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The effects of Capsaicin on the function of human α7 Nicotine Acetylcholine receptors expressed in Xenopus Oocytes.

Asma Hassan Ali Muftah Alzaabi

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The Effects of Capsaicin on the Function of Human α7 Nicotinic Acetylcholine Receptors Expressed in Xenopus Oocytes

Asma Hassan Ali Muftah Alzaabi

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

Under the Supervision of Professor Murat Oz

March 2018
Declaration of Original Work

I, Asma Hassan Ali Muftah Alzaabi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "The Effects of Capsaicin on the Function of Human α7 Nicotinic Acetylcholine Receptors Expressed in Xenopus Oocytes", hereby, solemnly declare that this thesis 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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: 16/5/12
Approval of the Master Thesis

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Signature [Signature] Date 5/16/2018

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Declaration of Original Work

I, Asma Hassan Ali Muftah Alzaabi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “The Effects of Capsaicin on the Function of Human α7 Nicotinic Acetylcholine Receptors Expressed in Xenopus Oocytes”, hereby, solemnly declare that this thesis 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 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: ________________
Abstract

Capsaicin is a naturally occurring alkaloid derived from chili pepper fruits. Capsaicin is a unique compound due to its ability to trigger a desensitization after the initial neuronal excitation in the nerve terminal expressing TRPV1 as well as generation of long-lasting nerve defunctionalization at sufficient dosing. Capsaicin has a beneficial role in the treatment of pain, cancer, obesity, diabetes, gastrointestinal, cardiovascular, dermatological conditions, and several other pathologies. In this study, we investigated the effect of capsaicin on the function of a cloned α7 subunit of human nicotinic acetylcholine receptor (α7-nAChR) expressed in Xenopus laevis oocytes using the two-electrode voltage clamp method. Capsaicin showed maximum potency of inhibition of ACh-induced currents with an IC50 value of 8.6 μM (with 50% inhibition). The mechanisms of capsaicin’s action on the α7-nAChR were further investigated and found to be independent of membrane potential. Furthermore, capsaicin (10 μM) did not affect the activity of Ca2+ dependent Cl− channels since the extent of inhibition by capsaicin was unaltered by the intracellularly injected Ca2+ chelator BAPTA and perfusion with Ca2+ free bathing solution containing 2 mM Ba2+. The effect of capsaicin was associated with decreased ACh efficacy, and the inhibition was not reversed by increasing ACh concentrations, suggesting a non-competitive inhibition of nicotinic receptors. Capsaicin not only had an inhibitory effect on α7-nAChRs but also on the other members of cys-loop family of ligand-gated ion channels including: α3β2, α4β4, α4β2, α1β1, and α3β4 nACh receptors, 5HT3 receptor, and glycine α1 and α3-receptor, while, it caused potentiation of glycine α2-receptor function. In conclusion, our results indicate that capsaicin inhibits the function of the α7-nACh receptor, and emphasizes the importance of α7-nACh receptor for future pharmacological/toxicological profiling.

Keywords: Capsaicin, Xenopus laevis oocytes, nicotinic acetylcholine receptors.
أثار الكابسيسن على صفات مستقبلات ألفا 7 الاستيتابول كولين النيكوتينية (Xenopus laevis) المستنسخة في بويضات ضفادع القيطم الأفريقي (Xenopus laevis) المفصل

الكابسيسن هو قلوي طبيعي يستخرج من فاكهة الفلفل الحار. الكابسيسن مركب فريد من نوعه بسبب قدرته على تحفيز إزالة الحساسية بعد الإثارة العصبية الأولية في محطة العصب التي تحتوي على مستقبلات فانيلويد 1 (TRPV1)، وكذلك تتبث في أفقاد وظيفة الأعصاب لمدة طويلة الأمد عندما يواجهه كابسيسن. الكابسيسن لها دور مفيد في علاج الألم والسرطان والشجاعة والسكري، الجهاز الهضمي، القلب والأوعية الدموية، والأمراض الجلدية، والعديد من الأمراض الأخرى. في هذه الدراسة، نحن نقوم بالتحقيق في صفات الكابسيسن على مستقبلات الاستيتابول كولين النيكوتينية المكونة من وحدات ألفا 7 المستنسخة في بويضات ضفادع القيطم الأفريقي (Xenopus laevis) باستخدام تقنية تثبيت الجهد الكهربائي باستخدام قطبين كهربائيين.

وقد ظهر أن الكابسيسن يثبط فاعلية التيار الأساسي بتلك النسبة 60% بعد إجراء تجارب إضافية للتحقق من صفات الكابسيسن على مستقبلات الاستيتابول كولين النيكوتينية المكونة من وحدات ألفا 7، تبين أن تأثير الكابسيسن لا يتمسك على جهد الغشاء الخلوي. و علاوة على ذلك، الكابسيسن لم يؤثر على قنوات الكالسيوم المعتمدة على الكالسيوم المتواجدة في غشاء البويضات حيث ان درجة تثبيت الكابسيسن لم تتغير في البويضات التي حققت بدارة الحساسة للكالسيوم BAPTA و عندما نضحت بحلول خالي من الكالسيوم. كما تبين ان تأثير الكابسيسن (10 ميكرومول) قلل من قوة الاستيتابول كولين ولم يكن يمكن ادراك التثبيت من خلال زيادة تراكيز الاستيتابول كولين، مما يدل على عدم وجود تناقص ما بين الكابسيسن واستيتابول كولين على المستقبلات النيكوتينية ألفا 7. وجدنا كذلك أن الكابسيسن ليس فقط لا تأثير مثبط على على المستقبلات النيكوتينية ألفا 7 ولكن يؤثر أيضا على وظيفة الأعضاء الأخرى من عائلة حلقة-سيس من القنوات النيكوتينية المعتمدة على وجود مؤثر (cys-loop family of ligand-gated ion channels). في الختام، نتائجنا أن الكابسيسن يثبط وظيفة المستقبلات النيكوتينية ألفا 7، و سوف نركز على الأهمية المستقبلية للمستقبلات النيكوتينية ألفا 7 من الناحية الدوائية و السمية.

مفهوم البحث الرئيسي: كابسيسن، ضفادع القيطم الأفريقي (Xenopus laevis) ، مستقبلات الاستيتابول كولين النيكوتينية
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Special thanks go to my husband, parents, family and friends who helped, encouraged, and supported me spiritually throughout the way, and who provided me constant faithful prayers. I am sure they suspected it was endless.
Dedication

To my beloved husband, parents, family and friends,
who have consistently supported me
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<th>Description</th>
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<tr>
<td>5-HT</td>
<td>Serotonin or 5-hydroxytryptamine</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Acetyl Coenzyme A</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AChEIs</td>
<td>Acetylcholinesterase inhibitors</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Akt or PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>CaCCs</td>
<td>Ca$^{2+}$-activated Cl$^{-}$ channels</td>
</tr>
<tr>
<td>CDKs</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Cys-loop LGICs</td>
<td>Cysteines-loop superfamily of ligand-gated ion channels</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptors</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase pathway</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IC/PBS</td>
<td>Interstitial cystitis/Painful bladder syndrome</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>IND</td>
<td>Indomethacin</td>
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<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LO</td>
<td>Lipoxygenases</td>
</tr>
<tr>
<td>LRP1</td>
<td>LDL-related protein 1</td>
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<tr>
<td>mACHR</td>
<td>Muscarinic acetylcholine receptors</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<td>MLA</td>
<td>Methyllycaconitine</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>nACHR</td>
<td>Nicotinic acetylcholine receptors</td>
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<td>NAR</td>
<td>Non-allergic rhinitis</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa b</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
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<td>NO</td>
<td>Nitric oxide</td>
</tr>
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<td>oxLDL</td>
<td>Oxidized low-density lipoprotein</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PKA</td>
<td>Protein kinase A</td>
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<td>PLA2</td>
<td>Phospholipase A2</td>
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<td>Abbreviation</td>
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<tr>
<td>PLCβ3</td>
<td>Phospholipase C-β3</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTX</td>
<td>Resiniferatoxin</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid 1</td>
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<tr>
<td>VAT</td>
<td>Vesicle-associated transporter</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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<tr>
<td>α-CBT</td>
<td>α-cobratoxin</td>
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<tr>
<td>α-BTX</td>
<td>α-bungarotoxin</td>
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<td>β-ARs</td>
<td>β-arrestin</td>
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Chapter 1: Introduction

1.1 Capsaicin

1.1.1 Natural Source and Historical Viewpoint

Capsaicin is the major pungent ingredient derived from the fruit of plants that belong to the *genus Capsicum* (Figure 1), family *Solanaceae* (Frias & Merighi, 2016). *Capsicum* is known by many names such red pepper, chili pepper, hot pepper, cayenne, paprika, and tabasco pepper (Barnes et al., 2007). Worldwide, there are more than 200 varieties belonging to more than 30 species of *genus Capsicum* (Reddy et al., 2016).

Chili pepper crops are an excellent source of high-value compounds, such as flavonoids and capsaicinoids, as well as ascorbic acid (vitamin C), tocopherols (vitamin E), and carotenoids (provitamin A), compounds with well-known antioxidant properties (Materska & Perucka, 2005). Capsaicin represents up to 1% of the total mass of the capsicum fruits (Fattori et al., 2016). Capsaicin is believed to be synthesized by the chili pepper plants during seed germination as a repellent substance for animals (Thiele et al., 2008). Rodents and other mammals are averted to eating large amounts of pepper due to its unpleasant taste which helps to increase the chances of germination as the pepper seeds could be harmed by the digestive system of mammals making them unable to germinate. On the other hand, birds cannot feel the unpleasant taste of chili peppers, and the pepper seeds are not harmed in their digestive tract, (Tewksbury et al., 2008). Moreover, capsaicin is used by the plant as a defense substance against the attacks of parasites, such as insects and mould. Humans have been using this crop for its property as an anti-infective agent against different diseases, and as a food preservative (Cichewicz & Thorpe, 1996; Veloso et al., 2014; Ziglio & Gonçalves, 2014).
Figure 1: Plant of the *genus Capsicum* [From shutterstock.com]

The history of the chili peppers started in America, and has become popular in both medicinal products and foods (Szolcsányi, 2014). Together with salt, they represent the most widely-consumed condiments throughout the world (Fattori et al., 2016) due to their unique pungency, color, and aroma (Arora et al., 2011). Besides its use as a food additive, it has been useful in a broad range of other applications such as ornamental plants, self-defense products (pepper spray), self-protectant lotions to ward off sharks, insect repellent, repelling squirrels, chemical weapons, and protecting crops from roving herds of elephants (chili-dung bombs) (Buck & Burks, 1986). In tropical areas, people consumed chili peppers to cope with the high temperatures by increasing the regulation of heat loss with capsicum-induced skin vasodilatation and “gustatory sweating” (Lee, 1954). Moreover, capsicum has a long history of medicinal use to treat cough, sore throat, tonsillitis, toothache, gastric ulcers, cramps, diarrhea, dyspepsia,
backache, cholera, water retention, gout, rheumatism, appetite stimulation, and hair growth restoration (Conway, 2008; Szallasi, 1995).

The history of the discovery of capsaicin dates back to the early decades of the nineteenth century when Christian Friedrich Bucholz first isolated the compound in impure crystalline form and named it ‘capsicin’ (Bucholz, 1816; Pertwee, 2014). In 1876, Thresh extracted this compound in almost pure form. He renamed it as ‘capsaicin’ (Thresh, 1876). Capsaicin was first obtained in pure form in 1898 by Micko (Micko, 1898), but its chemical structure was first described in 1919 by Nelson (Nelson, 1919). The structure (Figure 2), and the physical, chemical, and biological properties of capsaicin is now well defined. In 1930, Spath and Darling were the first scientists who reported the original synthesis of the compound (Späth & Darling, 1930). Since then, similar substances have been isolated from Capsicum genus and grouped as capsaicinoids (Kosuge, 1961).

1.1.2 Capsaicin Structure and Analogs

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) belongs to a group of chemicals known as vanilloids such as vanillin from vanilla, zingerone from ginger, and eugenol from bay leaves and cloves (Hayman & Kam, 2008; Kaiser et al., 2018). Capsaicin has similar functional groups as the other vanilloids. The structure of capsaicin consists of a benzene ring and a long hydrophobic carbon tail linked by a polar amide group (Figure 2) (Hayman & Kam, 2008). The pharmacological effects of capsaicin are associated with the exact combination of these functional groups (Rios & Olivo, 2014); the hydrophobic tail required for high potency; vanillyl group for pungency and adverse effects in clinical use; and the vanillyl and amide bond groups associated with maintaining sensory neurons excitation (Basith et al., 2016).
Capsaicin contains three important chemical regions; vanillyl group (methylcatechol) (the Head) and a fatty acid chain (the Tail) linked by a central amide group (the Neck). [From Yang & Zheng, 2017]

Capsaicin is a highly volatile, pungent, hydrophobic, colorless and odorless white crystalline powder with a molecular weight of 305.4 kDa., melting point of 62–65°C and molecular formula C\textsubscript{18}H\textsubscript{27}NO\textsubscript{3} (Gonzalez-Zamora et al., 2013; Hayman & Kam, 2008) Capsaicin is not water-soluble due to its nonpolar phenolic structure. Thus, nonpolar solvents such as dimethyl sulfoxide (DMSO), acetone, benzene, and ether are used to dissolve, extract, and maintain the properties of capsaicin. Ethanol can also dissolve capsaicin due to its mixed properties (can dissolve both polar and non-polar substances due the presence of hydroxyl group and ethyl group, respectively) (Fattori et al., 2016).

Several capsaicin-related compounds are produced by plants of the genus *Capsicum*. These analogs are grouped in a family called capsaicinoids (Figure 3). The main capsaicinoid is capsaicin (48.6%), followed by dihydrocapsaicin (36%), nordihydrocapsaicin (7.4%), homodihydrocapsaicin (2%), and homocapsaicin (2%) (Barnes et al., 2007). All capsaicinoids share structural and activity similarities with capsaicin (Fattori et al., 2016), but varying only in the degree of saturation (presence or absence of double bonds) in the alkyl side chain region, arborization, or length of the long aliphatic side chain (Rios & Olivo, 2014). Capsaicin and its analogs possess
analgesic, anti-inflammatory (Anand & Bley, 2011; Choi et al., 2011), anti-oxidant (Henderson et al., 1999), anti-cancer (Aggarwal & Shishodia, 2006), and anti-obesity properties (Janssens et al., 2014; Reinbach et al., 2009), plus benefits for cardiovascular (Yang et al., 2010) and gastrointestinal systems (Satyanarayana, 2006; Yeoh et al., 1995).

Besides capsaicinoids, there are other groups of compounds that show physiological functions similar to capsaicinoids such as capsinoids from bell peppers (CH-19 Sweet) (He et al., 2009). These molecules differ slightly in their structure and have an ester central linking group (Figure 3) (Appendino, 2007). Capsinoids are non-pungent, not stable in the aqueous environment (can be easily broken down) (Basith et al., 2016), and unlike capsaicinoids, do not produce unpleasant discomfort because of their higher lipophilicity or hydrolysis before reaching the nerve endings which desensitize the sensory neurons (Rios & Olivo, 2014). Capsinoids are less toxic than capsaicinoids (Luo et al., 2011), and possess very light analgesic properties (Rios & Olivo, 2014). Capsinoids possess anti-cancer, anti-oxidant and anti-obesity properties (Basith et al., 2016).
Figure 3: The general chemical structure of capsaicinoids and capsinoids

Both groups have a common vanillyl group but different central linkage (shown in red) and R-group. [Modified from Basith et al., 2016]
1.1.3 Capsaicin Pharmacology

1.1.3.1 Pharmacokinetics of Capsaicin

Capsaicin can be administered by various routes, but only a few have importance in humans including the topical route mainly in the form of creams and patches as well as perineural, intrarticular, or soft tissue injection (Giacalone et al., 2015). Indeed, capsaicin is a non-water-soluble compound, very lipophilic, and resists diffusion into the aqueous blood phase (Baranidharan & Bhaskar, 2015). Topically applied capsaicin is rapidly and effectively absorbed through the epidermal and dermal layers of the skin, and maximal cutaneous concentration is rapidly achieved, but shows limited potential for systemic absorption (Baranidharan & Bhaskar, 2015; Hayman & Kam, 2008). Cutaneous absorption depends on the duration and surface area of exposure (McCormack, 2010). The half-life of capsaicin is approximately 24 hours (Pershing et al., 2004).

