also abrogated the phenylephrine-induced iPLA$_2$ expression. Betulin inhibits the maturation of SREBP while PF-429242 selectively inhibits S1P, an enzyme involved in the release of mature activated SREBP (Hawkins et al., 2008; Tang et al., 2011). Based on our results, it was then suggested that SREBP-2 is essential for iPLA$_2$ expression induction and is similarly regulated by alpha-1 adrenergic receptors and the MAPK/ERK signaling pathway.

A previous study showed a link between SREBP-2 and the iPLA$_2$ promoter, which contains the SRE sequence. A mutant cell line that constitutively produces mature SREBP proteins showed increased iPLA$_2$ expression and activity (Seashols et al., 2004). EMSA analysis showed that mature SREBP-2 forms a complex with a 30-mer EMSA probe corresponding to the SRE sequence in the iPLA$_2$ promoter region (Seashols et al., 2004). Based on these seminal findings, EMSA analysis was carried out in our study using a non-radioactive iPLA$_2$ EMSA probe with the same oligonucleotide sequences as used in the aforementioned study (Seashols et al., 2004). Our

results confirmed that SREBP-2 indeed binds to the SRE region of the iPLA$_2$ promoter and treatment with both maprotiline and phenylephrine significantly increased this binding. The increase in binding was abolished by co-treatment with prazosin, consistent with a role of alpha-1 adrenergic receptors in causing an increase in SREBP-2 and iPLA$_2$ expression. Results from our western blot analysis also confirmed that maprotiline treatment increased SREBP-2 expression and this increase was significantly blocked by co-treatment with prazosin. Immunocytochemical staining showed an increase in iPLA$_2$ immunostaining after maprotiline treatment, and this increase was significantly abolished by blockade of alpha-1 adrenergic receptors with prazosin and inhibition of the SREBP pathway with betulin. These results corroborate the RT-PCR and EMSA findings and further emphasize the role of alpha-1 adrenergic receptors and SREBP pathway in iPLA$_2$ expression.

Overall, results from the present study indicate that stimulation of alpha-1 adrenergic receptors causes increased iPLA$_2$ expression via the MAPK/ERK signaling pathway and SREBP-2 protein. It is important to note however, that this study was carried out under *in vitro* conditions and further study is needed to validate these findings in an *in vivo* setting. Nonetheless, our findings may potentially provide a basis for further research on the regulation of iPLA$_2$ expression and possible crosstalk between sterol and glycerophospholipid mediators that may play a role in physiological or pathophysiological processes in the brain and other organs.

Chapter 5: Effect of Antidepressant Treatment on 15-LOX Expression

1. Introduction

We have established earlier that antidepressant treatment, especially those with strong noradrenaline reuptake inhibition activity, induced an up-regulation in $iPLA_2$ expression. Increasing $iPLA_2$ expression and activity will lead to enhanced production of DHA which can then be metabolized by 15-LOX to produce resolvins and neuroprotectins (Farooqui, 2011). 15-LOX is a member of the non-heme LOX family of enzymes which have dioxygenase activity and a molecular weight of around 75-78 kDa (Radmark and Samuelsson, 2009). They are essential for the dioxygenation or the insertion of molecular oxygen into PUFAs in lipids (Radmark and Samuelsson, 2009). The molecular structure of LOX consists of a single polypeptide chain which folds into two domains (Brash, 2001). Besides 15-LOX, there are three other types of LOX: 5-LOX, 8-LOX and 12-LOX. LOX enzymes are categorized according to the position of oxygen insertion where the dioxygenation takes place, either at carbon 5, 8, 12, or 15 of the aliphatic chain (Phillis et al., 2006). Out of the four different LOX enzymes, only three are present in the brain: 5-LOX, 12-LOX and 15-LOX.

15-LOX is also known as arachidonate 15-LOX and is encoded by the *ALOX15* gene. As stated earlier, 15-LOX catalyzes the metabolism of DHA into docosanoids consisting of resolvins and neuroprotectins. DHA is a precursor for the production of D-series resolvins and DHA is first converted into 17S-hydroperoxy-DHA (17S-H(p)DHA) with the involvement of 15-LOX (Serhan and Chiang, 2008; Farooqui, 2011; Ji et al., 2011). 17S-H(p)DHA is then catalyzed into several different bioactive compounds such as RvD1 (Fig. 5.1). 15-LOX is also involved in the conversion of NPD1 from

DHA in the brain via epoxide intermediates with the epoxy group located at the 16(17) position (Hong et al., 2003; Farooqui, 2011). Resolvins are suggested to act via specific receptors called resolvin D receptors which can be found in neural as well as non-neural cells and they are essential in stimulating strong immunoregulatory and anti-inflammatory activities (Serhan et al., 2008a; Serhan et al., 2008b). DHA and its metabolites have been shown to have a significant role on synaptic functions and resolvins were suggested to normalize the spinal synaptic plasticity linked with pain hypersensitivity (Xu et al., 2010; Farooqui, 2011). RvD1 was also shown to have pro-resolving and anti-inflammatory properties in ocular surface inflammation and cigarette smoke-induced lung inflammation (Hsiao et al., 2013; Erdinest et al., 2014). Furthermore, resolvins and neuroprotectins can modulate the effects of proinflammatory eicosanoids derived from AA (Hong et al., 2003). Any changes in 15-LOX expression will then impact the generation of resolvins and neuroprotectins, affecting their overall anti-inflammatory and neuroprotective effects.

