United Arab Emirates University
Scholarworks@UAEU

Theses   Electronic Theses and Dissertations

11-2015

The Effects of Thujone on the Function of Nicotinic Acetylcholine Receptors

Ahmed Salaheldin Mustafa Ali Sultan

Follow this and additional works at: https://scholarworks.uaeu.ac.ae/all_theses

 Part of the Medical Pharmacology Commons

United Arab Emirates University

College of Medicine and Health Sciences

Department of Pharmacology

THE EFFECTS OF THUJONE ON THE FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS

Ahmed Salaheldin Mustafa Ali Sultan

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Professor Murat Oz

November 2015

Declaration of Original Work

I, Ahmed Salaheldin Mustafa Ali Sultan, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled *"The Effects of Thujone on the function of Nicotinic Acetylcholine Receptors"*, hereby, solemnly declare that this thesis/dissertation is my own original research work that has been done and prepared by me under the supervision of Professor Murat Oz, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis/dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature _____   Date _____

Advisory Committee

1) Advisor: Dr. Murat Oz

Title: Professor

Department of Pharmacology

College of Medicine and Health Sciences

2) Co-advisor: Dr. Chris Howarth

Title: Professor

Department of Physiology

College of Medicine and Health Sciences

3) Member: Dr. Bassem Sadek

Title: Associate Professor

Department of Pharmacology

College of Medicine and Health Sciences

4) External Member: Dr. Imad Damaj

Title: Professor

Virginia Commonwealth University, USA

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Murat Oz

   Title: Professor

   Department of Pharmacology

   College of Medicine and Health Sciences

   Signature: _____   Date: _____

2) Member: Dr. Chris Howarth

   Title: Professor

   Department of Physiology

   College of Medicine and Health Sciences

   Signature: _____   Date: _____

3) Member: Dr. Bassem Sadek

   Title: Associate Professor

   Department of Pharmacology

   College of Medicine and Health Sciences

   Signature: _____   Date: _____

4) Member (External Examiner): Dr. Imad Damaj

   Title: Professor

   Department of Pharmacology and Toxicolgy

   Institution: Virginia Commonwealth University, USA

   Signature: _____   Date: _____

This Master Thesis is accepted by:

Dean of the College of Medicine and Health Science: Professor Dennis Templeton

Signature: _____   Date: _____

Dean of the College of Graduate Studies: Professor Nagi T. Wakim

Signature: _____   Date: _____

Copy _____ of _____

Abstract

Thujone is a natural compound found in plants such as "wormwood" and "sage" and it's also found in the alcoholic drink "Absinthe". Thujone has been suggested as a neurotoxic compound and reported to modulate $GABA_A$ receptors. In this study, we investigated the pharmacology of Thujone on nicotinic receptors expressed in *Xenopus* oocytes using the two electrode voltage clamp method. Thujone (100μM) caused an 80% inhibition of Acetylcholine (ACh) induced currents in human α7-nAChRs expressed in frog oocytes while only 30% inhibition currents in α4β2 expressed oocytes and no effect on α3β4, α3β2 and α4β4 nAChRs. The mechanisms of Thujone effect on the α7-nAChR were further investigated and found to be independent of membrane potential and did not compete with ACh. Furthermore, Thujone did not affect the activity of endogenous Ca^{2+} dependent Cl^- channels. In conclusion, Thujone inhibits human nAChRs with different potencies.

Keywords: Thujone, Xenopus oocytes, nicotinic acetylcholine receptors.

Title and Abstract (in Arabic)

أثارالمركب ثوجون على صفات مستقبلات الاسيتايل كولين النيكوتينية

ملخص

تم العثور على مركب طبيعي ثوجون في النباتات مثل " مرارة " و " حكيم " وانها وجدت أيضا في المشروبات الكحولية " Absinthe " . وقد اقترح أن ثوجون قد يؤثر على الأعصاب وهذا بعمله على مستقبلات $GABA_A$. في هذه الدراسة، نحن نقوم بالتحقيق في صفات ثوجون على مستقبلات الاسيتايل كولين النيكوتينية المكونة من ألفا 7 و المستنسخة في بويضات ضفادع (Xenopus) و ذالك باستخدام تقنية تثبيت الجهد الكهربائي في البويضة باستخدام قطبين كهربائين. ثوجون ($100μM$) تسبب في تثبيط 80 ٪ من حجم التيار هت الاصلية. في حين أن ثوجون قام فقط بتثبيط 30 ٪ من حجم التيار الأصلي على بويضات مستنسخة بقنوات الاسيتيل كولين النيكوتينية المكونة من $α4β2$ كما ليس له تأثير على بويضات مستنسخة بقنوات الاسيتيل كولين $α3β4$، $α3β2$، $α4β4$. و عند مزيد التحقيق في صفات ثوجون على مستقبلات الاسيتايل كولين النيكوتينية المكونة من ألفا 7 وجدت أن التثبيت ليس له علاقة بالتيار الكهربائي لدى الخلية وغشاء المحتملة ، ولم يتنافس مع أدن تشافيز . وعلاوة على ذلك ، ثوجون لم يؤثر على قنوات الكلوريد المعتمدة على الكالسيوم و المتواجدة في غشاء البويضة الذاتية. وفي الختام، ثوجون لديه فعالية وصفات ذاتية مختلفة على مستقبلات الاسيتايل كولين النيكوتينية.

مفاهيم البحث الرئيسية: ثوجون ، البويضات لضفادع (Xenopus)، مستقبلات الأستيل كولين النيكوتينية .

Acknowledgments

I would like to take this chance and deeply express my gratitude to thank and appreciate everyone that helped me and truly inspired me to pursuit my Master's degree and help motivate me along the way.

First of all, I would like to thank my kind and understanding supervisor, Professor Murat Oz in the Department of Pharmacology, College of Medicine and Health Science. I really appreciate his support and guidance and his undivided attention. He has guided me along the way and inspired me to become passionate about seeking science and knowledge.

I would like to appreciate the Chairman of Department of Pharmacology, College of Medicine and Health Science Professor. Salim Bastaki for his support and enthusiasm and Dr. Samir Attoub the coordinator for the post graduate studies for all his support and feedback.

I want to express my graduation to all my doctors and professors in the pharmacology department who have been very keen on asking me all the right questions and to insure my proper education.

Furthermore, I would like to thank the external evaluator Dr. Imad Damaj from Virginia Commonwealth University for coming all the way to Alain to review and correct my thesis and for his thoughtful remarks.

I must also appreciate all the guidance and support I have received from Dr. Syed Muhammad Nurulain, Medical Research Specialist for all his dedicated hard to support me along the way and to teach me to perform admirably in the laboratory.

I would like to thank all my friends and colleagues for their support and advice Ali Saad, Eglal Mahgoub, Ahlam Said and Aisha Alkatbi and a special thanks to my dear friend Mohamed Omer Mahgoub for he is one the reasons why I chose this program.

Dedication

I dedicate this to my beloved family and my lovely parents firstly to my mother for raising me to become the man I am today and for loving me the way she did and especially to my beloved Father for he is a professor of Analytical Chemistry and he has always supported me to fulfill my goals weather emotionally or financially, I hope by this I can make you proud as I am a proud son.

Finally, I would like to thank my sisters and brothers for their encouragement, without them I will not be who I am today.

Table of Contents

List of Tables

List of Figures

Glossary

5-HT	Serotonin or 5-hydroxytryptamine
ACC	Antibody Dependent Complement Mediated Cytotoxicity
AcCoA	Acetyl Coenzyme A
ACh	Acetylcholine
AChE	Acetylcholineesterase
AD	Alzheimer's Disease
ADCC	Antibody Dependent Cellular Cytotoxicity
BAPTA	(1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid)
CNS	Central Nervous System
CTL	Cytotoxic T lumphocyte
DLA	Daltons Lymphoma Ascites
EMA	European Medicines Agency
GABA	Gamma γ-Aminobutyric Acid
GPCR	G-Protein Coupled Receptors
Hb-IPN	Habenulo-interpeduncular Pathway
HEK	Human Embryonic Kidney cells
IL-2	Interleukin 2
INF-γ	Interferon Gamma
LGIC	Ligand-Gated Ion Channels
mAChRs	Muscarinic Acetylcholine Receptors
MBS	Modified Barths Solution
MG	Myasthenia Gravis
mIPSC	Miniature Inhibitory Postsynaptic Currents
NA	Noradrenaline or Norepinephrine
nAChRs	Nicotinic Acetylcholine Receptors
NB	Nucleus Basalis

NK	Natural Killer Cell
NOEL	No Observed Effect Level
NTP	National Toxicology Program
PNS	Peripheral Nervous System
SOC	Store-operated Ca^{2+} Channels
TEVC	Two Electrode Voltage Clamp
VGCCs	Voltage Gated Ca^{2+} Channels
VGIC	Voltage-Gated Ion Channels

Chapter 1: Introduction

1.1 Artemisia absinthium L (Wormwood)

The Artemisia Absinthium shown in Figure 1.1, named after the Roman "goddess Artemis" also called "wormwood", is a well-known herb used for medicine and culinary purposes over many centuries. It comes from the family called "Asteraceae" that has 22 different Artemisia species such as: Artemisia vulgaris, Artemisia arbotanum and Artemisia santonicum. It was also given the name "wormwood" due to its bitterness and strong taste and it has been included in the alcoholic beverage "Absinthe" which means undrinkable. (Belhattab, Amor, Barroso, Pedro, & Figueiredo, 2014; Tobyn, Denham, & Whitelegg, 2011)

Figure 1.1: Artemisia absinthium L (wormwood) (Tobyn et al, 2011)

Since the ancient Egyptian times (460-377 B.C.), wormwood extracts have been used in the treatment of menstrual pain and rheumatism (Lachenmeier, 2010). During

the 18th century alcoholic wormwood extracts were produced and in the beginning of the 19th century, the alcoholic distillates of Absinthe and similar drinks were produced on a larger scale in Europe. (Baydar, Sangun, Erbas, & Kara, 2013; Lachenmeier, 2010). "Absinthe" also named "the green fairy, Green Muse and Green Goddess" became a very popular drink especially in France among many artists such as: Vincent van Gogh, Paul Verlaine, Pablo Picasso, Charles Baudlaire and Oscar Wilde. Absinthe is a highly alcoholic beverage (45-75%) and it mostly contains essential oil extracts from different herbs such as: wormwood (Artemisia absinthium), peppermint (Mentha piperita), fennel seed (Foeniculum vulgare) and angelica (Angelica archangelica) (Lachenmeier, 2010; Dawidowicz and Dybowski, 2012).

1.2 The plant Sage Lamiaceae

The plant Sage of the family Lamiaceae also called "Labiateae" is made of about 2500 different species with the essential oils as the major component. The Sage herb produces its highest content of Thujone during the late autumn season. The Sage can also be called the Salvia herb and include the following:

1.2.1 Dalmatian Sage: Extracts of the dried leaves of this plant contains Salvia officinalis, which can be found all over Europe making it the most widely used sage, mainly due to its high content of Thujone reaching up to 65%, 1,8-ceniole up to 22% and camphor up to 38% of the total oil content as in Figure 1.2.

Figure 1.2: Salvia officinalis L. (Picture taken from Wikipedia on 21/12/2014)

1.2.2 **Spanish Sage:** As its name implies it is produced in Spain from the shrubs of the plant named Salvia lavandulifolia vahl. This plant has a considerably low content of Thujone of 1.3%. However, it has very high contents of 1,8-ceniole and camphor of the total oil (56% and 37%, respectively).

1.2.3 **Greek Sage:** Salvia fruticosa miller has the highest contents of 1,8-ceniole of 66% of the total oil and much lower concentrations of Thujone and camphor.

1.2.4 **Cretan Sage:** Salvia pomifera has a high Thujone content between 58%-83% of the total oil content with minimum camphor and 1,8-ceniole quantities. It's found mainly in Turkey and Greece (Badawy & Abdelgaleil, 2014; Baydar et al., 2013; Kokkini, Karousou, & Hanlidou, 2003; Lachenmeier, 2010; Tobyn et al., 2011).

1.3 Thuja C. species (Red cedar)

This plant comes from the family Cupressacaea and it is subdivided into two main species; First is Thuja occidentalis shown in Figure 1.3, also called the "Eastern Abrovitae" or the "Atlantic red cedar" while the second is the Thuja plicata donn which is called the "Western Abrovitae" or the "Pacific red cedar". These two subdivisions contain high amounts of Thujone, 50-52 % of the total oil content for Thuja occidentalis and 54-63% of the total oil content in the Thuja plicata donn. (Bouajaj et al., 2013; Tsiri et al., 2009; Zhang, Schneidmiller, & Hoover, 2013)

Figure 1.3: Thuja occidentalis L. (picture taken from Wikipedia on 21/12/2014)

1.4 Monoterpene Ketones

Thujone is a monoterpene ketone found as one of the main constituent's (almost 90% of total oil) in the essential oils of different kinds of plants. The essential oils are the lipophilic extracts or mixtures that are produced after the plant has been processed by different methods such as distillation. The monoterpenes are composed of 10 carbon atoms with at least one double bond derived from two isoprene subunits (2-methyl-1, 3-butadiene) that has a molecular formula of $(C_5H_8)_n$ (n referring to the isoprene units). An example of major essential oil families are: Asteraceae, Apiaceae, Lamiaceae, Pinaceae and Rutaceae. Monoterpenes are grouped into regular which is more common, and irregular such as those found in the family Astraceae e.g. Chrysanthemic acid. The regular monoterpenes are subdivided into ketones, alcohols, aldehydes and ethers such as those in the Figure 1.4 below. (Akhgar, Rajaei, & Alizadeh-Saljoughi, 2013; Aldred, Buck, & Vall, 2009; Amri et al., 2013; Badawy & Abdelgaleil, 2014; Belhattab et al., 2014; Lachenmeier, 2010)
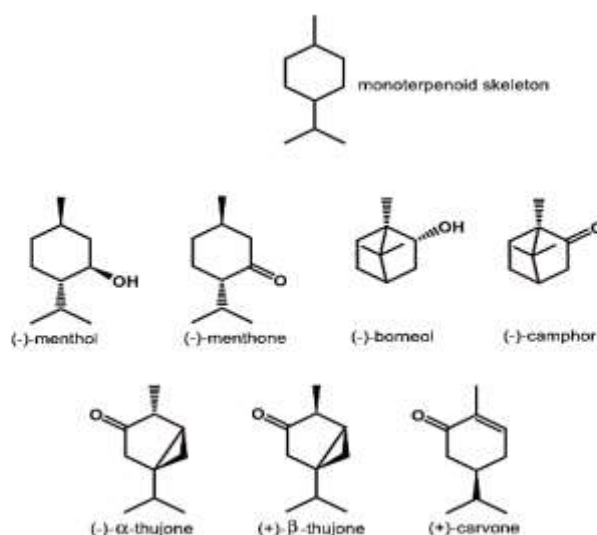
Figure 1.4: Structures of monoterpene aldehydes and ketones in essential oils (taken from Hall et al., 2004)

1.5 Thujone

Thujone, as previously mentioned, is available in large amounts from different plants. Thujone is a lipophilic bicyclic monoterpene ketone usually present in nature in two stereoisomers α-Thujone also called (-)-3-Thujone (CAS No. 546-80-5) and β-Thujone also named (+)-β-Thujone (CAS No. 471-15-8) as in Figure 1.5 (Oz, Lozon, Sultan, Yang, & Galadari; Suramya Waidyanatha et al., 2013)
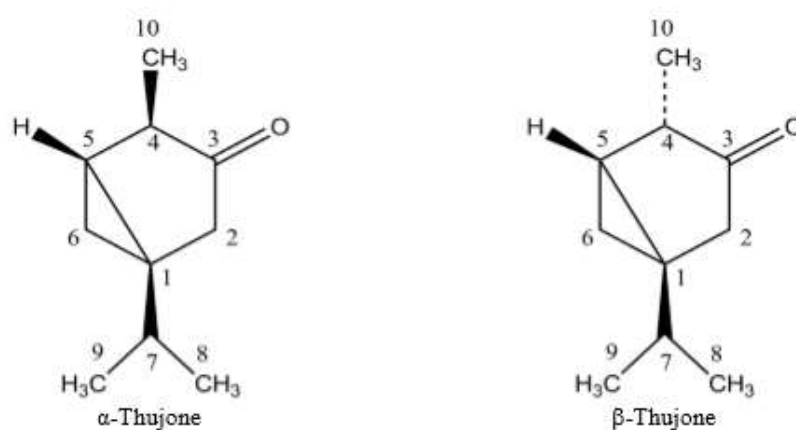
Figure 1.5: Two dia-stereoisomers of Thujone (taken from Waidyanatha et al, 2013)

Thujone has been reported to have a neurotoxic effect in various earlier studies (Hold, Sirisoma, Ikeda, Narahashi, & Casida, 2000; Pelkonen, Abass, & Wiesner, 2013; Polatoglu, Karakoc, & Goren, 2013; S. Waidyanatha et al., 2013). It has been proposed that Thujone intake causes mental deterioration and "Absinthism" which is manifested by hallucinations, depression and psychotic symptoms. Although large quantities of Absinthe were required for Absinthism to occur, prolonged exposure to Thujone has been reported to lead to neurotoxic effects similar to those seen in Absinthism (Czyzewska & Mozrzymas, 2013; Lachenmeier, Emmert, Kuballa, & Sartor, 2006; Lachenmeier & Uebelacker, 2010).