Capsaicin is not suitable for systemic administration in pharmacological doses because of the side effects including nausea, vomiting, bronchospasm, hypertension, and hypothermia (Giacalone et al., 2015; Premkumar & Sikand, 2008). On the other hand, oral intake of capsaicin with food is safe, but it does not have pharmacologic effects (Giacalone et al., 2015). After oral administration, absorption of capsaicin occurs in the stomach, jejunum, and ileum by non-active processes (Kawada et al., 1984), where nearly 94% of orally administered capsaicin was absorbed and maximum blood concentration was attained 1hr after administration (Figure 4) (Suresh & Srinivasan, 2010). Moreover, a maximum distribution of 24.4% of administered capsaicin in blood, liver, kidney and intestine was attained in 1hr, which decreased notably until no intact capsaicin was detected after 4 days (Suresh & Srinivasan,
The human pharmacokinetic profile of oral administration of capsaicin was reported after ingestion of 5 g of *C. frutescens* (capsicum) (equipotent to 26.6 mg of pure capsaicin); the pharmacokinetic parameters were \( C_{\text{max}} = 2.47 \pm 0.13 \, \text{ng/ml}, \ t_{\text{max}} = 47.1 \pm 2.0 \, \text{min}, \) and \( t_{1/2} = 24.9 \pm 5.0 \, \text{min} \) (Figure 4) (Chaiyasit et al., 2009; O'Neill et al., 2012). In animals, capsaicin is distributed widely in unchanged form to the brain, spinal cord and liver after intravenous and subcutaneous administration (O'Neill et al., 2012).

Figure 4: Pharmacokinetics of capsaicin after oral administration in humans and rats [From O'Neill et al., 2012]

In the case of oral ingestion, capsaicin undergoes a rapid metabolism in the liver by cytochrome P450 enzymes giving rise to specific macrocyclic, alkyl
dehydrogenated, omega-, and omega-1-hydroxylated metabolites (Reilly & Yost, 2005). Three major metabolites have been identified for capsaicin: 16-hydroxycapsaicin, 17-hydroxycapsaicin and 16,17-dihydrocapsaicin, whereas vanillin is a minor metabolite (Chanda et al., 2008). Capsaicin and its metabolites are excreted mainly by the kidneys, with a small untransformed proportion excreted through the urinary and bile routes (Hayman & Kam, 2008). The elimination half-life of oral capsaicin is 24.9 min (Baranidharan & Bhaskar, 2015). In experiments in human skin, capsaicin undergoes slow biotransformation in the skin and only a small fraction is metabolized to vanillylamine and vanillyl acid as most capsaicin remains unchanged suggesting that cytochrome P450 enzymes participate minimally in their metabolism when given by this route (Chanda et al., 2008). Dermally applied capsaicin and its metabolites are mainly excreted by the kidney (O'Neill et al., 2012) with an elimination half-life of 1.64 hr (Baranidharan & Bhaskar, 2015).

1.1.3.2 Pharmacodynamics of Capsaicin

Capsaicin acts through the activation of transient receptor potential vanilloid 1 (TRPV1) (Cortright & Szallasi, 2004). This receptor is expressed in the plasma membrane of nerve fibers of nociceptive neurons, mainly the Aδ and C fibers (McCormack, 2010). TRPV1 is widely expressed in the brain, sensory nerves, dorsal root ganglia, blood vessels, gut and several other cell types (For reviews (Matsumoto et al., 2009; Zhu et al., 2011)). TRPV1 belongs to a ‘superfamily’ of channel-receptors called transient receptor potential (TRP). In mammals, TRP channels are divided into six subfamilies, based on their domain structure and details of their sequences (Caterina, 2015), including the TRPV (vanilloid; TRPV1–6), TRPC (canonical; TRPC1–7), TRPM (melastatin; TRPM1–8), TRPML (mucolipin; TRPML1–3), TRPA
(ankyrin; TRPA1), and TRPP (polycystin; TRPP2, TRPP3, TRPP5) subfamilies (Nilius & Owsianik, 2010; Nilius et al., 2007). TRP channels have a role in auditory, visual, taste, and pain signal transduction pathways. In addition, they are also involved in the regulation of cell survival and growth, cardiovascular function, body fluid balance, mineral absorption, and gut motility (Sun et al., 2016).

Figure 5: Structural and physiological function of TRPV1
(A) Activation of TRPV1 after topical application of capsaicin results in sensory neuronal depolarization leading to the sensations of heat, burning, stinging, or itching, (B) Schematic diagram showing the topology of a TRPV1 subunits (ARD: Ankyrin-repeat like domain). [Modified from Anand & Bley, 2011; Yang & Zheng, 2017]

TRPV1 is a widely studied TRPV subfamily member because of its significant role in the pain sensory system (Oh, 2006). Moreover, the channel has an important
role in physiological homeostasis (Sun et al., 2016). TRPV1 is a homo-tetrameric non-selective ligand-gated cationic channel that opens with exposure to agonists (McCormack, 2010). TRPV1 contains 838 amino acids and has a molecular weight of 95 kDa in humans (Arora et al., 2011). The channel consisting of both N and C termini of each subunit located intracellularly and six transmembrane helices per subunit (S1 to S6) which form the transmembrane core region (Figure 5) (Yang & Zheng, 2017). The channel is non-selectively activated by multiple physical and chemical stimuli such as heat (>43°C) (Caterina et al., 1997), divalent cations such as Mg²⁺ and Ba²⁺ (Ahern et al., 2005; Yang et al., 2014), inflammatory mediators (Ma & Quirion, 2007), and extracellular pH (<5.9) (Tominaga et al., 1998), as well as endogenous and exogenous vanilloids (Giacalone et al., 2015; Hayman & Kam, 2008). All these compounds are lipophilic and therefore act at intracellular binding sites (Yang et al., 2003).

The activation of TRPV1 by capsaicin leads to depolarization of the nociceptive neurons associated with Na⁺ and Ca²⁺ influx; although the TRPV1 channel is nonselective, it has been shown to have a high preference for Ca²⁺ (Caterina et al., 1997). Depolarization is associated with the firing of action potentials in nociceptive fibers which propagate into the spinal cord and brain, and are associated with burning, itching, or stinging sensations (Figure 5) (Anand & Bley, 2011). Activation of TRPV1 by capsaicin also results in the release of proinflammatory peptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) from the peripheral terminals of these afferent neurons, promoting vasodilation, vascular leakage, and inflammation (Kim-Katz et al., 2010). In contrast to transient activation which follows endogenous
chemicals, capsaicin has a more persistent effect on the TRPV1 channel (Anand & Bley, 2011; Baranidharan et al., 2013).

Binding of capsaicin to TRPV1 causes opening of the ion channel. However, with constant stimulation, the receptor becomes desensitized preventing permeability to ions (Desensitization state; ligand-bound state). Following desensitization, the channel should shift to the closed state, after which the channel is again ready to open (Basith et al., 2016). There are two types of desensitization: “classic pharmacological desensitization”, and “functional desensitization”. Pharmacological desensitization occurs due to prolonged repeated administrations of capsaicin which leads to a gradual decrease in the size of a subsequent response to that agonist. On the other hand, functional desensitization refers to a reduction or loss of responsiveness of sensory neurons to other stimuli, following short-term local application to nerve endings; functional desensitization results from a high concentration of capsaicin (Figure 6) (Baranidharan & Bhaskar, 2015).

The TRPV1 channel is highly permeable to calcium ions. The calcium:sodium permeability ratio is increased from a baseline of 8:1 to 25:1 during prolonged capsaicin exposures, which allows a massive influx of $\text{Ca}^{2+}$ down the electrochemical gradient into nerve fibers. In addition, as TRPV1 is also expressed on intracellular organelles such as the endoplasmic reticulum, capsaicin administration can cause a release of calcium from internal stores through calcium-dependent calcium release resulting in excessive intracellular calcium accumulation (Baranidharan & Bhaskar, 2015; Baranidharan et al., 2013). Consequently, sustained high levels of intracellular calcium causes activation of calcium-dependent protease enzymes leading to cytoskeletal breakdown. Depolymerization of cytoskeletal components such as
microtubules causes an interruption in fast axonal transport (Chard, et al., 1995; Han et al., 2007). Moreover, osmotic swelling is caused by chloride influx which accompanies the influx of positively charged ions (Baranidharan et al., 2013). Furthermore, at concentrations in excess of those required to activate TRPV1, capsaicin can cause a direct inhibition of electron transport chain resulting in disruption of mitochondrial respiration, and subsequent mitochondrial impairment (Shimomura et al., 1989). Thus, capsaicin ultimately induces the loss of cellular integrity, and nociceptor fiber ‘defunctionalization’ (Baranidharan & Bhaskar, 2015; Baranidharan et al., 2013), temporary loss of membrane potential, impaired cellular transport, and reversible retraction of nerve fiber endings (Anand & Bley, 2011). Subsequently, the release of all the neuropeptides including substance P (SP) from nociceptive nerve endings is decreased (Anand & Bley, 2011).

![Figure 6: Capsaicin induces defunctionalization by multiple mechanisms](image)

High concentrations of capsaicin or continuous low concentrations increase the levels of intracellular calcium in TRPV1-expressing sensory nerve fibers resulting in enzymatic, cytoskeletal, and osmotic changes, and the disruption of mitochondrial respiration leading to impaired local nociceptor function for extended periods. [From Anand & Bley, 2011]
Originally, SP depletion from nociceptive nerve terminals was thought to be responsible for the capsaicin-induced effect (analgesia), but this is no longer believed to be the primary mechanism (Anand & Bley, 2011; Rudin, 2013). In accord with these widely recognized effects, if high concentrations or repetitive low-concentration of capsaicin is administered, increased levels of intracellular calcium in TRPV1-expressing sensory nerve fibers and other subsequent processes leads to local nociceptor impairment for extended periods (Anand & Bley, 2011). Furthermore, capsaicin has several TRPV1-independent pharmacological effects which led to a debate on the possible existence of other receptor targets of capsaicin (Frias & Merighi, 2016; Sharma et al., 2013). Some of these effects have been discussed in the following section.

1.1.4 Therapeutic Potential

Capsaicin is unique among naturally occurring irritant compounds because of its ability to trigger a desensitization after the initial neuronal excitation in the nerve terminal expressing TRPV1 receptors as well as generation of long-lasting nerve defunctionalization. The major part of capsaicin pharmacology focuses on pain management (Basith et al., 2016). Furthermore, there is considerable evidence that TRPV1 activation by capsaicin have a beneficial role in the treatment of various cancers, obesity, diabetes, gastrointestinal, cardiovascular and dermatological conditions, and several other pathologies. Some of these roles have been reviewed in the following sections (summary shown in Figure 7).
1.1.4.1 Chronic Pain Conditions

Pain is one of the most common reasons for people to seek medical assistance (Curtiss & McKee, 2004), yet chronic pain is still considered a major treatment challenge for medical providers because of its vague etiology, complex history, and poor response to treatment (Basith et al., 2016). Examples of chronic painful disorders include neuropathic pain, musculoskeletal pain, inflammatory bowel disease (IBD), bone cancer pain, and migraine. The search for new and more effective analgesics to manage chronic pain is still ongoing (Basith et al., 2016). Topical capsaicin preparations have been used for many years to treat diverse pain disorders (Hayman & Kam, 2008). However, evidence for the effectiveness or safety of capsaicin use in the management of pain is still controversial (Bode & Dong, 2011). Capsaicin has both pronociceptive and antinociceptive effects (Basith et al., 2016) e.g. topical application of capsaicin enhanced sensitivity of to the TRPV1 channel to noxious stimuli, followed by a period with reduced sensitivity and, after repeated exposure to capsaicin,
persistent desensitization (Bode & Dong, 2011; Kim-Katz et al., 2010). This desensitization is the reason for using topical capsaicin for the management of chronic pain (Kim-Katz et al., 2010). Capsaicin has been used in several clinical settings as a topical medication to treat pain derived from different conditions such as rheumatoid arthritis, osteoarthritis, neuralgias, diabetic neuropathy, and other conditions including neural dysfunction (detrusor hyperreflexia, reflex sympathetic dystrophy, and rhinopathy), and painful or itching cutaneous disorders resulting from surgery, injury, or tumors such as postmastectomy pain syndrome, amputation stump pain, and skin tumor (Hautkappe et al., 1998).

Currently, over-the-counter creams are available with capsaicin content of 0.025%, 0.075%, or 0.1%, and approved for muscle and joint pain, arthritis, and neuralgia (Groninger & Schisler, 2012; Rudin, 2013). Creams are applied by patients or caregivers three to five times per day over two to six weeks; this therapy results in a variable degree of effectiveness (Giacalone et al., 2015). The applications may be painful and provoke erythema and uncomfortable burning, stinging, or itching (Groninger & Schisler, 2012), and thus patients may become non-compliant with this treatment (Giacalone et al., 2015). Over repeated applications, these unpleasant sensations decrease; due to progressive neuronal defunctionalization (Groninger & Schisler, 2012). Capsaicin is also available in the form of a high-dose (8%) capsaicin patch to be administered for 60 minutes by a medical professional in a hospital or specialized facility (Groninger & Schisler, 2012; Rudin, 2013). An 8% capsaicin patch was reported to be effective and safe in the management of neuropathic pain resulting from several conditions, with clear improvement in pain attacks, sleep duration and quality of life (Frias & Merighi, 2016). As the application of capsaicin may initially
evoke pain, the area is pretreated with lidocaine cream to anesthetize the skin (Rudin, 2013). The pain relieving effect usually has a long duration (up to several weeks) (Giacalone et al., 2015).

1.1.4.2 Cancer

Despite several advances in therapies, cancer is still one of the leading causes of morbidity and mortality worldwide (Fattori et al., 2016). In the past decades, the anticancer activity of capsaicin has been evaluated. The proposed anticancer mechanisms of capsaicin include decrease in cancer cell growth, increase in cell-cycle arrest, and programmed cell death in a variety of cancer types, but the exact cellular mechanisms are still not fully understood (Basith et al., 2016; Clark & Lee, 2016).

Apoptosis serves as a natural barrier against cancer development and progression, and the loss of apoptotic signaling is highly related to malignancy (Hanahan & Weinberg, 2011). Various cancer types disrupt apoptotic pathways and enhance anti-apoptotic ones which make cancer cells resistant to apoptosis (Clark & Lee, 2016). Capsaicin has been recognized to induce apoptosis in a wide variety of tumor and cancer cell lines, including colon adenocarcinoma (Kim et al., 2007), pancreatic cancer (Pramanik et al., 2011), hepatocellular carcinoma (Lee et al., 2004), prostate cancer (Mori et al., 2006), breast cancer (Chou et al., 2009), and many others, while leaving normal cells unharmed (Bley et al., 2012). Recent reviews noted that capsaicin can induce apoptosis in over 40 different types of cancer cell lines (Bley et al., 2012; Clark & Lee, 2016). The mechanism that underlies capsaicin-induced apoptosis in cancer cells is not fully understood but includes several intracellular mechanisms (see Figure 8) such as increase in intracellular Ca$^{2+}$, generation of reactive oxygen species (ROS), and disruption of mitochondrial membrane transition potential,
as well as pathways including AMP-dependent kinase and autophagy (Díaz-Laviada & Rodríguez-Henche, 2014).

Figure 8: Scheme of the main signaling pathways involved in capsaicin-induced apoptosis in cancer cells [Modified from Díaz-Laviada & Rodríguez-Henche, 2014]

Capsaicin mediates apoptosis via activation of TRPVs (mainly via TRPV1 in many types of cancers) resulting in Ca\(^{2+}\)-mediated mitochondrial damage and cytochrome c release (Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016). In most cancer cell types, the increase in intracellular Ca\(^{2+}\) is secondary to ER stress-dependent responses rather than a massive Ca\(^{2+}\) entry by a Ca\(^{2+}\)-permeable channel (Díaz-Laviada & Rodríguez-Henche, 2014). Moreover, studies performed in pancreatic cancer cells have shown that capsaicin induced apoptosis was associated with generation of ROS, persistent disruption of mitochondrial membrane potential,
release of cytochrome c in the cytosol and activation of caspase-3 cascade; suggesting that capsaicin can activate apoptosis via non-receptor mechanisms (Zhang et al., 2008).