In view that $iPLA_2$ expression was increased after maprotiline and nortriptyline treatment, leading to increased production of DHA which is the precursor for resolvins and neuroprotectins, it is possible that 15-LOX may also be affected by antidepressant treatment and regulated in a similar way as $iPLA_2$. We then postulate that besides inducing $iPLA_2$ expression, antidepressants especially those with strong noradrenaline reuptake inhibition activity will similarly stimulate the expression of 15-LOX enzyme. In this part of the study, we studied the effect of antidepressant treatment on 15-LOX expression.

Real-time RT-PCR and Western blot analyses were carried out to investigate the effect of antidepressant treatment on 15-LOX expression in SH-SY5Y cells. Co-treatment with alpha-1 adrenergic receptor blocker, prazosin, was also performed to study the involvement of alpha-1 adrenergic receptors in 15-LOX expression.

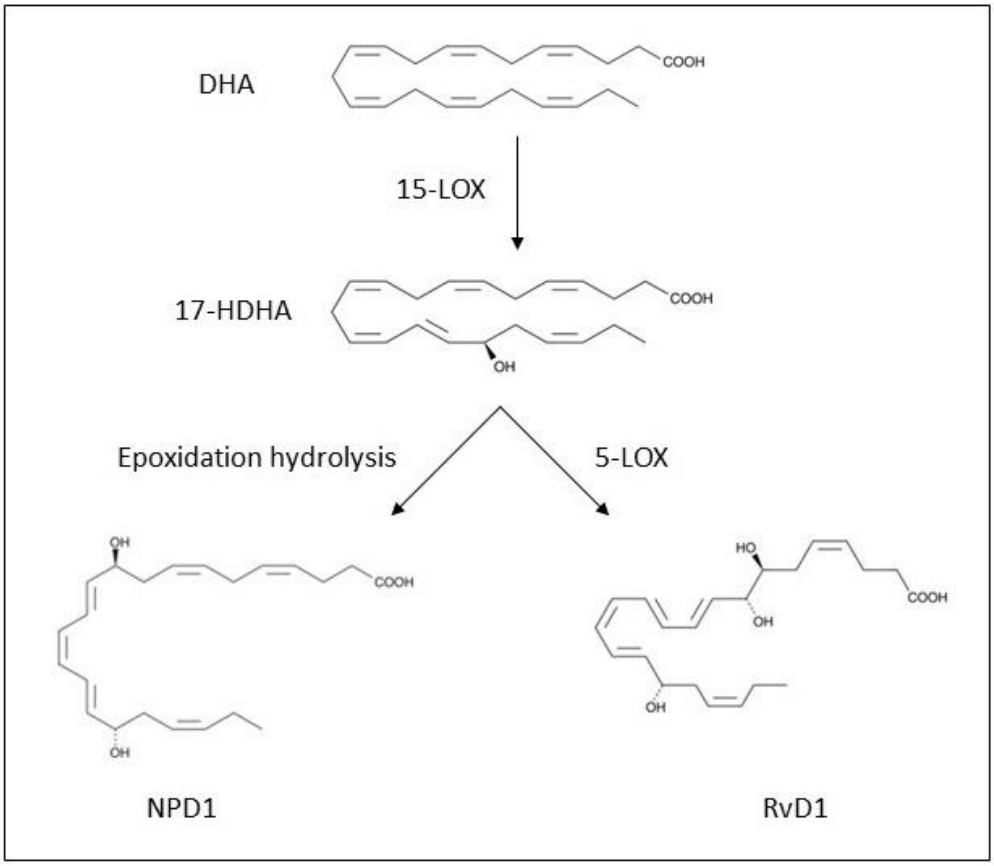


Fig. 5.1 The metabolic steps involved in generation of neuroprotectin D1 (NPD1) and resolvin D1 (RvD1) from DHA. Adapted from (Rius et al., 2012)

2. Materials and method

2.1. Cell culture

For this part of the study, SH-SY5Y cells were similarly obtained from ATCC (Manassas, VA, U.S.A.) and used for cell culture experiments. The

cells were cultured until reaching approximately 80% confluency and maintained in DMEM supplemented with 10 % fetal bovine serum, 1 % penicillin-streptomycin solution at 37 °C with 5 % CO_2 aeration. Cells were then separated into treatment groups where each group consisted of five 100 mm culture plates for subsequent analyses (n = 5).

2.1.1. Treatment with antidepressants

Treatment with different antidepressants was first carried out to investigate their effect on 15-LOX expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 25 µM maprotiline 3) Treatment with 25 µM amitriptyline 4) Treatment with 25 µM nortriptyline. Incubation was carried out for 24 hours, after which the cells were washed in PBS and harvested for real-time RT-PCR analysis.

2.1.2. Treatment with maprotiline and alpha-1 adrenergic receptor blocker

Treatment with maprotiline and alpha-1 adrenergic receptor blocker was performed to determine their effect on 15-LOX expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with vehicle, DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 25 µM maprotiline 4) Treatment with 25 µM maprotiline and 10 µM prazosin. Prazosin pre-treatment was carried out for 1 hour followed by maprotiline or vehicle incubation for another 24 hours, after which the cells were harvested for real-time RT-PCR and Western blot analyses.

2.1.3. Treatment with nortriptyline and alpha-1 adrenergic receptor blocker

Treatment with nortriptyline and alpha-1 adrenergic receptor blocker was carried out to study their effect on 15-LOX expression. SH-SY5Y cells were divided into 4 groups: 1) Treatment with DMSO 2) Treatment with 10 µM prazosin 3) Treatment with 25 µM nortriptyline 4) Treatment with 25 µM nortriptyline and 10 µM prazosin. Prazosin pre-treatment was carried out for 1 hour followed by nortriptyline or vehicle incubation for another 24 hours, after which the cells were harvested for real-time RT-PCR analysis.