The popularity of Absinthe declined when it was associated with several psychotic effects, seizures, hallucinations, or mental prostration; all of which were summarized under the term Absinthism. In particular, the monoterpene Thujone was considered the active ingredient of absinthe. As a consequence, Absinthe was banned in Belgium in 1905, and soon after by Switzerland (1908), USA (1912), Italy (1913), France (1915), and Germany (1923). After its decline in popularity, Absinthe experienced a resurrection in 1988, when the European Commission allowed the processing of Thujone-containing plants to maximal concentration of 35 mg/ml in alcoholic spirits (European Council, 1988). Consequently, in recent years, research on Thujone and Thujone containing products increased significantly (Höld, Sirisoma, & Casida, 2001; Padosch, Lachenmeier, & Kroner, 2006). For example, it was reported that Thujone can bind to $GABA_A$ receptors, inhibit its function and cause convulsions (Hold et al., 2000).

1.5.1 Clinical applications of Thujone herbs

Thujone containing herbal products have been demonstrated to show clinically beneficial effects in the treatment of the acute common cold and cough (Naser, Bodinet, Tegtmeier, & Lindequist, 2005). It has also been shown that Thujone have some antibacterial activity against bacteria such as: E. coli, K pneumonia, S Aureus and P aeruginosa (Tsiri et al., 2009).

The European Medicines Agency (EMA) also mentioned some of the pharmacological actions of the herb Artemisia absinthium in promoting relief for gastric dyspepsia. It was also suggested that Thujone has antipyretic, anthelmintic and analgesic properties. (EMEA/HMPC/234444/2008) The EMA also reported that the use of the herb Salvia officinalis is indicated for the inhibition of perspiration or

sweating making the herb antihidrotic. It was also reported that sage extracts have memory enhancing and gland secretion enhancing effects (EMA/HMPC/330383/2008)

In an earlier study, Thujone, when administered by intra peritoneal injection to mice, caused increased plaque forming cells in spleen, increased natural killer cell (NK) activity, increased total white blood cell (WBC) count, antibody dependent cellular cytotoxicity (ADCC) and enhanced cytotoxic T lymphocyte generation (Siveen & Kuttan, 2011). It was proposed that Thujone enhances the release of cytokines such as interleukin 2 (IL-2) and interferon gamma (IFN-γ). This is confirmed by the reduction of Dalton's Lymphoma Ascites (DLA) induced solid tumor formation through modulation of immune mechanisms (Lachenmeier & Uebelacker, 2010; Siveen & Kuttan, 2011).

1.5.2   Thujone toxicity studies

Over the years many groups have tested the toxicity profile of Thujone using different techniques; although most of these techniques were not performed in humans, animal studies on mice or rats can be used to help understand our human bodies. In 2010, *Lachenmeier et al* reported that gavage application of a racemic mixture of α, β-Thujone to four groups of 20 different weanling rats at doses of 0, 12.5, 15, 50 mg/kg/day for a period of 13 weeks resulted in convulsions at the higher dose groups. The NOEL (No Observed Effect Level) for males was 12.5 mg/kg/day and it was not determined for females since convulsions happened early on in the experiment with the middle dose adjustments. In other experiments four groups of 20 rats received the Thujone by gavage for 6 days a week for a period of 14 weeks with doses of 0, 5, 10, 20 mg/kg. Results show that 6 male and 9 female rats convulsed

when given a high concentration of Thujone and 4 rats died due to convulsions. It was suggested that the NOEL was 5 mg/kg/day for the females and 10 mg/kg/day for the males (Dawidowicz & Dybowski, 2012; Lachenmeier & Uebelacker, 2010). In another study, the reported NOEL was 10 mg/kg in males and 5 mg/kg in females (Pelkonen et al., 2013).

In an earlier study, Rice and Wilson (1976), identified the subcutaneous LD_{50} (lethal dose in 50 percent of the population) of α-Thujone on mice to be 134 mg/kg while β-Thujone to be 442 mg/kg. Another single dose toxicity study by Pinto-Sconamiglio (1967) suggested that the intra peritoneal dose of Thujone of 180 mg/kg body weight in rats would lead to lethal effects and convulsions. (Pelkonen et al., 2013) However, another study reported that the intra peritoneal LD_{50} in mice was 45 mg/kg body weight (Hold et al., 2000).

The results of United States NTP (National Toxicology Program) in 2011 indicated that gavage doses of both α-Thujone and a mixture of α, β-Thujone resulted in neurotoxicity (represented by showing signs of hyperactivity, tonic-clonic seizures and tremors) and increased mortality rates on B6C3F1 mice and Fischer 344 rats. The doses that were given were 0, 1, 3, 10, 30, or 100 mg/kg/day for a period of 14 days. The study also performed a 3 month toxicity experiment by giving the animals both α-Thujone and a mixture of α, β-Thujone in doses of 0, 6.25, 12.5, 25, 50 or 100 mg/kg/day for 13 weeks by gavage. The higher dose groups showed a higher seizure rate along with high mortality rates when compared to the control and lower dose groups (Suramya Waidyanatha et al., 2013) (Lachenmeier & Uebelacker, 2010; Pelkonen et al., 2013).

Chronic toxicity studies were also performed by the NTP by administering low doses of a racemic mixture of Thujone by gavage for a period of 2 years. The doses that were given were 0, 3, 6, 12 and 25 mg/kg per day for the B6C3F1 mice and doses of 0, 12.5, 25 and 50 mg/kg per day for the Fischer 344 rats. Results show increase mortality and seizures in both groups at the higher doses. They also show increased non-neoplastic lesions in the spleen and brains of Fischer 344 rats. Lesions were also present in the kidneys of male rats and in the female's pituitary glands at the higher dose groups. The seizure and mortality NOEL for the rats was 12.5 mg/kg per day and 12 mg/kg per day for the mice (Pelkonen et al., 2013; Suramya Waidyanatha et al., 2013).

These studies also revealed the carcinogenicity potential for Thujone since there was significant increase in the female's B6C3F1 mice micro nucleated erythrocytes when they were given the racemic mixture for a period of 3 months. It is also worth mentioning that Fischer F344 rats had an increase in the formation of benign pheochromocytoma of the adrenal medulla as well as perpetual gland neoplasms in the males when given high doses (Ashoor et al., 2011; Davis & de Fiebre, 2006; Pelkonen et al., 2013; Suramya Waidyanatha et al., 2013; Wu, Gao, & Taylor, 2014).

1.5.3   Molecular and Cellular mechanisms of Thujone

As mentioned earlier, Thujone was found to cause neurotoxicity (represented by showing signs of hyperactivity, tonic-clonic seizures and tremors) and there have been several *in-vivo* and *in-vitro* investigations to clarify the mechanisms underlying the neurotoxic effects of Thujone.

1.5.3.1 The effect of Thujone on Ligand Gated Ion Channels

The ligand-gated ion channels allow the passage of ions by means of extracellular binding of ligands or neurotransmitters like Acetylcholine (ACh). In (Figure 1.6), Ligand-gated ion channels have been divided into five major families: 1) Cys-loop superfamily, 2) Glutamate (Glu)-gated family, 3) Adenosine Triphosphate ATP (Purinergic)-gated family, 4) Transient receptor family (TRP) family and 5) Acid-sensitive ion channel (ASIC) family. The Cys-loop superfamily has also been subdivided into many receptor subtypes including: A) Nicotinic Acetylcholine Receptors, B) Serotonin type 3 Receptors, C) $GABA_A$ and $GABA_C$ Receptors, D) Glycine Receptors and E) Zinc-Gated Ion channels. (Briggs & Gopalakrishnan, 2007; Imoto, 2004; McGivern & Worley Iii, 2007; Millar & Gotti, 2009; Suppiramaniam, Abdel-Rahman, Buabeid, & Parameshwaran, 2010).
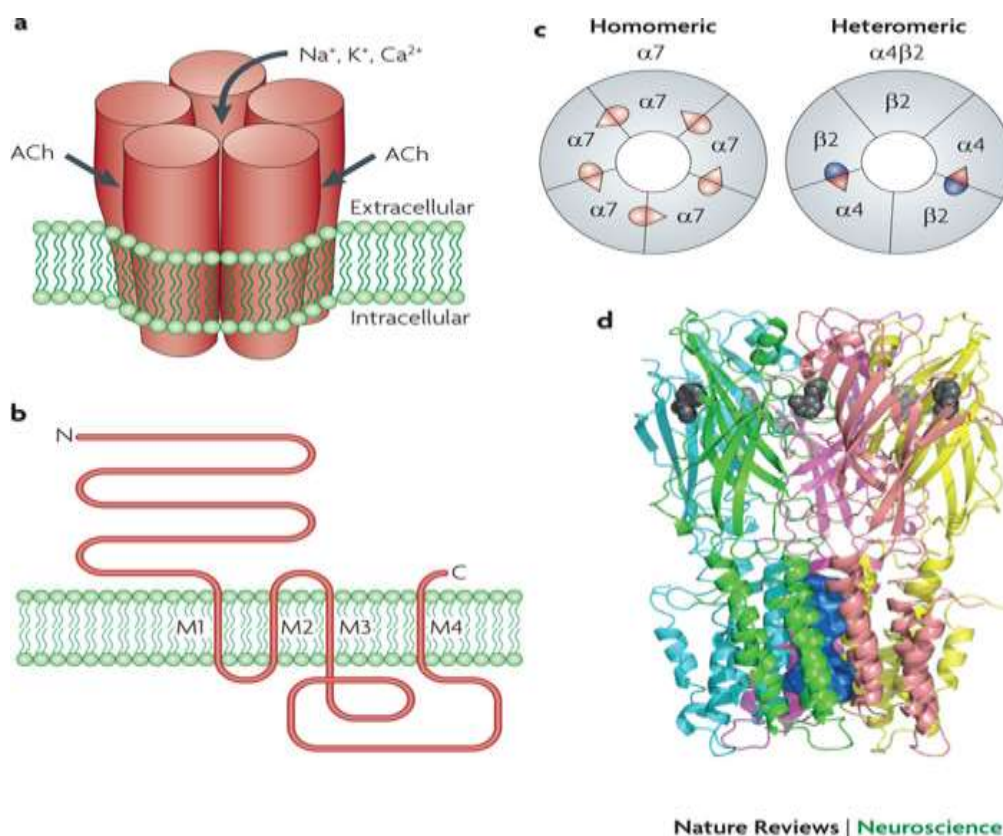
Figure 1.6: Ligand gated ion channels (picture taken from Nature Reviews)

The Cys-loop superfamily is composed of a large N-terminal domain where the agonist will bind at one end and the C-terminus at the other making this adjacent cysteine pair important for channel gating, four transmembrane α-helices segments (M1-M4) and a variable intracellular loop between M3 and M4 shown in Figure 1.6. Some of these channels are excitatory receptors such as nicotinic acetylcholine and serotonin type 3 (5-HT$_3$) receptors. They permeate mainly cations such as Ca^{2+} and Na^+ causing depolarization of the membrane. While others are named inhibitory such as GABA$_A$ and glycine receptors, and they selectively permeate anions such as Cl^- causing hyperpolarization or inhibition of neuronal firing s (Briggs & Gopalakrishnan, 2007; Hold et al., 2000).

In an early study conducted to understand the mechanisms of Thujone action it was found that this compound acts on dorsal root ganglia of mice by inhibiting GABA$_A$ receptors that are mainly distributed in the central nervous system (CNS) (Hold et al., 2000). This effect was also seen in the (Hall et al., 2004) experiments involving *Xenopus* oocytes injected with α$_1$β$_2$γ$_2$ GABA$_A$ receptors. The results of the study show that Thujone dia-stereoisomers have an inhibitory effect on these receptors. In another study done recently in cultured hippocampal neurons, α-Thujone (100 μM) was able to inhibit miniature inhibitory postsynaptic currents (mIPSC) in patch clamp experiments (Szczot, Czyzewska, Appendino, & Mozrzymas, 2012); suggesting that Thujone effects the function of GABA$_A$ receptors by down regulating phasic or synaptic GABA$_A$ transmission. However, subsequent studies showed that (Czyzewska & Mozrzymas, 2013) α-Thujone has an inhibitory effect on the phasic as well as the tonic or extra-synaptic GABA$_A$ transmission by its actions on different subunits of GABA$_A$ receptors. It was found that Thujone acted on α1β2δ receptors with highest potency, suggesting that tonic currents might be

more sensitive to Thujone than phasic currents (Czyzewska & Mozrzymas, 2013; Szczot et al., 2012). In another study on GABA_A receptors in neonatal chicks, α-Thujone was able to induce "anxiogenic-like" effects on behavior when administered centrally; the authors proposed that Thujone reduced the postsynaptic membrane receptors and caused suppression in stress induced increase of GABA_A receptor recruitment (Höld et al., 2001; Lachenmeier & Uebelacker, 2010; Rivera, Cid, Zunino, Baiardi, & Salvatierra, 2014).

The effects of Thujone are not limited to GABA_A receptors. In patch clamp studies, Thujone was able to reduce serotonin type 3 receptor (5-HT_3) activity by enhancing the binding of the ligand serotonin and strengthening the channel blocking potency. It was reported that, Thujone was involved in receptor desensitization in cultured human embryonic kidney (HEK 293) cells (Deiml et al., 2004; Qiao, Sun, Clare, Werkman, & Wadman, 2014).

Both, 5-HT_3 and GABA_A receptors are members of the LGIC family. However, nicotinic receptors are the prototype for LGIC, and the effects of Thujone on these receptors are currently unknown.

1.5.3.2 The effect of Thujone on G-Protein Coupled receptors

G-protein coupled receptors also named "7TM receptors" are the largest group of receptors that have been identified to date with 800 different types. They are the most common site for drugs and chemicals. They are made of a single polypeptide that has an intracellular C-terminal domain and an extracellular N-terminal domain between these two domains consisting of seven hydrophobic trans-membranes (TM1-TM7) (Figure 1.7). These trans-membrane domains are also linked by intracellular and extracellular loops. Most GPCRs involve second messenger systems such as GS

(Stimulatory) and GI (Inhibitory) such as those involved in the binding of α-GTP which activates or inhibits adenylyl cyclase (AC) which ultimately modulates the synthesis of the second messenger cyclic adenosine monophosphate (cAMP) and eventually leads to a biological response (S. P. Alexander et al., 2013; Clark, 2012; Gotti et al., 1988; Oz, 2006)
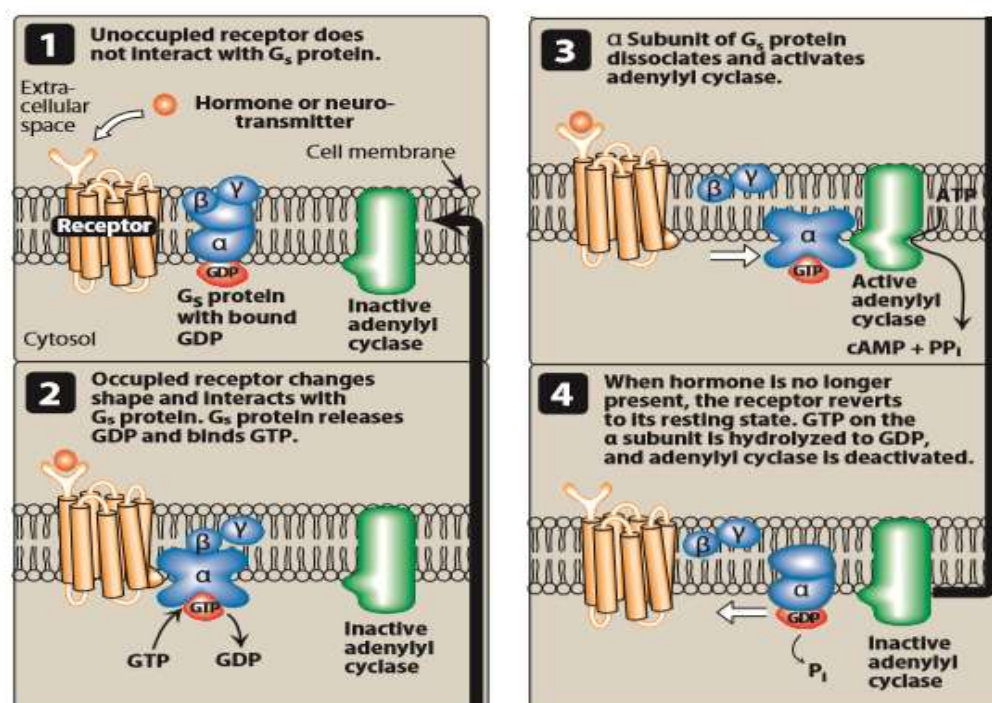
Figure 1.7: GPCR Signal transduction (picture taken from Clark, 2012)

Cannabinoid receptors are a member of the GPCR class of receptors. Since "Absinthsm" has several symptoms (such as cognitive deficits) common with cannabis toxication, it was initially thought that Thujone modulates cannabinoid receptors. However, Thujone has failed to evoke responses in Cannabinoid receptors although it has a low binding affinity for these receptors since it was able to displace the binding of the agonist [^{3}H]CP55940 with a K_i of 130 μM and K_i of 115 μM at the Cannabinoid receptor CB_1 from rats P2 isolated membranes and CB_2 in human isolated tonsil

membranes expressing the cannabinoid receptors, respectively (Meschler and Howlett, 1999; Gotti and Clementi, 2004). Thus it has been concluded that Thujone does not evoke cannabinoid receptor responses. (Gotti & Clementi, 2004; Gotti, Fornasari, & Clementi, 1997; Gotti et al., 1988; Mahgoub et al., 2013; Meschler & Howlett, 1999; Oz, 2006; Oz, Al Kury, Keun-Hang, Mahgoub, & Galadari, 2014)

1.6 Cholinergic system

The nervous system is a widely studied complex network with many different structures; it is divided into the central nervous system (CNS) which consists of the brain and spinal cord and the peripheral nervous system (PNS) which consists of the neurons outside the brain and spinal cord either named: "afferent" that bring or carry the signal towards the CNS from the periphery or "efferent" that carry the signal away from the CNS to the periphery. The "efferent" is also subdivided into "Somatic" which includes the voluntary neurons involved in motor control; and "Autonomic" which are involved in the involuntary neurons that control requirements of the vital functions of the body and everyday unconscious activities such as breathing and digestion (Clark, 2012; Clarke, 2008a; Cuello, 2009).