Cell proliferation is the process whereby cells reproduce themselves by growing and then dividing into two equal copies via the cell cycle, which is divided into G0/G1, S and G2/M phases which has DNA checkpoints to ensure the integrity of DNA replication (Berridge, 2014; Machida & Dutta, 2005). The checkpoint proteins and repair pathways, together coordinate cell cycle progression and facilitate cellular responses to DNA damage (Zannini et al., 2014). Essential parts involved in the cell-cycle machinery are the cyclins, cyclin-dependent kinases (CDKs) and the CDK inhibitors. The CDKs provide a driving force for the cells to move from one phase to the next but if CDK activity is inhibited, cell cycle arrest occurs (Clark & Lee, 2016). The CDKs and cyclins are highly activated in most cancer types, and are therefore, considered as targets for cancer therapy to prevent uncontrolled proliferation of cancer cells. Capsaicin was found to induce G0/G1 phase arrest in human esophageal carcinoma cells with an increase of p21 and p53 which are inhibitors of Cdk2 and cyclin E complex (Wu et al., 2006). Moreover, Chen et al. found that capsaicin induced cycle arrest and inhibition of the proliferation of bladder carcinoma cells (5637 cell line) by inhibition of Cdk2, Cdk4 and Cdk6 (Chen et al., 2012).

1.1.4.3 Obesity

Obesity is an escalating public health challenge globally (Chan & Woo, 2010; Vaidya et al., 2010). Obesity is a chronic disease where individual gains too much body fat which could have a harmful impact on their health. The individual is considered obese if the body weight is 20% more than normal (Basith et al., 2016). In the last decade, several laboratory and clinical studies support the therapeutic role of
capsaicin as an anti-obesity agent (Basith et al., 2016; Leung, 2014). Several human and animal studies have shown that capsaicin and its TRPV1 receptor are involved in energy expenditure providing a potential strategy to treat obesity (Janssens et al., 2013; Sun et al., 2016). Yet, the underlying mechanism of action is not presently fully understood. The proposed mechanisms for the anti-obesity effects of capsaicin are: (1) increasing lipid oxidation and prevention of adipogenesis; (2) activation of brown adipose tissue (BAT - burns fat into energy), browning of white adipose tissue (WAT - stores energy as fat) and induction of thermogenesis; (3) suppression of appetite and increasing satiety and fullness regulated by neuronal circuits in the hypothalamus; (4) modulation of gastrointestinal tract function and gut microbiome (see Figure 9) (Zheng et al., 2017). Lee et al. found that after topical application of 0.075% capsaicin cream to the skin of male mice fed a high-fat diet for 8 weeks, there was a significant reduction in weight gained and the mesenteric adipose tissue weighed less than that of the control obese mice (Lee et al., 2013). The serum levels of fasting glucose, cholesterol, and triglycerides were also lower in the capsaicin-treated mice (Lee et al., 2013). Moreover, very recent studies by Baskaran et al. showed that activation of TRPV1 channels by dietary capsaicin triggered browning of WAT to overcome obesity (Baskaran et al., 2016). Additionally, Shen et al. demonstrated that anti-obesity effect of capsaicin in mice fed with high-fat diet was associated with a modest modulation of gut microbiome and an increase in the population of gut bacterium Akkermansia muciniphila (Shen et al., 2017).
Diabetes mellitus (DM) has emerged as one of the most important public health challenges (Chen et al., 2011). The pathophysiology of type 2 diabetes mellitus is multifactorial and complex, involving oxidative stress, endoplasmic reticulum stress, and inflammation which facilitates insulin resistance and β-cell dysfunction (Sun et al., 2016). Activation of TRPV1 alleviates insulin resistance and attenuates abnormal glucose homeostasis by suppressing inflammation (Sun et al., 2016). Dietary intake and supplementation with capsaicin has beneficial effects on glucose and insulin homeostasis in humans (Ahuja et al., 2006b; Chaiyasit et al., 2009; Yuan et al., 2016). Crossover studies showed that the postprandial hyperinsulinemia was attenuated after regular consumption of capsaicin-containing chili in healthy adults (Ahuja et al., 2006b), and capsaicin supplementation was associated with a decrease in plasma...
glucose levels and maintenance of insulin levels shortly after oral administration in an oral glucose tolerance test (Chaiyasit et al., 2009).

Animal studies have also shown that capsaicin administration has beneficial effects on glucose and insulin homeostasis. Dietary capsaicin was found to reduce obesity-induced glucose intolerance by markedly reducing adipose tissue macrophages and levels of inflammatory adipocytokines, including tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, and leptin, and normalizing fasting glucose levels in obese mice (Kang et al., 2010). Obesity-related inflammatory proteins can affect insulin sensitivity by blocking insulin signaling pathways (Fattori et al., 2016; Makki et al., 2013; Xu et al., 2003); therefore, capsaicin may decrease glucose tolerance by suppressing inflammatory protein production in obese mice (Fattori et al., 2016). Another study showed that dietary capsaicin consumption enhanced insulin sensitivity during hyperglycemic states in diabetic rats (Kwon et al., 2013). Capsaicin treatment was found to reduce body weight gain, visceral fat accumulation, and serum leptin levels, and improved glucose tolerance, as well as, protect β-cell mass by increasing proliferation and decreasing apoptosis (Kwon et al., 2013). Moreover, capsaicin supplementation ameliorated abnormal glucose homeostasis and increased antihyperglycemic hormone glucagon-like peptide-1 (GLP-1) levels in the plasma and ileum of wild type but not TRPV1-/- mice. Chronic capsaicin supplementation improved glucose tolerance, insulin levels, daily blood glucose profiles and increased plasma GLP-1 levels (Wang et al., 2012). Taken together, these beneficial effects of dietary capsaicin may represent a promising intervention for individuals at high risk of diabetes.
1.1.4.5 Gastrointestinal Diseases

Afferent neurons are responsible for maintenance of gastric mucosal integrity (Holzer, 1998). The arterial wall in the stomach receives a dense supply of TRPV1 bearing nerve fibers suggesting that the TRPV1 may be involved in gastric protection and inflammation (Hayman & Kam, 2008; Mózsik et al., 2005). Capsaicin has a dual effect in the prevention and aggravation of gastric mucosal injury depending on the applied doses (Ares & Outt, 1998; Mózsik, 2014). There are four different stages of capsaicin action depending on the dose and duration of the exposure to the compound: Stage 1 (Excitation) and Stage 2 (Sensory blocking effect) are reversible, followed by Stage 3 (Long-term selective neurotoxin impairment) and Stage 4 (Irreversible cell destruction) which are irreversible compound-induced actions on capsaicin sensitive afferent nerves. These different stages of capsaicin-induced actions can be detected in the gastrointestinal tract in animal experiments (Mózsik et al., 2001; Mózsik, 2014). The gastroprotective mechanism of capsaicin is due to the activation of TRPV1 in the foregut which results in the production of gastric hyperemia and enhanced mucus and HCO\textsubscript{3}\textsuperscript{-} secretion (Premkumar & Sikand, 2008). In a prospective study of 84 young healthy volunteers, it was shown that capsaicin has gastroprotective properties against gastric injuries induced by ethanol or indomethacin (IND) (Mózsik et al., 2005). It was suggested that the mucosal protective effect induced by capsaicin was due to acute stimulatory effects resulting in mucosal hyperemia rather than the desensitizing effects (Mózsik et al., 2005). The vasodilation and increased mucosal blood flow caused by capsaicin has been suggested to be mediated by nitric oxide (NO) and CGRP (vasoactive peptide) released by TRPV1 at gastric sensory neurons (Hayman & Kam, 2008; Mózsik et al., 2005).
1.1.4.6 Cardiovascular Diseases

Capsaicin-sensitive sensory nerves (rich in TRPV1) are densely distributed in the cardiovascular system which seems to regulate a series of complex cellular events contributing to physiological and pathological myocardial function (Peng & Li, 2010). Activation of TRPV1 has been repeatedly reported to exert hypotensive activity or cardioprotective effects (Bone & Mills, 2013). Various studies have shown the beneficial effects of capsaicin on cardiovascular diseases such as atherosclerosis, angina, hypertension, and cardiac hypertrophy (McCarty et al., 2015).

Low-density lipoprotein (LDL) oxidation is associated with the genesis and progression of atherosclerosis (Ahuja et al., 2006a; Mitra et al., 2011; Stocker & Keaney, 2004). *In vitro* studies showed that capsaicin increases the resistance of LDL to oxidation by delaying the initiation and/or slowing down the rate of oxidation (Ahuja et al., 2006a). Moreover, chronic activation of TRPV1 by capsaicin significantly reduces lipid storage and atherosclerotic lesions in aortic sinus and thoracoabdominal aorta of mice. Additionally, activation of TRPV1 by capsaicin impedes foam cell formation through autophagy induction (via AMP-activated protein kinase (AMPK) signaling pathway) (Figure 10) in oxidized low-density lipoprotein (oxLDL)-treated vascular smooth muscle cells (VSMCs) and ultimately slows down the process of atherosclerosis (Li et al., 2014).

Capsaicin has been shown to exhibit anti-aggregating effects on platelets (Adams et al., 2009; Harper et al., 2009; Mittelstadt et al., 2012) which may also provide protection against cardiovascular diseases such as atherosclerosis (Harper et al., 2009). This effect on platelets is attributed to the ability of capsaicin to pass through plasma membrane of platelets and alter membrane fluidity (Meddings et al., 1991).
Moreover, Harper et al. revealed that TRPV1 is present in human platelets. However, they found that TRPV1 involved in pro-aggregating effect of capsaicin, and plays a role in capsaicin-evoked Ca\(^{2+}\) signal generation and serotonin release; Ca\(^{2+}\) release from intracellular platelet stores and consequently result in adenosine diphosphate (ADP)- and thrombin-induced platelet activation (Harper et al., 2009). Therefore, further investigation is needed to clarify the mechanisms involved in the anti-hemostatic properties of capsaicin.

Figure 10: Schematic diagram showing the autophagy in VSMC foam cell formation

Activation of TRPV1 by capsaicin rescued the autophagy impaired by oxLDL via activating AMPK signaling pathway, and subsequently inhibiting the formation of foam cells. [From Li et al., 2014]

The cardiovascular system is rich in capsaicin-sensitive sensory nerves that help in the regulation of cardiovascular function (Peng & Li, 2010). Activation of TRPV1 by capsaicin has been suggested to exert an anti-hypertensive effect by stimulating the release of NO from endothelial cells and CGRP from capsaicin-sensitive sensory nerves (Sun et al., 2016; Yang et al., 2010). CGRP is a very potent
vasodilator neuropeptide and plays an important role in the initiation, progression and maintenance of blood pressure under both physiological and pathophysiological conditions (Deng & Li, 2005). Studies have shown that capsaicin stimulates the release of CGRP and subsequent decrease in blood pressure through the activation of TRPV1 (Peng & Li, 2010; Zhou et al., 1999). Yang et al. reported that chronic activation of endothelial TRPV1 by dietary capsaicin mediates an increased $\mathrm{Ca}^{2+}$ influx and subsequent phosphorylation of cAMP-dependent protein kinase (protein kinase A (PKA)) and endothelial nitric oxide synthase (eNOS), and therefore, increases in NO production which accounts for the potentiation of the endothelium-dependent relaxation and regulation of blood pressure in genetically hypertensive rats (Yang et al., 2010).

### 1.1.4.7 Urological Disorders

Capsaicin-sensitive sensory nerves have also been identified in the bladder and urethra (Andersson, 2011). Many studies have shown that capsaicin-sensitive nerves are involved in different urological diseases/disorders, including neurogenic and idiopathic detrusor overactive bladder and interstitial cystitis/painful bladder syndrome (IC/PBS) (Andersson, 2011; Foster Jr & Lake, 2014).

Capsaicin has been studied as an alternative therapy for the relief of the symptoms of neurogenic bladder, a urological disorder that interferes with the normal functioning of the lower urinary tract and seriously affects the quality of life of patients (Foster Jr & Lake, 2014). Neurogenic bladder is commonly associated with neurological disorders such as spinal cord injury (SCI), multiple sclerosis (MS), cerebrovascular accident and other neurological pathologies (Foster Jr & Lake, 2014). Spinal cord injury may result in increasing levels of nerve growth factor (NGF) and
subsequent upregulation of TRPV1 in urothelial cells and C-fibers suggesting that activation of these receptors may be associated with the resulting detrusor overactivity (Dorsher & McIntosh, 2012; Hayman & Kam, 2008). Intravesical instillation of vanilloid solutions, such as capsaicin or resiniferatoxin (RTX; an ultrapotent capsaicin analog), has been shown to increase bladder capacity and reduce urge urinary incontinence in patients with neurogenic and non-neurogenic detrusor hyper-reactivity, e.g. in case of detrusor hyperreflexia due to spinal cord lesions (Andersson, 2017; Hayman & Kam, 2008). These drugs reduce detrusor overactivity by reducing uptake of NGF through sensory neurons, and thereby reducing TRPV1 over-expression (Andersson, 2017).

1.1.4.8 Airway Diseases

TRPV1 channels are widely expressed in human nasal epithelium and neuronal tissue, and have a role as a sensor of inhaled irritants (Singh & Bernstein, 2014). Hypersensitivity of these channels may be responsible for inducing non-allergic rhinitis (NAR) symptoms (Singh & Bernstein, 2014). Stimulation of type C nociceptive sensory nerves (hypersensitive TRP ion channels) by nonspecific inhaled irritants result in the release of neuropeptides, such as SP and CGRP, from the nerve endings via an axonal response system as an immediate protective mucosal defense mechanism against these noxious irritants (antidromic reflex). Subsequently, these cause excessive nasal glandular secretions resulting in mucosal edema. Moreover, activation of TRP channels can also cause an increase in blood flow to the cerebral circulation resulting in other symptoms, such as headaches (frequent complaints of NAR patients) (Singh & Bernstein, 2014).
Capsaicin has been shown to hyposensitize the sensory neurons responsible for relaying pain signals from the nasal cavity. This results in significant reduction of neurogenic pain signals (Singh & Bernstein, 2014). Additionally, capsaicin causes ablation in TRPV1-SP nociceptive signaling pathways in the nasal mucosa and therefore relieve chronic painful sinus conditions; as SP may dilate nasal blood vessels and enhance secretions from mucosal membranes (Singh & Bernstein, 2014; Van Gerven et al., 2014). Capsaicin can also decrease mucosal permeability without compromising the nasal mucosal epithelial integrity (Jeon et al., 1995). Surgical denervation of sensory or autonomic nerves and intranasal capsaicin treatment has been found to be successful in reducing the symptoms of NAR, including glandular secretions and mucosal permeability (Baraniuk, 1992; Norlander et al., 1996; Singh & Bernstein, 2014). Studies showed that intranasal capsaicin is safe, and can cause significant and long-term reductions in visual analog scale scores for overall nasal symptoms, rhinorrhea, and nasal blockage (Van Wijk et al., 2000; Van Rijswijk et al., 2003).

1.1.4.9 Dermatological Disorders

Several studies suggest a possible link between histamine-related itch and TRPV1. A previous study showed that histamine excited the TRPV1 expressed on sensory neurons both in vivo and in vitro via downstream stimulation of phospholipase A2 (PLA2) and lipooxygenases (LO) suggesting that the TRPV1-expressing neurons may act as the main sensors and mediators of itch (Figure 11) (Shim et al., 2007). Additionally, Imamachi et al. found that phospholipase C-β3 (PLCβ3) is a critical intracellular mediator in C-fiber nociceptors that link the histamine H1 receptor to histamine-induced itch (Imamachi et al., 2009). In summary, both the PLCβ/PKC
pathway and the PLA2/lipoxygenase (LO) pathway have been suggested to link the Gq coupled histamine receptor and TRPV1 (Figure 11) (Sun & Dong, 2016).

Figure 11: Schematic diagram showing signal transductions of histamine itch [Modified from Sun & Dong, 2016]

Topical capsaicin has been used in treating several dermatological conditions including chronic, localized pruritic disorders, particularly those of neuropathic origin, such as aquagenic pruritus, brachioradial pruritus, notalgia paresthetica, prurigo nodularis, postherpetic neuralgia, and pruritus associated with chronic kidney disease (Boyd et al., 2014). The antipruritic effects of topical capsaicin application are thought to be achieved via the defunctionalization of TRPV1-expressing primary afferents, induced by direct desensitization of the channel in the short term, and retraction of nerve fiber endings due to excitotoxic terminal damage mediated by excessive Ca\(^{2+}\),
and inhibition of mitochondrial respiration in the long term (Wilson & Bautista, 2014). Furthermore, immunohistochemical studies using antibodies to nerve terminal proteins reported that the application of capsaicin induces localized loss of nociceptive nerve fiber terminals in the epidermis and dermis (Polydefkis et al., 2004; Wilson & Bautista, 2014).