2.2. Real-time RT-PCR

SH-SY5Y RNA samples were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as described previously. Quantitative real-time PCR amplification was then carried out in the 7500 Real-time PCR system (Applied Biosystems, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA) and Applied Biosystem TaqMan® probes for human 15-LOX (ALOX15) and beta-actin (Hs00609608_m1 and #4326315E respectively). All reactions were carried out in triplicates and the PCR conditions were as described previously.

2.3. Western blot analysis

Proteins were extracted from SH-SY5Y cells and their concentrations were measured as stated previously. Subsequent western blot analysis was carried out as described earlier using 30 µg of protein and mouse monoclonal antibody to 15-LOX (ab119774, Abcam, New Territories, HK; diluted 1:500).

2.4. Statistical analysis

The mean and standard errors of the values were calculated for each experimental group, and possible significant differences between the means were examined using one-way ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Real time RT-PCR

3.1.1. Antidepressant treatment

Real-time RT-PCR showed a significant increase in 15-LOX mRNA expression in SH-SY5Y cells after maprotiline (2.09 fold change) and nortriptyline (1.96 fold change) treatment for 24 hours. However, there was no significant increase in 15-LOX expression after amitriptyline treatment (0.96 fold change) (Fig. 5.2).

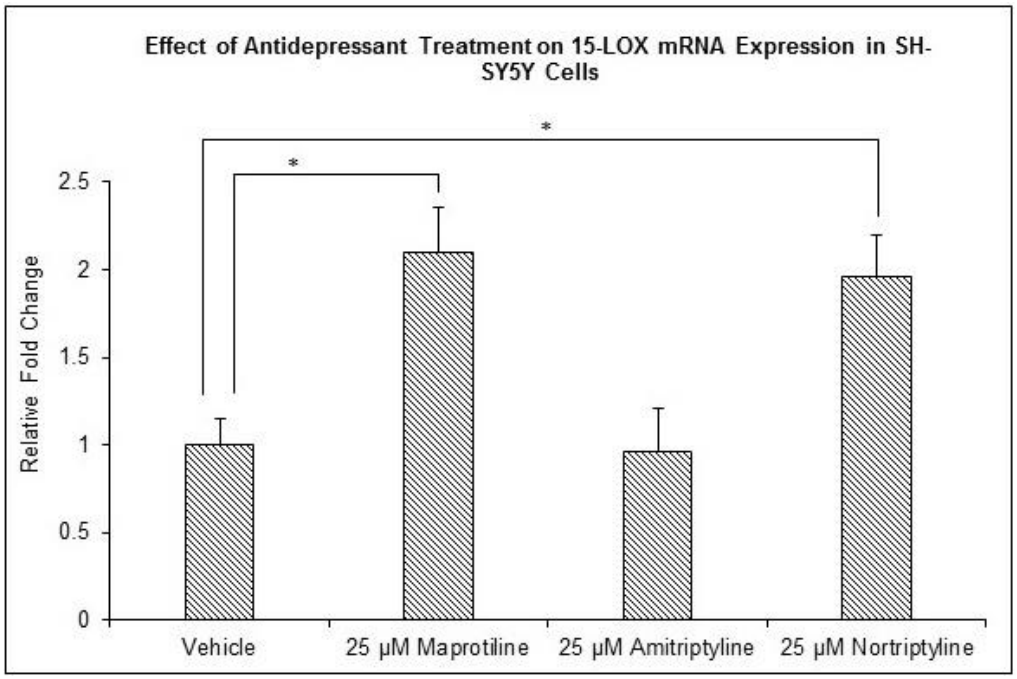


Fig. 5.2 Real time RT-PCR results. Effect of antidepressant treatment on 15-LOX mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.2. Treatment with maprotiline and alpha-1 adrenergic receptor blocker

Real-time RT-PCR showed a significant 1.7 fold increase in 15-LOX mRNA expression after maprotiline treatment (Fig. 5.3). Treatment with alpha-1 adrenergic receptor blocker, prazosin, significantly abolished this increase (0.96 fold change). Results suggest the involvement of alpha-1 adrenergic receptors in 15-LOX expression induction.