Over the past century scientists were able to map most of these systems and the main neurotransmitters involved in the mechanisms of actions of these systems. Our focus in this study is the cholinergic system with acetylcholine (ACh) being the most abundant neurotransmitter involved in the central and peripheral nervous systems. It is synthesized in the synaptic axon and the neuronal cytoplasm by choline acetyltranferase enzyme which requires the presence of both acetyl coenzyme A and choline as substrates. Once formed ACh is stored in synaptic vesicles in the axon. Membrane depolarization will cause an increase in the intracellular Ca^{2+} and the release of the neurotransmitter ACh from the vesicles into the synaptic cleft by

exocytosis (Figure 1.8). Once released ACh can either bind to receptors on pre or post-synaptic membranes or get hydrolyzed by the enzyme Acetylcholineesterase (AChE) into Acetate and Choline which will be taken up by the axon (Clementi, Fornasari, & Gotti, 2000; Cooper, Bloom, & Roth, 2003; Hendrickson, Guildford, & Tapper, 2013; Mufson, Counts, & Ginsberg, 2009; Westfall, 2009).
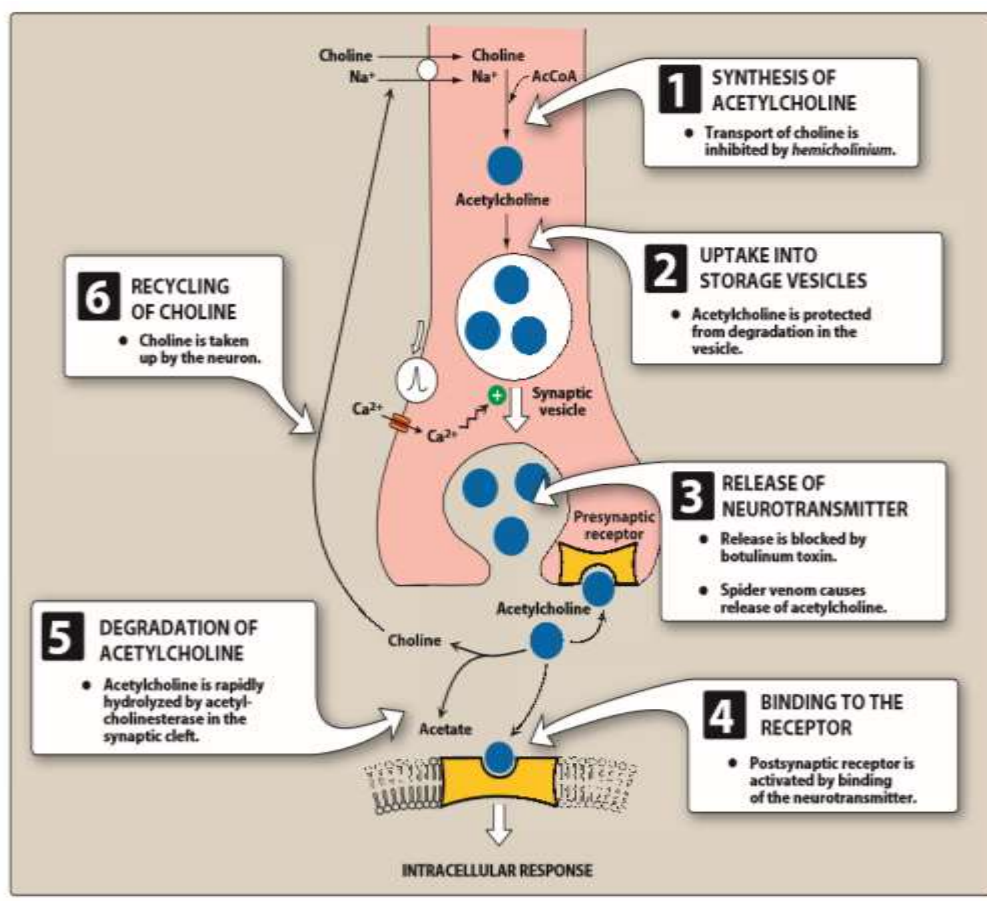
Figure 1.8: Summary of synthesis and release of Acetylcholine from cholinergic neuron. AcCoA: Acetyl coenzyme A. (picture taken from (Clark, 2012; Clarke, 2008b).

The brain contains several ACh innervations called cholinergic pathways. It has been shown that ACh is the neurotransmitter at all autonomic ganglionic neurons, neuromuscular junctions and parasympathetic post-ganglionic synapses (Cooper et al., 2003; Gotti et al., 2007). In the central nervous system (CNS) the

neurotransmitter ACh plays a role in several physiological functions such as learning, attention, motor control and memory through multiple and complex cholinergic pathways. These cholinergic pathways have been recently mapped through various immuno-histochemical staining techniques and it has been shown that these pathways originate from the basal forebrain. Scientists then introduced the Ch1-Ch4 nomenclature of these cholinergic neurons. Ch1 represents the cholinergic neurons of the medial septum, Ch2 consist of the diagonal band of Broca also referred as the "Vertical limb". Ch3 neurons compromises the "horizontal limb" and finally, Ch4 nomenclature contains the largest group of cholinergic neurons almost 90% which are in the cells of the nucleus basalis (NB) (Figure 1.9). The nucleus basalis projects to the cerebral cortex but the cortex doesn't contain cholinergic neurons, it receives projections from the limbic, amygdala and paralimbic projections; thus the term "nucleus basalis" can be referred to the cholinergic and non-cholinergic components, however, only Ch4 is used in cholinergic innervations. The second group of cholinergic pathways can be described by their presence in the upper brain stem projecting from the rostral midbrain to the midpontine level. This group can also be subdivided into Ch5 mainly in the pedunculopontine nuclei, Ch6 in the lateral dorsal tegmental nucleus connecting to the thalamus. Ch7 neurons innervate the interpeduncular nucleus in the medial habenula of the epithalamus. Finally, Ch8 neurons are in the mesopontine region inside the parabigeminal nucleus innervating the superior colliculus. (Figure 1.9) summarizes these cholinergic pathways. (Cooper et al., 2003; Cuello, 2009; Gotti & Clementi, 2004; Gotti et al., 1997; Keverne & Ray, 2005; Mesulam, 2009; Mufson et al., 2009; Oz, Lorke, & Petroianu, 2009)
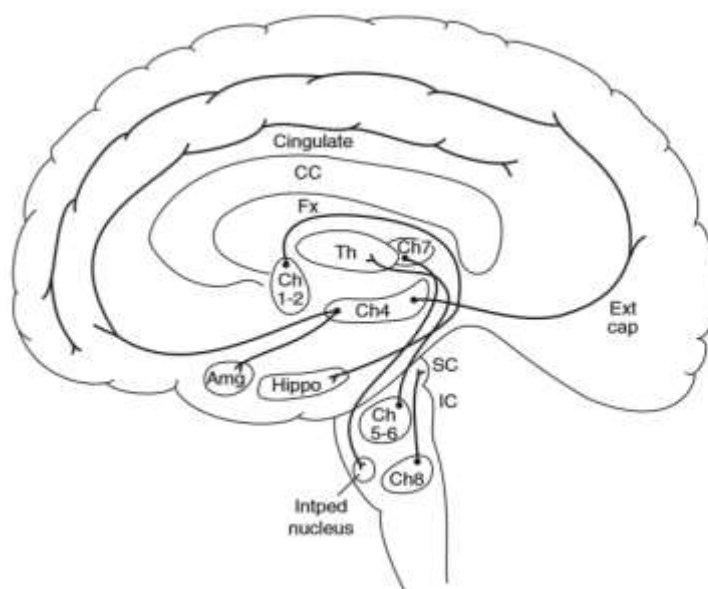
Figure 1.9: Summary of the Ch1-Ch8 neurons. Amg:amygdala, CC: corpus callosum, Fx: fornix, Hippo: hippocampus, Ext cap: external capsule, IC: internal capsule, Th: thalamus, SC: superior colliculus, intped nucleus: interpeduncular nucleus (Taken from: (Mufson et al., 2009).

1.7 Cholinergic receptors "Cholinoceptors"

After acetylcholine has been released into the synaptic cleft, it binds to the cholinergic receptors either on the pre-synaptic or on the post-synaptic nerve terminal. There are two different types of "Cholinoceptors"; the muscarinic "mAChR" and nicotinic receptors "nAChR" with different affinities and efficacies for ACh. The muscarinic receptors are G-protein coupled receptors and they have the highest affinity for the alkaloid found in mushrooms called: "Muscarine" and lower affinities for nicotine. The nicotinic receptors on the other hand have a higher affinity for nicotine and they are ligand gated ion channels (Clark, 2012; Cuello, 2009; Eaton, Ospina, Rodríguez, & Eterović, 2004; Keverne & Ray, 2005; Mesulam, 2009).

1.7.1   Muscarinic receptors

As it was mentioned previously, these receptors are G-protein coupled receptors and their signal transduction mechanism involves the activation of G-proteins. There are five subtypes of the muscarinic receptors M1-M5 as have been described by binding studies, gene cloning and pharmacological methods. These metabotropic receptors are located in almost all organs and tissues which are innervated by parasympathetic nerves. The M1 is located mainly in the gastric parietal cells, whereas the M2 receptors are located in smooth muscle cells and cardiac muscle. M3 receptors are located in exocrine gland, bladder and smooth muscles. M1, M3 and M5 function by activating Gq leading to activation of phospholipase C. Hydrolysis of phosphatidylinositol-(4,5)-bisphosphate by phospholipase C leads to production of diacylglycerol and inositol-(1,4,5)-trisphosphate which are second messengers that increase the intracellular Ca^{2+} levels. On the other hand, M2 and M4 receptors are coupled to inhibitory Gi protein that will inhibit adenylyl cyclase 2 and increase K^+ conductance and inhibit cyclic adenosine monophosphate cAMP (Figure 1.10). Both of these mechanisms of action will ultimately lead to a physiological response by the effector organ (Clark, 2012; Eglen, Choppin, & Watson, 2001; Gotti & Clementi, 2004; Mufson et al., 2009; Paterson & Nordberg, 2000; Westfall, 2009).
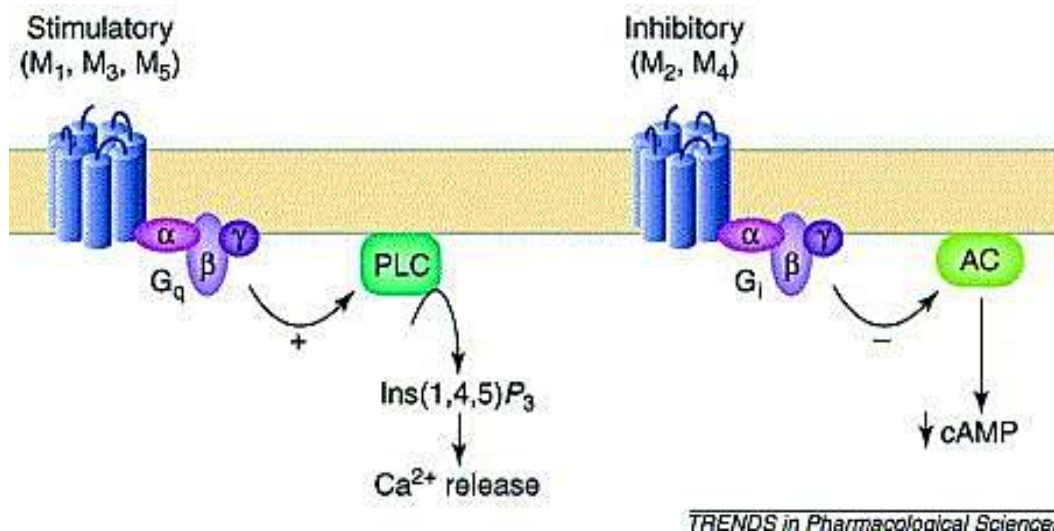
Figure 1.10: Muscarinic receptors and their signal transduction (taken from Eglen et al., 2001).

1.7.2   Nicotinic receptors

The nAChR is an ionotropic pentameric protein belonging to the Cys-loop superfamily of ion channel complexes. Other members of the Cys-loop family include the $GABA_A$, $5-HT_3$ and glycine receptors. A number of nAChR genes have been identified that encode a total of 17 subunits (α1-10, β1-4, γ, δ and ε). Each gene encodes a protein structure of a large N-terminal extracellular domain, an extracellular C-terminus domain, four α-helixes (M1-M4) and intracellular loop between M3 and M4 (Figure 1.11). The Acetylcholine binds to a site near the α-helix where two cysteine tandem residues are located. The "orthosteric" binding site for ligands is formed by at least three different peptides the α-helix and three different adjacent subunits (complimentary components). The receptor also contains a binding site for several compounds with diverse structure that is different from Acetylcholine and referred to as the allosteric binding site. Both positive and negative allosteric modulators have a separate binding site on the receptor. Nicotinic receptors assemble in different combination for example α (2-6) and β (2-4) subunits which influence

the receptors pharmacology and biophysical properties. The β3 and α5 subunits fail to produce function when alone or paired; however, they can produce function when they are expressed as an extra subunit with α and β subunit pairs e.g. α4β2β3, α5α6β2. The α7, α8, and α9 subunits can form homo-oligomers and hetero-oligomers by adding another subunit such as: α7β2 and α9α10 (Figure 1.11) (S. P. H. Alexander et al., 2013; Ashoor et al., 2011; Baginskas, Kuraitė, & Kuras, 2015; Davis & de Fiebre, 2006; Hendrickson et al., 2013; Wu et al., 2014; Zwart, Strotton, Ching, Astles, & Sher, 2014).
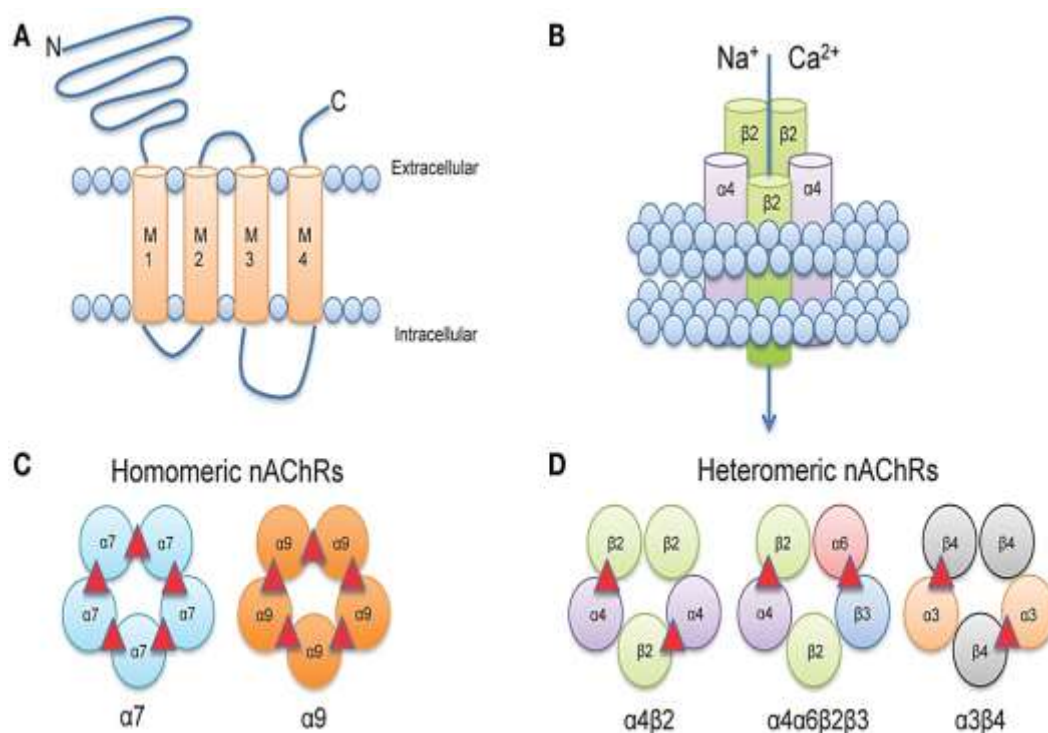
Figure 1.11: Structure of Nicotinic acetylcholine receptors and nomenclature taken from: (Hendrickson et al., 2013)

1.8 Neuronal nicotinic receptors

The term "neuronal" implies that these receptors are located in the central nervous system but not on the peripheral nervous system. The most common receptors which have been identified to be neuronal in nature are the α7 and α4β2 nicotinic acetylcholine receptors. They are pentameric in structure which suggests they are composed of five subunits. The α7 have been associated with cognitive functions and psychosis, while the α4β2 have been linked with analgesia, tobacco addiction, learning and memory, although not exclusively. The α7 nicotinic receptor is "homomeric" which means it is composed of five α7 subunits while the α4β2 is "heteromeric" and is composed of various ratios of α4 and β2 subunits shown in (Figure 1.11). The α7 receptor allows entry of Ca^{2+} while the α4β2 allows the entry of both Ca^{2+} and Na^+. Mutations of these receptors would result in various forms of disease such as epilepsy associated with α4β2 neuronal receptor and schizophrenia associated with α7 mutations (Grady et al., 2010). Up-regulation of the α4β2 receptors that have high affinity to nicotine in smokers was reported after chronic exposure to nicotine both *in- vitro* and *in-vivo* (Gotti et al., 2007). The α4β2 was also found to be selectively reduced in the cortex of patients with Alzheimer's disease (Gotti et al., 2009).