1.1.5 Toxicity of Capsaicin

Over the years, concerns have been raised over the safety of capsaicin. Although the benefits appear greater than its risks, it is necessary to evaluate the therapeutic limitation of its use (Basith et al., 2016). Capsaicin-containing products such as creams and patches have been used in the clinical setting for many years in treating a variety of painful conditions. Nevertheless, their effectiveness as an analgesic is still highly debated, and some adverse side effects have been reported (Bode & Dong, 2011). Common adverse effects noted in clinical studies, which are associated with the topical application of capsaicin, appear to be small, transient, and application-site reactions; including pain, skin erythema, and papules, as well as pain-related transient increases in blood pressure, and airway irritation and coughing due to accidental inhalation of capsaicin (FDA, 2009). Unfortunately, capsaicin is a potent skin irritant even at low concentrations (Basith et al., 2016). Therefore, it has been observed that people with direct, continuous and prolonged exposure to chili peppers develops a painful contact dermatitis condition known as ‘Hunan Hand Syndrome’ (Basith et al., 2016; Williams et al., 1995). Moreover, coronary vasospasm and subsequent acute myocardial infarction were also reported with the use of the capsaicin patch to relieve lower back pain (Akçay et al., 2009). Eye exposure to capsaicin results in intense tearing, pain, conjunctivitis and blepharospasm (Hayman
Systemic application of capsaicin caused a marked reduction in the body temperature (hypothermic effect) due to a coordinated heat loss response along with vasodilatation, salivation, and fall in metabolic rate at cool ambient temperature (Szolcsanyi, 2004). Additionally, when large quantities of capsaicin are ingested, it can trigger abdominal pain, burning diarrhea, nausea and vomiting (Hayman & Kam, 2008). Because of its poor tolerance, clinical studies have shown that up to 30% of patients withhold capsaicin treatment (Papoiu & Yosipovitch, 2010).
1.2 Acetylcholine and Cholinergic Pathways

1.2.1 Historical Viewpoint

Acetylcholine (ACh) was the first identified neurotransmitter (Ettinger, 2017; Leroy, 2003). In 1914, ACh was discovered in ergot-containing plant material. Henry Hallett Dale found that this ACh induces the same effect as that of the stimulation of parasympathetic nerves when applied to peripheral tissues (Fisher & Wonnacott, 2012). Later, ACh was confirmed as a neurotransmitter by Otto Loewi, who originally termed it as ‘vagusstoff’ (vagus stuff) due to its ability to mimic the electrical stimulation of the vagus nerve (Gonzalez et al., 2016). Both scientists received the 1936 Nobel Prize in Physiology or Medicine for isolating ACh and for their discoveries relating to chemical transmission of nerve impulses (Leroy, 2003).

1.2.2 Synthesis, Storage and Release of Acetylcholine

ACh is a principal neurotransmitter in various levels of the nervous system (Gonzalez et al., 2016). The availability of ACh for cholinergic transmission consists of an extremely coordinated process of ACh biosynthesis, storage in vesicles, release, hydrolysis and reuptake into the presynaptic nerve terminal. Within the cholinergic presynaptic terminal, ACh is synthesized in a one-step reaction from acetyl-CoA and choline catalyzed by enzyme choline acetyltransferase (ChAT) (Figure 12). Even though the reaction is reversible, the equilibrium is strongly shifted toward the formation of ACh (Fisher & Wonnacott, 2012).
The two substrates utilized by ChAT are acetyl-CoA (Acetyl Coenzyme A) and choline. Acetyl-CoA is synthesized via the pyruvate dehydrogenase complex in mitochondria which are present in large numbers in the nerve endings (Cooper et al., 2003; Katzung, 2012). Acetyl-CoA is translocated from the mitochondria into the cytoplasm through an unknown mechanism (Fisher & Wonnacott, 2012). In contrast, choline is present in plasma at a high concentration (about 10 mM) (Augustine, 2004) and is derived from different sources; endogenous synthesis (principally in liver) (Gonzalez et al., 2016), dietary sources (red meat, eggs, poultry, and milk, etc.) (Gonzalez et al., 2016; Yonemori et al., 2013), and membrane phospholipids (stored as phosphorylcholine) (Atri et al., 2017). Choline does not readily cross the blood-brain barrier; it appears that the major source of choline for ACh synthesis is generated by acetylcholinesterase (AChE)-mediated hydrolysis of ACh in the synaptic cleft (Atri et al., 2017; Fisher & Wonnacott, 2012). Choline is taken up from the extracellular fluid into nerve terminal by a high-affinity Na\(^+\)/choline transporter (Atri et al., 2017; Augustine, 2004; Katzung, 2012) which is dependent on both Na\(^+\) and Cl\(^-\), and can be inhibited by hemicholinium (Cooper et al., 2003; Fisher & Wonnacott, 2012). Transport of choline into the neuron is the rate-limiting step in the production of ACh (Ducis, 1988; Radhakrishnan, 2015).
Once synthesized, ACh is transported from the cytoplasm into the synaptic vesicles by a specific vesicle-associated transporter (VAT; antiporter) that couples ACh entry into the vesicle with proton efflux (Figure 13). This antiporter can be selectively inhibited by the research drug vesamicol (Fisher & Wonnacott, 2012; Katzung, 2012). These vesicles not only contain ACh but also proteoglycan as well as adenosine triphosphate (Radhakrishnan, 2015). Upon axon terminal depolarization by
an action potential and the opening of voltage-dependent calcium channels, Ca\(^{2+}\) enters the presynaptic terminal and facilitates the fusion of the vesicle membrane with the plasma membrane, and subsequently the release of ACh into the synaptic cleft via exocytosis. This process can be inhibited by botulinum neurotoxins (Fisher & Wonnacott, 2012; Katzung, 2012; Radhakrishnan, 2015). ACh diffuses into the synaptic cleft, and can bind to postsynaptic receptors on the target cell, to presynaptic receptors on the membrane of the neuron which released the ACh, or to other targeted presynaptic receptors (Figure 13) (Radhakrishnan, 2015). There are two classes of receptors that bind ACh; nicotinic acetylcholine receptors (nAChR) which are ligand-gated ion channels, and muscarinic acetylcholine receptors (mAChR) which are seven transmembrane helix G-protein coupled receptors (GPCRs) (Atri et al., 2017). Finally, the neurotransmitter is rapidly eliminated from the CNS and peripheral synapses by acetylcholinesterase (AChE) through enzymatic hydrolysis into choline and acetate (Fisher & Wonnacott, 2012). This process helps to regulate the amount of ACh available in the synaptic cleft, and to rapidly terminate its effects on receiving neurons (Ettinger, 2017). Choline produced by ACh hydrolysis can be taken back into the presynaptic neurons by a sodium coupled, high-affinity uptake system, and used to resynthesize ACh (Augustine, 2004; Radhakrishnan, 2015).

1.2.3 Cholinergic Distribution

Cholinergic neurons are widely distributed throughout the CNS (Fisher & Wonnacott, 2012; Strominger et al., 2012). The major central cholinergic pathways are shown in Figure 14. They include:

1) Projection neurons of the basal forebrain located in the medial septal nucleus and the vertical limb of the diagonal band of Broca sending axons to the limbic cortex and
hippocampus; in the horizontal nucleus of the diagonal band and the magnocellular preoptic area whose axons terminate in the amygdala, olfactory bulb and limbic cortex; and in the nucleus basalis of Meynert that project to all parts of the neocortex (Fisher & Wonnacott, 2012) which are involved in cortical activation and memory function, particularly consolidation of short-term memory. They often appear to be damaged in patients with Alzheimer’s disease (AD) (Felten et al., 2015).

2) Projection neurons of pedunculopontine tegmental nucleus and the dorsolateral tegmental nucleus to different areas of the CNS, mainly to thalamus (Fisher & Wonnacott, 2012) which are involved in modulation of cortical arousal and the sleep-wake cycle, and in the initiation of REM sleep (Felten et al., 2015).

3) Cholinergic interneurons located in the striatum which may participate in basal ganglia function, and are of importance in controlling tone, posture, and initiation of movement or selection of wanted patterns of activity. These cholinergic neurons can become overactive in Parkinson’s disease (Felten et al., 2015; Fisher & Wonnacott, 2012).

In addition to above functions, ACh is used as a key neurotransmitter in autonomic neurons and lower motor neurons in the spinal cord and brain stem (see Figure 15) (Felten et al., 2015). In the autonomic nervous system (ANS), it is the neurotransmitter of the preganglionic sympathetic and parasympathetic neurons, of the adrenal medulla, of all the parasympathetic postganglionic innervated organs, and sympathetic postganglionic fibers innervating sweat glands and piloereector muscle. In the peripheral nervous system (PNS), ACh is the neurotransmitter at the neuromuscular junction between motor nerves and skeletal muscle (Gonzalez et al., 2016).
Figure 14: Cholinergic pathways in the human brain

The major concentration of cholinergic cells located in the basal forebrain group (red) and in the brainstem tegmental group (blue). Septal nuclei (SE); diagonal band of Broca (DBB); basal nucleus of Meynert (M); lateral dorsal tegmental nucleus (Ldt); pedunculopontine nucleus (PPn). [From Strominger et al., 2012]

<table>
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<tr>
<th>CNS</th>
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<td>Motor Neurone</td>
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<td>Para symp.</td>
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<td>Sympathetic</td>
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<td>Autonomic</td>
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Figure 15: The general outlay of the neurotransmitters released, types of receptors, and types of neurons within the somatic and autonomic nervous systems

ACh = Acetylcholine, NA = Noradrenaline, N = Nicotinic, M = Muscarinic, α = α-adrenergic, β = β-adrenergic [From Tripathi, 2013]
The nicotinic acetylcholine receptor (nAChR) belongs to the cysteines-loop superfamily of ligand-gated ion channels (cys-loop LGICs), which also includes the inhibitory GABA$_A$ and glycine receptors as well as the excitatory 5-HT$_3$ receptors (Corradi & Bouzat, 2016; Fisher & Wonnacott, 2012; Millar & Gotti, 2009; Wouden & Miller, 2012). Structurally, the cys-loop LGICs members comprise five homologous subunits (Sine & Engel, 2006; Wouden & Miller, 2012), each of which contains a conserved pair of disulfide-bonded cysteines separated by 13 amino acid residues (Corradi & Bouzat, 2016; Jones & Sattelle, 2008). nAChRs are non-selective cation channels that generate excitatory postsynaptic responses which are named after the agonists nicotine, a CNS stimulant derived from a common tobacco plant (*Nicotiana tabacum, Solanaceae*) (Augustine, 2004; Pohanka, 2012). They are widely expressed in the central and peripheral nervous system, in addition to non-neuronal tissues (Corradi & Bouzat, 2016; English & Jones, 2012).

Overall, nAChRs are pentameric proteins (Figure 16) with intrinsic central ion-conducting pore that is permeable to cations such as Na$^+$, K$^+$ and Ca$^{2+}$ when the receptors are activated (Wu et al., 2015). In vertebrates, genes encode a total of 17 subunits ($\alpha1$-$10, \beta1$-$4, \gamma, \delta$ and $\epsilon$) (Millar & Gotti, 2009; Peng & Ding, 2015; Pohanka, 2012) and nAChRs subunits can be either homomeric (identical) or heteromeric (variable) (Figure 17) (Corradi & Bouzat, 2016). Thus, various subunit compositions may be involved in a diversity of physiologic functions and pharmacological effects unique for receptor subtypes (Kim et al., 2015; Peng & Ding, 2015).
The nAChR has a molecular mass of approximately 280 kDa (Fisher & Wonnacott, 2012). The single subunit structure consists of: 1) an N-terminal extracellular domain that makes up the ACh-binding site; 2) four hydrophobic transmembrane spanning alpha-helical domains that make up the ion channel portion of the receptor called M1, M2, M3 and M4 with a long cytoplasmic loop between M3 and M4, and other shorter loops connecting the domains (see Figure 16) (Atri et al., 2017; Augustine, 2004; Fisher & Wonnacott, 2012). The ACh-binding site is
composed of three protein loops on the α subunit (known as principal subunit) and three loops from the adjacent subunit (known as complementary subunit) (Fisher & Wonnacott, 2012).

nAChRs mediate cholinergic transmission through a process known as direct ligand-gated conductance. The binding of two ACh molecules to the extracellular domain of nAChR causes a conformational change in the receptor allowing the opening of the channel which enables flow of cations across the cell membrane. This results in a net inward current that depolarizes the postsynaptic cell. Stimulation of various nAChRs may depolarize the cell sufficiently to elicit fast changes in the membrane electric potential (action potential) and to open voltage-dependent calcium channels, and subsequently activation of several intracellular signaling pathways (Atri et al., 2017). Upon continuous exposure to agonists, most nAChRs desensitize. The open state is not maintained, and the receptor is locked in a closed conformation (agonist remains bound in the desensitized states; see Figure 18) which provides a protective and regulatory mechanism to prevent over-excitation of the target cell (Changeux & Paas, 2010; Fisher & Wonnacott, 2012; Taylor, 2012). The M2 membrane-spanning domains of the five subunits are symmetrically arranged around the central axis of the molecule and together form the transmembrane channel regulating ion transport through the nicotinic channel (Atri et al., 2017; Hammond, 2015); three negatively charged rings of five amino acids from each M2 subunit draw cations through the channel, and an uncharged leucine ring found at the center of the channel participates in closing the channel during desensitization (Figure 19) (Atri et al., 2017).
Muscle nAChR are found in vertebrate skeletal muscles, where they mediate a central role in neuromuscular transmission at the neuromuscular junction (NMJ), and is considered a target of muscle relaxants (Corradi & Bouzat, 2016), as well as in fish electric organs of the electric ray *Torpedo* (*Torpedo* nAChR) (Hammond, 2015; Taylor, 2012; Zouridakis et al., 2009). Five nAChRs subunits are expressed in skeletal muscle (α1, β1, γ, δ and ε); nAChRs in embryonic muscle have the subunit composition (α1)β1γδ, like that in *Torpedo* (α2βγδ), whereas in adult muscle, the
composition is \((\alpha_1)\beta_1\delta\varepsilon\) (Figure 20) (Millar & Harkness, 2008; Zouridakis et al., 2009). As a consequence of a developmental switch in gene transcription, the \(\gamma\) subunit is expressed in embryonic muscle, whereas the \(\varepsilon\) subunit is expressed in adult muscle (Millar & Harkness, 2008). Muscle-type nAChRs are found in very high density localized in the motor end plate (postsynaptic muscle membrane) (Fagerlund & Eriksson, 2009; Taylor, 2012), as well as at the presynaptic nerve ending at NMJ, where they mediate fast chemical transmissions of electrical signals from invading motor neurons (Taylor, 2012; Zouridakis et al., 2009). Mutations in muscle-type nAChRs give rise to congenital myasthenic syndromes (Engel et al., 2015; Fagerlund & Eriksson, 2009; Taylor, 2012).

Figure 20: Nicotinic receptor subtypes [From Mohammed, 2014]
In contrast to the homogeneous population of nAChRs found at the NMJ, subunit compositions of the receptors expressed in the nervous system are far more complex (Millar & Harkness, 2008; Taylor, 2012). Neuronal nAChRs are generated from α (α2–10) and β (β2–4) subunits (Dani & Bertrand, 2007) which are either homomers (e.g., α7, α8, α9, and α10) or heteromers of α and β subunits (e.g., α4β2, α3β4, and α4α2β3) but can also be two different α subunits (e.g., α7α8, α9α10) (Zouridakis et al., 2009). α4β2 is the most prevalent nAChR subtype in the CNS (more than 90%) (Buckingham et al., 2009; Taylor, 2012), which is found in the cerebral cortex, thalamus, hippocampus, substantia nigra, striatum and cerebellum (Buckingham et al., 2009), and α7-nAChR is widely distributed in several CNS areas (Buckingham et al., 2009; Taylor, 2012) which seem to be mainly involved in cognitive functions (Buckingham et al., 2009). Neuronal nAChRs play a key role in modulating post-, pre-, and/or extrasynaptic signaling (Posadas et al., 2013; Zouridakis et al., 2009). They are associated with a wide range of CNS disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), anxiety, depression, schizophrenia, Tourette’s syndrome and epilepsy (Posadas et al., 2013).

