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THE LEVEL OF GABA\textsubscript{B} RECEPTOR SUBUNITS INCREASES IN THE RAT’S LIMBIC STRUCTURES FOLLOWING SODIUM SALICYLATE APPLICATION

By

Fareeha Ashraf

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2016

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THE LEVEL OF GABA$\text{B}$ RECEPTOR SUBUNITS INCREASES IN THE RAT'S LIMBIC STRUCTURES FOLLOWING SODIUM SALICYLATE APPLICATION

by

Fareeha Ashraf

APPROVED BY:

______________________________
Dr. N. Azar
Department of Kinesiology

______________________________
Dr. L.A. Porter
Department of Biological Sciences

______________________________
Dr. H. Zhang, Advisor
Department of Biological Sciences

June 16, 2016
AUTHOR’S DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

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ABSTRACT

Tinnitus is a phantom sensation of sounds in the absence of external acoustic stimuli. This hearing abnormality can be caused by ototoxic drugs such as sodium salicylate (SS) and lead to emotional responses such as stress. It is likely that tinnitus is caused by a change in the activity in the central nervous system, which is dependent on excitatory/inhibitory neurotransmission. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain that activates type-B GABAergic (GABA_B receptors), which in turn can regulate the excitation/inhibition throughout the nervous system. I hypothesize that there will be a change in the GABA_B receptors in the rat’s nervous system, specifically in the limbic system which is responsible for emotion and stress. Immunohistochemistry results indicate an increase in the level of GABA_B receptors after SS exposure. Tinnitus is commonly understood to cause an increase in activity in the central nervous system. An increase in GABA_B receptors can be indicative of an increase in pre-synaptic GABA_B receptors, which regulate the release the GABA.
DEDICATION

This thesis is dedicated to my loving parents who believed in me since kindergarten. I have truly been blessed with amazing parents.
ACKNOWLEDGEMENTS

First and foremost I would like to thank Allah for my accomplishments. Nothing is possible without His will.

I received help, guidance, and support from many people throughout my study. I would like to thank my supervisor Dr. Huiming Zhang. He was always there for guidance and motivation. He provided insight to many aspects of my research.

I would also like to thank my committee members, Dr. Lisa Porter and Dr. Nadia Azar for their guidance. I really appreciate them spending time to give me to important feedback and suggestions to make my thesis project better.

I also want to thank past and present lab members of the Zhang lab for their help, support, and motivation. I would especially like to thank Sehrish Butt, Nam Vo, Basma Alyashae, Jeff Aguilar, Mathiang Ghai, Dennis Chan, and Joshua Wright. Not only did these people help me through the academic aspect but also made the experience enjoyable. I wouldn’t have been able to do it without them.

Also, I would like to thank Dr. Barbara Zielinski for allowing me to use her lab equipment and space. I would also like to thank some of the lab members, especially Tina Suntres for being very supportive and informative in helping me with the equipment and making me feel welcomed.

I would like to thank the technical staffs that were always there to help me solve my problems. I would like to especially thank Linda Sterling, Melissa Gabrieau, Bob Hodge, Ingrid Churchill, Rodica Leu, and Nancy Barkley.

Last but not least, I would like to thank my friends and family for their constant support throughout the years. They have encouraged me to keep working during tough
times and take a break when needed. My parents have been very supportive of my education throughout the years. I could have not done this without their constant love and support. I would like to thank my husband, Rafey Ansar for being extremely understanding when long hours were spent in the lab and when I had to miss out on a few occasions. You have truly been by my side throughout this process and I appreciate you for that. I would like to thank my sisters, Uzma, Maham, and Zahra for helping me overcome stressful times. Without all these people, my research would not have been possible.
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LIST OF ABBREVIATIONS

AC: auditory cortex
AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BLA: basolateral nuclei of the amygdala
BMA: basomedial division of the amygdala
CA1: cornus ammonis-1
CA2: cornus ammonis-2
CA3: cornus ammonis-3
CAS: central auditory system
CBM: cerebellum
CeN: central nuclei of the amygdala
CN: cochlear nucleus
CNS: central nervous system
DG: dentate gyrus
GABA: gamma-aminobutyric acid
GAD: GABA decarboxylase
IC: inferior colliculus
IHC: immunohistochemistry
LaN: lateral nuclei of the amygdala
MGN: medial geniculate nucleus
NLL: nucleus of the lateral lemniscus
NMDA: N-methyl-D-aspartic acid
PNS: peripheral nervous system

SOC: superior olivary complex

SUBd: dorsal division of the subiculum

SUBv: ventral division of the subiculum

SS: sodium salicylate
1. Introduction

1.1 Hearing and hearing abnormalities

Hearing is the ability of an organism to sense and perceive sound vibrations. It is one of the five basic senses (Carlson, 2001). Hearing is important for the biological fitness of animals, including humans. It is required by many animal behaviours such as mating and social communication. Hearing is not only dependent on the ears but also dependent on a group of brain structures collectively called the central auditory system (Pickles, 2012).

Structural, neurochemical, and neurophysiological changes in the ear and/or the central auditory system can lead to hearing abnormalities, such as deafness, hyperacusis, and tinnitus. While hyperacusis is heightened sensitivity to sound, tinnitus is phantom sensation of sound in the absence of external acoustic stimulation (See section 1.7 for details).

Recent studies indicate that auditory neural processing centers receive inputs from other brain regions such as limbic structures (Kraus and Canlon, 2012). In individuals with tinnitus, the neural activity increases in limbic structures. Due to the neural connectivity, changes in the inputs from these structures can affect neural responses in the auditory system, which can also lead to hearing abnormalities (Kraus and Canlon, 2012; Chen et al., 2014).

1.2 The auditory periphery

The peripheral auditory system, i.e., the ear, consists of the outer, middle, and inner ears. The outer ear consists of the pinna and the ear canal. The middle ear contains three oscicles, i.e., the malleus, incus, and stapes. The inner ear contains the cochlea, a
spiral-shaped structure that has the appearance of a coiled snail’s shell (Silverthorn, 2007; The peripheral auditory system, 2016).

The cochlea has a hard bony shell encompassing a membranous system. The membrane important for sensory transduction, i.e., the conversion of mechanical vibrations into electrical neural signals, is called the basilar membrane. The basilar membrane separates the fluid-filled interior of the cochlea into two major parts. The lower part is called the scala tympani and is filled with perilymph, which is low in $K^+$ concentration and high in $Na^+$ concentration (Johnstone and Sellick, 1972; The peripheral auditory system, 2016). The upper part is further divided by the Reissner’s membrane into two compartments. The top compartment is known as the scala vestibule, which contains perilymph in similar concentration to the scala tympani. The other compartment is known as the scala media, which contains endolymph that is high in $K^+$ concentration and low in $Na^+$ concentration (Bosher and Warren, 1968).

The Organ of Corti is an auditory sensory organ that is found in the basilar membrane. It has two types of mechanoreceptor cells: inner hair cells (IHCs) and outer hair cells (OHCs). These mechanoreceptors have stereocilia on their apical surfaces. The stereocilia are connected to one another by tip-links. When a sound reaches the ear, it eventually vibrates the basilar membrane and an overlaying membrane named tectorial membrane. The relative movements between the two membranes cause the stereocilia of hair cells to bend and mechanical force-gated ion channels attached to the tip-links to open. $K^+$ ions then flux from the endolymph into a hair cell, causing depolarization of the cell (Hudspeth, 1982; The peripheral auditory system, 2016). In this way, mechanical signals are transduced (i.e., converted) into electrical signals, which can be sent through
the cochlear nerve to the central auditory system (CAS) for processing (Silverthron, 2007).

1.3 The central auditory system (CAS): Major structures and interconnections

There are six major structures in the central nervous system with primary functions related to hearing. These structures include the cochlear nucleus (CN), superior olivary complex (SOC), nucleus of lateral lemniscus (NLL), inferior colliculus (IC), medial geniculate nucleus (MGN), and the auditory cortex (AC). These six major auditory structures form an ascending pathway, with CN at the bottom receiving auditory signals from the cochlea in the inner ear and the AC at the top of the hierarchy. Such a hierarchy has been conserved in mammals throughout evolution and thus a homology to humans exists (Figure 1).

1.3.1 Ascending auditory pathway

1.3.1.1 Cochlear nucleus (CN)

The first brainstem auditory structure along the ascending pathway is the CN (Figure 2). It consists of two major subdivisions, the dorsal CN (DCN) and the ventral CN (VCN). The VCN can be further subdivided into the anterior VCN (AVCN) and posterior VCN (PVCN) (Malmierca and Merchan, 2005). The CN receives direct inputs from the peripheral auditory system through the auditory nerve (i.e., the 8th cranial nerve). It sends projections to the SOC, NLL, IC, and MGN (Schofield and Coomes, 2005). There are different cell types in each subdivision of the CN. These cells have different cell morphology and temporal patterns of action potential discharge in response to acoustic stimulation. For instance, globular and spherical bushy cells in the AVCN fire
Figure 1: Visual representation of the ascending and descending projections in the nervous system a human and rat brain. (A) Visual representation of the ascending projections found in the human brain (left) and the rat brain (right). (B) Descending projections found in the human brain (left) and the rat brain (right). [Courtesy of Dina Kokh].
over the whole duration of a stimulus. Octopus cells found in the PVCN only fire at the onset of a stimulus. Fusiform and pyramidal cells in the DCN fire at the offset of a stimulus (Gil-Layzaga et al., 2013). The CN is involved in analyzing the intensity, duration, and on- and offset of an acoustic stimulus (Gil-Layzaga et al., 2013).