Other Neuronal subtypes have also been identified, for instance, *Fowler at al. (2011)* reported that α5 nAChR in the (Hb-IPN) habenulo-interpeduncular pathway subunit plays an important role in nicotinic dependence in rodents by regulating nicotine intake and self-administration, this was demonstrated by α5 knockout mice. α3β2 which is involved in the visual pathway mainly in the retina and its target tissues the Superior Collicolus (SC) and Lateral Ganiculate Nucleus (LGN). On the other hand, the α3β4 subtype has been found to be expressed in the pineal gland,

cerebellum, retina and involved in the release of noradrenaline in the hippocampus (Gotti et al., 2007). Finally, the α4β4 receptor subtype is expressed in both rat and chick retinas (Baginskas et al., 2015; Briggs & Gopalakrishnan, 2007; Clementi et al., 2000; Davis & de Fiebre, 2006; Fowler, Lu, Johnson, Marks, & Kenny, 2011; Gotti et al., 2009; Gotti et al., 1997; Gotti et al., 2007; Grady et al., 2010; Mufson et al., 2009; Westfall, 2009)

This project will focus on the α7 receptor subtype while testing the effect of Thujone on the other α4β2, α3β2, α3β4 and α4β4 nicotinic receptor subtypes (Figure 1.12).
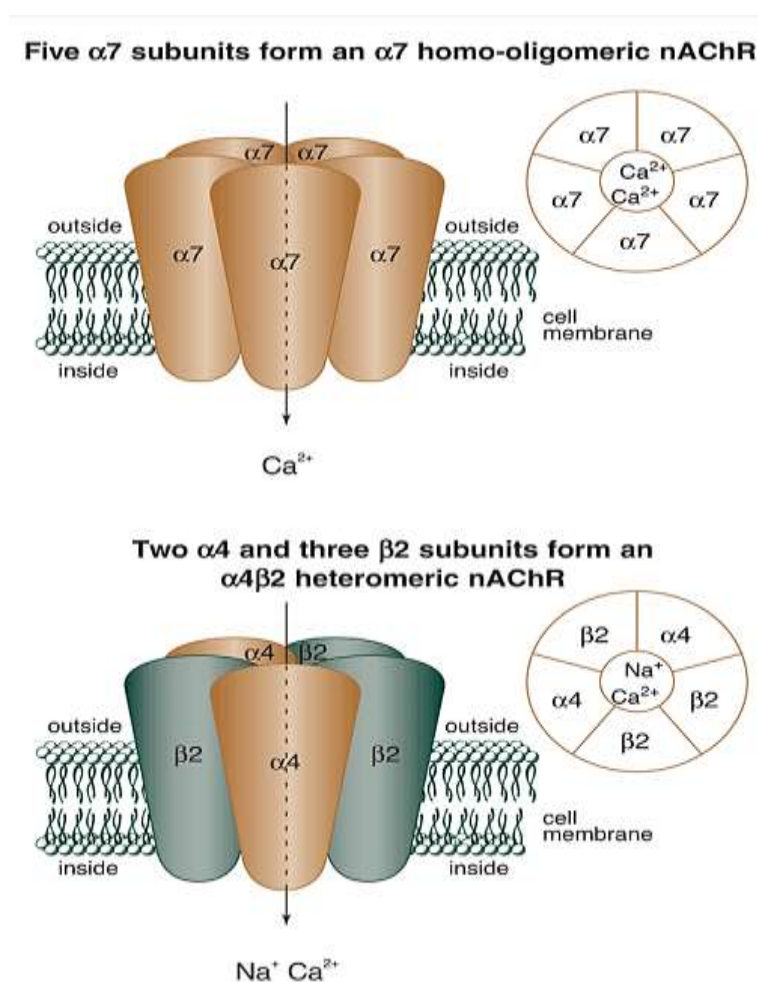
Figure 1.12: Neuronal nicotinic acetylcholine receptors taken from: (Davis & de Fiebre, 2006).

Chapter 2: Aims and Objectives

Our laboratory was interested on the effects of different monoterpenes and their role on nAChRs. Earlier studies revealed the effect of the monoterpene Thujone on different types of LGICs such as 5-HT$_3$, and GABA$_A$ receptors. Since the effects of Thujone on the function of neuronal nicotinic nAChR is unknown; our main aim was to investigate the effects of Thujone's on human neuronal nAChR expressed in *Xenopus* oocyte systems. The expression system has the following advantages:

A. *Xenopus* oocyte expression systems have been widely employed and well described in many previous studies.

B. We can obtain large number of oocytes with the expression levels necessary to conduct the experiment.

C. Using the two-electrode voltage clamp method along with the *Xenopus* oocytes allows for sensitive yet reliable results and conclusions.

D. The Two-electrode voltage clamp allows for long periods of recording which is important in long-lasting experiments when using oocyte systems.

The detailed objectives of the experiments are as follows:

A. To investigate the function of Thujone on different neuronal nAChRs expressed in oocytes using the two-electrode voltage clamp.

B. To investigate the role of intracellular Ca^{2+} on the effects of Thujone on neuronal α7 nAChR expressed oocytes.

C. To determine the voltage dependence of Thujone on the α7 nAChR expressed oocytes.

D. To investigate the binding site of the Thujone on the nAChR.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1   Chemicals

All the chemicals (Table 3.1) used in the experiment were purchased from pharmaceutical companies and they were in research grade purity.

Table 3.1: Chemicals

Compounds	Molecular weight	Manufacturer	Catalogue number
Acetylcholine chloride	181.7	Sigma, USA	A6625
BAPTA	764.68	Sigma Aldrich	A-1076
Barium chloride	244.28	BDH, England	R:20-25 S:45
Benzocaine	165.2	Sigma, USA	E-1501
Calcium chloride anhydrous ($CaCl_2$)	110.99	Panreac Quimica, SA	Lot. #73917PCP
Collagenase – A (from Clostridium histolyticum EC.3.4.24.3	---------	Roche Diagnostic Corporations, USA	Lot. #93211521
Gentamicin reagent solution	Conc. 50mg/ml	Introgen Corporation, China	Cat. Number 15750-060

HEPES	238.3	Sigma, USA	H3375
Hydrogen chloride (HCL)	37%	Sigma Aldrich, USA	25.814.B
Magnesium chloride (MgCl$_2$)	95.22	Sigma, USA	M4880
Magnesium Sulfate (MgSO$_4$)	246.5	Sigma, USA	M9391
Penicillin G	356.4	Sigma, USA	P3032
Potassium chloride (KCL)	74.55	Sigma, USA	P9541
Sodium Chloride (NaCl)	58.4	Sigma, USA	S-3014
Sodium Bicarbonate (NaHCO$_3$)	84.01	Sigma, USA	S6014
Sodium Hydroxide (NaOH)	40	Amresco, USA	Lot. #214613010
Sodium Pyruvate	110.04	Sigma Aldrich, USA	P2256
Streptomycin	1457.4	Sigma, USA	S9137
α-Bungarotoxins	~8500	Sigma	T-0195
α-Thujone	152.23	Sigma Aldrich	546-80-5
α,β Thujone	152.23	Sigma Aldrich	76231-76-0

3.2 Female *Xenopus laevis* frogs

Electrophysiological recordings from *Xenopus* laevis frog oocytes have been commonly used for the investigation of ion channels. Importantly, it was demonstrated that oocytes have few endogenous channels and they can express foreign proteins when injected with their respective RNAs (Eaton et al., 2004; Miledi, Parker, & Sumikawa, 1983; Oz, Spivak, & Lupica, 2004; R. L. Papke & Smith-Maxwell, 2009; Weber, 1999; Zwart et al., 2014).

Mature African wild type female clawed *Xenopus laevis* frogs (Figure 3.1) were obtained from Xenopus Express, Haute-Loire, France and held in reserve in a de-chlorinated tap water aquarium (dimensions: 100 cm height, 130 cm length, 66 cm width) where the water was changed twice per week. The aquarium was placed in an ambient room temperature of 18 $^{\circ}$C and a 12 hour alternating dark and light cycle. The frogs were fed twice a week with special frog's food pellets that were also supplied from Xenopus Express, France. Animal handling and care for all the animals and frogs were in accordance with institutional guidelines and approved by the animal Ethics Committee of the College of Medicine and Health Sciences (CMHS) of the United Arab Emirates University (UAEU).

Figure 3.1: African clawed female *Xenopus laevis* frog (taken from: xenopus.com)

3.2.1   Experimental configuration

Two-electrode voltage clamp (TEVC) method was used in the study. This electrophysiological method clamps the membrane potential by a feedback amplifier to allow measurement of the current passing through the membrane at clamped potential. Using this method, the pharmacological properties of membrane proteins such as ion channels can be investigated. The solutions and drugs were applied by gravity flow perfusion tubes that were connected directly to the perfusion chamber where the oocyte was placed as shown in (Figure 3.2).
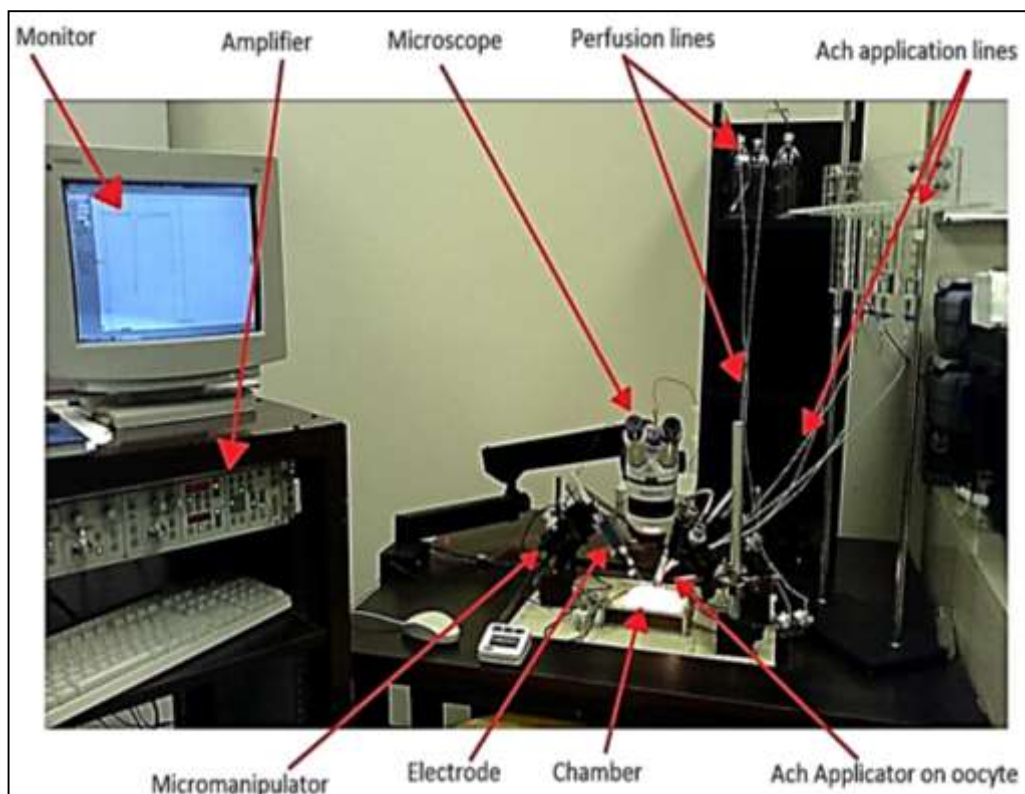
Figure 3.2: Two-electrode voltage clamp system

Table 3.2: Components of the Two-electrode voltage clamp system

Device Name	Company of purchase
GeneClamp500B amplifier	Axon Instruments, Molecular Devices, Inc, Sunnyvale, CA, USA
Current Head stages	HS-2A Head stage, Gain 10 MG
Voltage Head stages	HS-2A Head stage, Gain 1 MG, Axon instruments, Molecular devices, Inc, Sunnyvale, CA, USA
Magnetic Holding device	Kanetec USA corporation, Bensenville IL, USA
Manual micromanipulator	M33, Märzhäuser, Wetzlar, Germany

The two electrode voltage clamp consists of the following items:

The Computer hardware for data acquisition (Compaq personal computer, Compaq Corporation, Wynyard, UK) and analog-digital converter BNC 2081 (National Instruments, Austin Texas, USA).

Recording chamber illumination used in the recording chamber was supplied by an optic fiber light source (Figure 3.2) (Fiber life, Hight intensity illuminator series 180, Dolan, Jenner Industries Inc., MA, USA)

A low-power stereo-dissection microscope was employed for visual observation of the recording chamber (Olympus, Tokyo, Japan, SZ-STB1m 100 AL0.5 Xm WD186).

The perfusion apparatus consisted of perfusion tubes and bottles containing extracellular solutions connected to the recording bath by silicon tubing (Cole Parmer Instrument Company, O.D. 1/8 inch, WALL 1/32 inch and I.D. 1/16 inch, Vernon Hills, Illonois, USA). The flow rate of perfusion was set to 3 – 5 ml/minute.

Another multichannel perfusion was used for drug applications that consisted of C-flex tubing (Cole Parmer Instruments Company, O.D. 3/32 inch, WALL 1/32 inch and I.D.1/32 inch, Vernon Hills, Illinois, USA), 50 ml glass syringes, plastic valves and coupling devices. The drug application system was based on gravity flow usually through a micropipette that sits directly 2-3 mm from the perfusion chamber where the oocyte was placed. On the other end of the recording chamber, a glass suction tubing was connected directly to the waste disposal flask that was observed and cleaned routinely (Figure 3.3).
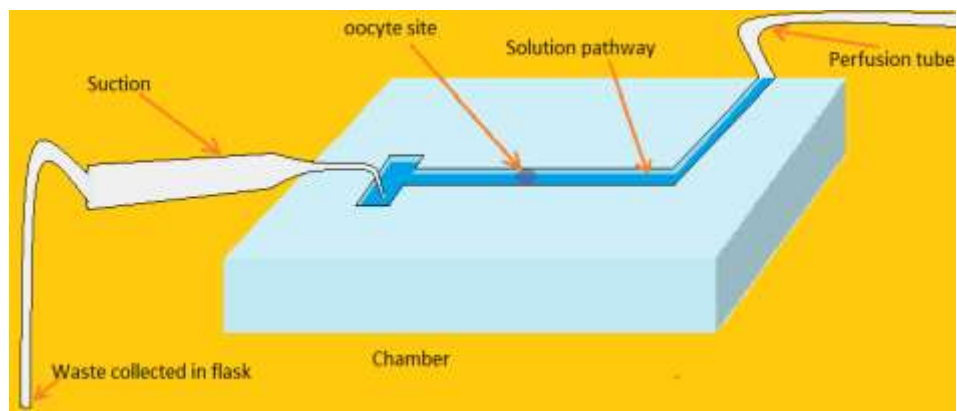
Figure 3.3: Oocyte perfusion chamber schematic diagram

3.2.2 Other material used

Table 3.3: Other devices and materials

Device or Material	Specifications	Company of Purchase
Automatic Nano-liter injector	Nanoject	Drummond Scientific Company, Broomall, PA, USA
Borosilicate Glass tubing for microelectrodes	Glass thin-wlled w/filament 1.5 mm, Catalog #TW150F-4	World Precision Instruments, Sarasota, FL, USA
Electrode Holder	---	World Precision Instruments, Sarasota, FL, USA
Magnetic stand and manipulators	Catalog #7739	Narishige, Tokyo, Japan

Microfill filling syringe	---	World Precision Instruments, Sarasota, FL, USA
Micro-4 Microsyringe pump controller	Model UMC4-C	World Precision Instruments, Sarasota, FL, USA
Petri Dishes	Catalog #127,60mm	Sterillin, Newport, UK
pH Meter	Model 450	Corning pH meter, Albany, NY, USA
Picofuge	Catalog #400550	Stratagene, Santa Clara, CA, USA
RNAase free water in 1.8 ml Eppendorf tubes	Lot #M25/80502	Epicenter Biotechnologies Madison, Wisconsin, USA
Silver Wires	---	World Precision Instruments, Sarasota, FL, USA
Surgical Accessories	Scissors, forceps, scalpels	World Precision Instruments, Sarasota, FL, USA
Surgical Sutures	Catgut chrom, reverse cutting 3/8 circle, USP 4/0, SMI	SMI, DemeTech Corporation, Miami, Florida, USA

Stirrer	Rotomix type 50800, Model #M50825	Barnstead/thermolyne, Dubuque, IA, USA
Vertical Puller	Model 700D, heater adjusted to 48 and solenoid adjusted to 70 ºC to get resistance of 1-2 MΩ	David Kopf Instruments, Tujunga, CA, USA

3.3 Methods

3.3.1   Preparation of Modified Barth's Solution (MBS)

The Modified Barth's Solution was mainly used during isolation of the oocytes and will be discussed in later chapters. The composition of MBS is shown in (Table 3.4)

A. Calcium free MBS solution:

Table 3.4: MBS Solution

Compound	Concentration (mM)	1x (Weight in Grams)	10x (Weight in Grams)
HEPES	10	2.38	23.8
KCl	1	0.075	0.75
MgSO4	0.8	0.2	2
NaCl	88	5.14	51.4
NaHCO3	2.4	0.2	2

All the above compounds were dissolved in 1 L of distilled water using a magnetic stirrer and plate and the pH was adjusted using NaOH to 7.5.

B. MBS with Calcium:

To prepare this solution, 0.22 g and 2.2 g of $CaCl_2$ was added to the above components to make stock solutions of 1x and 10x respectively, and they were dissolved in 1 L of distilled water and the pH was adjusted to 7.5 using NaOH.

3.3.2 Preparation of oocyte storage solution

The storage solution was used to keep the oocytes in incubators at 18° degrees for 7- 10 days. The composition of the storage solution is shown in (Table 3.5)

Table 3.5: Oocyte storage solution

Compounds	Concentration (mM)	Weight in Grams
$CaCl_2$	2	0.22
Gentamycin	50 mg/L	2 mL
HEPES	10	2.38
KCl	1	0.075
$MgSO_4$	0.8	0.2
NaCl	88	5.14
$NaHCO_3$	2.4	0.2
Penicillin G	100000 U/L	0.019
Sodium Pyruvate	2	0.22
Streptomycin	10 mg/L	0.01
Theophylline	0.5	0.09

3.3.3   Preparation of Frogs Ringer Solution

The Frogs Ringer solution (ND96) is the working extracellular solution that was used in the two-electrode voltage clamp system to record the nAChR mediated ion currents. The composition of the (ND96) is shown in Table 3.6. Working solutions of 1x were adjusted to pH 7.5 using NaOH before the experiment.