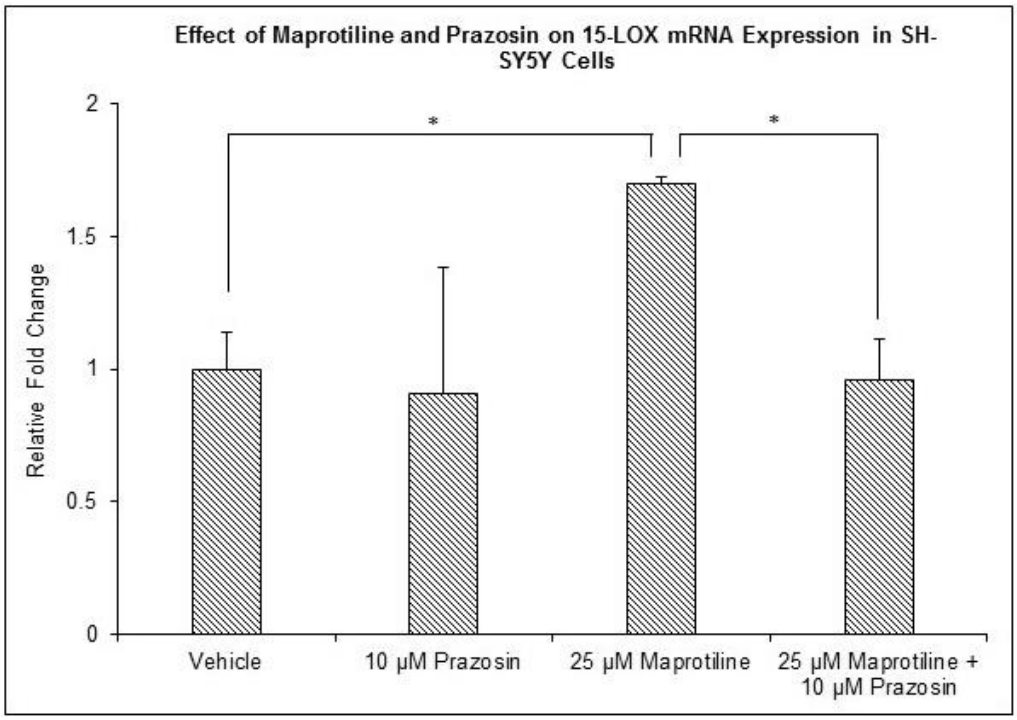


Fig. 5.3 Real time RT-PCR results. Effect of maprotiline and alpha-1 adrenergic receptor blocker, prazosin, on 15-LOX mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.1.3. Treatment with nortriptyline and alpha-1 adrenergic receptor blocker

Real-time RT-PCR showed a significant 1.79 fold increase in 15-LOX mRNA expression after nortriptyline treatment (Fig. 5.4). Treatment with alpha-1 adrenergic receptor blocker significantly abolished this increase (1.19 fold change). Results showed that besides being involved in maprotiline-induced 15-LOX expression, alpha-1 adrenergic receptors are also essential for the nortriptyline-induced expression of 15-LOX.

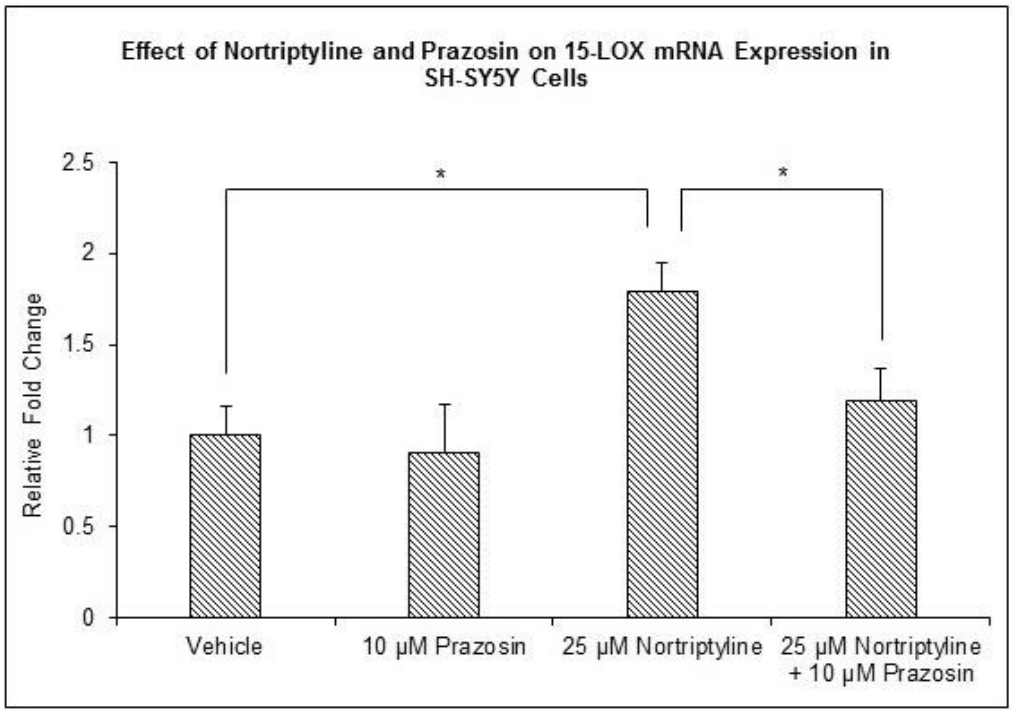


Fig. 5.4 Real time RT-PCR results. Effect of nortriptyline and alpha-1 adrenergic receptor blocker, prazosin, on 15-LOX mRNA expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$. $N = 5$.

3.2. Western blot analysis

Western blot analysis showed the presence of a band at approximately 75 kDa which corresponds to the band size of 15-LOX protein. Densitometric analysis of the protein blots showed a significant 2.10 fold change increase in 15-LOX protein expression in the SH-SY5Y cells after maprotiline treatment, compared to vehicle controls (Fig. 5.5). The increase in 15-LOX expression was significantly abolished after treatment with the alpha-1 adrenergic receptor blocker, prazosin (0.47 fold change).