1.3.1.2 Superior olivary complex (SOC)

The SOC is the second brainstem structure along the ascending auditory pathway (Figure 2). The SOC is composed of six small subdivisions (Malmierca and Merchan, 2005). These subdivisions include the lateral nucleus of the trapezoidal body (LNTB), the ventral nucleus of trapezoidal body (VNTB), the medial nucleus of the trapezoidal body (MNTB), the lateral superior olivary nucleus (LSO), the medial superior olivary nucleus (MSO), and the superior paraolivary nucleus (SPN). SOC receives inputs from the CN and sends projections to the NLL and IC (van Adel and Kelly, 1998). Major functions of the SOC are related to the localization of sounds (van Adel and Kelly, 1998). Two of the major subdivisions, the MSO and LSO, are especially important for these functions. While the MSO is critical for localizing sounds by using interaural-time difference (ITD) cues, the LSO is essential for localizing sounds by using interaural-level difference (ILD) cues (Tollin, 2003). Other functions of the SOC are related to the suppression of echoes of sounds from the environment (Malmierca and Merchan, 2005).

1.3.1.3 Nucleus of the lateral lemniscus (NLL)

The NLL is the last brainstem structure along the ascending auditory pathway (Figure 2). This structure consists of two different groups of neurons associated with two different innervation patterns. Neurons in the ventral NLL (VNLL) receive inputs driven
Figure 2: Schematic diagram showing the major ascending projections in the central auditory structures. Abbreviations: AC: auditory cortex, MG: medial geniculate body, IC: inferior colliculus, NLL: nucleus of lateral lemniscus, SOC: superior olivary complex, CN: cochlear nucleus [Modified from Winer and Schreiner, 2005].
by only the ear on the contralateral side. These monaural neurons are involved in processing temporal characteristics of sounds (Gil-Layzaga et al., 2013). Neurons in the dorsal NLL (DNLL) receive diverse inputs, with some of them driven by the contralateral ear while others driven by both ears. Neurons in the DNLL are involved in sound localization (Aitkin et al., 1970; Bruggae et al., 1970, Bajo et al., 1998; Gil-Layzaga et al., 2013).

1.3.1.4 Inferior Colliculus (IC)

The IC is a midbrain auditory structure (Figure 2). It receives projections from all the major brainstem structures (CN, SOC, and NLL) and forebrain structures (MGN and AC, see section 1.3.1.5 and 1.3.1.6). The IC is the primary source of projections for the MGN, which relays auditory information to the AC (Malmierca and Merchan, 2005). The IC includes three subdivisions: the central nucleus of the IC (ICc), the dorsal cortex of the IC (ICd), and the external cortex of the IC (ICx). Neurons in the ICc are organized in such a way, that neurons sensitive to low frequencies are located in the dorsolateral parts while neurons sensitive to high frequencies are located in the ventromedial part of the structure. This topographic organization (i.e., tonotopic organization) allows for processing of frequency information of sound. Among other acoustic characteristics processed by the ICc are the ITD and ILD. The involvement of this collicular subdivision in processing these aboustic characteristics suggests that it contributes to the localization of sounds (Gil-Layzaga et al., 2013). The ICd and ICx are involved in processing higher order information, such as language and identifying novel sounds.

1.3.1.5 Medial geniculate nucleus (MGN)
The MGN is a thalamic structure and it is the only structure that sends direct projections to the AC (Figure 2). The MGN, along with the AC, is a forebrain auditory structure. The MGN can be subdivided into the ventral division of the MGN (MGv), the dorsal division of the MGN (MGd), and medial division of the MGN (MGm).

The MGv

The MGv receives major projections from the contralateral ICc and ipsilateral ICc, ICd, and ICx (Bordi and LeDoux, 1994; Gonzalez-Hernandez et al., 1991; Malmierca and Merchan, 2005). It sends projections to the AC as well as the IC (Winer and Schreiner, 2005). The MGv is involved in the processing of basic characteristics of sounds including temporal structures and frequency content (Bordi and LeDoux, 1994).

The MGd

The MGd receives projections from the ICd, ICx, and somatosensory system (Winer, 1985). It sends projections to the ICx and secondary AC areas (Winer, 1992; Malmierca and Merchan, 2004). The main function of the MGd is most likely related to the processing of complex sounds, including the recognition of sound patterns (Bordi and LeDoux, 1994).

The MGm

The MGm is a small subdivision receiving projections from many structures. It provides outputs to a variety of other structures. Auditory input projections include those from the ICx, ICd, CN, SOC, VNLL, and the reticular thalamic nucleus (Winer, 2005). The MGm communicates with structures outside the major auditory pathway, which include the somatosensory cortex (Spreafico et al., 1981), visual system (Malmierca et al., 2002), and the amygdala (Otterson and Ben-Ari, 1979; Otterson, 1981; LeDoux,
1985, 1987). The complex connections render the MGNm a variety of functions. It is believed that the structure is involved in multisensory integration (Bordi and LeDoux, 1994) and processing of emotional characteristics of sounds (LeDoux et al., 1984).

1.3.1.6 Auditory Cortex (AC)

The AC is a part of the temporal cortex. Along with the MGN, the AC is a key forebrain auditory center (Figure 2). It can be subdivided into the primary auditory cortex (A1), the secondary auditory cortex (A2), and association auditory cortices. The A1 is sometimes also referred to as the core area of the auditory cortex, while the other areas are sometimes also referred to as the belt areas (Gil-Layzaga et al., 2013). The AC can be subdivided into six layers. From the surface to the deep part of the structures are layers I through VI (Gil-Layzaga et al., 2013). The AC plays a critical role in the sensation, perception, and cognition of sounds (Gaese and Ostwald, 1995). Neurons in the A1 are arranged topographically based on frequency that they best respond to. The ones that respond best to low frequencies correspond to the apex of the cochlea and the ones that respond best to high frequencies correspond to the base of the cochlea. Such a tonotopic organization is important for the processing of frequency information of sounds. The belt areas of the AC do not have tonotopic organizations. Neurons in this subarea are involved in sound localization, auditory memory, and understanding complex sounds such as language. Neurons in the belt regions also contributes to the integration of auditory information with other sensory systems (Gil-Layzaga et al., 2013).

1.3.2 Descending and commissural projections in the central auditory system

Major auditory structures not only provide ascending projections to higher structures in the hierarchy of the auditory system, they also provide descending
Figure 3: Schematic diagram showing the major descending projections in the central auditory system. Abbreviations: AC: auditory cortex, MG: medial geniculate body, IC: inferior colliculus, NLL: nucleus of lateral lemniscus, SOC: superior olivary complex, CN: cochlear nucleus [Modified from Winer and Schreiner, 2005].
projections to lower structures and commissural projections to the contralateral side of the brain (Figure 3).

1.3.2.1 Major structures providing descending projections

1.3.2.1.1 Auditory cortex (AC)

Being at the very top of the hierarchal auditory pathway, the auditory cortex sends many descending projections to other structures. Neurons from both layer V and VI send ipsilateral projections to the MGNv and MGNd (Winer and Prieto, 2001). Neurons in the layer V of the AC also send descending projections to the IC (Schofield, 2009). These cortico-colliclular connections are of critical importance for auditory processing. Projections from the AC are also sent to the CN (Schofield and Coomes, 2005).

1.3.2.1.2 Medial geniculate nucleus (MGN)

The MGN does not send many descending projections. However, there are some ipsilateral projections to the IC. Specifically, these projections are sent to the ICx subdivision of the IC (Malmierca et al., 1999).

1.3.2.1.3 Inferior colliculus (IC)

The IC sends descending projections to the CN (Schofield and Coomes, 2005). It can send projections further downward to the periolivary medial olivocochlear cells, which are cells that innervate the OHCs (Faye-lund, 1986; Caicedo and Herbert, 1993; Saldana, 1993; Vetter et al., 1993).

1.3.2.1.4 Nucleus of lateral lemniscus (NLL)

The NLL is a small structure that does not have many descending projections. The CN is the only one auditory structure that receives descending projections from the NLL (Schofield and Coomes, 2005).
1.3.2.1.5 Superior olivary complex (SOC)

The only central target structure of descending projections from the SOC is the CN (Schofield and Coomes, 2005). Descending neural projections from the SOC are also sent to the cochlea of the inner ear. These projections provide protection to sensory receptor cells in the cochlea. When these cochlear cells are exposed to a loud sound (>80dB), the descending projections from the SOC provide signals to cause middle ear muscles to contract and the basilar membrane to become rigid, thereby reducing the movement of the basilar membrane and decreasing the energy being provided to the inner ear (Gil-Layzaga et al., 2013).