Table 3.6: Frogs Ringer Solution (ND96)

Compounds	Concentration (mM)	1x (Weight in Grams)	10x (Weight in Grams)
$CaCl_2$	1.8	0.2	2.0
$BaCl_2$ Or $CaCl_2$	1.8	0.439	4.39
HEPES	5	1.19	11.9
KCl	2	0.15	15
$MgCl_2$	1	0.10	1.0
NaCl	96	5.61	56.1

3.3.4   Isolation of Oocytes from *Xenopus Laevis*

Once a week, surgery was performed on a frog for oocyte collection. After a 2-3 month recovery period the same frog was used again. Each frog could be used for 4 oocyte collection procedures. The frog was brought in 1 L of cold tap water from the animal house aquarium where all the frogs are located and securely stored. The frog was anesthetized by immersion in 0.03% w/v p-aminobenzocaine prepared by dissolving 300 mg of ethyl p-aminobenzocaine in 15 mL of 70% ethanol which were immersed in the 1 L of cold tap water which contains the frog. The frog was then monitored for the unset of anesthesia by pinching of the lower limbs and usually takes about 5 minutes for the animal to be completely anesthetized. Afterwards, the frog was placed on a bed of ice to keep its body temperature low during the surgery.

To perform the surgery, all the surgical instruments were sterilized using 70 % ethanol. A 1.5 cm incision was made using a surgical scalpel through the epidermis vertically around the right, middle or left of the lower abdominal area. Another deeper incision was made to the muscle layer where the oocyte lobes are located using surgical scalpel and scissors. A single lobe or pair of lobes of ovaries containing oocytes were removed (Figure 3.4) and placed on a petri dish containing Calcium Free MBS solution. Subsequently, the muscle layer of the abdomen and epidermal layer were sutured using absorbable Catgut sutures. The frog was then placed in a container of water and monitored during recovery. All the procedures conducted in these experiments were approved by the Animal Ethics Committee of College of Medicine and Health Sciences (CMHS), United Arab Emirates University (UAEU).
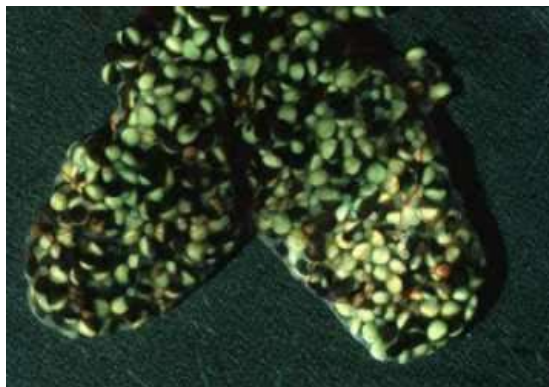
Figure 3.4: Two oocyte containing ovaries in different stages of development taken from: (http://ibmmsrvlakitu.unibe.ch/sigel/xenopicts.html

3.3.5 Oocyte preparation

After extraction of the oocyte lobes from the frog, the epithelial and follicular layers on the oocyte surface were removed by treatment with collagenase solution at room temperature. The collagenase was prepared by dissolving 50 mg of Collagenase, type A (from Clostridium histolyticum, Roche Diagnostic Corporations, USA) in 25 mL of Calcium Free MBS solution and transferred in two separate small conical flasks. The oocytes were placed in two 12.5 mL Collagenase containing flasks for two hours stirring slowly at 60-80 rotations/minute. At the end of the 1st hour, collagenase solution was replaced with fresh collagenase solution for the 2nd hour. Subsequently, the oocytes were washed 6 times using Calcium Free MBS and another 6 times with Calcium containing MBS solution. Then the oocytes were placed in a Calcium MBS containing petri dish for oocyte separation and selection. In the selection step, the oocytes are selected under a dissecting microscope (Bunton Instruments Co Inc, Model GSZ, Rockville, MD, USA). All the selected oocytes were stage V and VI which were larger in size with round shape, 1.0 mm - 1.2 mm diameter and clearly separated dark brown and yellow vegetal animal poles as shown in (Figure 3.5).

Figure 3.5: Individual oocyte isolated from *Xenopus laevis* frog after treatment with collagenase taken from: (http://ibmmsrvlakitu.unibe.ch/sigel/xenopicts.html)

3.3.6   Ion channels endogenously expressed in the oocytes

The *Xenopus* oocytes can express a wide number of receptors, ion channels and transporter proteins. Nevertheless, oocytes also contain various endogenously expressed transporter proteins, ion channels that could interfere with the expression level and the functional properties of exogenously expressed channels or proteins, the first type is voltage-gated ion channels (VGIC) which can further include: 1) Voltage-Gated Ca^{2+} channels, 2) Inducible Na^+ channels, 3) Transient Na^+ channels, 4) Amiloride-sensetive N^+ channels, 5) Rapidly inactivating K^+ channels, 6) Delayed rectifier ISDN-sensitive K^+ channels and 7) Hyper-polarization-activated Cl^- channels. The second type of endogenously expressed receptors are Ligand-activated ion channels (LGIC) which include: 1) Ca^{2+} activated Cl^- channels, 2) Na^+ channels, 3) K^+ channels, 4) Store-operated Ca^{2+} channels (SOC) and 5) iGluRs containing endogenous. (Terhag, Cavara, & Hollmann, 2010(Roger L. Papke, Boulter, Patrick, & Heinemann, 1989; Sands, Costa, & Patrick, 1993; Weber, 1999). Our experiments were designed to eliminate the contribution of Ca^{2+} activated Cl^- channels mediated inward currents which will be discussed in later chapters.

3.3.7 Advantages and disadvantages of oocyte expression system

3.3.7.1 Advantages

- Large number of oocytes can be isolated from each frog.

- The cells are large in size and can be easily injected with cRNA.

- The cells can remain alive for periods up to 2 weeks.

3.3.7.2 Disadvantages

- The small number of endogenous expressed proteins can interfere with the actual current measurements.

- The oocytes can deteriorate when exposed to high temperatures so the recording must be performed at temperatures of 18-23 ºC.

- Expressed channels can function differently from native cells due to posttranslational modifications.

3.4 Synthesis of cRNA

The Capped cRNA transcripts were synthesized using mMASSAGE mMACHINE kit *in-vitro* (Ambion, Austin, TX, USA) and analyzed by using 1.2% formaldehyde agarose gel to check the quality and size of the transcripts. This procedure for cDNA cloning of human nAChRs was provided by Dr. J. Lindstorm (University of Pennsylvania, PAM, USA). The final concentration synthesized for the human nAChRs mRNA was 3.7 µg/µL.

3.4.1   Microinjection of cRNA

The human nAChRs mRNA was kept in 1 µl aliquots in a -80 °C freezer and stored there for microinjection. After oocyte preparation one aliquot of RNA was transferred to the laboratory on an ice bucket. The bench was sterilized using 70 % ethanol and masks and gloves were worn during microinjection to minimize RNAase contamination. Using a micro-centrifuge the tube was centrifuged at 1200 rpm for 1-3 minutes and 8 µL of RNAase and DNAase free water was added to the 1 µl cRNA pellet by the use of a sterile pipette and RNAase DNAase free pipette tips (Denville Scientific Inc., Metuchen, NJ, USA). During the microinjection only 3-4 µL of diluted cRNA was required for each batch of oocytes and the remaining cRNA was returned to the freezer for storage. Microelectrodes used for micro-injection were prepared with a horizontal puller PIL-1 (World Precision Instruments, Sarasota, FL, USA) using autoclaved glass capillaries (World Precision Instruments, Sarasota, FL, USA). The fine needle shaped tips of the glass microelectrodes were broken by applying a gentle pressure using a fine pair of forceps (Fine Science Tools Inc., Vancouver, Canada) under a dissecting microscope (Bunton Instruments Co., Rockville, MD, USA).

Subsequently, the electrode needle was back filled with mineral oil (Sigma, St. Louis, MO, USA) using a 1 mL glass syringe. The electrode needle was fixed in the micro-dispenser which was controlled by the micromanipulator. The next step was to withdraw the 3 µL of diluted cRNA or distilled water drop by the micro-injector and the mineral oil filled glass. Using the withdrawal knob of the micro-dispenser controller the mRNA containing aqueous phase was drawn into the needle. This step was performed under the dissecting microscope to make sure that the tip of the needle was always at the center of the droplet while drawing the solution (Figure 3.6).
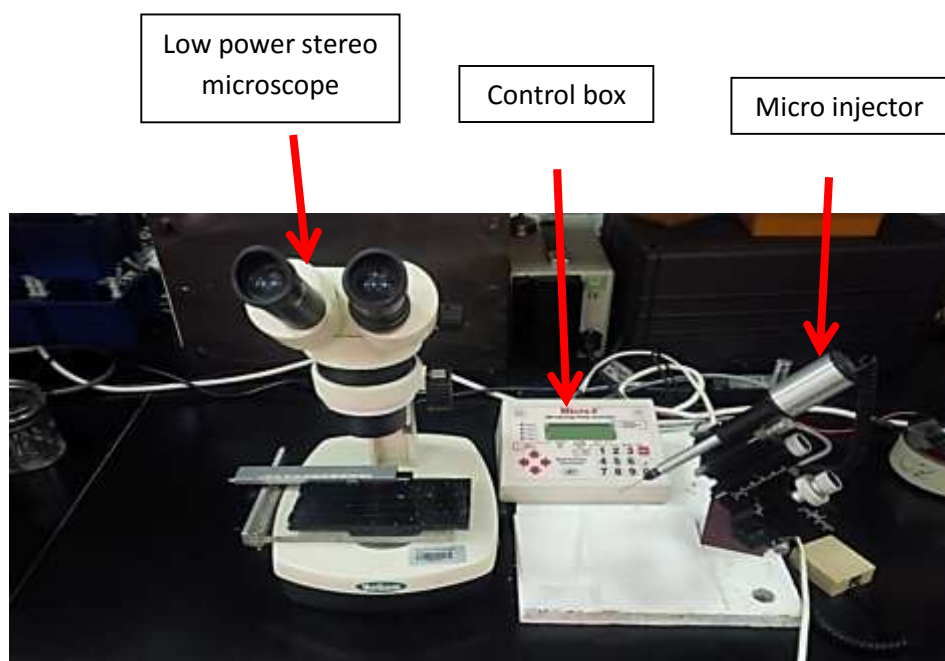
Figure 3.6: Micro injector (Nano-injector) and dissecting microscope. The oocyte micro injector unit consisted of a micro injector mounted in the micro-manipulator. A control box to adjust the amount of solution and a low power dissecting microscope.

The selected oocytes were placed in a U shaped arrangement in a round mesh-bottom petri dish that provided mechanical support to the oocytes during the injection. The oocytes were impaled by the microelectrode needle and 25-30 nL mRNA or distilled water were injected into each oocyte using the nano-liter injector (micro-injector) guided by a Micro-4 micro syringe pump controller. Each petri dish filled with injected oocytes was then labeled and stored in oocyte storage solution at 18 °C in a fridge. The oocytes were divided into two groups, the first group contained cells injected with cRNA encoding human nAChRs and the second group of was the control which was injected with distilled water. The groups were stored in 18 °C fridge for 48-72 hours prior to two-electrode clamp experiments to achieve maximal expression of receptors. The injected oocytes were used for experiments for 7 - 10

days during which the storage solution was replaced on a daily basis and the cells with poor quality were removed under the dissecting microscope and were not used in experiments.

3.4.2 Drug preparation

Stock solutions of the test compounds were all prepared in ND96 solution using the following formula:

$$\text{Weight (mg)} = \text{(MW)} \times \text{(Volume (L))} \times \text{(concentration (mM))}$$

Further dilutions were also prepared using the Charles equation:

$$C1 \times V1 = C2 \times V2$$

C1 = concentration of stock solution

V1 = volume of stock solution

C2 = concentration of the solution to be prepared

V2 = volume of C2 of the solution to be prepared

Required dilutions and fresh stock solutions were all prepared fresh before the start of experiments.

3.5 Electrophysiological recording

The two-electrode voltage clamp technique is a widely used method. It measures the electric current generated by the flow of ions across the cell membrane while clamping the trans-membrane potential at a specific value (in our experiments usually -70 mV). This is done by using a feedback amplifier. This technique allows the evaluation of the functional properties of different proteins expressed in *Xenopus* oocytes. The membrane potential (measured by the voltage sensing electrode and high input resistance amplifier) is compared with a command voltage and the difference is brought to zero by a feedback amplifier. The second electrode measures the total

current generated by the amplifier to keep the oocyte at the clamped potential (Figure 3.7).
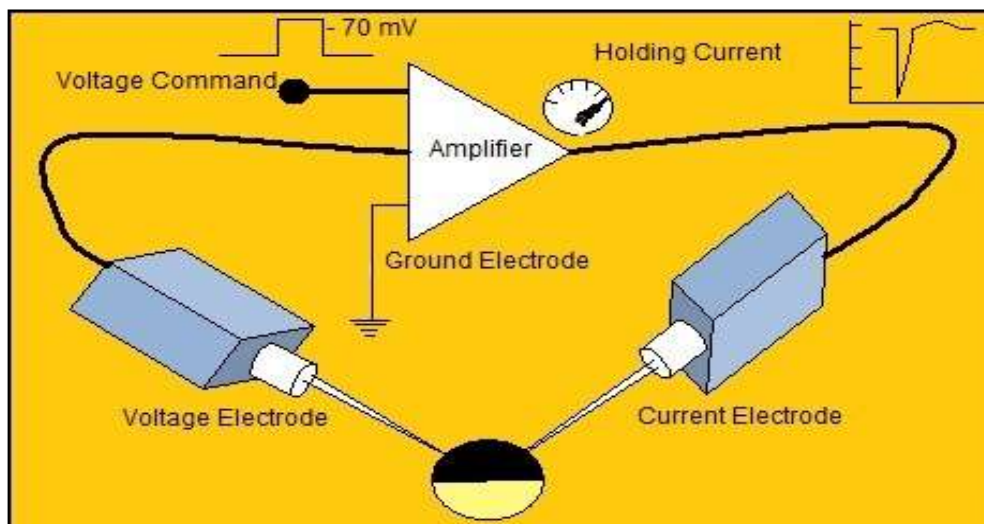
Figure 3.7: Illustration of electrical circuitry connecting oocytes to the recording set-up (Schematic Diagram)

The oocytes were transferred to the perfusion chamber and bathed in ND96 solution at a perfusion rate of 5-7 mL/minute (Figure 3.2). Prior to each experiment, two glass microelectrodes were prepared by a vertical puller (David Kopf Instruments Tujunga, CA, USA) and the animal pole (the dark side) was impaled by the two glass microelectrodes filled with 3M KCL solution with a resistance of 1-2 MΩ. Drugs were applied by a micropipette placed about 2 mm away from the oocytes, and operated by gravity flow based multichannel application system. Two-electrode voltage clamp was performed using a Geneclamp 500 amplifier (Axon Instruments, Molecular Devices, Inc, Sunnyvale, CA, USA) that was interfaced to a PC computer equipped with data acquisition software (Win WCP, University of Strathclyde, Glasgow, UK).

3.6 Tested electrophysiological parameters

αβ and α Thujone purchased from Sigma Aldrich were dissolved in absolute Ethanol to prepare a 100 mM stock solution. Further final dilutions were prepared using distilled water. Working dilutions of drug perfusion and ND96 must not contain Ethanol concentrations of more than 0.3% (v/v) for the nAChRs because our published results indicate that concentrations of Ethanol higher than 0.3% interferes with the function of nicotinic receptors.

3.6.1 Concentration response curves

Acetylcholine (ACh) was selected as it is the endogenous agonist of nicotinic receptors. Furthermore, ACh has less desensitizing properties on nAChRs compared with other agents like nicotine.

Control readings of ACh induced currents (3-5 recording) at 5 min intervals were obtained followed by wash out with regular ND96 solutions. The average of 3 or 4 readings were calculated and recorded as the control. Subsequently, the oocytes were perfused for a total of 15 minutes with the selected concentrations of Thujone. The average of two to three responses at the end of the 15 min drug application were calculated to determine the effect of Thujone co-application with ACh. Finally, drug perfusion was stopped and ND96 perfusion was restarted to record the recoveries of ACh induced currents.

By comparing the maximum current amplitudes in the absence and presence of Thujone, the percent inhibition of the drug was calculated. The values were normalized and displayed as percent changes compared to controls. Using different concentrations of Thujone, it was possible to construct dose response curves. For each concentration of Thujone, averages of 5-6 oocytes were used.

The concentration of Thujone that caused a 50 % inhibition of ACh-induced currents (IC_{50}) was calculated by nonlinear curve-fitting and regression fits (logistic equation) using statistical software (Origin, OriginLab Corp., Northampton, MA, USA).

3.6.2   Competitive and non-competitive inhibition

In this experiment, different concentrations of ACh, ranging from 1 μM to 1mM, were used in the absence and presence of a fixed concentration of α-Thujone. Cells were clamped at a holding potential of -70 mV using the GeneClamp500. An average of 5-6 oocytes were used for each concentration and the percent inhibition was recorded and calculated. Concentration response curves were plotted using computer software (Origin, OriginLab Corp., Northampton, MA, USA).

3.6.3   Voltage dependency of drug inhibition

In these experiments, the membrane potential was held at different values ranging from +20 mV to -120 mV for 30 seconds just before current recording to observe the effect of drug at different membrane potentials. After each recording the potential was returned back to -70 mV and the recordings were performed at 5 min intervals. The ACh concentration was fixed at 100 μM at all tested holding potentials. The relationship between voltage and current (I-V relationship) for the ACh induced currents were determined in the absence and presence of α-Thujone.