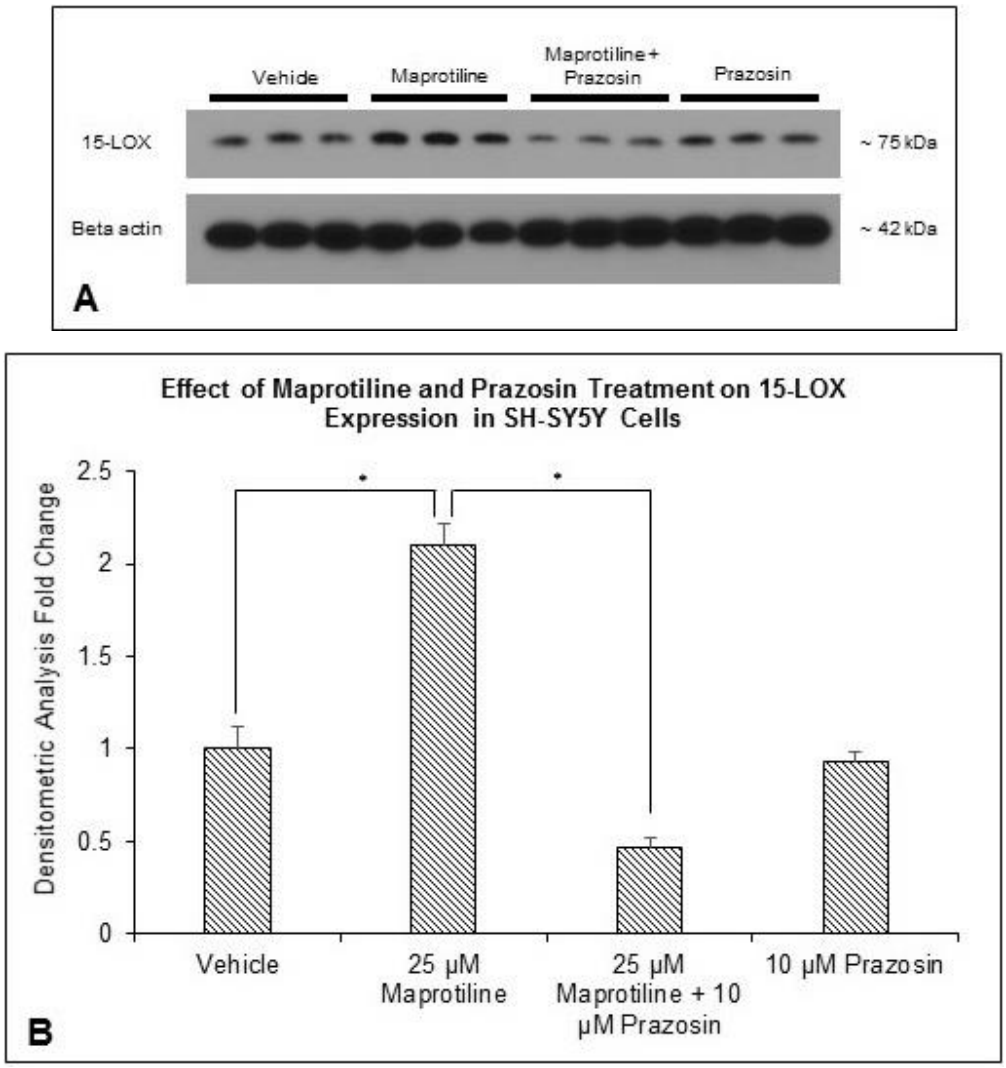


Fig. 5.5 Western blot densitometric analysis results. (A) Western blot and (B) densitometric analysis on the effect of maprotiline and alpha-1 adrenergic receptor blocker, prazosin, treatment on 15-LOX expression in SH-SY5Y cells. Analyzed by one-way ANOVA with Bonferroni post-hoc test. Asterisks (*) indicate significant differences at $P < 0.05$.

4. Discussion

This part of the study was carried out to investigate the effect of antidepressant treatment on 15-LOX expression in SH-SY5Y cells. Administration of maprotiline significantly increased 15-LOX mRNA expression. Nortriptyline treatment showed a similar significant increase in 15-LOX expression. Treatment with amitriptyline, however, did not affect 15-

LOX expression. The effect of antidepressant treatment on 15-LOX expression was found to have a similar trend as the antidepressant action on $iPLA_2$ expression where maprotiline and nortriptyline increased both $iPLA_2$ and 15-LOX expression but amitriptyline did not have any effect. Thus, it is possible that 15-LOX and $iPLA_2$ both share common signaling pathways or mechanisms in regulating their expression induction.

As stated previously, maprotiline and nortriptyline both have strong effects on the noradrenaline transporter and weaker activity on the serotonin transporter as compared to amitriptyline (Frazer, 1997a). Thus, like $iPLA_2$, 15-LOX expression was also suggested to involve activation of the alpha-1 adrenergic receptors by maprotiline and nortriptyline treatment. This was further investigated using the alpha-1 adrenergic receptor blocker, prazosin. Blockade of the alpha-1 adrenergic receptor by prazosin significantly abolished maprotiline and nortriptyline-induced 15-LOX expression, thus suggesting that alpha-1 adrenergic receptor activation is indeed essential for antidepressant-induced 15-LOX expression. Real-time RT-PCR results was supported by Western blot results where maprotiline treatment increased 15-LOX protein expression and this increase was abolished by prazosin treatment.