1.3.2.1.6 Cochlear nucleus (CN)

The CN is at the very bottom of the hierarchy. Due to this, it does not provide any descending projections to other central auditory structures. However, it is one of structures that receive many descending projections. It receives projections from the AC, IC, and SOC (Schofield and Coomes, 2005).

1.3.2.2 Major commissural projections

Commissural projections exist throughout the CNS. These are projections that connect structures from both sides by crossing the midline (Gonzalez-Hernandez et al., 1986; Saldana and Merchan, 1992). The mammalian forebrain consists of two main cortical hemispheres. Interconnections exist between the two due to major white matter tracts, known as the corpus callosum (Robichaux et al., 2016). There exists commissure of Probst that are found between the DNLL of both sides and also from the DNLL to the ICc. Binaural responses in DNLL are mostly influenced by these projections (Beyerl, 1978; Adams, 1979; Zook and Casseday, 1982; Bajo et al., 1993; Merchan et al., 1994).
There also exist commissure projections between the two IC structures on both sides of the brain. These are known as the commissure of the inferior colliculus and include many excitatory fibres and some inhibitory fibres (Hernandez et al., 2006).

1.4 The limbic system and its relation with the auditory system

The auditory system is regulated by other systems in the brain. Such regulation aids in hearing (Galazyuk et al., 2012; Kraus and Canlon, 2012). The systems involved in the regulation of auditory functions include the limbic system (Figure 4). Existing evidence indicate that major limbic structures including the amygdala and hippocampus either directly or indirectly innervate auditory structures including the MG and IC (Moller and Rollins, 2001; Kraus and Canlon, 2012). The projections to the MG are likely involved in the gating of auditory signals relayed from the MG to the AC (Muhlau et al., 2006; Rauschecker et al., 2010). The MGN sends direct projections to the amygdala, which innervates the hippocampus. The hippocampus sends direct projections to the amygdala, which innervates the IC (Kraus and Canlon, 2012). The reciprocal innervations between the auditory and limbic system provide structural bases of limbic regulation of hearing.

1.4.1 Amygdala

The amygdala is an almond-shaped structure found in the temporal lobe of the brain. The structure is well known for its role in emotions such as stress (Sah et al., 2003). Besides, the structure is involved in the processing of information related to all major sensory modalities including audition, olfaction, somatosensation, gustation, and vision (Sah et al., 2003). The amygdala can be subdivided into four subdivisions (Figure 5): the lateral nucleus (LaN), central nucleus (CeN), basolateral nucleus (BLN), and
Figure 4: Neural connectivity between auditory and limbic systems. Green: represents the auditory structures. Purple: represents the amygdala. Blue: represents the hippocampus. [Kraus and Canlon, 2012]
Figure 5: Major subdivisions in the amygdala. Blue: represents the lateral and basolateral subdivisions. Green: represents the central and basomedial subdivisions. [Sah et al., 2003]
basomedial nucleus (BMN) of the amygdala (Le Doux, 2007). These subdivisions differ in cytoarchitectonics and connectivity (Krettek and Price, 1978; Pitkanen, 2000).

**Basolateral Amygdala (BLA)**

The BLA, sometimes referred to as the basal amygdala, receives major projections from the subicular regions of the hippocampus (see below). Among all the amygdaloid subnuclei, the BLA is the main target for fiber projections from the hippocampus (Canteras and Swanson, 1992). The BLA sends projections to the hippocampus, prefrontal cortex, nucleus accumbens, and the thalamus (Pare et al., 1995).

The cells of the BLA have been well defined based on Golgi impregnation staining. Almost 70% of the cells in the BLA are pyramidal cells (Hall, 1972; Millhouse and DeOlmos, 1983; Washburn and Moises, 1992). These pyramidal neurons have randomly oriented apical dendrites (McDonald, 1992; Rainnie et al., 1993; Pare et al., 1995; Faber et al., 2001). The second types of cells found in the BLA look like the nonspiny stellate cells found in the neocortex (Hall, 1972). Other cells found in the BLA include the extended neurons, cone cells, chandelier cells, and neurogliaform cells (Faulkner and Brown, 1999; Kamal and Tombol, 1975; McDonald, 1982).

**Lateral nucleus of the amygdala (LaN)**

The LaN serves as a point of entry of the amygdala. Most of the structures innervating the amygdala contact the LaN, which relays neural signals to the other subdivisions of the amygdala (Sah et al., 2003). The LaN receives inputs from MGN (LeDoux et al., 1990; LeDoux et al., 1991; Turner and Herkenham, 1991). Retrograde tract-tracing studies revealed no direct projections from the Te1 to the amygdala (Mascagni et al., 1993; Shi and Cassell, 2001). However, projections from other cortical...
regions such as Te3 to the LaN do exist (LeDoux et al., 1991; Shi and Cassell, 2001). In addition to these connections with forebrain auditory structures, the LaN has connections with brain areas responsible for long-term memory. These areas include the perirhinal cortex, entorhinal cortex, parahippocampal cortex, and the hippocampus (Macdonald, 1998; Milner et al., 1998; Pitkanen et al., 2000).

The cell types found in the LaN are quite similar to those found in the BLA. Generally, cells in the LaN are smaller than those in the BLA. The cells in the LaN have an average soma size of 10-15\(\mu\)m, whereas the cells in the BLA have an average soma size of 15-20\(\mu\)m (McDonald, 1982; Millhouse and DeOlmos, 1983).

**Basomedial amygdala (BMA)**

The BMA receives fiber innervations from the LaN and BLA (Sah et al., 2003). It sends fiber outputs to the CeN, which acts as the output station and relays neural signals to other structures in various regions of the CNS, including the auditory system (Pitkanen et al., 1997).

There is only one known cell type in the BMA. These cells have relatively small or medium sizes and ovoid shapes. Most neurons have two to four spiny primary dendrites in this structure (McDonald, 1992).

**Central nucleus of the amygdala (CeN)**

The CeN sends projections to many target structures that are responsible for various different functions. Among these targets, the hypothalamic nucleus is involved in the activation of the autonomic system (Kapp et al., 1982; LeDoux et al., 1988). Other target structures include the periaqueductal gray, which can modify vocal and cardiovascular functions (Behbehani, 1995; Rizvi et al., 1991). The parabrachial nucleus
Figure 6: Major subdivisions in the hippocampus. (A) shows a rostral slice of the hippocampus. Major subdivisions are colour coordinated with the legend at the bottom. (B) shows a caudal slice of the hippocampus. Major subdivisions are colour coordinated with the legend at the bottom. [Modified from Kjonigsen et al., 2011]
is involved in pain processing (Gauriau and Bernard, 2002; Moga and Gray, 1985). In addition, the CeN also send fiber projections to the stria terminalis (Dong et al., 2001) and nuclei in the midbrain, pons, and medulla (Veening et al., 1984).

There are three different cell populations in the CeN. The predominant cell type is known as the medium spiny neurons (McDonald, 1982; Hall, 1972). Another type has larger soma than the medium spiny neurons and a thick aspiny dendrite (Schiess et al., 1999; Cassell and Gray, 1989). The third type of neurons are aspiny neurons. They exist throughout the CeN but are small in number (Cassell and Gray, 1989). The three types of cells exist homogenously throughout the CeN (Sah et al., 2003).

1.4.2 Hippocampus

The major function of the hippocampus is related to the formation of memory (Tulving and Markowitsch, 1998), although the structure is also involved in other high functions such as stress (McEwen, 1999).

The structure receives projections from other structures. One of these other structures is the amygdala, which sends many projections to the different subdivisions of the hippocampus (Ottersen, 1982; Kishi et al., 2006; Cenquizca and Swanson, 2007).

Target structures of projections from the hippocampus include olfactory bulb (Cenquizca and Swanson, 2007), primary visual cortex (Miller and Vogt, 1984), entorhinal cortex (Burwell and Amaral, 1998), and hippocampal formation (Verwer et al., 1997). Major subdivisions of the hippocampus include the cornus ammonis 1-3 (CA1-CA3), dentate gyrus (DG), and subicular area (SUB) (Figure 6) (O’Mara, 2005; Amaral et al., 2007; Mohedano-Moriano et al., 2007). The CA1-CA3 and the DG are collectively
called the hippocampus proper, while the SUB is sometimes also referred to as the hippocampal formation.

*Cornus ammonis I (CA1)*

The CA1 is a large part of the hippocampus proper. Extensive projections have been found between this area and other neural subdivisions and structures. Major projections to CA1 are from other regions of the hippocampus and subdivisions of the amygdala. Strong projections originating from within the hippocampus to the CA1 include those from the CA3 (Swanson and Cowan, 1977) and subicular region, specifically the dorsal SUB (Witter et al., 1990). Strong projections originating from outside the hippocampus include those from the BMA (Canteras et al., 1992) and BLA (Savander et al., 1995) of the amygdala. Moderate projections to CA1 are from the LaN (Petrovich et al., 2001), while weak projections to CA1 are from the dentate gyrus (Swanson et al., 1978).