3.6.4   Effect of Ca^{2+} on drug action

These experiments were conducted to determine the role of intracellular Ca^{2+} on α-Thujone inhibition of ACh induced currents. 50 nL of 100 mM stock solution of BAPTA (1,2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid) was injected into each oocyte just before the electrophysiological recording (Sands, Costa and Patrick 1993b). Subsequently, the oocytes were kept in ND96 for 5-10 min and

then transferred to ND96 solution containing 2 mM Ba^{2+} instead of Ca^{2+}. The stock solutions of BAPTA were prepared in distilled water and the pH was adjusted by CsOH to 7.4 pH. The ND96 contained 1.8 mM $BaCl_2$ instead of 1.8 mM $CaCl_2$.

3.7 Statistical data analysis

Origin version 8.1 (Origin Lab Corp., Northampton, MA, USA) was used for nonlinear curve fitting. The average values were calculated as the mean ± standard error means (SEM). ANOVA or Student's t-test was used for statistical analysis. Specific *p value* of p≤0.05 was considered significant. Concentration response curves were acquired by fitting the data to the logistic equation:

$$y = E_{max} / (1 + [x / EC_{50}]^{n})$$

E_{max} = maximal response (Keverne & Ray, 2005)

x = drug concentration

y = response

EC_{50} = half the maximal concentration

n = slope factor (apparent Hill coefficient)

Chapter 4: Results

4.1 Effects of ACh and α-bungarotoxin on α7 nAChRs

The endogenous nAChR agonist ACh was used to activate the nicotinic receptors. In oocytes injected with cRNA of human α7 nAChRs, the application of 100 μM of ACh for 3-4 seconds caused fast inward currents (Figure 4A, control currents) indicating that ACh induced currents were mediated by a protein synthesized by the injection of human α7 nAChRs. In line with these findings, the bath application of ACh for 4 seconds did not elicit a response on the oocytes injected with distilled water (Figure 4B).

In the same experiment, α-bungarotoxin which is a selective and potent inhibitor of the α7 nAChRs obtained from snake venom was used to verify the pharmacology of these receptors. ACh-induced currents were significantly inhibited by 30 nM α-bungarotoxin (Figure 4A, 4B). The recoveries were slow and incomplete during the washout period (Figure 4A, right panel).
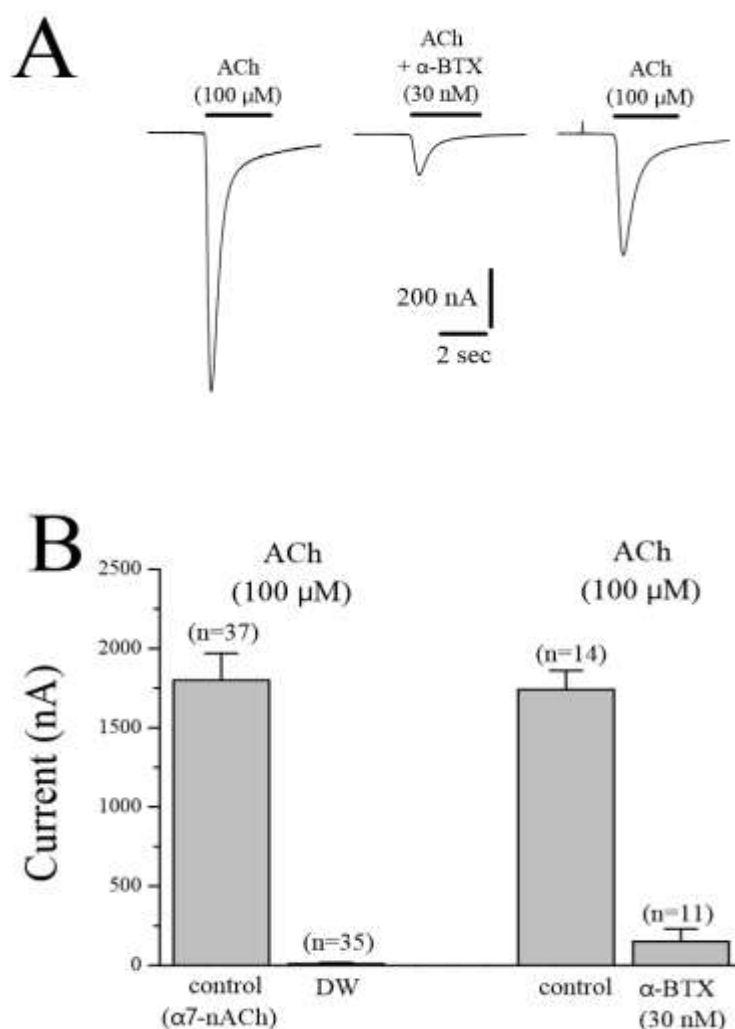
Figure 4.1: Control experiments demonstrating the functional characterization of ACh induced currents. (Figure 4.1.A) Current traces of control 100 µM ACh (left panel), after 10 minutes bath application of 30 nM α-bungaratoxin (middle panel) and following 15 minute washout 100 µM ACh (right panel). Bars represent the means of maximal amplitudes of effects of ACh-induced currents vs distilled water injected oocytes and human α7 nAChR 50 ng cRNA injected oocytes are shown in (Figure 4.1.B). The numbers of oocytes tested were represented for each group on top of each bar (n) in (Figure 4.1.B). In oocytes injected with human α7 nAChR cRNA, the mean maximal amplitudes of ACh-induced currents in the presence and absence of α-bungaratoxin were presented on the right side of the (Figure 4.1.B)

4.2 Time and Concentration effects of α-Thujone on α7 nAChR

The following experiments (Figure 4.2) involves the time and concentration effects of α-Thujone on the α7 nAChR induced currents.

In the Figure 4.2.A the current traces show the effects of 100 μM ACh in control conditions (left panel), followed by 10 minutes bath application of α-Thujone (100 μM) co-applied with Acetylcholine (100 μM) and finally the recovery after 15 min α-Thujone wash out (right panel).

Figure 4.2.B shows the time course of the effect and the recovery of α-Thujone application, on the maximal amplitudes of currents induced by the application of 100 μM ACh for 15 min. Time course of the effect of vehicle (0.1% Ethanol; filled circles) and α-Thujone (100 μM; open circles) on the maximal amplitudes of ACh induced currents. Each data point represents the normalized mean ± S.E.M. of 6 - 8 experiments.

We have also investigated the time course of α-Thujone inhibition by comparing various pre-incubation times of α-Thujone on ACh induced currents Figure 4.2.C. The ACh induced currents in the presence of α-Thujone (co-application) without pre-incubation did not cause a significant change in the amplitudes of currents (as seen before break sign on the X-axis) and the small augmentation of currents was not statistically significant ($P > 0.05$ paired *t*-test. 6–7 oocytes). However, pre-incubation of α-Thujone 100 μM before the (ACh + α-Thujone) co-application caused a significant inhibition of the α7 nAChR mediated induced ion currents. The mean inhibition as a function of α-Thujone pre-incubation times was presented in the figure after the break sign on the X-axis and ultimately, the half maximal inhibition or ($\tau 1/2$) was calculated to be 1.4 ± 1 min (n= 6-7 oocytes). Usually in our experiments and

the majority of the time we pre-incubated with α-Thujone before co-application for 10 - 15 min to ensure equilibrium conditions.

Figure 4.2.D. demonstrates the concentration-response curve for α-Thujone. Increasing the concentrations of α-Thujone caused further decrease in the amplitudes of 100 μM ACh-induced currents and shows that α-Thujone inhibited the α7 nAChR function in a concentration dependent manner with an IC_{50} of 24.7 μM.
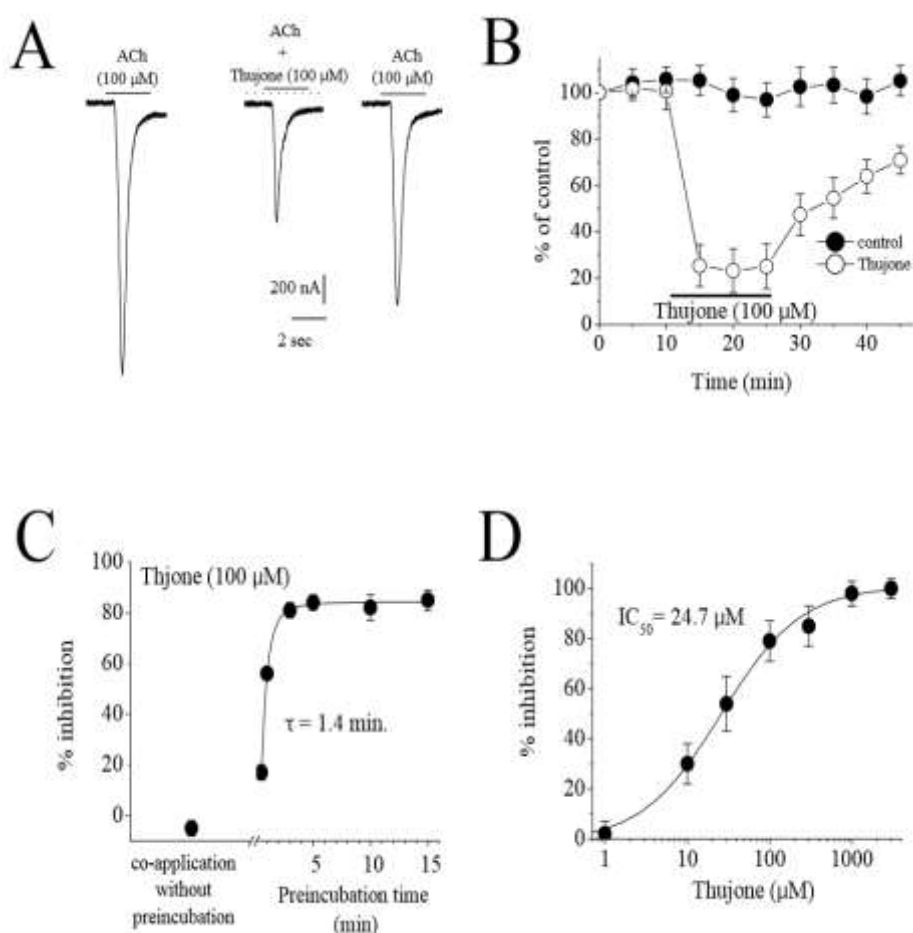
Figure 4.2: Effects of α-Thujone on α7 nAChR: (A) Records of currents activated by 100 μM ACh in control conditions (left panel), after 10 min pre-treatment with 100 μM α-Thujone co-application (middle panel), and 15 min washout (right panel). (B) Time-course of the effect of vehicle (0.1% Ethanol; filled circles) and α-Thujone (100 μM open circles) on the maximal amplitudes of ACh induced currents. Each data point represents the normalized mean ± S.E.M. of 6 – 8

experiments. The horizontal bar represents the application duration of both the Vehicle and α-Thujone. (C) The effect of pre-application of α-Thujone. Increased pre-application time significantly increased the inhibition of α7-nACh receptors. Each data point represents the mean ± S.E.M. of 6 – 7 oocytes. (D) The concentration dependence time course of α-Thujone inhibition. Each data point represents the mean ± S.E.M. of 6 – 9 oocytes. The curve is best fit of the data to the logistic equation described in the methods section.

4.3 Effects of α-Thujone on endogenous Ca^{2+} dependent Cl^- Channels and voltage dependence

In order to understand the mechanisms involved in α-Thujone's inhibition, further investigations were conducted on α7-nAChRs. It has been shown in previous studies that the α7-nAChRs have a high permeability for Ca^{2+} cations. Entrance of Ca^{2+} through α7-nAChRs has been shown to activate endogenous Ca^{2+} dependent Cl^- channels (Sands et al., 1993; Seguela, Wadiche, Dineleymiller, Dani, & Patrick, 1993). In order to find whether α-Thujone acts on endogenous Ca^{2+} dependent Cl^- channels, we have recorded nAChR induced currents in Ca^{2+} free solution by replacing Ca^{2+} in the extracellular solution with Ba^{2+} since Ba^{2+} can pass through the receptors without activating these endogenous Ca^{2+} dependent Cl^- channels (Sands et al., 1993).

It has also been proposed by (Sands et al., 1993) that even when Ba^{2+} is present, endogenous Ca^{2+} dependent Cl^- channels are not completely eliminated. Therefore, we have blocked the activity of these channels by the intracellular injection of BAPTA (1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid) a Ca^{2+} chelating agent. Experiments were performed in BAPTA injected oocytes and perfused with an extracellular solution of 2 mM Ba^{2+} instead of Ca^{2+} cations (Sands

et al., 1993). The bath application of 100 µM α-Thujone caused the same level of

inhibition (80 ± 5 % in controls versus 78 ± 5 % in BAPTA injected oocytes;

ANOVA, P >0.05) on ACh induced currents when compared to BAPTA injected

oocytes recorded in 2 mM Ba^2 (Figure 4.3A).

It is possible that the inhibition of receptors is altered by changes in membrane

potential. Therefore, we have investigated if the inhibition of α-Thujone was effected

by membrane potential. As shown in Figure 4.3B in the presence (circles) and

absence (filled circles) of 100 µM α-Thujone, ACh induced currents were recorded

and normalized to controls at different voltages from -120 mV to 0 mV. The

membrane potential was held at voltages between -120 and 0 mV for 30 seconds

before each current recording and returned to the holding potential of -70 mV. Figure

4.3C show the extent of α-Thujone induced inhibition at different voltages. The

results reveal that the inhibition of α-Thujone 100 µM was not changed by alterations

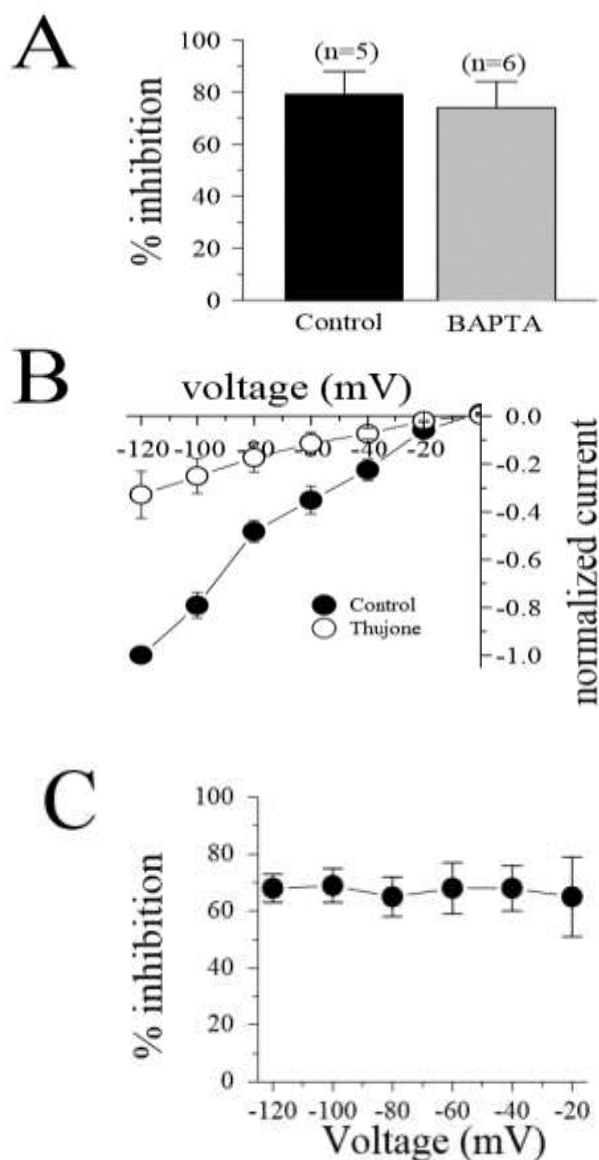in membrane potential (P>0.05, n=6, ANOVA).

Figure 4.3: Effects of α-Thujone on endogenous Ca^{2+} dependent Cl^- Channels and voltage dependence. In the presence of BAPTA, the effects of α-Thujone on α7-nAChRs are not dependent on intracellular Ca^{2+} levels and changes in membrane potential. The effects of 100 μM α-Thujone (10 min) on the maximal amplitudes of ACh induced currents in oocytes injected with 50 nl distilled water controls (n=5) or BAPTA (50 nl, 200 mM, n=6). Bars represent the means ± S.E.M. of 5 – 6 experiments. (B) Current-voltage relationship of ACh induced currents in the absence and presence of α-Thujone. Normalized currents activated by 100 μM ACh before

(control, filled circles) and after 10 min treatment with 100 μM α-Thujone (open circles). Each data point presents the normalized means ± S.E.M. of 5 – 6 experiments. (C) Quantitative presentation of the effect of α-Thujone as percent inhibition at different voltages. Each data point presents the normalized means ± S.E.M. of 5 – 6 experiments.

4.4 Non-competitive effects of α-Thujone on α7 nAChR

Furthermore, we examined the possible sites mediating the effect of α-Thujone on the α7-nAChRs. We investigated whether α-Thujone acts as a competitive or non-competitive antagonist of these receptors. ACh induced concentration response curves were plotted in the absence and presence of α-Thujone (Figure 4.4). The maximum response induced by ACh was reduced to 30 ± 5% (n= 8-12), whereas the EC$_{50}$ values remained unchanged. (93±8 μM in controls versus 98±7 μM α-Thujone, ANOVA, P>0.005, n=5-8). These results provide evidence that α-Thujone inhibits the function of α7-nAChRs and ACh-induced currents in a non-competitive manner.