Moreover, ganglia of the autonomic nervous system express neuronal nAChRs with α3, α5, α7, β2 and β4 subunits (Hamill et al., 2012; Mohammed, 2014; Wang et al., 2002), and play an important role in signal transmission in autonomic ganglia (Wang et al., 2002). α3β4 are the most prevalent nAChR subtype expressed by ganglionic neurons (Skok, 2002; Taylor, 2012). Ganglion nACRs are located primarily on the postsynaptic dendrite and nerve cell body, but they are also found presynaptically to control the release of ACh from the presynaptic nerve ending (Taylor, 2012).
1.2.5 α7 Nicotinic Acetylcholine Receptors

The α7-nAChR is composed of five identical α7 subunits (Ren et al., 2017), and is one of the few mammalian nicotinic subunits that are homopentameric (Hone & McIntosh, 2018). The α7-nAChR exhibits unique functional properties that distinguish it from other nicotinic receptors including very low probability of channel opening, high calcium permeability, and fast desensitization after binding with agonists that occur on the submillisecond time scale. However, this is reversible (Corradi & Bouzat, 2016; Ren et al., 2017; Williams et al., 2011). Both ACh and choline acting as its endogenous ligands, activate the channel with similar single-channel open time and conductance, however, choline dissociates from the receptor more rapidly and, subsequently, induces a less stable state of desensitization compared with ACh (Albuquerque et al., 2009). Additionally, α-bungarotoxin (α-BTX; antagonists) exhibits a high-affinity binding for the ligand binding site of α7-nAChRs (Barrantes, 2014; Corradi & Bouzat, 2016).

α7-nAChR is one of the most widely distributed nAChRs throughout the nervous system. It is expressed in many non-neuronal cells including astrocytes, microglia, oligodendrocyte precursor cells, and endothelial cells; playing an important role in immunity, inflammation, and neuroprotection (Corradi & Bouzat, 2016; Dineley et al., 2015; Hone & McIntosh, 2018).

As mentioned previously, α7 nAChR is widely distributed in the CNS, such as hippocampus, cortex, and several subcortical limbic regions, and seem to be particularly involved in cognitive functions and memory (Corradi & Bouzat, 2016). Even though α7-nAChRs may serve as postsynaptic receptors mediating classical neurotransmission, they appear to be involved in the modulation of other
neurotransmitter release such as GABA, glutamate, norepinephrine, and dopamine by acting as presynaptic modulators, therefore, affecting different neurobiological functions (Baranowska & Wisniewska, 2017). Thus, decreased expression and function of α7-nAChR is associated with several neuropsychiatric and neurologic disorders including Alzheimer disease, autism, learning disability, attention deficit hyperactivity disorder, schizophrenia, bipolar disorder, and epilepsy (Baranowska & Wisniewska, 2017).

![Dual ionotropic/metabotropic nature of α7-nAChRs](image)

**Figure 21: Dual ionotropic/metabotropic nature of α7-nAChRs**

DAG, diacylglycerol; GPCR, G protein-coupled receptor; PKC, protein kinase C; ROS, reactive oxygen species [From Corradi & Bouzat, 2016]

α7-nAChRs operate as a dual metabotropic/ionotropic receptor (Bouzat et al., 2017; Corradi & Bouzat, 2016; Kabbani et al., 2013). α7-nAChR agonists generate a transient ion flux response; allows flux of Na⁺ and K⁺, as well as high permeability to Ca²⁺ (Corradi & Bouzat, 2016). The high permeability of α7-nAChRs to calcium underlies most of their functions including facilitation of neurotransmitter release, depolarization of postsynaptic cells, and initiation of various cellular processes by acting as a second messenger (Corradi & Bouzat, 2016; Gotti & Clementi, 2004). The
transient increase in intracellular Ca\(^{2+}\) is further sustained by the calcium release from intracellular stores via a calcium-induced calcium release mechanism, a process involving IP\(_3\) and ryanodine receptors (Figure 21). Owing to its metabotropic nature, activation of α7-nAChRs triggers a series of calcium-dependent intracellular signaling pathways that can persist beyond the expected time course of channel activation (Figure 21) (Corradi & Bouzat, 2016). α7-nAChRs bind both G\(\alpha\) and G\(\beta\gamma\) proteins through the M3–M4 loop of the receptor (Corradi & Bouzat, 2016; Kabbani et al., 2013; King et al., 2015).

α7-nAChRs have been implicated in various immunological processes including the modulation of inflammation in different pathologic conditions, including sepsis, ischemia/reperfusion, rheumatoid arthritis, and pancreatitis (Corradi & Bouzat, 2016; Hone & McIntosh, 2018). α7-nAChRs have a crucial role in the “cholinergic anti-inflammatory pathway” that is a link between vagal efferent fibers and the innate immune system (Martelli et al., 2014). In some immune cells, α7-nAChRs modulate intracellular signal pathways including: 1) recruitment and activation of Janus kinase 2/signal transducer and activator of transcription (JAK2/STAT3) leading to the inhibition of nuclear factor (NF)-kB nuclear translocation, and subsequently, proinflammatory cytokine production inhibition; 2) activation of phosphoinositide 3-kinase (PI3K)/Akt pathway promoting the master regulator of oxidative stress Nrf-2 translocation to the nucleus, and overexpression of heme oxygenase 1 (HO-1). Both pathways result in potent anti-inflammatory effects (Figure 21) (Baez-Pagan et al., 2015; Corradi & Bouzat, 2016; Egea et al., 2015; Hone & McIntosh, 2018).

Furthermore, alternative hypotheses suggest that the link from the vagus to the spleen in the cholinergic anti-inflammatory pathway is non-neural (Figure 22) (Fujii...
Many immune cells, including T cells, are capable of synthesizing and releasing ACh (Fujii et al., 2017). Activation of vagal efferent fibers or vagal afferent sensory fibers stimulate mobilization of lymphocytes including ACh-synthesizing T-cells into the spleen. ACh released from the incoming T-cells acts on α7-nAChRs on sympathetic nerve terminals in the spleen which then induces noradrenaline release. Noradrenaline acts on β2-adrenoceptors on splenic macrophages, thus suppressing production of TNF-α (Fujii et al., 2017; Martelli et al., 2014). Pharmacological stimulation of α7-nAChRs has been shown to have anti-inflammatory effects in several inflammatory disease models (Bertrand et al., 2015; Hone & McIntosh, 2018).

Figure 22: Non-neural cholinergic anti-inflammatory pathway [From Martelli et al., 2014]

Additionally, high levels of α7-nAchRs have been found to be associated with formation of various cancers such as lung, bladder, and colon cancers (Wu et al., 2011). α7-nAChR serve as important channels for promoting the survival, proliferation, and
angiogenesis of cancer cells with possible contribution from the epidermal growth factor receptors (EGFR) and/or β-arrestin (β-ARs) which stimulate tumor-promoting signaling cascades (Figure 23) (Schaal & Chellappan, 2014).

Figure 23: Schematic representation of the relation between α7-nAChR and cancer Components of tobacco smoke (Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)) stimulates tumor-promoting signaling cascades by activating the nAChR, mainly the α7-nAChRs. [From Schaal & Chellappan, 2014]

Activation of α7-nAChR induces the secretion of growth factors such as epidermal growth factor (EGF), neurotransmitters such as adrenaline and noradrenaline, and angiogenic factors such as vascular endothelial growth factor (VEGF) (Schaal & Chellappan, 2014). Secretion of EGF via nAChRs results in the
transactivation of EGFR leading to the activation of a variety of signaling cascades including the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (MAPK/ERK), phosphoinositide 3-kinase (PI3K)/AKT pathway, (Schaal & Chellappan, 2014; Wu et al., 2011) and Janus-activated kinase (JAK)/STAT signaling (Schaal & Chellappan, 2014). Similarly, the release of adrenaline and noradrenaline, which are the physiologic ligands for β-ARs further stimulate secretion of EGF to further transactivate EGFRs which contribute to the development and progression of cancer (Schaal & Chellappan, 2014). Moreover, activation of α7-nAChR recruits of Src kinase in β-arrestin-1 leading to inactivation of Rb protein, and thus results in the expression of E2F1-regulated proliferative genes which subsequently, enhances the proliferation of cells, and renders them resistant to apoptosis induced by different agents (Schaal & Chellappan, 2014).
Chapter 2: Aims and Objectives

Capsaicin has been shown to exhibit anti-inflammatory, analgesic and anti-cancer properties. Considering the importance of α7-nAChRs in inflammatory pain and pathogenesis of cancer, the main aim of this project was to investigate the effect of capsaicin on the functional properties of α7-nAChRs expressed homomerically in *Xenopus* oocytes. The effect of Capsaicin was tested electrophysiologically on receptor activity induced by ACh using the two-electrode voltage-clamp technique. The main objectives of this study were as follows:

1) Evaluating the functional expression of human α7-nAChRs in *Xenopus* oocytes.
2) Exploring the effect of capsaicin on the function of human α7-nAChRs.
3) Assessing the involvement of intracellular Ca\(^{2+}\) level on the effects of Capsaicin on α7-nAChRs.
4) Determining the effect of capsaicin on passive membrane properties of *Xenopus* oocytes.
5) Defining the voltage-dependence of capsaicin action on the α7-nAChRs.
6) Investigating the nature of the interaction between capsaicin and α7-nAChR (competition studies).
7) Examining the effect of capsaicin on other members of the Cys-loop ligand-gated ion channel family.
Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Maintenance of *Xenopus laevis* Frogs

*Xenopus laevis*, African clawed frog (Figure 24), is a member of the anuran family Pipidae (Burton & Burton, 2002). Following the key finding that exogenous RNA injected into *Xenopus laevis* oocyte can be translated into proteins by Gurdon et al. in 1971 (Gurdon et al., 1971), the use of *Xenopus laevis* oocyte has been used extensively as a heterologous expression system. Expression of cloned ion channels in *Xenopus* oocyte was first introduced in 1982 by Miledi (Miledi et al., 1982). Subsequent studies have shown that, in fact, channel proteins can be expressed in the plasma membrane by microinjecting the oocyte with mRNAs transcribed from an isolated gene that codes for that channel (Sigel, 1990). Due to its large size, high degree of expression of ion channels, and the relative absence of endogenous channels. The two-electrode voltage-clamp technique using *Xenopus laevis* oocytes has become important technique for the investigation of ion channel structure, function, and regulation (Dascal, 1987).

Mature Female *Xenopus laevis* frogs were purchased from Xenopus Express, (Haute-Loire, France). Frogs were housed in an aquarium filled with chlorine-free tap water (aquarium dimensions; 32 cm width, 130 cm length, and 66 cm height), in a temperature-controlled room at approximately 18 °C and maintained in a constant light-dark cycle of 12 hrs each to avoid seasonal variation of oocyte quality. Frogs were fed twice per week with special frog’s food pellets, supplied by Xenopus Express Inc. (Brooksville, FL, USA). Tank water was changed and cleaned twice weekly (after
feeding). Experiments conducted in this study were in accordance with institutional guidelines and approved by the Animal Ethic Committee of the CMHS/UAEU.

Figure 24: *Xenopus laevis* frog distinctive characteristics

### 3.1.2 Electrophysiology Rig

Electrophysiological techniques used to study functional properties of ion channels in the cell membranes are based on the fact that ion channels conduct electrical current carried by ions which can be detected by using highly sensitive electronic apparatus (Petkov, 2009). In general, this technique allows ion flow through the cell membrane to be recorded as electric current while the membrane potential is held at constant value with the feedback amplifier. The voltage clamp technique was
first developed by Cole (1949) and Hodgkin (1952) for use with the squid giant axon (Clay, 1985). Since then, different types of voltage clamp techniques have emerged and have been used in a wide range of tissues/cells.

The electrophysiological technique used in this study is the two-electrode voltage-clamp using *Xenopus laevis* oocyte. The apparatus used in the two-electrode voltage-clamp technique (Figure 25) were as follows: Gene Clamp-500B amplifier (Axon Instruments, Molecular devices, Inc., Sunnyvale, CA, USA) which is used to record the current traces, magnetic holding devices (Kanetec USA corporation, Bensenville IL, USA) and manual micromanipulator (M33; Märzhäuser, Wetzlarm Germany). Head-stages; for voltage (HS-2A Head-stage, Gain1 MG, Axon Instruments, Molecular Devices, Inc, CA, USA) and current (HS-2A Head-stage, Gain10 MG) were attached to the manipulators. The two glass electrodes were inserted in electrode holders and then connected to the head-stages. Micromanipulators were used to control the electrodes and impale the oocyte.

The perfusion apparatus was composed of perfusion tubes, plastic connectors, plastic valves, 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, Illinois, USA). The flow rate of perfusion was set to 3 - 5 ml/minute. For drug application, a second perfusion line was used. This system contained tubing (C-Flex tubing, Cole Parmer Instruments Company, I.D.1/32 inch, O.D. 3/32 inch, and WALL 1/32 inch, Vernon Hills, Illinois, USA), 50 ml glass syringes, and coupling devices. The drug application micropipette was placed 2-3 mm from the oocyte in the recording chamber (Warner Instruments LLC, Hamden, CT, UK) (Recording chamber is where the oocyte is placed, impaled with microelectrodes,
and perfused with solution). The flow of the drug through the micropipette into the recording chamber was controlled by gravity. The perfusion solution in the recording chamber was removed by using a glass suction tube connected to an adjustable vacuum source, and collected in the waste tank.

Figure 25: Two-electrode voltage-clamp recording from *Xenopus* oocytes

A fiber optic light source was employed for the illumination of the recording chamber (Fiber life, High intensity illuminator series 180, Dolan, Jenner Industries Inc., MA, USA). A low-power stereo-dissection microscope was used for visual observation of the recording chamber (Olympus, Tokyo, Japan, SZ-STB1m 100 AL0.5 Xm WD186). Computer set up for data acquisition consisted of a Compaq personal
computer (Compaq Corporation, Wynyard, UK) and analog-digital converter BNC 2081 (National Instruments, Austin Texas, USA).

### 3.1.3 Chemicals

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Formula weight</th>
<th>Manufacturer/ Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)- Capsaicin</td>
<td>305.42</td>
<td>Tocris/ 7A/176841</td>
</tr>
<tr>
<td>α-Bungarotoxins</td>
<td>~ 8500</td>
<td>Sigma / T-0195</td>
</tr>
<tr>
<td>Acetylcholine Chloride</td>
<td>181.7</td>
<td>Sigma, USA/ A6625</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>387.4</td>
<td>Sigma, USA / H-7752</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>Sigma, USA / G7126</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>58.44</td>
<td>Sigma, USA/ S-3014</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.3</td>
<td>Sigma, USA/ H3375</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>74.55</td>
<td>Mallinckrodt, USA/ 6858</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO$_4$)</td>
<td>246.5</td>
<td>Sigma, USA/ M9391</td>
</tr>
<tr>
<td>Sodium Bicarbonate (NaHCO$_3$)</td>
<td>84.01</td>
<td>Sigma, USA/ S6014</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl$_2$)</td>
<td>95.22</td>
<td>Sigma, USA/ M8266</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl$_2$)</td>
<td>110.9</td>
<td>Sigma-Aldrich, USA/C-4901</td>
</tr>
<tr>
<td>Barium Chloride (BaCl$_2$)</td>
<td>244.28</td>
<td>BDH, England/R-20-25 S:45</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH)</td>
<td>40</td>
<td>Amresco, USA/ Lot.#214613010</td>
</tr>
<tr>
<td>Hydrogen Chloride (HCl)</td>
<td>37%</td>
<td>Sigma-Aldrich, USA/ 25.814.B</td>
</tr>
<tr>
<td>Gentamycin Sulphate salt (mixture of 3 major components designated as C1, C1a, and C2)</td>
<td>C1 = 477.6, C1a = 449.5, C2 = 463.6</td>
<td>Sigma-Aldrich, USA/ G1264</td>
</tr>
<tr>
<td>Theophylline</td>
<td>180.2</td>
<td>Sigma/ T-1633</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>110.04</td>
<td>Sigma-Aldrich, Japan/P5280</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>356.4</td>
<td>Sigma, USA/ P3032</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>1457.4</td>
<td>Sigma-Aldrich, USA/ S9137</td>
</tr>
<tr>
<td>BAPTA</td>
<td>764.68</td>
<td>Sigma-Aldrich / A-1076</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>165.2</td>
<td>Sigma, USA/ E-1501</td>
</tr>
<tr>
<td>Collagenase-A CLS-1 (from <em>Clostridium histolyticum</em>)</td>
<td>Concentration 200 u/mg</td>
<td>Worthington, biochemical corporation, NJ, USA / LS004196</td>
</tr>
</tbody>
</table>