The CA1 sends projections to the four major amygdaloid subdivisions including the BLA, BMA, CeN, and LaN (Ottersen, 1982; Kishi et al., 2006; Cenquizca and Swanson, 2007). The CA1 provides weak projections to the primary auditory cortex (Paperna and Malach, 1991).

Pyramidal cells are the main type of cells found in the CA1 area. The pyramidal cells found in the CA1 are morphologically identifiable specifically as CA1 pyramidal cells. They differ in cytoarchitecture from other pyramidal cells found throughout the hippocampus, i.e CA2-CA3, SUB pyramidal cells (Amaral and Lavenex, 2007).

*Cornus ammonis 2 (CA2)*
The CA2 is found between CA1 and CA3 and is the smallest region of the hippocampus proper. It is not studied as extensively as the other subdivisions. Nevertheless, there evidence showing that the CA2 has projections to and from other structures. Most incoming projections are from the amygdala, specifically the BLA and BMA (Petrovich et al., 2001). Neurons in the CA2 project to the SUBd (Swanson and Cowan, 1977). Other projections from the CA2 are relatively moderate/weak. Targets of these projections include the primary auditory cortex (Paperna and Malach, 1991), primary motor cortex (Reep et al., 1990), and primary visual cortex (Miller and Vogt, 1984).

The major type of cells in the CA2 are pyramidal cells. Morphologically these appear smaller than the pyramidal cells found in the CA1, CA3, and SUB regions. This allows for identification of CA2 pyramidal cells to be made possible. (Amaral and Lavenex, 2007).

*Cornus ammonis* 3 (CA3)

The CA3 covers a larger area of the hippocampus proper. Major projections to this hippocampal region include those from other hippocampal regions and from structures outside the hippocampus. Other hippocampal regions providing projections to the CA3 include the dentate gyrus (Swanson et al., 1978), the subiculum (Kohler, 1985), and the CA1 (Swanson et al., 1981). Non-hippocampal regions sending major projections to the CA3 include the BLA and BMA of the amygdala (Pikkarainen et al., 1999). The CA3 sends projections to the CA1, ventral and dorsal SUB, CA2, and dentate gyrus (Swanson and Cowan, 1977). CA3 also send projections to the olfactory bulb, dorsal auditory cortex (Swanson and Cowan, 1977), and the visual cortex (Miller and Vogt,
Furthermore, projections from CA3 are sent to the BLA and BMA of the amygdala (Swanson and Cowan, 1977).

The CA3 pyramidal cells are the main type of cells found in the CA3 region. These cells branch out more than those found in CA1-CA2 regions (Amaral and Lavenex, 2007).

**Dentate Gyrus (DG)**

The DG is the largest subdivision within the hippocampus proper. Inputs to the DG include those from the CA3 (Swanson and Cowan, 1977) and subiculum (Kohler, 1985). The BLA, BMA, and CeN of the amygdala also send projections to the dentate gyrus (Petrovich et al., 2001). Major projections from the DG are sent to the CA3. Projections are also sent to the subiculum and CA1 (Swanson et al., 1978) as well as to the BLA and LaN regions of the amygdala (Ottersen, 1982).

Granule cells are the main type of cells in the DG. These cells are morphologically very different from pyramidal cells found in other areas of the hippocampus. Granule cells in the DG are not as densely packed and have different orientations, in contrast to pyramidal cells in other hippocampal regions, which are aligned with similar orientations in a thin sheet (Amaral and Lavenex, 2007).

**Subiculum**

The subiculum is considered to be a part of the hippocampus. However, it is not a part of the hippocampus proper. The subiculum can be further subdivided into the dorsal subiculum (SUBd) and the ventral subiculum (SUBv) (Verwer et al., 1997; Cenquizca and Swanson, 2007). Differences exist between the two regions of the subiculum in fiber innervation.
The SUBd receives projections from the CA2 (Swanson and Cowan, 1977), CA3 (Swanson and Cowan, 1977), and DG (Swanson et al., 1978). The SUBd sends projections to the CA1 and the SUBv (Swanson and Cowan, 1977). It also sends projections to the BLA and BMA. Also, weak projections are provided by the subiculum to the dorsal auditory cortex (Witter et al., 1990).

The SUBv receives projections from the CA3 (Swanson and Cowan, 1977) and DG (Swanson et al., 1978). The SUBv sends major projections to the amygdala, including the BLA, BMA (Kishi et al., 2006), LaN (Ottersen, 1982), and CeN (Canteras and Swanson, 1992).

Projecting pyramidal cells are the major type of cells found in the SUB. These cells are not as densely packed as the pyramidal cells in the CA1-CA3 region. However, they do have a specific aligned orientations as seen in those regions. (Amaral and Lavenex, 2007).

1.4.3 Auditory-limbic connections

The auditory and limbic structures are connected with one another through some major areas. Overall, the auditory structures form direct connections with the amygdala and indirect connections with the hippocampus (Kraus and Canlon, 2012). The MGN is the key structure that sends direct projections to the LaN of the amygdala (Graybiel, 1972; Aitkin, 1986). The AC sends projections to the LaN (Kraus and Canlon, 2012). The LaN then sends projections to the BLA, which sends direct projections to the CA1, CA3 and indirect connections to the SUB (Kraus and Canlon, 2012).

Projections towards the auditory system from the limbic system involve direct projections from the amygdala and indirect projections from the hippocampus. The CA1
and SUB send projections to the LaN, BMA, and BLA in the amygdala. The BLA then sends direct projections to the IC in the auditory pathway (Sah et al., 2003; LeDoux, 2007; Kraus and Canlon, 2012).

1.5 Neurotransmission in the central nervous system

Relay of signals from one neuron to the next is dependent on a synapse. At a synapse, molecules of a specific neurotransmitter are released from a presynaptic terminal and bind onto postsynaptic neurotransmitter receptors. There are a variety of neurotransmitters found in the CNS including glutamate and gamma-aminobutyric acid (GABA) (Silverthorn, 2013).

Neurotransmitter receptors can be either ionotropic or metabotropic. An ionotropic receptor is also an ion channel. When bound by a neurotransmitter, such a receptor opens the ion channel and allows for the movement of ions across the cell membrane. An ion flux changes the membrane potential, leading to either an excitatory or inhibitory effect depending on the species of ions moving across the cell membrane. Generally, such an ion flux and membrane voltage change is short-lived (Purves et al., 2001; Silverthorn, 2013).

A metabotropic receptor itself is not an ion channel. Such a receptor is coupled to a guanine nucleotide-binding protein (G-protein). When a neurotransmitter molecule binds onto the receptor, G-protein molecules are activated. Subunits of the G-protein dissociate from each other. Dissociated G-protein subunits can directly modify ion channels to either enhance or reduce their opening probability. The subunits can also activate target proteins to produce second messengers, which in turn can initiate various intracellular signaling cascades and lead to modification of ion channels. As a result,
opening probabilities of these ion channels can be enhanced or suppressed. Enhanced or suppressed ion fluxes can change the cell membrane voltage. Thus, a cell can be excited or inhibited. Multiple steps between the activation of a metabotropic receptor and changes in the opening probability of ion channels lead to a slow and long-lived membrane voltage alteration (Purves et al, 2001; Silverthorn, 2013). Furthermore, binding between one single receptor by a neurotransmitter can affect multiple ion channels.

1.5.1 Glutamatergic neurotransmission

Glutamate is an amino acid neurotransmitter. Both ionotropic and metabotropic glutamatergic receptors exist in the nervous system. Major ionotropic glutamatergic receptors include the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, the N-methyl-D-aspartic-acid (NMDA) receptor, and the kainate receptor (Silverthorn, 2013). Metabotropic glutamate receptors are divided into Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3), and Group III (mGluR4, mGluR6, mGluR7 and mGluR 8) (Ferraguti and Shigemoto, 2006).

1.5.1.1 AMPA receptors

AMPA receptors are permeable to monovalent cations. After binding by glutamate, an activated AMPA receptor allows sodium ions to move into a cell and potassium ions to leave the cell. At or near the resting potential of a neuron, more sodium ions enter the neuron than potassium ions leaving the neurons. The net ion influx leads to an excitatory post-synaptic potential (EPSP) and a depolarization of the cell membrane (Silverthorn, 2013).

1.5.1.2 NMDA receptors
The NMDA receptors are different from the other ionotropic glutamatergic receptors (i.e., AMPA and kainite receptors) in a few aspects. First, these receptors are permeable to not only sodium and potassium ions, but also calcium ions. Second, the activation of the receptors is dependent not only on binding by glutamate, but also on other factors including binding by a co-agonist glycine and removal of blockage by magnesium ions. The later is dependent on membrane depolarization, which is normally provided by activation of AMPA receptors on the cell membrane. An activated NMDA receptor allows for an influx of sodium and calcium ions and efflux of potassium ions. The resulting net influx of cations leads to an EPSP and depolarization of the cell membrane. As calcium can serve as a second messenger and affect many aspects of intracellular signalling, the permeability of the NMDA receptor to this ion makes the effect of activation of the receptor long lasting. It has been found in many structures of the brain that tetanic stimulation of synaptic inputs can enhance the excitatory response of neurons and the enhancement of response can last for hours. Such an enhancement is known as long-term potentiation and is largely dependent on the activation of the NMDA receptors (Silverthorn, 2013).