Overall, our preliminary results showed an increase in 15-LOX expression after antidepressant treatment and this increase involved alpha-1 adrenergic receptor activation. Increasing 15-LOX expression coupled with higher DHA production due to antidepressant-induced $iPLA_2$ expression will subsequently lead to enhanced levels of resolvins and neuroprotectins, potentially enhancing neural plasticity. Antidepressant treatment has been

shown to enhance neurogenesis and increase plasticity in the adult human brain (Warner-Schmidt and Duman, 2006; Pittenger and Duman, 2008; Castren and Hen, 2013). In addition, RvD1 was shown to attenuate NMDA receptor phosphorylation and normalize the spinal synaptic plasticity under pain conditions (Xu et al., 2010; Quan-Xin et al., 2012). Together, our results suggest that besides inducing $iPLA_2$ and increasing the production of DHA, antidepressants with strong noradrenaline reuptake inhibition activity also has an effect on enzymes affecting DHA, most notably 15-LOX. The increase in both $iPLA_2$ and 15-LOX may then lead to an overall increase in DHA and its metabolites which may activate the prefrontal cortex to stimulate the PAG and subsequent descending pain inhibitory pathway. Thus, it is possible that besides involving $iPLA_2$, the effects of antidepressants on plasticity and antinociception could also potentially include activation of 15-LOX and subsequent production of resolvins and neuroprotectins although additional work is needed to investigate this further.

Chapter 6: Conclusions

Conclusions

In the first part of this study, we investigated the role of prefrontal cortical $iPLA_2$ in antidepressant-induced antinociception. Mice that received maprotiline injection showed increased $iPLA_2$ mRNA and protein expression in the prefrontal cortex, and reduced nociceptive responses in a carrageenan mouse model of inflammatory orofacial pain. Injection of antisense oligonucleotide to $iPLA_2$ in the dorsolateral prefrontal cortex abolished the antinociceptive effect of maprotiline. In contrast, $iPLA_2$ antisense injection in the somatosensory cortex had no effect on antinociception. Lipidomic analysis showed decreased PC and increased LPC lipid species in the prefrontal cortex after maprotiline treatment, indicating increased $iPLA_2$ enzymatic activity and endogenous release of DHA and EPA. These changes were blocked by intracortical $iPLA_2$ antisense injection. Together, results from the first part of this study indicate an important role of prefrontal cortical $iPLA_2$ in the antinociceptive effect of maprotiline, and suggest the involvement of $iPLA_2$ not only in the antidepressive, but also antinociceptive effects of maprotiline and possibly other similar antidepressants.

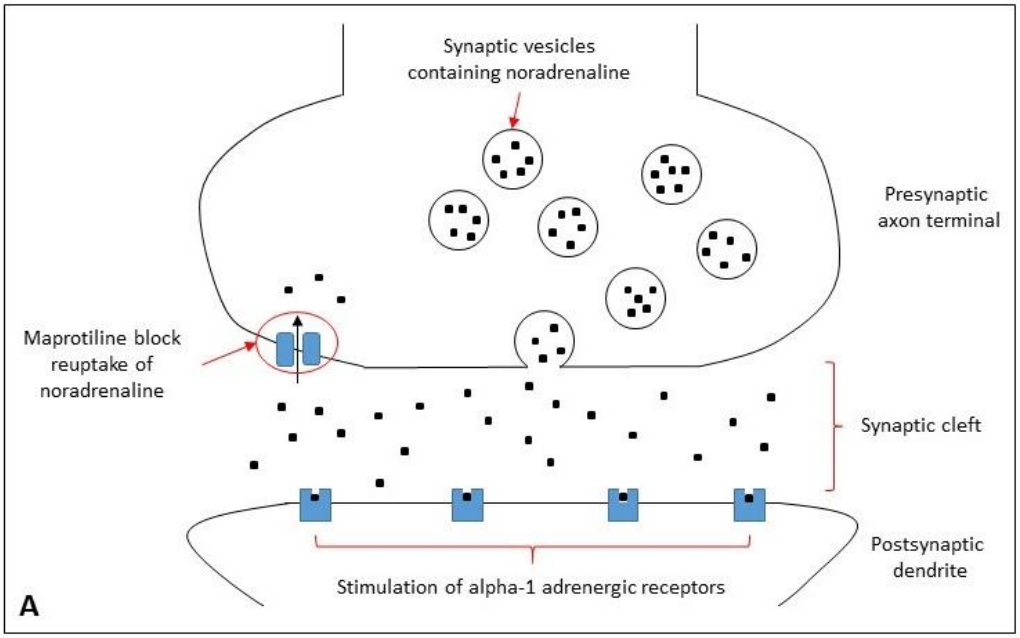
In the second part of this study, we elucidated the potential cellular mechanisms involved in the regulation of $iPLA_2$ expression induction. Treatment of SH-SY5Y neuroblastoma cells with maprotiline and another TCA with strong noradrenaline reuptake inhibition activity, nortriptyline, as well as the alpha-1 adrenergic receptor agonist, phenylephrine, resulted in increased $iPLA_2$ expression. This increase was blocked by inhibitors of the MAPK/ERK signaling cascade, SREBP pathway and the alpha-1 adrenergic receptor. In addition, maprotiline and phenylephrine increased the binding of

SREBP-2 to the SRE region on the $iPLA_2$ gene, as determined by EMSA. Together, our results indicate that stimulation of alpha-1 adrenergic receptors causes increased $iPLA_2$ expression via MAPK/ERK and SREBP pathways.

We have shown that stimulation of alpha-1 adrenergic receptors by maprotiline and nortriptyline led to an increase in $iPLA_2$ expression and activity. Thus, in the third part of this study, we studied the possibility that other enzymes affecting DHA such as 15-LOX may also be regulated in the same way by maprotiline and other similar antidepressants. Overall, our results showed an increase in 15-LOX expression after maprotiline and nortriptyline treatment and this increase was suggested to be associated with alpha-1 adrenergic receptor activation. Increasing 15-LOX expression coupled with more DHA production due to antidepressant-induced $iPLA_2$ expression may subsequently lead to enhanced levels of resolvins and neuroprotectins.