Table 1: Chemicals used for the experiments
### 3.1.4 Other Materials

<table>
<thead>
<tr>
<th>Device or Material</th>
<th>Specifications</th>
<th>Company of Purchase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrode Holder</td>
<td>--</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Magnetic stand and manipulators</td>
<td>Catalog #7739</td>
<td>Narishige, Tokyo, Japan</td>
</tr>
<tr>
<td>Silver Wires</td>
<td>--</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Borosilicate Glass tubing for microelectrodes</td>
<td>Glass thin-walled with filament 1.5 mm Catalog #TW150F-4</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Vertical Puller</td>
<td>Model 700D, heater adjusted to 48 and solenoid adjusted to 70 °C to get optimal resistance of 1-2 MΩ</td>
<td>David Kopf Instruments, Tujunga, CA, USA</td>
</tr>
<tr>
<td>Microfill filling syringe</td>
<td>--</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Micro-4 Microsyringe pump controller</td>
<td>Model UMC4-C</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Automatic Nano-liter injector</td>
<td>Nanoject</td>
<td>Drummond Scientific Company, Broomall, PA, USA</td>
</tr>
<tr>
<td>RNAase free water in 1.8 ml Eppendorf tubes</td>
<td>Lot #M25/80502</td>
<td>Epicenter Biotechnologies Madison, Wisconsin, USA</td>
</tr>
<tr>
<td>pH Meter</td>
<td>Model 450</td>
<td>Corning pH meter, Albany, NY, USA</td>
</tr>
<tr>
<td>Stirrer</td>
<td>Rotomix type 50800, Model #M50825</td>
<td>Barnstead/ thermolyne, Dubuque, IA, USA</td>
</tr>
<tr>
<td>Picofuge</td>
<td>Catalog #400550</td>
<td>Stratagene, Santa Clara, CA, USA</td>
</tr>
<tr>
<td>Petri Dishes</td>
<td>Catalog #127,60mm</td>
<td>Sterillin, Newport, UK</td>
</tr>
<tr>
<td>Surgical Accessories</td>
<td>Scissors, forceps, scalpels</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Surgical Sutures</td>
<td>Catgut Chrom, reverse cutting 3/8 circle, USP 4/0, SMI</td>
<td>DemeTech Corporation, Miami, Florida, USA</td>
</tr>
<tr>
<td>Dissecting microscope</td>
<td>Model GSZ</td>
<td>Bunton Instruments Co Inc, Rockville, MD, USA</td>
</tr>
</tbody>
</table>

Table 2: Other materials and devices used in the study
3.2 Methods

3.2.1 Preparation of Required Solutions

3.2.1.1 Modified Barth’s Solution (MBS)

The Modified Barth’s Solution was mainly used during the process of oocytes isolation. The composition of MBS is shown in table below:

a) Calcium free MBS solution:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>1x (Weight in grams)</th>
<th>10x (Weight in grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>88</td>
<td>5.14</td>
<td>51.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>2.38</td>
<td>23.8</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.1</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>0.075</td>
<td>0.75</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8</td>
<td>0.2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3: Calcium free MBS solution composition

The above components were dissolved in distilled water to get total volume of 1 L and the pH was adjusted to 7.5 using NaOH.

b) Calcium containing MBS solution:

In addition to substances mentioned in (Table 3), 0.22 g and 2.2 g of CaCl₂ (2mM) was added to make stock solutions of 1x and 10x, respectively. All the compounds were dissolved in distilled water to get total volume of 1 L and the pH was adjusted to 7.5.

3.2.1.2 Oocyte Storage Solution

The storage solution was prepared as follows: 1) 100 ml of 10x Calcium containing MBS solution was placed in a measuring cylinder; 2) Distilled water was
added to produce a total volume of 900 ml; 3) Antibiotic cocktail shown in (Table 4) were dissolved in the solution; 5) The pH was adjusted to 7.5; 6) The mixture was filtered and stored in sterile container.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Weight in grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>10,00 U/L</td>
<td>0.02</td>
</tr>
<tr>
<td>Gentamycin Sulphate salt</td>
<td>50mg/L</td>
<td>0.1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10mg/L</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>2mM</td>
<td>0.22</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.5mM</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 4: Antibiotic materials

3.2.1.3 Extracellular Solution

ND96 extracellular solution was used in the two-electrode voltage-clamp technique to record ion currents mediated by the nAChRs, and normal Ringer’s solution was used to record ion currents mediated by the glycine and 5-HT3 receptors.

The recipes for both ND96 and normal Ringer’s solution used were:

a) ND96 solution:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (mM)</th>
<th>1x (Weight in grams)</th>
<th>10x (Weight in grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>96</td>
<td>5.61</td>
<td>56.1</td>
</tr>
<tr>
<td>KCl</td>
<td>2</td>
<td>0.15</td>
<td>1.5</td>
</tr>
<tr>
<td>MgCl2</td>
<td>1</td>
<td>0.10</td>
<td>1.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>1.19</td>
<td>11.9</td>
</tr>
<tr>
<td>CaCl2 or BaCl2</td>
<td>1.8</td>
<td>0.20</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>0.439</td>
<td>4.39</td>
</tr>
</tbody>
</table>

Table 5: ND96 solution composition

The above components (1x) were dissolved in distilled water to produce a total volume of 1 L and the pH was adjusted to 7.5 using NaOH.
b) Normal Ringer’s solution:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (mM)</th>
<th>1x (Weight in grams)</th>
<th>10x (Weight in grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>115</td>
<td>6.72</td>
<td>67.2</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>0.074</td>
<td>0.74</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.8</td>
<td>0.199</td>
<td>1.99</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>2.38</td>
<td>23.8</td>
</tr>
</tbody>
</table>

Table 6: Normal Ringer’s solution composition

The above components (1x) were dissolved in distilled water to get total volume of 1 L and the pH was adjusted to 7.2 using NaOH.

3.2.2 Drug preparation

Stock solutions of the test compounds were prepared in either ND96 solution when recording ion currents mediated by the nAChRs, or in normal Ringer’s solution when recording ion currents mediated by glycine and serotonin receptors using the following formula:

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

Further dilutions were prepared using the Charles equation:

\[
C_1 \times V_1 = C_2 \times V_2
\]

Where \(C_1\) is the concentration of stock solution, \(V_1\) is the volume of stock solution, \(C_2\) is the concentration of the solution to be prepared, and \(V_2\) is the volume of \(C_2\) of the solution to be prepared.

Stock solutions and required dilutions were prepared freshly before starting each experiment.
3.2.3 Isolation and Maintenance of Oocytes from *Xenopus laevis*

Surgery to harvest oocytes from the *Xenopus laevis* is a relatively straightforward procedure (Figure 26) performed on a clean bench top. As the oocytes are susceptible to bacterial contamination, all the surgical implements were sterilized with 70% ethanol prior to use. Surgery was done once weekly. Oocytes were collected up to 4 times per animal (three survival surgeries and one terminal procedure), alternating between the right and left ovaries for each procedure, and allowing for 2-3 months recovery period between surgeries. All the procedures conducted in this study were in accordance with institutional guidelines and approved by the Animal Ethic Committee of the CMHS/UAEU.

*Xenopus laevis* female frog was anesthetized via immersion in 0.03% w/v benzocaine bath (prepared by dissolving 300 mg of ethyl p-aminobenzoate in 15 mL of 70% ethanol, and then adding that to 1 L of cold tap water). The depth of anesthesia was determined by gently pinching the lower limbs of the frog and ensuring that the frog is non-responsive to noxious stimuli. It usually took approximately 5-15 minutes for the animal to be completely anesthetized. The anesthetized frog was then placed on the top of a bed of crushed ice covered with wet paper towel to avoid drying of the skin while maintaining low core body temperature during the surgery. Abdominal laparotomy was performed with a 1.5 cm vertical incision to the left or right of the midline using a surgical scalpel. Another similar incision was made through the fascia and muscle to visualize the oocyte lobes using a surgical scalpel and scissors. 1-2 ovarian lobes were removed and placed on a petri dish containing calcium free MBS solution. After ensuring that there was no bleeding from the surgical site, the muscle layer of the abdomen and epidermal layer were sutured together as a single layer using
absorbable catgut sutures. Immediately after surgery, the frog was placed in a container filled with tap water with head elevated (to prevent drowning) and the rest of the body submerged, and monitored frequently, based on free swimming behavior. Recovery from the anesthesia usually took up to 1 hour. After 3-4 hours of recovery, the frog was transferred to the main frogs’ aquarium tank.

The lobes were cut into small pieces by removing the thecal, epithelial and follicular layers manually using fine surgical forceps and digested with collagenase A solution. The enzyme collagenase solution was prepared by dissolving 80 mg of collagenase, type A, in 25 ml of Ca^{2+} free MBS solution. The oocyte clumps were incubated in a small conical flask containing 12.5 ml collagenase solution with constant stirring (60-80 rotations/minute) at room temperature for 1.5 h. After that,
the collagenase solution was replaced with fresh collagenase solution 12.5 ml and kept stirring for another 1.5 hour. Since collagenase damages the vitelline and plasma membrane, the oocytes were washed gently; 5 times using Ca\(^{2+}\) free MBS, then 5 times with Ca\(^{2+}\) containing MBS solution. Subsequently, oocytes were transferred to a petri dish filled with Ca\(^{2+}\) containing MBS for oocyte selection.

![Diagram of Xenopus oocyte stages](image)

**Figure 27:** Sorting *Xenopus* oocytes
(A) Stages of oocyte development, (B) Stage V or VI oocyte as found in an ovarian lobe, (C) Only stage V and VI were selected. [Modified from Rasar & Hammes, 2006; Bianchi & Driscoll, 2006; Jullien, 2015]

Only healthy looking mature oocytes (stage V-VI) were selected under a dissecting microscope. Stage V and VI oocytes are characterized by larger size with
1.0 mm - 1.2 mm diameter, rounded shape, and clear dark brown animal pole and yellow vegetal pole divisions (Figure 27) were selected.

### 3.2.4 In Vitro cRNA Synthesis

The cDNA clone of human α7-nAChR was provided by Dr. J. Lindstorm (University of Pennsylvania, PAM, USA). The mMessage mMachine kit used to synthesize the capped cRNA transcripts was from Ambion (SP6 kit, Austin, TX, USA). The synthesized capped cRNA transcripts were analyzed on 1.2% formaldehyde agarose gel to check the quality and size of the transcripts. The cDNA for human α3, α4, β2, and β4 nAChR subunits were kindly provided by Dr. Isabel Bermudez (Oxford Brookes University, Oxford, UK). Subunit combinations, α and β subunits were injected at 1:1 ratio.

### 3.2.5 Microinjection of cRNA into Oocytes

The mRNA concentration of synthesized human α7-nAChRs and other subunits used in this study are shown in (Table 7). They were stored as 1μl aliquots in freezer at -80°C. Once the oocytes were prepared and sorted, only one RNA aliquot was transferred in an ice bucket to the laboratory. To maintain a RNase free environment while working with cRNA, gloves and masks were put on during the experiment and the bench surface was sterilized using 70% ethanol. The tube was centrifuged using a microcentrifuge at 1200 rpm for 1-3 min, and then by using a sterile pipette and RNase/DNase free pipette tips (Denville Scientific Inc., Metuchen, NJ, USA), and resulting cRNA pellet was diluted to final concentration of 10 ng/50 nl in RNase-/DNase-free water. Only 3 to 4 μL of diluted cRNA was used for each batch of oocytes, and the remaining cRNA was returned to the freezer. A vertical puller
(Model 700 D, David Kopf Instruments Tujunga, CA, USA) was used for making glass microelectrodes with fine needle shaped tip from autoclaved glass capillaries (World Precision Instruments, Sarasota, FL, USA). The tip of each glass microelectrode was broken by applying gentle pressure using a fine pair of forceps (Fine Science Tools Inc., Vancouver, Canada) under a dissecting microscope (Bunton Instruments Co., Rockville, MD, USA). The glass needle was backfilled with mineral oil (Sigma, St. Louis, MO, USA) using a 1 ml glass syringe. Then the glass needle was fitted into a microdispenser connected to the micromanipulator (Figure 28).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Subunit</th>
<th>Main stock conc. (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nACh receptors</td>
<td>β2</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>β4</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>α3</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>α4</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>α7</td>
<td>3.7</td>
</tr>
<tr>
<td>Glycine receptor</td>
<td>α1</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>α2</td>
<td>1.678</td>
</tr>
<tr>
<td></td>
<td>α3</td>
<td>1.595</td>
</tr>
<tr>
<td>5-HT3 receptor</td>
<td>A</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 7: Initial concentration of all subunits

Subsequently, 3 to 4 µL of diluted cRNA or distilled water was dropped on the center of a mineral oil drop placed on parafilm (American National Can Co., Greenwich, CT, USA). By focusing on the drop under the dissecting microscope, the tip of the glass needle was placed at the center of the droplet, and the aqueous phase was carefully withdrawn into the glass needle using the withdrawal option on the microdispenser controller. After loading the sample, the sorted oocytes were arranged in a U-shaped pattern in a small petri dish, containing MBS, with mesh-bottom to hold the oocytes in place during the microinjection procedure. Each oocyte was impaled by
the glass needle and gently injected with 50 nL of cRNA solution or distilled water using the microinjector (driven by a Micro-4, micro syringe pump controller).

Figure 28: cRNA microinjection setup. Consist of stereomicroscope, micromanipulator and control unit [From Nakagawa & Touhara, 2013]

Figure 29: The process of expressing exogenous proteins in *Xenopus* oocytes
Following the injection, the oocytes were stored in 25 ml petri dishes filled with oocyte storage solution and incubated at 18°C. In initial experiments, the oocytes were divided into two groups; oocytes that were injected with cRNA encoding the human $\alpha_7$-nAChR, and oocytes that were injected with distilled water (control). Xenopus oocytes were used in the electrophysiology study 2-3 days after microinjection to allow maximal expression of receptors (Figure 29). Healthy oocytes were transferred to fresh dishes with new storage solution daily. A bottle of storage solution was stored in the incubator with the oocytes. The oocytes were used for about 7-10 days.

3.2.6 Two-electrode Voltage-clamp Method

Two-electrode voltage-clamp is used to record current while the membrane potential is held at constant value with a feedback amplifier. In this study, the two-electrode voltage-clamp was used in combination with exogenous mRNA expression in Xenopus laevis oocytes for ion channel recording (Figure 30). This system is well established and has been widely used to evaluate the functional properties of different receptors or ion channels of interest expressed in the Xenopus laevis oocytes. The advantages and disadvantages of using oocytes in two-electrode voltage-clamp experiments are the following (Goldin, 2006):

Advantages:

- Relatively straightforward and easy to operate.
- Fast to perform and ideal for screening purposes.
  - Perfusion of the external solution can be easily changed multiple times.
- Recordings can be stable over long periods of time.
- Possible to impale multiple electrodes into the same oocyte.
• It is very sensitive; records currents through whole cell ion channels.

Disadvantages:

• The large size and extensive membrane invaginations result in a very large membrane capacitance which may result in slow clamp settling time when the membrane potential is changed.

• Difficult to perform quantitative studies (such as examining selective permeability) as there is no control of the internal cellular environment.

Figure 30: Schematic illustration of two-electrode voltage-clamp setup using *Xenopus* oocytes expressing the receptor of interest [Modified from Nakagawa & Touhara, 2013]

The oocyte is simultaneously impaled with two microelectrodes; one electrode measures the voltage across the membrane while the other is to inject enough current to maintain the transmembrane potential at the desired/command voltage. The voltage
electrode is connected to the membrane potential amplifier to measure the membrane potential ($V_m$). This potential amplifier is fed into feedback amplifier which compares between the recorded membrane potential ($V_m$) and the command potential ($V_c$). If there is any difference between the two potential values, the feedback amplifier will pump the difference as current into the cell via the current electrode to clamp the membrane potential at the predetermined desired potential ($V_c$). The magnitude of this current ($I_m$) will be measured.

All electrophysiological experiments using Xenopus oocytes were performed at room temperature. The two-electrode voltage clamp setup is illustrated in Figure 25. A single oocyte was placed in the oocyte recording chamber and bathed with ND96 solution at a perfusion rate of 5-7 ml/min and solution delivery was controlled by using a stopcock. Drug application was through a simple gravity-fed system calibrated to deliver solution at the same rate. Glass microelectrodes were pulled to have a very sharp tip using a vertical microelectrode puller (with the following settings; heated value=52.5, solenoid value=52.5) (Model 700 D, David Kopf Instruments Tujunga, CA, USA) and backfilled with 3M KCl solution (Figure 31).