1.5.1.3. Kainate receptors

Kainate receptors are similar to AMPA receptors. They exist in many structures such as the hippocampus, cortex, spinal cord, and retina. In many cases, kainate receptors are co-localized with AMPA and NMDA receptors. However, there are structures in which kainate receptors exist without co-localied AMPA and NMDA receptors. These structures include the retina, in which a synapse between a cone cell and an off-centre bipolar cell is mediated by kainate receptors (Huettner, 2003).
1.5.1.4. Metabotropic glutamatergic receptors

Metabotropic glutamate receptors are G-protein coupled receptors. These receptors are found throughout the mammalian CNS. Activation of a metabotropic glutamatergic receptor can lead to either an excitatory or an inhibitory response depending on downstream signaling cascades including the specific cell membrane ion channels modified (Ferraguti and Shigemoto, 2006).

1.5.2 GABAergic neurotransmission

GABA is a major inhibitory neurotransmitter found throughout the brain (Silverthron, 2013). Almost 40% of the synapses in the brain are GABAergic (Malcangio et al., 1996).

There are three subtypes of receptors to which GABA binds. These three are the type-A, type-B, and type-C GABA receptors (GABA_A, GABA_B, and GABA_C receptors) (Enna, 2001). GABA_A and GABA_B receptors are found in all the major central auditory processing centres, limbic structures including the amygdala and hippocampus, and cerebellum (Chu et al., 1990; Wamsley et al., 1986; McCabe and Wamsley, 1986; Zezula et al., 1988; Jamal et al., 2012; Liu et al., 2014). GABA_C receptors, however, are mostly found in the retina of vertebrae and are believed to be important for the processing of visual information (Bormann and Feigesnspan, 1995).

1.5.2.1 Type-A and type-C GABA receptors

Both GABA_A and GABA_C receptors are ionotropic receptors that are permeable to chloride ions upon activation. There are many isoforms of GABA_A and GABA_C receptors. Each functional receptor is made up of five subunits that surround a central channel, which is selective for chloride ions. Upon binding by GABA, the channel
Figure 7: GABA\textsubscript{B} receptor mechanism pre- and post-synaptically. After the GABA molecule binds to the GABA\textsubscript{B} receptor, the \(\beta\gamma\)-subunit dissociates from the \(\alpha\)-subunit. It moves to the \(K^+\) or \(Ca^{2+}\) channels postsynaptically or presynaptically, respectively, and leads to the opening of \(K^+\) channels and the closing of \(Ca^{2+}\) channels. [Chalifoux and Carter, 2011]
Figure 8: Protein conformation of a functional GABA$_B$ receptor with both subunits. Blue: shows the GABA$_B$R1 subunit. Yellow: shows the GABA$_B$R2 subunit. Both subunits have a venus flytrap (VFT) module and a heptahelical domain (HD). The VFT module of the GABA$_B$R1 contains an area for GABA binding. The HD of the GABA$_B$R2 contains area associated with the G-protein. [Pin et al., 2003].
becomes open, allowing negatively charged chloride ions to enter the cell and to cause an inhibitory post-synaptic potential (IPSP) (Sigel and Steinmann, 2012).

1.5.2.2 Type-B GABA receptors

The GABA$_B$ receptor is a metabotropic receptor with more complex functions than the ionotropic GABAergic receptors (Benke et al., 1999). GABA$_B$ receptors exist at both presynaptic and postsynaptic sites. In general, binding of a GABA$_B$ receptor by GABA leads to activation of a G-protein and subsequent modification of calcium or potassium ion channels (Filippov et al., 2000).

Activation of a presynaptic GABA$_B$ receptor can regulate the release of neurotransmitters including glutamate and GABA (Scholz and Miller, 1991; Mintz and Bean, 1993, Obreitan and Van Den Pol, 1999). Following binding of a GABA$_B$ receptor by GABA, a G-protein trimer dissociates into the $\beta\gamma$-subunit and $\alpha$-subunit. The $\beta\gamma$-subunit modifies a voltage-gated calcium channel, decreasing influx of calcium ions and thereby decreasing the release of neurotransmitters (Figure 7) (Herlitze et al., 1996; Ikeda, 1996). Activation of postsynaptic GABA$_B$ receptors can result in an IPSP. After GABA binds to a GABA$_B$ receptor, a G-protein dissociates into the $\beta\gamma$-subunit and $\alpha$-subunit. The $\beta\gamma$-subunit modifies the inward rectifier potassium channels (GIRK), which lead to enhanced potassium ion efflux and an IPSP (Figure 7) (Luscher et al., 1997; Ulrich and Bettler, 2007).

1.5.2.2.1 Type-B GABA receptor subunits

A functional GABA$_B$ receptor is a heterodimer comprised of two subunits, GABA$_B$R1 and GABA$_B$R2 (Kaupmann et al., 1998). The GABA$_B$R1 contains a venus flytrap (VFT) module, a domain that is capable of binding to the ligand GABA (Figure 8)
(Malitschek et al., 1999; Pin et al., 2004). The GABA\(_B\)R2 is coupled with a G-protein at its heptahelical domain (HD) (Figure 8) (Robbins et al., 2001; Pin et al., 2004). The GABA\(_B\)R1 has a retention signal that keeps it bound to the endoplasmic reticulum after the subunit is made (Pin et al., 2004). The two subunits interact with their coiled-coil domains. Once a heterodimer is formed between the two subunits, the retention signal is masked and the dimeric receptor can be brought towards the plasma membrane to become functional (Margeta-Mitrovic et al., 2000; Pagano et al., 2001).

GABA\(_B\)R1 consists of two splicing variants: GABA\(_B\)R1\(\alpha\) and GABA\(_B\)R1\(\beta\) (Bowery et al., 2002). They differ in the lengths of their N-terminus. GABA\(_B\)R1\(\alpha\) contains 163 amino acids in its N-terminus, whereas GABA\(_B\)R1\(\beta\) contains 47 amino acids. The levels of the two splicing variants change over different developmental stages. GABA\(_B\)R1\(\alpha\) is highly expressed before birth, whereas GABA\(_B\)R1\(\beta\) is highly expressed in adults (Fritschy et al., 1999). It is possible that GABA\(_B\)R2 has some variants as well, however, these have not been characterized yet (Chalifoux and Carter, 2011).

1.5.2.2 Distribution of Type-B GABA receptors in auditory and limbic systems

GABA\(_B\) receptors are found throughout all the major structures in the central auditory system. Structures showing a high level of GABA\(_B\) receptors include the ICd, DCN, MGN, and AC (Fubara et al., 1996; Jamal et al., 2012). Lower levels are found in the ventral and lateral parts of the IC, the NLL, the SOC, and ventral part of the CN. The GABA\(_B\) receptors also exist throughout the limbic system including both the hippocampus and amygdala. The hippocampus contains GABA\(_B\) receptors in all subdivisions, however, the strongest labeling is found in the CA1 and CA3 pyramidal
cells (Kulik et al., 2003). Immunohistochemistry and western blotting experiments have confirmed the localization of GABA_B receptors in the amygdala (Sah et al., 2003).

1.6 Neurotransmission and functions of the auditory and limbic systems

A balance between excitatory and inhibitory neurotransmission is critical for the normal function of the brain. An imbalance between excitation and inhibition can lead to changes in neural activity, which can have various behavioural consequences (Bauer et al., 1999; Gerken et al., 1984). The pre- and postsynaptic functions of the GABA_B receptor makes this receptor especially important for regulating the balance between excitation and inhibition.

In the central auditory system, a balance between excitation and inhibition is also critical for maintaining appropriate activities and functions of auditory neurons (Jastreboff and Sasaki, 1986; Chen and Jastreboff, 1995; Kenmochi and Eggermont, 1997). An abnormal reduction in inhibition and/or increase in excitation can lead to epileptiform firing of action potentials in an auditory neuron. Such a type of neural firing can certainly affect an auditory perception (Guitton et al., 2003; Guitton and Puel, 2004).

Functions of the hippocampus (Salvi et al., 2011) and the amygdala (De Ridder et al., 2006) also rely on the balance between excitation and inhibition. The main functions of the amygdala and hippocampus are emotion and memory, respectively. A proper balance between excitation and inhibition is required for these structures to function. A change to the balance can lead to differences in the functionality. An increase in activity in both structures is possible, which can be due to an increase in excitatory neurotransmission or a decrease in inhibitory neurotransmission (Chen et al., 2012).
Physiological studies have revealed that GABA_B receptors are involved in the regulation of sound-driven responses in auditory structures (Faingold et al., 1989; Vaughan et al., 1996; Szcepanial and Moller, 1996). GABA_B receptors in limbic structures are important for the activity of neurons, which are implicated in higher functions of the brain including stress and anxiety (Cryan and Kaupmann, 2005). Therefore, a change in GABA_B receptors can affect auditory functions as well as stress and anxiety.