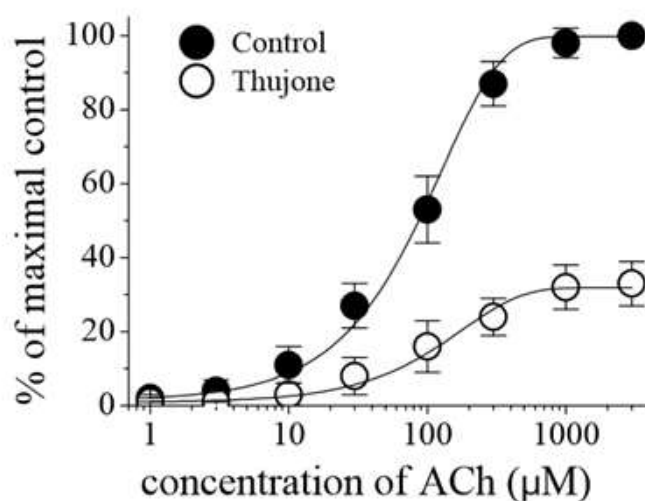Figure 4.4: Concentration-response curve for α-Thujone during increasing concentrations of ACh. Filled circles indicate the ACh induced currents. Open circles

indicate the responses in the presence of α-Thujone. Responses are normalized to maximal ACh induced currents. Each data point presents the normalized means ± S.E.M. of 5 – 6 experiments.

4.5 Effects of Thujone Sterioisomers on α7 nAChR

Since Thujone has two major stereoisomers, we have investigated whether these stereoisomers would behave differently on the human α7 nAChRs, so we compared the percent of inhibition of both 100 µM α-Thujone and 100 µM αβ-Thujone (Figure 4.5). The level of inhibition of the current traces was similar for both compounds with no statistical significance (ANOVA, P>0.05, n=5-6).
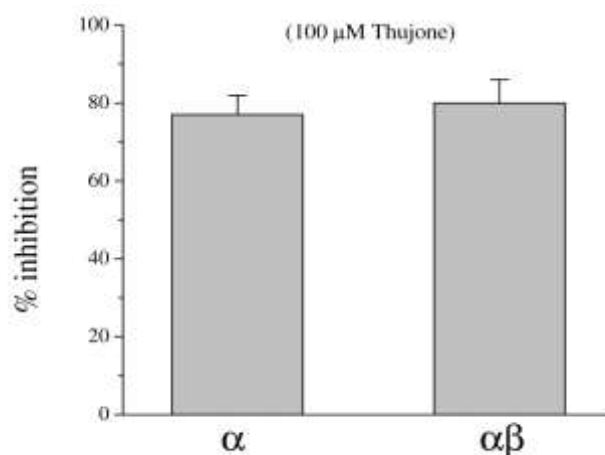

Figure 4.5: Percent of Inhibition of α-Thujone and αβ-Thujone on human α7-nAChRs induced currents with 100 µM Acetylcholine. Each data point presents the normalized means ± S.E.M. of 5 – 6 experiments.

4.6 Effects of Thujone on different nAChRs

In the last set of experiments were compared. The effects of α-Thujone on different subtypes of the nAChRs were compared. The level of inhibition between different subtype combinations of nAChRs indicated that α-Thujone has the highest potency for α7 nAChRs. Although α4β2 was also significantly inhibited, the extent of inhibition by α-Thujone was significantly less than that of α7 nAChRs (Figure 4.6.)
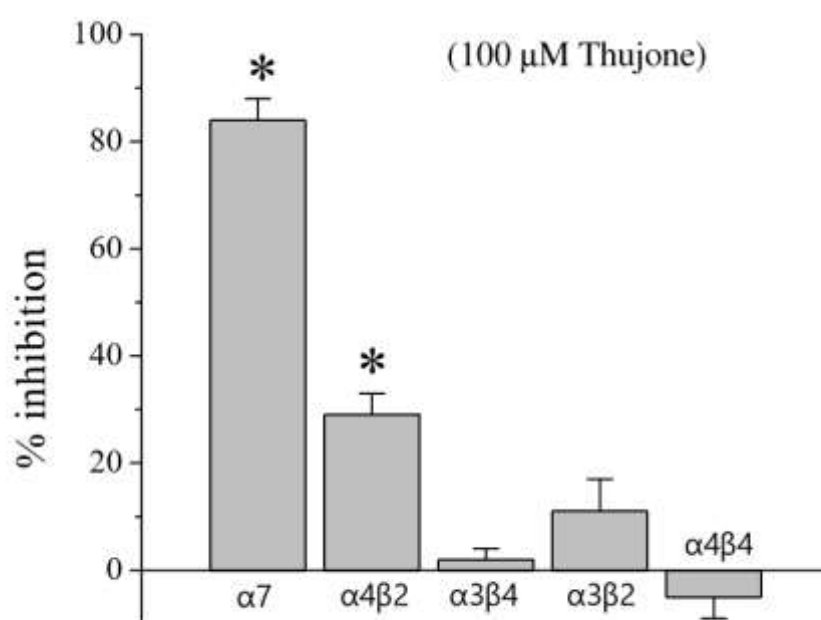

Figure 4.6: Percent of inhibition of 100 μM α-Thujone co-applied with 100 μM ACh on different nAChRs induced oocyte currents. Each data point presents the normalized means ± S.E.M. of 5 – 6 experiments.

Chapter 5: Discussion

The results of our study indicate that Thujone binds non-competitively to α7-nACh receptors and causes time and concentration dependent inhibition.

In an earlier study the mechanism of Thujone neurotoxicity was investigated (Hold et al., 2000). It was demonstrated that the poisoning signs of Thujone (and their alleviation by diazepam and phenobarbital) in mice are similar to those of the classical antagonist picrotoxin suggesting that $GABA_A$ receptors are involved in Thujone toxicity. Secondly, a strain of Drosophila specifically resistant to chloride channel blockers was found to be tolerant to Thujone. Third it was shown that Thujone is a competitive inhibitor of a $GABA_A$ receptor ligand, [^{3}H]ethynylbicycloorthobenzoate binding to mouse brain membranes. In addition, peak currents induced by GABA in rat dorsal root ganglion neurons are suppressed by Thujone with an IC_{50} value of 21 μM (Hold et al., 2000). The action of Thujone on GABAergic miniature inhibitory post-synaptic currents (mIPSCs) and on responses to exogenous GABA applications was also studied in cultured hippocampal neurons (Szczot et al., 2012). Thujone reduced mIPSC frequency and amplitude and also moderately affected their kinetics, indicating both pre- and postsynaptic mechanisms. Analysis of current responses to exogenous GABA revealed that Thujone reduced their amplitude, affecting their onset, desensitization, and deactivation, suggesting an effect on the gating of $GABA_A$ receptors (Szczot et al., 2012). However, $GABA_A$ receptors are highly heterogeneous and the effects of Thujone have also been investigated on different recombinant $GABA_A$ receptors (α1β2γ2L, α1β2, α1β2δd and α4β2δ) relevant to phasic or tonic forms of $GABA_A$ responses. It was shown that α-Thujone, in the concentration range of 100 to 300 μM, inhibits all receptor subtypes by a qualitatively similar mechanism

but the strongest effect was observed for α1β2δ receptors, suggesting that tonic currents might be more sensitive to Thujone than the phasic ones. Moreover, tonic currents, mimicked by response to a sub-μM GABA concentration (0.3 μM) in cultured neurons, showed a substantially larger sensitivity to Thujone than responses elicited by higher [GABA] (more similar to phasic currents) or inhibitory postsynaptic currents in the same preparation. Importantly, the extent of tonic current inhibition by α-Thujone was as strong as in the case of currents mediated by α1β2δ receptors. Collectively, these data provide evidence that different $GABA_A$ receptor subtypes show distinct sensitivities to α-Thujone and suggest that this compound may differentially affect tonic and phasic components of GABAergic inhibition (Czyzewska and Mozrzymas, 2013).

Studies on the neurotoxic actions of Thujone have also been extended to fear-anxiety behaviors (Rivera et al., 2014). Higher doses of Thujone were convulsant by eliciting a toxic and excitatory action. Their results showed that a dose of 78 nmol of Thujone had an anxiogenic-like effect observed as an increased latency to ambulate and a reduced locomotor activity in open field experiments. Nevertheless, only the central administration of Thujone reversed the increase induced by acute stress in the flunitrazepam-sensitive $GABA_A$ receptor recruitment. These findings demonstrated that Thujone, when intracerebroventricularly administered, suppressed the $GABA_A$ receptor recruitment induced by acute stress, may be due to Thujone blocking the benzodiazepine binding site or another site of the $GABA_A$ complex (Rivera et al., 2014).

The effects of α-Thujone have also been investigated on $5\text{-}HT_3$ receptors, another member of ligand-gated ion channel family. In earlier studies (Deiml et al., 2004),

patch-clamp experiments revealed an inhibitory action of Thujone on both homomeric (5-HT_{3A}) and heteromeric (5-HT_{3AB}) $5HT_3$ receptors expressed in HEK-293 cells. Their results indicated that in homomeric receptors, α-Thujone enhanced the inherent channel-blocking potency of the natural ligand, 5-HT. The IC_{50} value for α-Thujone was found to be 60 μM. On the other hand, in heteromeric receptors, α-Thujone recruited an additional channel-blocking component of the agonist. It was suggested that α-Thujone reduced 5-HT_3 receptor activity by an effect on mechanisms involved in receptor desensitization, which depend on receptor subunit composition.

Although, the α7 nAChRs are known to cause an increase in the intracellular Ca^{2+} levels and to activate endogenous Ca^{2+} dependent Cl^- channels, our results indicate that α-Thujone does not activate these channels indicating that the effects of α-Thujone observed in our experiments are not due to its effects on these channels.

In addition, α-Thujone was able to bind and inhibit the α7 nAChRs in a non-competitive manner. Our results show that even high concentrations of ACh cannot reverse the inhibitory effect of α-Thujone, indicating that α-Thujone does not compete with ACh. α-Thujone is a monoterpene ketone, and these compounds have highly volatile and non-polar (lipophilic) structures that usually allow high membrane penetration (Clarke, 2008a). Considering the relatively slow onset of α-Thujone action (τ=1.4 min), lack of voltage-dependency, and the non-competitive nature of α-Thujone inhibition the results suggest that a hydrophobic binding site located in transmembrane domains of nAChR is likely to mediate the effects of α-Thujone on nAChRs.

Monoterpenes such as Thujone possess variable basic skeletons and exhibit stereoisomerism. It has also been shown that some of the biological effects of monoterpenes (Clarke, 2008b) and Thujone (Rivera et al., 2014) show stereo specific

action in *in-vitro* studies. However, our results indicate that α-Thujone and the racemic αβ-Thujone (β-Thujone is commercially unavailable) caused similar extents of inhibition suggesting that the effect of Thujone does not require stereospecific binding for its inhibitory actions in-vitro, because in-vivo models could behave differently.

Cholinergic nAChRs are involved in the pathogenesis of several diseases such as Alzheimer disease (AD), myasthenia gravis (MG), neuro-inflammation, and nicotine addiction (Gotti & Clementi, 2004; Keverne & Ray, 2005; Mufson et al., 2009). The results of these studies have not been conclusive possibly because more types of nAChRs are involved (for a review; Oz et al., 2013). In these studies, both inhibition and activation of nAChRs have been linked to pro-cognitive and pro-inflammatory effects. Many compounds such as Acetylcholinesterase AChE Inhibitors such as: Galantamine, Donepezil and Tacrine also target the same α7 nAChRs with actions of both stimulation and inhibition (Keverne & Ray, 2005). Other drugs involved in AD such as Memantine have also been reported to cause an inhibition on the α7 nAChRs (Eglen et al., 2001; Gangadhar, Mishra, Sriram, & Yogeeswari, 2014; Keverne & Ray, 2005).

Finally, in addition to α7-nAChR, other nicotinic receptors such as: α4β2, α3β2, α3β4 and α4β4 have been shown to be expressed in both the central and peripheral nervous systems. Therefore, we have investigated the effect of Thujone on these receptor subtypes. Our results indicated that, compared to other nAChR tested in our study, Thujone acted most potently on α7-nAChR suggesting that the effects of Thujone are somewhat specific to α7-nAChR.

Chapter 6: Conclusion

This study provides the first evidence for the effects of Thujone on different nAChRs, such as α7, α4β2 α3β2, α3β4 and α4β4. The study suggests that these receptors have pharmacologically different sensitivities to α-Thujone. Although α4β2 receptors were inhibited by 100 μM α-Thujone, the potency of Thujone on α7-nAChRs were significantly higher than α4β2 receptors. Inhibition by α-Thujone was concentration dependent with IC$_{50}$ of 24.7 μM. It appears that α-Thujone acts as a non-competitive antagonist on the α7 nAChRs. The effect of α-Thujone is not voltage dependent and does not involve endogenous Ca^{2+} dependent Cl$^-$ channels. There is no significant difference between α-Thujone and αβ-Thujone racemic mixtures regarding the extent of its inhibitory effect on nAChR. In conclusion, our results suggest that Thujone inhibits nAChRs and could lead to the development of new compounds effecting these receptors.

6.1   Limitations and Future directions:

In this study, we did not examine the effects of Thujone on mammalian cell lines and native neurons. It is possible that post-translational modification in *Xenopus* oocytes are different from mammalian cell lines. In future studies we could study the effects of Thujone on nicotinic receptors expressed in mammalian cell lines.

Bibliography

Akhgar, M. R., Rajaei, P., & Alizadeh-Saljoughi, O. (2013). Chemical Composition of the Essential Oil of Artemisia lehmanniana Bunge Growing Wild in Iran. *Journal of Essential Oil Bearing Plants, 16*(5), 641-645. doi:10.1080/0972060x.2013.854483

Aldred, E. M., Buck, C., & Vall, K. (2009). Chapter 22 - Terpenes. In E. M. A. b. B. Vall (Ed.), *Pharmacology* (pp. 167-174). Edinburgh: Churchill Livingstone.

Alexander, S. P., Benson, H. E., Faccenda, E., Pawson, A. J., Sharman, J. L., Spedding, M., . . . Harmar, A. J. (2013). The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. *Br J Pharmacol, 170*(8), 1459-1581. doi:10.1111/bph.12445

Alexander, S. P. H., Benson, H. E., Faccenda, E., Pawson, A. J., Sharman, J. L., Spedding, M., . . . Collaborators, C. (2013). THE CONCISE GUIDE TO PHARMACOLOGY 2013/14: LIGAND-GATED ION CHANNELS. *British Journal of Pharmacology, 170*(8), 1582-1606.

Amri, I., De Martino, L., Marandino, A., Lamia, H., Mohsen, H., Scandolera, E., . . . Mancini, E. (2013). Chemical Composition and Biological Activities of the Essential Oil from Artemisia herba-alba Growing Wild in Tunisia. *Natural Product Communications, 8*(3), 407-410.

Ashoor, A., Lorke, D., Nurulain, S. M., Al Kury, L., Petroianu, G., Yang, K.-H. S., & Oz, M. (2011). Effects of phenothiazine-class antipsychotics on the function of α7-nicotinic acetylcholine receptors. *European Journal of Pharmacology, 673*(1–3), 25-32. doi:http://dx.doi.org/10.1016/j.ejphar.2011.10.020

Badawy, M. E. I., & Abdelgaleil, S. A. M. (2014). Composition and antimicrobial activity of essential oils isolated from Egyptian plants against plant pathogenic bacteria and fungi. *Industrial Crops and Products, 52*, 776-782. doi:10.1016/j.indcrop.2013.12.003

Baginskas, A., Kuraitė, V., & Kuras, A. (2015). Nicotinic potentiation of frog retinotectal transmission in tectum layer F by α3β2, α4β2, α2β4, α6β2, or α7 acetylcholine receptor subtypes. *Medicina, 51*(2), 117-125. doi:http://dx.doi.org/10.1016/j.medici.2015.03.002

Baydar, H., Sangun, M. K., Erbas, S., & Kara, N. (2013). Comparison of Aroma Compounds in Distilled and Extracted Products of Sage (salvia officinalis L.). *Journal of Essential Oil Bearing Plants, 16*(1), 39-44. doi:10.1080/0972060x.2013.764175

Belhattab, R., Amor, L., Barroso, J. G., Pedro, L. G., & Figueiredo, A. C. (2014). Essential oil from Artemisia herba-alba Asso grown wild in Algeria: Variability assessment and comparison with an updated literature survey. *Arabian Journal of Chemistry, 7*(2), 243-251. doi:10.1016/j.arabjc.2012.04.042

Bouajaj, S., Benyamna, A., Bouamama, H., Romane, A., Falconieri, D., Piras, A., & Marongiu, B. (2013). Antibacterial, allelopathic and antioxidant activities of essential oil of Salvia officinalis L. growing wild in the Atlas Mountains of Morocco. *Natural Product Research, 27*(18), 1673-1676. doi:10.1080/14786419.2012.751600

Briggs, C. A., & Gopalakrishnan, M. (2007). 2.22 - Ion Channels – Ligand Gated. In J. B. T. J. Triggle (Ed.), *Comprehensive Medicinal Chemistry II* (pp. 877-918). Oxford: Elsevier.

Clark, M. A. (2012). *Lippincott's illustrated reviews*. Baltimore, MD: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Clarke, S. (2008a). Chapter 3 - Families of compounds that occur in essential oils. In S. Clarke (Ed.), *Essential Chemistry for Aromatherapy (Second Edition)* (pp. 41-77). Edinburgh: Churchill Livingstone.

Clarke, S. (2008b). Chapter 7 - Composition of essential oils and other materials. In S. Clarke (Ed.), *Essential Chemistry for Aromatherapy (Second Edition)* (pp. 123-229). Edinburgh: Churchill Livingstone.

Clementi, F., Fornasari, D., & Gotti, C. (2000). Neuronal nicotinic acetylcholine receptors: from structure to therapeutics. *Trends in Pharmacological Sciences, 21*(2), 35-37. doi:http://dx.doi.org/10.1016/S0165-6147(99)01423-6

Cooper, J. R., Bloom, F. E., & Roth, R. H. (2003). *The Biochemical Basis of Neuropharmacology*: Oxford University Press.