Figure 31: The glass microelectrodes
Mounted on electrode holder which contain a silver wire coated with silver chloride ($\text{AgCl}_2$) to allow the transmission of the signal from the potassium chloride (KCl) solution via the silver wire to the amplifier.
The two microelectrodes were mounted on micromanipulators; one connected to the current-electrode head stage and the other connected to the voltage-electrode head stage. Using the micromanipulator, the tips of the two electrodes were dipped into the bath solution, and the voltage of both electrodes was adjusted to 0 mV. Then, the animal pole (the dark pole) of the oocyte was impaled with the two microelectrodes (Figure 32). Pipette resistances ranged from 0.5-1.8 MΩ. The tip of the electrode was visualized using the microscope. After both electrodes were inserted, the amplifier was set in voltage-clamp mode. The oocytes were voltage clamped at a holding potential of -70 mV (command potential) using a Geneclamp 500 amplifier (Axon Instruments, Molecular Devices, Inc, Sunnyvale, CA, USA). Current responses were recorded and stored digitally for further analysis using Strathclyde Electrophysiology Software, WinWCP V4.0.8 (University of Strathclyde, Glasgow, UK). Perfusion of the ND96 was stopped immediately before the drug application and started immediately after drug application.

Figure 32: The oocyte impaled with two microelectrodes [Prof. Murat Oz Lab]
3.2.7 The Parameters Tested by Electrophysiological Recording

The agonist used during the study was ACh which is the endogenous agonist of nACh receptors. The advantages of using ACh over other agonists, such as nicotine, are; 1) has less desensitizing effect, and 2) can be easily removed during the washout due to its low hydrophobicity.

E-capsaicin purchased from Tocris was dissolved in dimethylsulfoxide (DMSO) to prepare a 100 mM stock solution. The final concentration of DMSO did not exceed 0.01% (v/v) which did not induce effects on the maximal amplitudes of ACh- induced currents.

1- Concentration Response Curve

For α7-nAChR, three to five recordings of current induced by ACh (100µM) were measured with 5 min intervals of wash out with ND96 solution. The average of stable readings was calculated and considered as control reading.

After obtaining the control readings, different concentrations of capsaicin were routinely applied, and 100µM ACh solution applied at the end of 5 min intervals which also included same concentration of capsaicin. The current induced by ACh + capsaicin application were recorded, and the average of 2-3 readings were calculated to determine the effect of capsaicin. Thereafter, drug application was stopped and the oocytes were washed with ND96 alone to obtain the recovery readings (100 µM ACh alone). Concentration-response data for each oocyte were normalized to the maximum current produced with 100 µM ACh for that oocyte, and percentage of inhibition was calculated by dividing the average of drugs-induced currents by the control values obtained before drug application. Capsaicin was used at varying concentrations to
construct dose response curves. For each concentration of capsaicin, averages of 5-6 oocytes were used. The concentration of capsaicin which produced a 50% inhibition of ACh-induced currents (IC$_{50}$) was obtained by nonlinear curve-fitting and regression fits (logistic equation) using computer statistical software v 8.5 (Origin Lab Corp., Northampton, MA, USA). Concentration of drug close to IC$_{50}$ was employed for further studies.

2- Effects of Capsaicin on α7-nAChR

The time-course of onset of capsaicin and the vehicle applications (0.01% DMSO) on the maximal amplitudes of ACh-induced currents were investigated. Capsaicin (10 µM) was co-applied in presence of 100 µM ACh for 5 min without any pre-application (0 pre-application time), and then washed out with ND96 to obtain the recovery readings (ACh alone). The time course of capsaicin inhibition was further tested by comparing the effect of varying capsaicin pre-application time on ACh-induced currents.

3- Ca$^{2+}$ Contribution to Observed Drug Action

One of the endogenous receptors expressed in the plasma membrane of Xenopus oocytes is Ca$^{2+}$ activated Cl$^{-}$ channels (CaCCs) which participate in many important physiological processes. Activation of α7-nAChR can causes significant increase in intracellular Ca$^{2+}$ which could activate the endogenous CaCCs (Sands et al., 1993) (Figure 33) resulting in alterations in the holding current under voltage-clamp conditions. To rule out the contribution of CaCC on the actions of capsaicin, intracellular Ca$^{2+}$ was buffered by Ca$^{2+}$ chelator BAPTA (1,2-bis (o-aminophenoxy) ethane-N, N, N’, N’-tetra acetic acid) by injecting 50 nL of 100 mM BAPTA into each oocyte. Thereafter, the oocytes were kept in ND96 for 5-10 min, and then placed in
ND96 solution containing 2 mM Ba\(^{2+}\) (instead of 2 mM Ca\(^{2+}\)) (Table 5). BAPTA stock solution was prepared in distilled water, and the pH was adjusted to 7.4 by using CsOH. To investigate the contribution of intracellular Ca\(^{2+}\) levels to drug observed effect, the effect of the capsaicin on ACh-induced currents was tested in BAPTA injected oocytes in the presence of ND96 perfusion solution, and the extent of drug effect was compared to those results obtained from control oocytes.

Figure 33: Sensitivity of CaCCs to intracellular Ca\(^{2+}\) levels

4- Voltage-dependency of Drug Inhibition

The voltage-dependency of capsaicin action on α7-nAChR was determined by holding the membrane potential at different values (ranging from -120 mV to 20 mV) for 30 s and then returned to -70 mV. The recordings were obtained at 5 min intervals. In these experiments, the concentration of ACh was 100 μM. Current–voltage (I-V) relationships of ACh-activated currents were determined in the absence and presence of 10 μM capsaicin.
5- Competitive and Non-competitive Inhibition

Concentration-response curves for ACh (conc. ranging from 1 μM- 1mM) were compared in the presence and absence (controls) of capsaicin (10 μM). The oocytes were voltage clamped at holding potential of -70 mV. The experiment was conducted in 6-8 oocytes, and the percent inhibition was calculated for each potential.

6- Effect of Capsaicin on Other Members of the Cys-loop Family of Ligand gated Ion Channel

The α7-nAChR belongs to the cys-loop family of ligand-gated ion channels. Therefore, the effect of capsaicin was investigated on the activity of other members of the cys-loop family of ligand-gated ion channel. Current induced by 100 μM ACh, 1 μM 5-HT, and 30 μM Gly in oocytes injected with cRNAs of nicotinic, serotonin type3 and glycine receptors, respectively. Current sizes were compared in the presence and absence of 10 μM capsaicin. The average of 2-3 readings in the presence capsaicin were calculated to determine the effect of capsaicin. Subsequently, drug application was stopped and the oocytes were washed with extracellular solution. Percentage of inhibition was calculated by dividing the average of currents in the presence of capsaicin by the control values obtained before drug application.

3.2.8 Statistical Data Analysis

The computer software Origin version 8.5 (Origin Lab Corp., Northampton, MA, USA) was used for nonlinear curve-fitting. Average values were calculated as the mean ± standard error means (SEM). Statistical significance was analyzed by using ANOVA or Student’s t-test as indicated. Specific p value of p≤0.05 was considered
significant. Concentration-response curves were obtained by fitting the data to the logistic equation,

\[ y = \frac{E_{\text{max}}}{1 + \left( \frac{x}{EC_{50}} \right)^n} \]

Where \( y \) is the response, \( E_{\text{max}} \) is the maximal response, \( x \) is the drug concentration, \( EC_{50} \) is half the maximal concentration, and \( n \) is the slope factor (apparent Hill coefficient).
Chapter 4: Results

4.1 Functional Expression of α7-nAChR

In the first set of experiments, bath application of ACh (100 µM) or capsaicin (10 µM) did not produce detectable currents in oocytes injected with distilled water (n=7). Application of 100 µM ACh for 3 to 4 sec activated fast inward currents that desensitized rapidly in oocytes injected with cRNA encoding the α7-subunit of human nAChRs (Figure. 34A and 34B). In addition, ACh-induced currents were significantly inhibited with 30 nM α-BTX (n=12, Figure 34B), indicating that these currents are mediated by the activation of α7-nAChRs.

Figure 34: Control experiments demonstrating the functional expression of α7-nAChRs in *Xenopus* oocytes

(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 minutes washout 100 µM ACh (right panel), (B) The mean maximal amplitudes of ACh-induced currents in distilled water injected oocytes and human α7 nAChR cRNA injected oocytes (left panel), and mean maximal amplitudes of ACh-induced currents in the presence and absence of α-bungaratoxin (30 nM) in oocytes injected with human α7 nAChR cRNA (right panel). The numbers of oocytes tested are represented for each group on top of each bar (n).
4.2 Time and Concentration Effect of Capsaicin on α7-nAChR

The time course and concentration dependency of capsaicin on α7-nAChRs were investigated. The effect of capsaicin (10 µM) was tested on 100 µM ACh-induced currents. Traces of currents induced by 100 µM ACh in control condition (left panel), followed by co-application of 10 µM capsaicin and ACh (100 µM) after 5 min pretreatment with 10 µM capsaicin (middle panel), and the recovery after 10 min capsaicin wash out (right panel) are presented in Figure 35A.

The time course of the effect of capsaicin and the vehicle (0.01% DMSO) on the maximal amplitudes of ACh (100µM)-induced currents are presented in Figure 35B. During the control experiment, vehicle (0.01% DMSO) alone did not alter the amplitude of the ACh-induced current. However, application of capsaicin for 15 min caused a significant inhibition of the ACh (100µM)-induced currents which was fully reversed after 10 to 15 min wash out period. These results suggested that capsaicin inhibits the function of α7-nAChRs in these cells.

Further investigation was conducted on the time course of capsaicin inhibition by comparing the effects of varying the capsaicin pre-incubation times on ACh-induced currents (Figure 35C). Co-application of capsaicin (10 µM) and ACh (100 µM) without capsaicin pre-incubation did not alter the maximal amplitudes of ACh-induced currents. However, when oocytes were pre-incubated with 10 µM capsaicin before the (ACh + capsaicin) co-application, maximal ACh-induced currents were inhibited by capsaicin in a time dependent manner reaching a maximal level between 2-5 min. The half-maximal inhibition ($\tau_{1/2}$) was reached at 0.32 ± 1 min (n= 6-7 oocytes). This result indicate that the inhibitory effect of capsaicin was significantly dependent on the application duration (time-dependent). To ensure equilibrium
conditions, 5 min capsaicin pre-application time was used routinely in the remaining of capsaicin experiments conducted in this study.

Figure 35: Effect of capsaicin on α7-nAChRs-mediated currents

(A) Records of currents activated by ACh (100 μM) in control conditions (left), during co-application of 10 μM capsaicin and ACh after 10 min pretreatment with 10 μM capsaicin (middle), and 10 min following capsaicin washout (right), (B) Time-course of the effect of vehicle (0.01% DMSO; filled circles) and capsaicin (10 μM; open circles) on the maximal amplitude of the ACh-induced currents. Each data point represents the normalized mean ± S.E.M. of 6-7 experiments, (C) Effect of pre-incubation time on the capsaicin inhibition of α7-nAChR; inhibition of the α7-nAChR-mediated currents increased with the prolongation of capsaicin pre-incubation time. Each data point represents the normalized mean ± S.E.M. of 6-7 experiments, (D) Capsaicin inhibits α7-nAChR function in a concentration-dependent manner. Each data point represents the normalized mean ± S.E.M. of 6-7 experiments. The curve is the best fit of the data to the logistic equation (described in the methods section).

The concentration-response curve for capsaicin is shown in Figure 35D. Capsaicin did not induce any response when applied alone. However, increasing the
concentrations of capsaicin caused greater inhibition of 100 μM ACh-induced currents indicating that capsaicin inhibits the function of α7-nAChR in a concentration dependent manner with an IC$_{50}$ = 8.6 μM.

4.3 Effect of Capsaicin on Endogenous Ca$^{2+}$ Dependent Cl$^{-}$ Channels

One of important physiological properties of α7-nAChRs is the high permeability to extracellular Ca$^{2+}$ (Uteshev, 2012). This property allows sufficient Ca$^{2+}$ influx to activate Ca$^{2+}$ activated Cl$^{-}$ channels (CaCCs) which are endogenously expressed in Xenopus oocytes (Figure 33). Therefore, I conducted further experiments to determine whether the effect of capsaicin was mediated by the inhibition of α7-nAChRs-mediated currents or by the inhibition of Cl$^{-}$ currents induced by Ca$^{2+}$ entry via α7-nAChRs. For this purpose, the extracellular Ca$^{2+}$ was replaced with Ba$^{2+}$. Ba$^{2+}$ can pass through α7-nAChRs without causing a significant activation of CaCCs (Sands et al., 1993). It has been reported that even in the presence of Ba$^{2+}$, a small Ca$^{2+}$ dependent Cl$^{-}$ current is observed which can be blocked by intracellular injection of Ca$^{2+}$ chelator BAPTA (Sands et al., 1993). Therefore, we investigated the effect of capsaicin on ACh-induced currents in extracellular solution containing 2 mM Ba$^{2+}$ in BAPTA-injected oocytes.

Bath application of 10 μM capsaicin induced the same level of inhibition (55 ± 3 % in controls versus 52 ± 5 % in BAPTA injected oocytes; ANOVA, P >0.05) of ACh-induced currents when BAPTA-injected oocytes were recorded in 2 mM Ba$^{2+}$ (Figure 36) suggesting that CaCCs are not significantly involved in capsaicin inhibitory effect on α7-nAChRs.
The effects of 10 μM capsaicin on the ACh-induced currents in α7-nAChRs expressing oocytes injected with 50 nL distilled water and recorded in 2 mM Ca\textsuperscript{2+} containing ND96 solution (control; n=8) or injected with 50 nL of BAPTA (100 mM) and recorded in 2 mM Ba\textsuperscript{2+} containing ND96 solution (BAPTA; n=9). Bars represent the means ± S.E.M. of 8 and 9 experiments.

4.4 Voltage-dependency of Drug Inhibition

To investigate whether inhibition of α7-nAChRs by capsaicin was affected by the membrane potential. ACh (100 μM)-induced currents were recorded at different voltages from -120 mV to 20 mV in the absence and presence of 10 μM capsaicin. Each tested membrane potential was held for 30 s before each current recording and then returned to holding potential of -70 mV. As shown in Figure 37A, the inhibitory effect of 10 μM capsaicin on 100 μM ACh-induced currents does not appear to be voltage sensitive, suggesting that the capsaicin-binding site is not affected by the transmembrane electric field. Evaluation of the current-voltage (I-V) relationship (Figure 37B) indicated that the extent of capsaicin (10 μM) inhibitory effect was not altered significantly at different holding potentials (P>0.05, n=7, ANOVA).
Figure 37: Inhibition of ACh-induced currents by capsaicin is independent of membrane potential

(A) Current-voltage (I-V) relationships of ACh (100 µM)-induced currents in the absence and presence of capsaicin (10 µM). Normalized currents activated by 100 µM ACh before (filled circles) and after 10 min treatment with capsaicin (opened circles). Each data point presents the normalized means ± S.E.M. of 6-7 experiments, (B) Quantitative evaluation of capsaicin effect is shown as percent inhibition at different membrane potential ranging from -120 mV to 20 mV.

4.5 Competitive and Non-competitive Inhibition

Capsaicin may exert its effect by competing with agonist (ACh) at the same binding site on the α7-nAChR, and may act as a competitive antagonist. Therefore, to investigate whether capsaicin acts as a competitive or noncompetitive antagonist, we examined the effect of capsaicin at different ACh concentrations (ranging from 1 µM-1mM). As shown in Figure 38, concentration-response curves for ACh were plotted in the absence and presence of 10 µM capsaicin. In the presence of capsaicin, the maximal response induced by ACh (efficacy) was reduced significantly (n=6–8) whereas ACh EC50 values (potency) remained unaltered (EC50 values were 89 ± 12 µM in the absence of capsaicin (control), 78 ± 14 µM in the presence of capsaicin (P>0.05, ANOVA, n=6–8), suggesting that capsaicin inhibits the α7-nAChRs in a non-competitive manner.
Figure 38: Concentration-response curves for ACh-induced currents in absence and presence of capsaicin

In controls, currents were induced by ACh (1 μM to 1 mM) (filled circles). Subsequently, ACh dose-response curve was plotted in the presence of capsaicin after 10 min pretreatment with capsaicin (open circles). Responses normalized to maximal amplitude of ACh-induced currents. Each data point presents the normalized means ± S.E.M. of 6-8 experiments.