1.7 Tinnitus

Tinnitus, i.e., ringing in the ear, is amongst the major types of hearing problems. An individual with tinnitus has a phantom sensation of sounds even when there is no stimulus present in the acoustic environment. Reported phantom sensations associated with tinnitus include ringing, buzzing, cricket-like sining, hissing, whistling, and humming (Canadian Academy of Audiology). Tinnitus affects a reported 360,000 Canadians, of which 150,000 having their quality of life severely compromised (Canadian Academy of Audiology).

A study conducted by Axelsson and Ringdahi (1989) on 3,600 randomly selected adults in the general population revealed that 14.2% of the subjects had chronic tinnitus throughout the day. The remaining individuals experienced transient tinnitus. Also, males were more affected than females and the left ear was more affected than the right. Two point four percent of the people in the study group were affected to such a degree that their quality of life was severely affected. Among the affected aspects of life is the quality of sleep. They found a positive correlation between tinnitus severity and sleep disturbances.
1.7.1 Objective vs. subjective tinnitus

1.7.1.1 Objective tinnitus

Objective tinnitus is a type of sensation caused by sounds generated within the peripheral auditory system (but not external environment) (Lockwood et al., 2002). There are three known causes of objective tinnitus. A tinnitus sensation with a pulsatile sound quality is usually caused by turbulent flow of blood in vessels near the cochlea (Weissman and Hirsch, 2000; Lockwood et al., 2002; Liyanage et al., 2006; Sonmez et al., 2007). The cause of this problem is linked to artery/vein malformations (or other vascular abnormalities), heart disease, high cardiac output, and other problems that may lead to turbulent blood flow (Lockwood et al., 2002). A tinnitus sensation with a clicking or low-pitched buzzing sound quality may be due to involuntary muscular contractions in the middle ear, specifically in the tensor tympani or stapedius muscle (Lockwood et al., 2002; Abdul-Baqi, 2004; Howsam et al., 2005). This can be caused by a spasm that occurs in the stapedius or tensor tympani muscle (Lockwood et al., 2002). A tinnitus sensation of environmental sound, may be due to irregular stimulation of outer hair cells. The cause of this is known to be spontaneous activation of acoustic emissions (Lockwood et al., 2002).

1.7.1.2 Subjective tinnitus

Subjective tinnitus is sometimes interchangeably referred to as tinnitus (Lockwood et al., 2002). People with this form of tinnitus report hearing phantom sounds when there is no actual acoustic stimulation presented either in the external environment or within the body.
Figure 9: Molecular structures of sodium salicylate and acetylsalicylic acid (i.e. aspirin).
It is believed that subjective tinnitus is due to changes in the peripheral and/or central nervous system (Jastreboff, 1990; Lockwood et al., 2002; Moller, 2003; Rauschecker et al., 2010). Evidence supporting the central origin of tinnitus comes from the fact that blocking inputs to the central auditory system does not eliminate phantom sensations of sounds (House and Brackman, 1981; Barrs and Brackman, 1984; Berliner et al., 1992; Kaltenbach et al., 2005).

Subjective tinnitus can be induced by otologic, neurologic, infectious, or drug-related factors. Otologic factors are associated with noise-induced hearing loss, presbycusis (i.e., age-dependent hearing loss), deafness, or any other cause of hearing loss. Neurological factors include head injury and multiple sclerosis. Infectious factors include otitis media, meningitis, syphilis, or any other infectious disease that can lead to hearing abnormalities. Drug-related factors include aminoglycoside antibiotics and various over-the-counter and prescription drugs that are used for treating inflammatory problems. Two examples of anti-inflammatory drugs are Aspirin and sodium salicylate (SS).

1.8 Aspirin- and SS-induced tinnitus

Aspirin is available in North America as both an over-the-counter drug and a prescription drug. Prescription aspirin is generally used for relieving pain caused by more serious problems such as rheumatoid arthritis, osteoarthritis, and other rheumatologic conditions. In North America alone, about one hundred and twenty billion pills are prescribed each year (Warner and Mitchell, 2002). Effects of the drug are related to the inhibition of cyclooxygenase, which decreases prostaglandin production (Gong et al., 2008). As a nonprescription drug, aspirin is used to relieve moderate pain from
headaches, menstrual cramps, toothaches, and muscle aches (NIH, 2016). The drug is capable of having such a widespread effect throughout the body because of its ability to reach systemic circulation (Rowland et al., 1972). After an oral dose is taken, the drug hydrolyzes to salicylic acids (Figure 9) and acetic acids (Rowland et al, 1972) of which the salicylic acid reaches peak plasma levels within 1-2 hours. The salicylic acid becomes widely distributed to many areas of the body including the central nervous system (Bayer Corp. USA).

Salicylate itself has been administered to individuals to help treat various health problems such as advanced myelodysplastic syndrome, acute myelogenous, leukemia, and chronic lymphocytic leukemia. It is reported that the drug can reduce the incidence of cancer and enhance the survival rate of cancer patients (Klimek et al., 2012).

Despite the benefits, Aspirin and other salicylic drugs have side effects include nausea, vomiting, stomachache, and heartburn. It also causes hives, rash, difficulty in breathing, and swelling. The drugs also cause hearing problems including transient tinnitus and hearing loss. For Aspirin, it is likely that its toxicity is produced by its metabolite salicylic acid. Therefore, it is sodium salicylate (SS) that is currently used by most of researchers in the study of ototoxic effects (e.g., tinnitus) of Aspirin and other salicylic drugs (Temple, 1978; Krause et al., 1992; Dargan et al., 2002).

1.8.1 SS induces tinnitus in humans

Many studies have been conducted in which patients have been study to understand how SS can cause the sensation of tinnitus. Furthermore, Studies have been conducted to find the plasma level of SS required to induce tinnitus in human subjects to understand the toxic levels. An early study based on salicylate injections revealed that SS
at a blood concentration of 200mg/L can lead to tinnitus in human subjects (Jagar and Always, 1946). When administered orally, salicylate can lead to deafness at a concentration of 250mg/L (Graham and Parker, 1948). Many subsequent studies also used oral administration of SS and found that tinnitus can be induced at blood concentrations of below 100mg/L to 250mg/L (Myers et al., 1965; McCabe and Day, 1965; Mongan et al., 1973; Halla et al., 1991; Janssen et al., 2000).

1.8.2 Tinnitus-like behaviour induced by SS in animals

Behavioural paradigms have been developed to assess the induction of tinnitus-like behaviour in animals. Some behavioural tests are based on conditioned suppression paradigms, which use water as a reward and foot-shocks as a punishment to shape animal behaviours. An animal is trained to obtain water reward through licking a drinking spout in the presence of a background sound and stop licking in the absence of the background sound. Failing to stop licking in the absence of the background sound following treatment of SS is interpreted as that the animal perceives a phantom sound in the absence of the background sound (i.e., showing a tinnitus behaviour). Using this behavioural paradigm, early studies found that SS applied systemically at a dosage of 300mg/kg could reliably induce tinnitus-like behavior in the rat (Jastreboff et al., 1988; Jastreboff and Sasaki, 1994). Late studies indicate that the degree of tinnitus seems to be correlated with the dosage of SS within a range from 50 to 300mg/kg. Researchers also examined the blood salicylate concentration required for inducing tinnitus in the rat. Results closely resemble those from human subjects.

1.8.3 Neurobiological changes caused by SS

1.8.3.1 SS-induced changes in the auditory sensory organ
In the peripheral auditory system, SS primarily affects outer hair cells in the cochlea. It is well known that outer hair cells can change their length in response to depolarization/hyperpolarization. Such an “electromotility” can enhance the sensitivity of the auditory sensory organ to sound stimulation. It has been found that SS can bind the motor protein prestin in the outer hair cells and reduce the electromotility of these cells (Shehata et al., 1991; Oliver et al., 2000; Chen et al., 2010). This can lead to a reduction in the output signal from the cochlea to the central nervous system. A reduction in cochlea output have been confirmed following systemic application (Cazals, 2000) as well as local application (in the cochlea) (Muller et al., 2009) of SS.

1.8.3.2 SS-induced neurobiological changes in the central auditory system

Tinnitus-like behaviour has been reported in animals even after the auditory nerve fibers are disconnected from the brain (Berliner et al., 1992). This leads one to believe that there are central changes that are taking place.

1.8.3.2.1 Effect of SS on the CN

The CN is the first auditory structure to receive information from the peripheral nervous system. Previous studies have shown the involvement of the CN in SS-induced tinnitus (Brozoski et al., 2002; Kaltenbach and Godfrey, 2008; Shore et al., 2007). Neurophysiological studies conducted using brain slice preparations have found that after SS application, the CN showed an increase in activity (Basta et al., 2008; Wei et al., 2010). Another study based on manganese enhanced functional magnetic resonance imaging found that systemic application of SS cased an increase in activity in the DCN but no change in the VCN (Holt et al., 2010).