Cuello, A. C. (2009). Cholinergic Pathways in CNS. In L. R. Squire (Ed.), *Encyclopedia of Neuroscience* (pp. 835-843). Oxford: Academic Press.

Czyzewska, M. M., & Mozrzymas, J. W. (2013). Monoterpene alpha-thujone exerts a differential inhibitory action on GABA(A) receptors implicated in phasic and tonic GABAergic inhibition. *European Journal of Pharmacology, 702*(1-3), 38-43. doi:10.1016/j.ejphar.2013.01.032

Davis, T. J., & de Fiebre, C. M. (2006). Alcohol's actions on neuronal nicotinic acetylcholine receptors. *Alcohol Research & Health, 29*(3), 179-185.

Dawidowicz, A. L., & Dybowski, M. P. (2012). Fast determination of α- and β-thujone in alcoholic beverages using solid-phase extraction and gas chromatography. *Food Control, 25*(1), 197-201. doi:http://dx.doi.org/10.1016/j.foodcont.2011.10.045

Deiml, T., Haseneder, R., Zieglgansberger, W., Rammes, G., Eisensamer, B., Rupprecht, R., & Hapfelmeier, G. (2004). alpha-thujone reduces 5-HT3 receptor activity by an effect on the agonist-induced desensitization. *Neuropharmacology, 46*(2), 192-201. doi:10.1016/j.neuropharm.2003.09.022

Eaton, M. J., Ospina, C. A., Rodríguez, A. D., & Eterović, V. A. (2004). Differential inhibition of nicotine- and acetylcholine-evoked currents through α4β2 neuronal nicotinic receptors by tobacco cembranoids in Xenopus oocytes. *Neuroscience Letters, 366*(1), 97-102. doi:http://dx.doi.org/10.1016/j.neulet.2004.05.019

Eglen, R. M., Choppin, A., & Watson, N. (2001). Therapeutic opportunities from muscarinic receptor research. *Trends in Pharmacological Sciences, 22*(8), 409-414. doi:http://dx.doi.org/10.1016/S0165-6147(00)01737-5

EMA/HMPC, 2008. Assessment report on Artemisia absinthium L., Herba. EMEA/HMPC/234444/2008. <http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2009/12/WC500017795.pdf >

EMA/HMPC, 2008. Assessment report on Salvia officinalis L., folium and Salvia officinalis L., aetheroleum. EMA/HMPC/330383/2008. <http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2010/02/WC500070850.pdf >

Fowler, C. D., Lu, Q., Johnson, P. M., Marks, M. J., & Kenny, P. J. (2011). Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. *Nature, 471*(7340), 597-601. doi:10.1038/nature09797

Gangadhar, M., Mishra, R. K., Sriram, D., & Yogeeswari, P. (2014). Future Directions in the Treatment of Neuropathic Pain: A Review on Various Therapeutic Targets. *Cns & Neurological Disorders-Drug Targets, 13*(1), 63-81.

Gotti, C., & Clementi, F. (2004). Neuronal nicotinic receptors: from structure to pathology. *Progress in Neurobiology, 74*(6), 363-396. doi:http://dx.doi.org/10.1016/j.pneurobio.2004.09.006

Gotti, C., Clementi, F., Fornari, A., Gaimarri, A., Guiducci, S., Manfredi, I., . . . Zoli, M. (2009). Structural and functional diversity of native brain neuronal nicotinic receptors. *Biochemical Pharmacology, 78*(7), 703-711. doi:http://dx.doi.org/10.1016/j.bcp.2009.05.024

Gotti, C., Fornasari, D., & Clementi, F. (1997). Human neuronal nicotinic receptors. *Progress in Neurobiology, 53*(2), 199-237. doi:http://dx.doi.org/10.1016/S0301-0082(97)00034-8

Gotti, C., Moretti, M., Gaimarri, A., Zanardi, A., Clementi, F., & Zoli, M. (2007). Heterogeneity and complexity of native brain nicotinic receptors. *Biochemical Pharmacology, 74*(8), 1102-1111. doi:http://dx.doi.org/10.1016/j.bcp.2007.05.023

Gotti, C., Sher, E., Chini, B., Fornasari, D., Esparis Ogando, A., & Clementi, F. (1988). The nicotinic receptors in the nervous system. *Pharmacological Research Communications, 20*(8), 637-662. doi:http://dx.doi.org/10.1016/S0031-6989(88)80112-7

Grady, S. R., Drenan, R. M., Breining, S. R., Yohannes, D., Wageman, C. R., Fedorov, N. B., . . . Marks, M. J. (2010). Structural differences determine the relative selectivity of nicotinic compounds for native α4β2*-, α6β2*-, α3β4*- and α7-nicotine acetylcholine receptors. *Neuropharmacology, 58*(7), 1054-1066. doi:http://dx.doi.org/10.1016/j.neuropharm.2010.01.013

Hall, A. C., Turcotte, C. M., Betts, B. A., Yeung, W.-Y., Agyeman, A. S., & Burk, L. A. (2004). Modulation of human GABAA and glycine receptor currents by menthol and related monoterpenoids. *European Journal of Pharmacology, 506*(1), 9-16. doi:http://dx.doi.org/10.1016/j.ejphar.2004.10.026

Hendrickson, L. M., Guildford, M. J., & Tapper, A. R. (2013). Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence. *Front Psychiatry, 4*, 29. doi:10.3389/fpsyt.2013.00029

Hold, K. M., Sirisoma, N. S., Ikeda, T., Narahashi, T., & Casida, J. E. (2000). alpha-thujone (the active component of absinthe): gamma-aminobutyric acid type A receptor modulation and metabolic detoxification. *Proceedings of the National Academy of Sciences of the United States of America, 97*(8), 3826-3831. doi:10.1073/pnas.070042397

Höld, K. M., Sirisoma, N. S., & Casida, J. E. (2001). Detoxification of α- and β-Thujones (the Active Ingredients of Absinthe): Site Specificity and Species Differences in Cytochrome P450 Oxidation in Vitro and in Vivo. *Chemical Research in Toxicology, 14*(5), 589-595. doi:10.1021/tx000242c

Imoto, K. (2004). Ion Channels. In L. Martini (Ed.), *Encyclopedia of Endocrine Diseases* (pp. 103-107). New York: Elsevier.

Keverne, J., & Ray, M. (2005). Neurochemistry of Alzheimer's disease. *Psychiatry, 4*(1), 40-42. doi:http://dx.doi.org/10.1383/psyt.4.1.40.58307

Kokkini, S., Karousou, R., & Hanlidou, E. (2003). HERBS | Herbs of the Labiatae. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 3082-3090). Oxford: Academic Press.

Lachenmeier, D. W. (2010). Wormwood (Artemisia absinthium L.)—A curious plant with both neurotoxic and neuroprotective properties? *Journal of Ethnopharmacology, 131*(1), 224-227. doi:http://dx.doi.org/10.1016/j.jep.2010.05.062

Lachenmeier, D. W., Emmert, J., Kuballa, T., & Sartor, G. (2006). Thujone—Cause of absinthism? *Forensic Science International, 158*(1), 1-8. doi:http://dx.doi.org/10.1016/j.forsciint.2005.04.010

Lachenmeier, D. W., & Uebelacker, M. (2010). Risk assessment of thujone in foods and medicines containing sage and wormwood – Evidence for a need of regulatory changes? *Regulatory Toxicology and Pharmacology, 58*(3), 437-443. doi:http://dx.doi.org/10.1016/j.yrtph.2010.08.012

Mahgoub, M., Keun-Hang, S. Y., Sydorenko, V., Ashoor, A., Kabbani, N., Al Kury, L., . . . Oz, M. (2013). Effects of cannabidiol on the function of α7-nicotinic acetylcholine receptors. *European Journal of Pharmacology, 720*(1–3), 310-319. doi:http://dx.doi.org/10.1016/j.ejphar.2013.10.011

McGivern, J. G., & Worley Iii, J. F. (2007). 2.21 - Ion Channels – Voltage Gated. In J. B. T. J. Triggle (Ed.), *Comprehensive Medicinal Chemistry II* (pp. 827-875). Oxford: Elsevier.

Meschler, J. P., & Howlett, A. C. (1999). Thujone Exhibits Low Affinity for Cannabinoid Receptors But Fails to Evoke Cannabimimetic Responses. *Pharmacology Biochemistry and Behavior, 62*(3), 473-480. doi:http://dx.doi.org/10.1016/S0091-3057(98)00195-6

Mesulam, M. M. (2009). Acetylcholine Neurotransmission in CNS. In L. R. Squire (Ed.), *Encyclopedia of Neuroscience* (pp. 1-4). Oxford: Academic Press.

Miledi, R., Parker, I., & Sumikawa, K. (1983). RECORDING OF SINGLE GAMMA-AMINOBUTYRATE-ACTIVATED AND ACETYLCHOLINE-ACTIVATED RECEPTOR CHANNELS TRANSLATED BY EXOGENOUS MESSENGER-RNA IN XENOPUS OOCYTES. *Proceedings of the Royal Society Series B-Biological Sciences, 218*(1213), 481-484. doi:10.1098/rspb.1983.0053

Millar, N. S., & Gotti, C. (2009). Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology, 56*(1), 237-246. doi:http://dx.doi.org/10.1016/j.neuropharm.2008.07.041

Mufson, E. J., Counts, S. E., & Ginsberg, S. D. (2009). Cholinergic System. In L. R. Squire (Ed.), *Encyclopedia of Neuroscience* (pp. 845-856). Oxford: Academic Press.

Naser, B., Bodinet, C., Tegtmeier, M., & Lindequist, U. (2005). Thuja occidentalis (Arbor vitae): A review of its pharmaceutical, pharmacological and clinical properties. *Evidence-Based Complementary and Alternative Medicine, 2*(1), 69-78. doi:10.1093/ecam/neh065

Oz, M. (2006). Receptor-independent actions of cannabinoids on cell membranes: Focus on endocannabinoids. *Pharmacology & Therapeutics, 111*(1), 114-144. doi:http://dx.doi.org/10.1016/j.pharmthera.2005.09.009

Oz, M., Al Kury, L., Keun-Hang, S. Y., Mahgoub, M., & Galadari, S. (2014). Cellular approaches to the interaction between cannabinoid receptor ligands and nicotinic acetylcholine receptors. *European Journal of Pharmacology, 731*(0), 100-105. doi:http://dx.doi.org/10.1016/j.ejphar.2014.03.010

Oz, M., Lorke, D. E., & Petroianu, G. A. (2009). Methylene blue and Alzheimer's disease. *Biochemical Pharmacology, 78*(8), 927-932. doi:http://dx.doi.org/10.1016/j.bcp.2009.04.034

Oz, M., Lozon, Y., Sultan, A., Yang, K.-H. S., & Galadari, S. Effects of monoterpenes on ion channels of excitable cells. *Pharmacology & Therapeutics*(0). doi:http://dx.doi.org/10.1016/j.pharmthera.2015.05.006

Oz, M., Spivak, C. E., & Lupica, C. R. (2004). The solubilizing detergents, Tween 80 and Triton X-100 non-competitively inhibit α7-nicotinic acetylcholine receptor function in Xenopus oocytes. *Journal of Neuroscience Methods, 137*(2), 167-173. doi:http://dx.doi.org/10.1016/j.jneumeth.2004.02.016

Padosch, S. A., Lachenmeier, D. W., & Kroner, L. U. (2006). Absinthism: a fictitious 19th century syndrome with present impact. *Substance Abuse Treatment Prevention and Policy, 1*, 14. doi:10.1186/1747-597x-1-14

Papke, R. L., Boulter, J., Patrick, J., & Heinemann, S. (1989). Single-channel currents of rat neuronal nicotinic acetylcholine receptors expressed in xenopus oocytes. *Neuron, 3*(5), 589-596. doi:http://dx.doi.org/10.1016/0896-6273(89)90269-9

Papke, R. L., & Smith-Maxwell, C. (2009). High Throughput Electrophysiology with Xenopus Oocytes. *Combinatorial Chemistry & High Throughput Screening, 12*(1), 38-50.

Paterson, D., & Nordberg, A. (2000). Neuronal nicotinic receptors in the human brain. *Progress in Neurobiology, 61*(1), 75-111. doi:http://dx.doi.org/10.1016/S0301-0082(99)00045-3

Pelkonen, O., Abass, K., & Wiesner, J. (2013). Thujone and thujone-containing herbal medicinal and botanical products: Toxicological assessment. *Regulatory Toxicology and Pharmacology, 65*(1), 100-107. doi:10.1016/j.yrtph.2012.11.002

Polatoglu, K., Karakoc, O. C., & Goren, N. (2013). Phytotoxic, DPPH scavenging, insecticidal activities and essential oil composition of Achillea vermicularis, A. teretifolia and proposed chemotypes of A. biebersteinii (Asteraceae). *Industrial Crops and Products, 51*, 35-45. doi:10.1016/j.indcrop.2013.08.052

Qiao, X., Sun, G. C., Clare, J. J., Werkman, T. R., & Wadman, W. J. (2014). Properties of human brain sodium channel alpha-subunits expressed in HEK293 cells and their modulation by carbamazepine, phenytoin and lamotrigine. *British Journal of Pharmacology, 171*(4), 1054-1067. doi:10.1111/bph.12534

Rivera, E. M., Cid, M. P., Zunino, P., Baiardi, G., & Salvatierra, N. A. (2014). Central α- and β-thujone: Similar anxiogenic-like effects and differential modulation on GABAA receptors in neonatal chicks. *Brain Research, 1555*(0), 28-35. doi:http://dx.doi.org/10.1016/j.brainres.2014.01.039

Sands, S. B., Costa, A. C., & Patrick, J. W. (1993). Barium permeability of neuronal nicotinic receptor alpha 7 expressed in Xenopus oocytes. *Biophysical Journal, 65*(6), 2614-2621. doi:http://dx.doi.org/10.1016/S0006-3495(93)81296-7

Seguela, P., Wadiche, J., Dineleymiller, K., Dani, J. A., & Patrick, J. W. (1993). MOLECULAR-CLONING, FUNCTIONAL-PROPERTIES, AND DISTRIBUTION OF RAT BRAIN-ALPHA-7 - A NICOTINIC CATION CHANNEL HIGHLY PERMEABLE TO CALCIUM. *Journal of Neuroscience, 13*(2), 596-604.

Siveen, K. S., & Kuttan, G. (2011). Augmentation of humoral and cell mediated immune responses by Thujone. *International Immunopharmacology, 11*(12), 1967-1975. doi:http://dx.doi.org/10.1016/j.intimp.2011.08.006

Suppiramaniam, V., Abdel-Rahman, E. A., Buabeid, M. A., & Parameshwaran, K. (2010). 13.09 - Ion Channels. In C. A. McQueen (Ed.), *Comprehensive Toxicology (Second Edition)* (pp. 129-171). Oxford: Elsevier.

Szczot, M., Czyzewska, M. M., Appendino, G., & Mozrzymas, J. W. (2012). Modulation of GABAergic Synaptic Currents and Current Responses by α-Thujone and Dihydroumbellulone. *Journal of Natural Products, 75*(4), 622-629. doi:10.1021/np200863q

Tobyn, G., Denham, A., & Whitelegg, M. (2011). CHAPTER 11 - Artemisia absinthium, wormwood *Medical Herbs* (pp. 105-121). Edinburgh: Churchill Livingstone.

Tsiri, D., Graikou, K., Poblocka-Olech, L., Krauze-Baranowska, M., Spyropoulos, C., & Chinou, I. (2009). Chemosystematic value of the essential oil composition of Thuja species cultivated in Poland-antimicrobial activity. *Molecules, 14*(11), 4707-4715. doi:10.3390/molecules14114707

Waidyanatha, S., Johnson, J. D., Hong, S. P., Robinson, V. G., Gibbs, S., Graves, S. W., . . . Smith, C. S. (2013). Toxicokinetics of alpha-thujone following intravenous and gavage administration of alpha-thujone or alpha- and beta-thujone mixture in male and female F344/N rats and B6C3F1 mice. *Toxicology and Applied Pharmacology, 271*(2), 216-228. doi:10.1016/j.taap.2013.05.001

Waidyanatha, S., Johnson, J. D., Hong, S. P., Robinson, V. G., Gibbs, S., Graves, S. W., . . . Smith, C. S. (2013). Toxicokinetics of α-thujone following intravenous and gavage administration of α-thujone or α- and β-thujone mixture in male and female F344/N rats and B6C3F1 mice. *Toxicology and Applied Pharmacology, 271*(2), 216-228. doi:http://dx.doi.org/10.1016/j.taap.2013.05.001

Weber, W.-M. (1999). Ion currents of *Xenopus laevis* oocytes: state of the art. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1421*(2), 213-233. doi:http://dx.doi.org/10.1016/S0005-2736(99)00135-2

Westfall, T. C. (2009). Cholinergic Neurotransmission in the Autonomic and Somatic Motor Nervous System. In L. R. Squire (Ed.), *Encyclopedia of Neuroscience* (pp. 827-834). Oxford: Academic Press.

Wu, J., Gao, M., & Taylor, D. H. (2014). Neuronal nicotinic acetylcholine receptors are important targets for alcohol reward and dependence. *Acta Pharmacologica Sinica, 35*(3), 311-315. doi:10.1038/aps.2013.181

Zhang, Q. H., Schneidmiller, R. G., & Hoover, D. R. (2013). Essential oils and their compositions as spatial repellents for pestiferous social wasps. *Pest Management Science, 69*(4), 542-552. doi:10.1002/ps.3411

Zwart, R., Strotton, M., Ching, J., Astles, P. C., & Sher, E. (2014). Unique pharmacology of heteromeric alpha 7 beta 2 nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *European Journal of Pharmacology, 726*, 77-86. doi:10.1016/j.ejphar.2014.01.031