4.6 Effect of the Capsaicin on Other Members of Cys-loop Family of Ligand gated Ion Channel

The effect of 10 μM capsaicin was examined on the activity of the other members of the cys-loop family of ligand-gated ion channels (Figure 39). Capsaicin caused a significant inhibitory action (approx. 60%) on the amplitudes of currents mediated by α3β2, α4β4, and α4β2 nACh receptors, modest inhibitory action (approx. 20%) on 5HT3 receptor, α1β1, and α3β4 nACh receptors, and a small inhibition (less than 10%) of glycine α1 and α3-receptor-mediated currents. However, it caused modest potentiation (approx. 20%) of glycine α2-receptor-mediated currents.
Figure 39: Percent of inhibition or potentiation of 10 μM capsaicin on the other members of the cys-loop family of ligand-gated ion channels induced currents

(A) Subtypes of the nAChRs, (B) α7-nAChR, 5HT{sub}_3 and α₁, α₂, and α₃ glycine receptor. Each data point presents the normalized means ± S.E.M. of 5-6 experiments.
Chapter 5: Discussion

Using electrophysiological methods, the study provides first evidence that the plant-derived capsaicin inhibits the function of human α7-nAChRs expressed in *Xenopus* oocytes. Capsaicin used in this study was employed in the concentration range of 1-300 μM. The inhibitory effect of capsaicin on ACh-induced currents was reversible, and time- and concentration-dependent with IC$_{50}$ value of 8.6 μM.

In human studies, oral administration of 26.6 mg capsaicin was calculated to have a maximum plasma concentration ranges of 2.47 ± 0.13 ng/ml (Chaiyasit et al., 2009; O'Neill et al., 2012). Interestingly, repeated applications of capsaicin cream (0.025–0.075%; or 82-246 μM) have been shown to induce a modest pain relief in most patients with painful conditions such as diabetic neuropathy, postherpetic neuralgia, and postmastectomy pain (Szallasi & Blumberg, 1999; Wang et al., 2007). *In vitro* studies in rats showed that 15 min application of capsaicin (1%, 0.1 mL) to the sciatic or saphenous nerve induced a long-lasting (more than 4 weeks) increase in nociceptive threshold to heat, and prevented neurogenic inflammation in the affected paw (Jancso et al., 1980). Furthermore, Capsaicin can be detected in both brain and spinal cord after intravenous and subcutaneous injection in animals (O'Neill et al., 2012). *In vitro* studies showed that capsaicin application causes increase in the permeability of many biological barriers such as the blood-brain barrier and intestinal barrier (Beggs et al., 2010; Kaiser et al., 2018). Intragastric application of capsaicin (3.2-640 μM) reduced the haemorrhagic lesions caused by 25% ethanol in a concentration-dependent manner. In addition, capsaicin caused inhibition of HL-60 cells (human myeloid leukemia cells) growth in a concentration-dependent manner over the range of 5-50 μM (Zheng et al., 2005). However, existing *in vitro* studies have
found that capsaicin concentration of ~300 μM was cytotoxic in various cell lines (Anand & Bley, 2011; Jordt & Julius, 2002; Kaiser et al., 2018; Pecze et al., 2008). These results indicate that the concentration of capsaicin (IC$_{50}$ = 8.6 μM) in \textit{in vitro} studies is in a similar range to earlier reports, and of pharmacologically relevant concentration for its action.

Application of 10 μM concentration of capsaicin significantly inhibited the amplitude of 100 μM ACh- induced current up to 60% of the control. Preincubation of capsaicin caused an increase in the extent of α7-nAChRs inhibition reaching a maximal level within 2-5 min with half time (τ$_{1/2}$) of 0.32 ± 1 min. The time course required for capsaicin to exhibit its effect on α7-nAChRs was relatively slow which might indicate the possible interaction of capsaicin with the lipid membrane. Capsaicin is hydrophobic compound, and several \textit{in vitro} studies suggests that capsaicin may influence the structure of biological membranes (Aranda et al., 1995).

Earlier studies showed that capsaicin induces an increase in intracellular calcium (Bevan & Docherty, 1996; Buck & Ehrlich, 2005). The α7-nAChRs are known to have a high calcium permeability (Corradi & Bouzat, 2016; Ren et al., 2017; Williams et al., 2011). Activation of those receptors expressed on \textit{Xenopus} oocyte can cause a significant increase in intracellular Ca$^{2+}$ which could activate the endogenous Ca$^{2+}$-activated Cl$^{-}$ channels (Sands et al., 1993) resulting in alterations in the holding current under voltage-clamp conditions. The extent of capsaicin inhibition remained unchanged in oocytes injected with BAPTA and recorded in a solution containing 2 mM Ba$^{2+}$, indicating that Ca$^{2+}$- activated Cl$^{-}$ channels were not involved in capsaicin inhibition of α7-nAChR responses. Since Ca$^{2+}$- activated Cl$^{-}$ channels are highly sensitive to intracellular Ca$^{2+}$ levels (For reviews (Hartzell et al., 2005; Marin, 2012)),
changes in intracellular Ca\textsuperscript{2+} levels may affect the holding current under voltage-clamp conditions. However, such a change was not observed in our experiments (did not alter holding currents/ baseline), suggesting that intracellular Ca\textsuperscript{2+} concentration was not changed by capsaicin.

Several mechanisms have been proposed to explain the inhibitory effect of drugs acting on ion channels. For example, inhibition may occur from the closed state of the channel where the blocker enhances and stabilizes the closed state or prevents the opening of the channel in response to stimuli leading to decreased rate and/or duration of the opened state; or open-channel blockade where the presence of the agonist is essential to allow the blocker to enter the channel and physically obstruct the channel transmembrane pore in the open state after an agonist-induced conformational change (Hille, 2001). Open-channel blockers enter the transmembrane pore region of the ion channel protein and transiently occlude the passageway by binding noncovalently with the hydrophobic pore-lining residues (Lin & Licht, 2014). In the presence of the blocker, the channel can open and close normally in response to various stimuli, yet transiently and reversibly impeding the current flowing throughout the open pore (Hille, 2001).

Open-channel blockade is a widely used model to describe the inhibition of ligand-gated ion channels (Hille, 2001). Nevertheless, this model does not seem to be consistent with the results of the present study. For an open-channel blocker, the presence of the agonist is required to allow the blocker to enter the channel after an agonist-induced conformational change. In contrast to open channel blockers, preincubation with capsaicin enhanced its effect on α7 -nAChRs activity, suggesting that capsaicin interacts with the closed state of the receptor. Furthermore, inhibition
by capsaicin is not voltage sensitive, indicating that the capsaicin-binding site is not charged and not affected by the transmembrane electric field. In addition, capsaicin did not significantly affect the reversal potential of ACh-induced current, indicating that the inhibition is not due to alteration in the ion channel selectivity.

Increasing the concentration of ACh did not reverse capsaicin inhibition of ACh-induced currents. Additionally, in the presence of capsaicin, the ACh efficacy was reduced significantly whereas the potency remained unaltered. Both results suggest that capsaicin inhibits the α7-nAChRs function in a non-competitive manner.

Several recent studies suggested the potential use of capsaicin for the prevention of Alzheimer’s disease (AD). AD is the most predominant worldwide neurodegenerative disease which is characterized by a progressive cognitive impairment and behavioral disturbances. The aggregation of the amyloid β-protein and phospho-tau are the hallmarks of AD and are linked to synapse loss, senile plaques, and neurofibrillary tangles (Gonzalez et al., 2016). It has been shown that capsaicin ameliorates stress-induced AD-like neuropathological alterations and cognitive impairments (Chen et al., 2017; Jiang et al., 2013). Capsaicin prevents stress-induced tau hyperphosphorylation by abolishing inhibition of protein phosphatase 2A (Jiang et al., 2013). In addition, dietary capsaicin appears to prevent the AD-associated tau hyperphosphorylation by increasing the activity of PI3K/AKT and inhibiting glycogen synthase kinase-3β in hippocampus of type 2 diabetes rats (Xu et al., 2017). In a human study, a capsaicin-rich diet has been shown to exert favorable effects on AD blood biomarkers (Amyloid-β Levels) and cognitive function in middle-aged and elderly adults (Liu et al., 2016).
α7-nAChRs have implications in the regulation of cognitive processes such as memory and attention, and the agonists of α7-nAChRs have shown promise as a therapeutic target for the treatment of multiple cognitive dysfunctions such as Alzheimer’s disease (Pohanka, 2012). There is evidence that amyloid-β may cause cholinergic dysfunction through action on of α7-nAChRs (Gonzalez et al., 2016; Schliebs & Arendt, 2011). This interaction results in intra-neuronal accumulation of amyloid-β/α7-nAChRs complexes, mediating tau phosphorylation, severe α7-nAChRs impairment, defect in cholinergic neurotransmission, and neuronal cell death (Toyohara & Hashimoto, 2010). An in vitro study demonstrated that amyloid-β increases acetylcholinesterase activity through α7-nAChRs (Fodero et al., 2004). Moreover, stimulation of α7-nAChRs is neuroprotective, and reduces amyloid-β-induced neurotoxicity (Hernandez et al., 2010; Sadigh-Eteghad et al., 2015). Yet, the only approved drugs for symptomatic treatment of AD are acetylcholinesterase inhibitors (AChEIs), which include tacrine, galantamine, rivastigmine, and donepezil. The enzyme inactivation prevents the hydrolysis of ACh, and thus resulting in the accumulation of ACh in the synaptic cleft prolonging its action on nicotinic and muscarinic receptors (Čolović et al., 2013). Selective α7-nAChR agonist can be considered as a potential therapeutic strategy for AD-related cognitive impairments (Bali et al., 2015; Toyohara & Hashimoto, 2010). Memantine is NMDA receptor antagonist approved for treatment of AD. Memantine is a α7-nAChRs antagonist with an IC₅₀ value of 0.34 μM (Aracava et al., 2005). However, it is still debatable whether considering the antagonism of memantine on α7-nAChRs as a negative phenomenon or beneficial in Alzheimer’s disease treatment (Pohanka, 2012).

Moreover, α7-nAChRs are associated with a cholinergic anti-inflammatory pathway. Activation of α7-nAChRs has been shown to have anti-
inflammatory effects in several inflammatory disease models (Bertrand et al., 2015; Hone & McIntosh, 2018). A selective α7-nAChRs agonist inhibits proinflammatory cytokine production in macrophages by preventing the phosphorylation of STAT3 and in turn preventing activation of the NF-kB pathway (Bertrand et al., 2015; Pena et al., 2010). However, capsaicin tested in our study showed antagonistic actions on α7-nAChRs suggesting that its anti-inflammatory effects would not be the result of a capsaicin’s effect on nicotinic receptors.

Capsaicin has been shown to possess chemopreventive effects in in vivo studies by decreasing the progression of several tumors in animal models, as well as an anti-tumorigenic activity (Bhutani et al., 2007; Clark & Lee, 2016; Lau et al., 2012; Lu et al., 2010; Pramanik et al., 2011; Zhang et al., 2008). The exact cellular mechanisms are still not fully understood (Basith et al., 2016; Clark & Lee, 2016). Capsaicin has been recognized to induce apoptosis in various human cancer cells types both in vitro and in mice models. Moreover, recent studies have focused on the potential therapeutic use of capsaicin in the management and treatment of human small cell lung cancer (SCLC), breast cancer, prostate cancer and colon cancer (Rollyson et al., 2014). Furthermore, capsaicin has synergistic anti-cancer activities with chemotherapeutic drugs and other agents (Clark & Lee, 2016). Capsaicin improved the therapeutic efficacy of 12-O-tetradecanoylphorbol-13-acetate (TPA) in some myeloid leukemia cells (Zheng et al., 2005). Recent studies showed that capsaicin and 3,3’-diindolylmethane, a major metabolite of indole-3 carbinol found in cruciferous vegetables, works synergistically to induce apoptosis and inhibit cell proliferation in human colorectal cancer cells (Clark et al., 2015). In addition, capsaicin enhanced the anti-proliferation efficacy of pirarubicin, an anthracycline drug, through TRPV1 activation in bladder cancer (Zheng et al., 2016). These results suggest the possibility
of using capsaicin as an adjuvant in cancer therapy with other chemotherapeutic drugs. Interestingly, inhibition, rather than activation, of α7-nAChRs has been suggested to prevent cancer progression in some earlier studies (Brown et al., 2013; Catassi et al., 2008; Davis et al., 2009; Paleari et al., 2009; Singh et al., 2011).

Cigarette smoking or environmental tobacco smoke is an important and the greatest preventable risk factor (Schaal & Chellappan, 2014; Zhao, 2016) for various types of cancers such as lung cancers, pancreatic cancers, and esophageal cancers (Hecht, 2003; Zhao, 2016). Several recent findings suggest that α7-nAChRs mediated oncogenic signaling plays an important role in promoting cell proliferation, invasion, and angiogenesis (Schaal & Chellappan, 2014; Wu et al., 2011; Zhao, 2016). Interestingly, the chronic exposure to nicotine or nicotine-derived carcinogenic nitrosamines (e.g. NNK) upregulates the α7-nAChR (Schuller, 2012; Zhao, 2016). Results from in vitro and in vivo animal studies suggest that the nicotine-induced proliferative effect can be reversed with the use of α7-nAChR antagonists such as snake neurotoxins; α-BTX or α-CBT (α-cobratoxin) (Brown et al., 2013; Catassi et al., 2008; Davis et al., 2009; Paleari et al., 2009; Singh et al., 2011). Moreover, methyllycaconitine (MLA- α7-nAChR antagonists) was also found to attenuate the mitogenic activity of nicotine (Brown et al., 2013). In several experimental models of angiogenesis, MG624 (α7-nAChR antagonists) was found to inhibit angiogenesis of human small cell lung cancer (SCLC) (Brown et al., 2012). The angiogenic effect of nicotine was blunted in α7-nAChR-deficient mice (Heeschen, 2002). Thus, α7-nAChRs are a promising target for cancer therapy (Schaal & Chellappan, 2014; Zhao, 2016).
Finally, capsaicin not only has an inhibitory effect on α7-nAChRs but also on the other members of cys-loop family of ligand-gated ion channels. Capsaicin caused a significant inhibitory action (approx. 60%) on the amplitudes of currents mediated by α3β2, α4β4, and α4β2 nACh receptors, modest inhibitory action (approx. 20%) on 5HT3 receptor, α1β1, and α3β4 nACh receptors, and a small inhibition (less than 10%) of glycine α1 and α3-receptor-mediated currents. However, it caused modest potentiation (approx. 20%) of glycine α2-receptor-mediated currents. The pharmacological importance of these actions currently remains unknown.
Chapter 6: Conclusion

Capsaicin displays a variable beneficial role in the treatment of various cancers, obesity, diabetes, gastrointestinal, cardiovascular and dermatological conditions, and several other pathologies. To our knowledge, this study provides the first evidence of the effect of capsaicin on the function of human α7-nAChRs as well as other members of cys-loop family of ligand-gated ion channels α3β2, α4β4, α3β4, α1β1, and α4β2 nicotinic receptors, 5HT3 receptor, and glycine α1, α2 and α3-receptors. Although therapeutic effects of capsaicin have been thought to be mainly mediated through their agonistic action on the TRPV1 receptor, our results suggest that modulation of the functions of neuronal nicotinic receptors may also contribute to pharmacological actions of capsaicin.

6.1 Limitations and Future Directions

In our study, we did not examine the effects of capsaicin 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 would like to investigate the effects of capsaicin in nicotinic receptors of native neurons such as acutely dissociated rat hippocampal neurons. I am also interested in conducting experiments to further study the structure-activity relationship of capsaicin as inhibitor human α7-nAChRs on mammalian cell lines, and to perform molecular docking studies to understand the binding interaction of capsaicin and the human α7-nAChRs.

The expression of α7-nAChRs have been described in several cancer cell lines (Wu et al., 2011). Their stimulation with specific agonists (for example nicotine) is associated with promoting cell proliferation, inhibiting apoptosis of cancer cells, and
stimulating tumor angiogenesis (Zhao, 2016). Recent studies suggest that inhibition of α7-nAChRs has significant suppressing effect on cancer progression (Brown et al., 2013; Catassi et al., 2008; Davis et al., 2009; Paleari et al., 2009; Schaal & Chellappan, 2014; Singh et al., 2011; Zhao, 2016). Thus, the effects of capsaicin in nicotinic receptor expression, on the proliferation of different types of cancer cells needs to be investigated.
References


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