1.8.3.2.2 Effect of SS on the IC
The IC is a key structure involved in the processing of auditory information. Studies have been conducted on this auditory structure to find how it’s involved in tinnitus (Manabe et al., 1997; Bauer et al., 2000). An increase in the expression of an immediate early gene c-fos has been observed in the IC following application of SS, suggesting that neural activity in this structure can be increase by the drug (Wu et al., 2003). A study based on manganese enhanced functional magnetic resonance imaging has supported that a systemic application of SS can increase the response in the IC, with the largest increase observed in the ICd (Holt et al., 2010). In vivo neurophysiological recordings confirmed a systemic application of SS can increase neural activities in the ICd and ICx (Jastreboff and Saskaki, 1986; Chen and Jastreboff, 1995; Manabe et al., 1997). Recordings from brain slice preparations showed that SS has a local target in the IC (Basta and Ernst, 2000). This result has been supported by our own results obtained by using in vivo neurophysiological recording/neuropharmacological manipulations (Patel and Zhang, 2014).

1.8.3.2.3 Effect of SS on the MGN

In spite of the importance of the MGN in auditory processing, not much attention has been paid to the role of this structure in the induction/generation of tinnitus. A study conducted by Basta and colleagues (2008) examined SS-induced changes in neural activity in the dorsal and ventral MGN using brain slice preparations. They found that SS enhanced activity in the MGN. In contrast to this finding, another brain slice study suggested that SS reduced firing of intrinsic action potentials in the MGN (Su et al., 2012) studied the activity in the MGN after SS-application.

1.8.3.2.4 Effect of SS on the AC
The effect of SS on neurons in the AC has been extensively studied. Many studies have yielded comparable results. It has been found that SS increases spontaneous neural activity in a brain slice preparation. The increase in neural activity was dose-dependent (Basta et al., 2008). An in vivo study indicates that a systemic application of salicylate can increase sound-evoked neural activity in the AC (Yang et al., 2007). In another study, they used systemic and local application of SS. They found that sound-evoked neural activity increased, however, the spontaneous firing had reduced (Lu et al., 2011).

1.8.3.3 Effects of SS on limbic structures

1.8.3.3.1 Effect of SS on the amygdala

Recent studies have examined the effect SS on limbic structures, including the amygdala. Chen and colleagues (2014) used a rat model to study the effect of a systemic application of SS. Behavioural tests were conducted to ensure that a tinnitus-like behaviour was induced and in vivo recordings were conducted in the LaN of the amygdala. An SS-induced increase in neural activity was found. Neurochemical changes in the amygdala were also examined following a systemic application of salicylate. Immunocytochemical experiments revealed that expressions of arg3.1 and c-fos, which reflect the level of neural activity, were increased substantially in the structure following the drug application (Mahlke and Wallhausser-Franke, 2004). The largest change was found in the CeN.

1.8.3.3.2 Effects of SS on the hippocampus

Studies have also examined the effect of SS on the hippocampus. In these studies, SS was applied systemically and behavioural tests were conducted to ensure that a tinnitus-like behavior was induced in experimental subjects. In vivo electrophysiological
recordings were then conducted in these animals. Results indicate that the drug causes an increase in activity in the hippocampus (Chen et al., 2014). An *in vitro* study conducted on the CA1 region of the hippocampus was also explored. Researchers found an increase in neuronal activation, following systemic application of salicylate (Gong et al., 2008).

1.9 Possible central mechanisms underlying SS-induced tinnitus

1.9.1 Tipping of balance between excitation and inhibition in auditory structures

It is believed by many researchers that tinnitus is dependent on neurophysiological changes in central auditory structures. These changes can include enhanced excitation and/or reduced inhibition, which can lead to excessive firing of action potential in the absence of external acoustic stimulation.

Previous research has studied the effects of SS on the neural activity in the cochlea and the major auditory structures involved in tinnitus. From *in vivo* studies it was found that following a systemic application of SS, there was a decrease in sound-evoked activity in the cochlea (Muller et al., 2009). Further studies found SS caused a decrease in the electromotility of OHCs, which caused a decrease in signal output (Shehata et al., 1991; Oliver et al., 2000; Chen et al., 2010). In contrast to the decrease in the output at the periphery, there is an increase in activity at the central structures. As mentioned in sections 1.8.3.2.1 – 1.8.3.2.4, there is generally an increase in activity of the auditory structures after SS-administration, with the exception of the MGN, which is indifferent (Basta and Ernst, 2000; Basta et al., 2008; Chen et al., 2014). Studying the structures in the ascending pathway could give better insight to this discrepancy.

An increase in neural activity in central auditory structures is supported by functional imaging studies. Using the manganese enhanced magnetic resonance imaging
technique, Holt and colleagues (2010) found a widespread increase in neural activity in the central auditory system with the increase being highest in the DCN and ICd. *In vitro* neurophysiological studies also support that SS can enhance neural activities in central auditory structures. The recordings showed a reduction in GABA-mediated inhibition, which lead to an increase in excitatory potential (Wang et al., 2008; Gong et al., 2008). Studies also focused on the GABAergic inhibition to understand the increase in activity in the AC. They found that there was a reduction here as well (Sun et al., 2009; Lu et al., 2011). However, there also exist inconclusive findings in which studies have shown a decrease or no significant change in activity (Basta and Ernst, 2004 Eggermont and Roberts, 2004; Ma et al., 2006). More studies are required to understand the molecular changes after tinnitus is induced (Knipper et al., 2009).

An SS-induced increase in auditory neural activity could be due to effects of the drug on the abundance of neurotransmitter and/or neurotransmitter receptors in auditory neural structures. Butt and colleagues (2016) have found a decrease in level of GABA$_B$ receptors in the IC after a systemic application of SS. Zou and Shang (2012) studied the neurochemical changes that occur in GABA$_A$ receptors and GABA decarboxylase (GAD), which is an enzyme required for synthesizing GABA. They found a reduction in both levels in the CAS after the application of the SS. Similar studies have been done by Basta and colleagues (2000), which also found a reduction in the GABA$_A$ receptors after SS-application.

Existing results have looked at how SS can cause changes in the expression of proteins. Some proteins are involved in contributing to both inhibitory and excitatory neurotransmission. These changes are most likely slower because they require the change
of cellular mechanisms. Researchers found a reduction in the mRNA expression of the 
GABA_{\alpha 1} subunit. They also found a reduction in the mRNA expression of the enzyme 
GAD67 (Zou and Shang, 2012). In other studies an increase in the mRNA expression of 
NMDA receptor subunit 2A was found in the CN after SS administration (Hu et al., 
2014).

1.9.2 Synchronization and bursting activity of auditory neurons

Other neurophysiological changes that may lead to a phantom sensation of sound 
include synchronization among activities generated by an ensemble of neurons. They also 
include enhanced bursting of action potential firing in auditory neurons. Both of these 
changes can be associated with reduction in inhibition. Following SS application, an 
increase in bursting activity was found in the IC (Chen and Jastreboff, 1995; Kwon et al., 
1999), while an increase in synchronization was seen in the auditory nerve (Cazals et al., 
1998). Studies based on electroencephalography and magnetoencephalography in human 
subjects supports an enhancement in synchronization in auditory cortical areas (Weisz et 
al., 2005; Schlee et al., 2009).

1.9.3 Involvement of limbic structures

The induction/manifestation of tinnitus may be dependent on regulation by other 
neural structures. Inputs from these structures may influence auditory neurons and cause 
abnormalities in physiological functions of these neurons. The limbic system is likely 
involved in the regulation of auditory neural activities (Lockwood et al., 1998; Mahlke 
and Walhausser-Franke, 2004; Vlassenko et al., 2010; Kraus et al., 2012). Early evidence 
supporting the involvement of the limbic system includes that tinnitus can be modulated 
emotional distress (Jastreboff, 1990; Sullivan et al., 1998; Dobie, 2003). Such
observations are supported by recent studies showing strong associations between tinnitus and emotional stress, anxiety, and depression (Langguth, 2011).

Anatomical studies indicate that the amygdala has direct connections with the MGN, while the hippocampus has indirect connections (See section 1.4). These connections likely serve as a gating mechanism that regulate the relay of neural signals from the MGN to the AC. Malfunction of this gating mechanism affect the relay of signals in the ascending auditory system (Leaver et al., 2011) which may contribute to the induction/manifestation of tinnitus. Such malfunction is likely important for the persistence of chronic tinnitus (Knipper et al., 2009; Galazyuk et al., 2012).

Knowledge about SS-induced changes in neurophysiological mechanisms and how these changes are dependent on neurochemical alterations in limbic structures is critical for understanding the involvement of these structures in the induction/manifestation of tinnitus. An important aspect of this knowledge is related to GABAergic neurotransmission. Researchers studied a molecule, i.e. GAD1, which is required to synthesize GABA. They found an increase, i.e. an upregulation in the level of this molecule (Im et al., 2007). It is equally importantly to study how SS affects the level of the GABAergic receptors in the limbic structures. It is especially important to study the level of the GABA_{B} receptors. A GABA_{B} receptor exists at both pre- and postsynaptic sites, the receptor possibly plays a major role in the regulation of excitation and inhibition in the limbic structures, which can consequently affect the level of activity in the central auditory system through limbic-thalamic projections.