In conclusion, it was suggested that prefrontal cortical $iPLA_2$ has an important role in the antinociceptive effect of antidepressants especially those with strong noradrenaline reuptake inhibition activity such as maprotiline. Our results also showed that the induction of $iPLA_2$ expression is potentially regulated by alpha-1 adrenergic receptors, MAPK/ERK signaling pathway and SREBP-2. Additionally, we found that 15-LOX is similarly increased by maprotiline and nortriptyline treatment. It is possible that antidepressant treatment with strong noradrenaline reuptake inhibition activity like maprotiline will first stimulate alpha-1 adrenergic receptors by increasing noradrenaline levels in the synaptic cleft (Fig. 6.1A). The stimulated alpha-1 adrenergic receptors will lead to activation of the MAPK/ERK signaling pathway, in particular ERK1/2, which then induce phosphorylation of SREBP-

2 as well as increase SREBP-2 expression and stimulate its transcriptional activity (Fig 3.47B). Activated SREBP-2 will enter the nucleus and bind to the SRE sequence on the $iPLA_2$ gene promoter, increasing $iPLA_2$ expression (Fig 3.47B). Increased $iPLA_2$ expression will lead to higher release of DHA from glycerophospholipids and the generated DHA can be further metabolized by 15-LOX which undergoes a concurrent increase in expression after antidepressant treatment (Fig. 6.1B). Increased DHA and its metabolites may then potentially facilitate activity or plasticity in the dorsolateral prefrontal cortex to stimulate the PAG and descending pain inhibitory pathway (Fig. 6.1C). The potential mechanisms and signaling pathways involved in antidepressant-induced antinociception is summarized in Fig. 6.2.



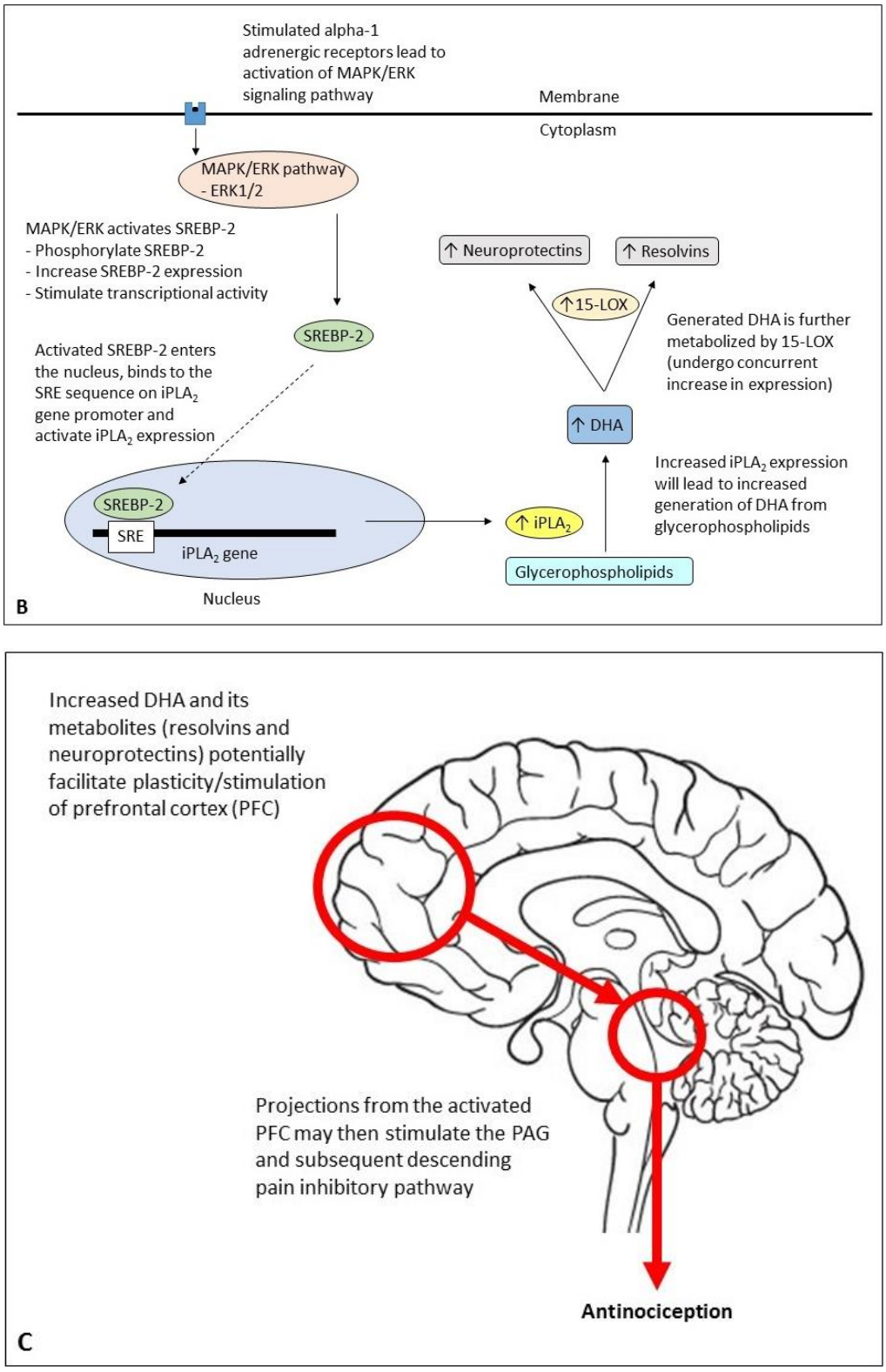


Fig. 6.1 Diagram showing the potential mechanisms and signaling pathways involved in antidepressant-induced antinociception in the (A) synaptic cleft, (B) neuronal cell and (C) brain.

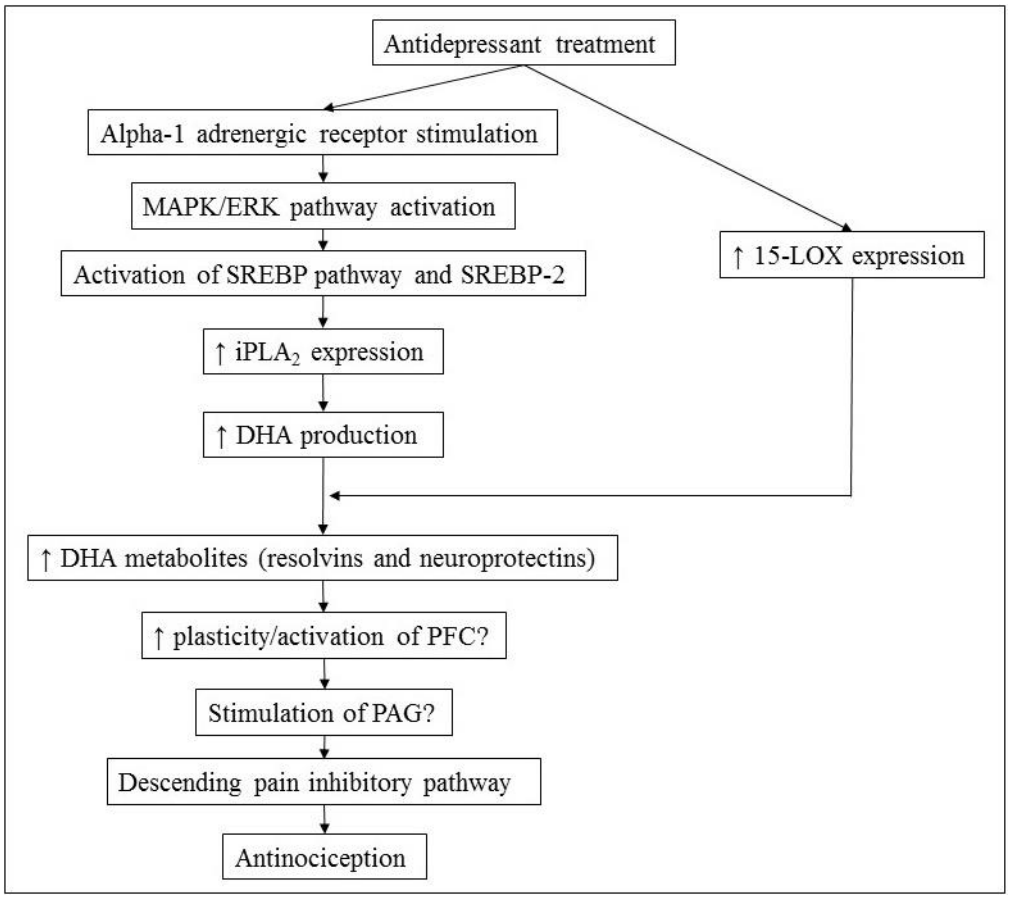


Fig. 6.2 Summary of the potential mechanisms and signaling pathways involved in antidepressant-induced antinociception.

Nonetheless, it is vital to bear in mind that there are some limitations to our current study and additional work is necessary to further validate the potential mechanisms as well as pathways involved in the antidepressant-induced antinociception suggested above. Future research directions include trying out other different antidepressants to investigate their effects on $iPLA_2$ and whether their antinociceptive activities are similarly affected by prefrontal cortical $iPLA_2$ knockdown. It is possible that nortriptyline, which was shown to increase $iPLA_2$ expression and has a similarly strong activity on noradrenaline reuptake inhibition as maprotiline, may likewise be affected by prefrontal cortical knockdown of $iPLA_2$. Our results also showed that the

antinociceptive effect of amitriptyline was unaffected by prefrontal cortical iPLA$_2$ inhibition, suggesting an alternative pathway and mechanism for antidepressant-induced antinociception which does not solely rely on prefrontal cortical iPLA$_2$. It is possible that the antinociceptive effect of antidepressants with strong noradrenaline reuptake inhibition activity such as maprotiline involves iPLA$_2$ while antidepressants with weaker noradrenaline reuptake inhibition activity and stronger serotonin reuptake inhibition activity such as amitriptyline induces antinociception without involving iPLA$_2$. Thus, it is imperative to examine other different antidepressants to study their effect on iPLA$_2$ and whether prefrontal cortical iPLA$_2$ is essential for their antinociceptive activities. We would also need additional *in vivo* prefrontal cortical data in the future to further support our *in vitro* findings.

Another possible future study is to supplement DHA to maprotiline-treated mice and determine whether supplementation of DHA can salvage and prevent the abolishment of maprotiline-induced antinociception when prefrontal cortical iPLA$_2$ is inhibited. More work can also be done on 15-LOX to investigate its role in pain and whether knockdown of 15-LOX will affect nociception. In addition, direct measurement of the levels of DHA and its metabolites in the prefrontal cortex can be performed to further substantiate our results in this study.

Chapter 7: References

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