1.10 Research objectives and hypothesis
The specific objective of this thesis was to understand how systemic application of SS affects the level of GABA_B receptors in the amygdala and hippocampus of a rat. I wanted to find whether the changes were localized to specific subdivisions of the amygdala and hippocampus. Based on previous findings, I hypothesized that the drug would cause an increase in the level of GABA_B receptors in both structures.

2 Experimental methods

Immunohistochemistry experiments were conducted to study the level of GABA_B receptors in the rat’s auditory structures. Techniques used in this study were well established by previous members in my lab (Jamal et al., 2011; Jamal et al., 2012; Butt et al., 2016).

2.1 Animal preparation

Experiments were conducted using 250-350g male Wistar rats (Rattus norvegicus) obtained from Charles River Laboratories Inc. (St. Constant, QC). They were housed in the University of Windsor Animal Care Facility (with a noise level of 50-60 dB SPL) for at least a week before experiments were conducted. All procedures were done in accordance with the Canadian Council on Animal Care guidelines.

Pairs of animals received either an intraperitoneal injection of SS (250mg/kg, i.p.) or an equivalent volume of physiological saline (PS). A survival period of three hours was given to allow SS-treated rats to develop a tinnitus-like behaviour (Jastreboff and Sasaki, 1994). Animals were euthanized with an overdose of sodium pentobarbital (120mg/kg, i.p.) and perfused with 200ml phosphate-buffered saline (PBS, 0.1M, pH 7.1) followed by 200ml paraformaldehyde (4% in PBS). Then the brains were extracted,
cryoprotected in 30% sucrose (in PBS), and sectioned along the coronal plane at 30µm using a cryostat (Leica Microsystems CM1050 S, Heidelberg, Germany).

2.2 Immunohistochemical (IHC) experiments

2.2.1 Experimental procedures

An experiment was conducted by using tissue sections from the hippocampal and amygdala from a pair of PS- and SS-treated animals. For each structure (i.e., amygdala or hippocampus) of each animal, the sections were taken from the whole rostrocaudal extent of the structure at 300µm intervals. Sections from similar rostrocaudal locations of PS- and SS-treated animals were placed next to each other on a microscope slide (Figure 10). All the sections from a pair of animals were placed on four microscope slides. Each slide contained two cerebellar sections from PS- and SS- treated animals, respectively. These cerebellar sections were used as a positive control (Benke et al., 2002; Charles et al., 2001; Ige et al., 2000). They were also used for providing references for normalization (Butt et al., 2016; also see Section 2.3). Throughout an entire immunohistochemical experiment, the four microscope slides were placed in the same slide jars to ensure that the same experimental conditions were applied to all the tissue sections.

For each reaction, the tissues were first incubated for 22 hours in a primary antibody (see “Antibodies” section) in 0.1M PBS containing 0.05% Triton and 5% normal donkey serum. After three rinses (10 minute each) with 0.1M PBS, the tissues were incubated in a secondary antibody (see “Antibodies” section) in 0.1M PBS with 2% normal donkey serum for 2 hours. After another three rinses of 0.1M PBS (10 minutes each), tissues were incubated in ExtraAvidin-Peroxidase (1:400 in 0.1M PBS, Sigma-Aldrich E2886, Oakville, ON) for 1.5 hours. The tissues were then rinsed in 0.1M PBS
Figure 10: Schematic diagram of a microscope slide showing the organization of brain slices. Gray area: naming system showing a sample naming. White area: showing where the slices were collected. PS CBM is the cerebellum from the PS-treated animal. SS CBM is the cerebellum from the SS-treated animal. PS 1-5 represent the limbic structures from the PS-treated animal. SS 1-5 represents the limbic structures from the SS-treated animal. The top row matches the bottom row in rostrocaudal location.
(10 minutes each) and stained with 3’3’ diaminobenzidine tetrahydrochloride in 0.1M-phosphate buffer. Distilled water was used to stop the staining reaction. The tissue was then dehydrated using an ethanol gradient (60%, 70%, 95%, 100%, and 100%, 5 minutes per concentration) and cleared with two charges of histosol (10 minutes each). Finally, slides were coverslipped with the mounting medium, Permount (Fisher Scientific SP-500, Ottawa, ON).

2.2.2 Antibodies

The primary antibody against GABA<sub>B</sub>R1 subunit was rabbit polyclonal GABA<sub>B</sub>R1 antiserum (Santa Cruz Biotechnology R-300, 1:500). The primary antibody against GABA<sub>B</sub>R2 subunit was guinea-pig polyclonal GABA<sub>B</sub>R2 antiserum (Chemicon AB5394, 1:1000).

The secondary antibody for probing GABA<sub>B</sub>R1 subunit was biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories 711-005-152, 1:400, Temecula, CA). The secondary antibody for probing GABA<sub>B</sub>R2 subunit was biotinylated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories 706-065-148, 1:400, Burlington, ON).

2.2.3 Positive and negative control experiments

Both negative and positive control experiments were conducted. Positive control experiments were conducted by using tissues from the cerebellum as previous studies have indicated that both GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 exist in this structure with the level being high in the molecular layer. My positive experiments revealed that antibodies for probing both GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits were effective.
A negative control experiment was conducted using the same procedures as that described in section 2.2.1 with a primary antibody (against GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2) or a secondary antibody (for probing GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2) omitted. Negative control experiments revealed no labeling in either cell body or neuropil regions in the limbic and cerebellar tissues, indicating that the labeling in limbic structures in normal IHC experiments was specific.

2.3 Data analysis

Images of the sections subjected to IHC reactions were taken using a CTR 6500 microscope and a DFC 425 digital camera (Leica Microsystems, Heidelberg, Germany). A piece of standard tissue was used to set light intensity and exposure. The same lighting condition was used across different imaging sessions to allow for quantitative comparison among images taken in different sessions.

Immunoreactivity was quantified using the LAS V4.5 software (Leica Microsystems, Heidelberg, Germany). An optic density (OD) was measured at each pixel to reflect the level of immunoreactivity. A mean OD was obtained for a target area or object (e.g., a cell body) by using the ODs obtained at all pixels within the target area or object. Immunoreactive cell bodies were identified using a threshold criterion. A threshold value was set for each individual slice as the mean OD of all the pixels in the neuropil region surrounding cell bodies minus three standard deviations of all the ODs at these pixels. For the amygdala, an additional length criterion was used. Only objects that passed the threshold criterion and with their longest axes in a range between 4 and 26 micrometer were included. Visual examination was also used to help identify the immunoreactive cell bodies.
To compare the level of immunoreactivity in a target area or a group of labeled neurons between a pair of SS- and PS-treated animals, the mean OD value of the target area or each neuron in the group was normalized using the OD values from the molecular layer and the white matter of the cerebellum. The level of GABA\(_B\) receptors in the cerebellum is not affected by the systemic application of SS (Butt et al., 2016)

\[
IR_N = (OD_{LS} - OD_{WM})/(OD_{Mol} - OD_{WM})
\]

where \(IR_N\) is a normalized level of immunoreactivity of a hippocampal or amygdaloid area or cell body. \(OD_{LS}, OD_{WM}, OD_{Mol}\) are the mean OD values of a cell body/area in the limbic structure of interest (i.e. hippocampus or amygdala), the white matter of the hippocampus, and the molecular layer of the cerebellum, respectively. The OD values from the cerebellar regions were used in normalization as our previous study indicated that these values are not affected by systemic application of SS (Figure 12; also see Butt et al., 2016).

2.3.1 Statistical analysis

All bar charts represent the mean OD ± standard deviation. Based on the specific data sets, a two-tail t-test or a two-way ANOVA was conducted. A level of \(p < 0.05\) was considered to be statistically significant.

3 Results

3.1 Control experiments and the effect of SS on GABA\(_B\) receptors in the cerebellum

Positive and negative control experiments were conducted to ensure the effectiveness of antibodies and the reliability of IHC results. To ensure that antibodies were effective in probing the GABA\(_B\)R1 or GABA\(_B\)R2 subunits, positive control experiments were conducted by using tissues from the cerebellum as both subunits exist
Figure 11: Positive and negative control using cerebellum tissue. M: molecular layer, G: granular layer, W: white matter. Top panel shows a positive control by showing the immunolabeling in the cerebellum by probing both GABA\(_B\)R1 and GABA\(_B\)R2. Middle panel shows a negative control by omitting the primary antibody for GABA\(_B\)R1 and GABA\(_B\)R2. Bottom panel shows a negative control by omitting the secondary antibody for GABA\(_B\)R1 and GABA\(_B\)R2. Scale bar: 100\(\mu\)m.
in this brain structure (Jamal et al., 2012). In all the six pairs of animals examined in this study, I conducted IHC experiments against the GABA$_B$R1 and GABA$_B$R2 subunits using pairs of cerebellar tissues from the PS- and SS-treated animals. These experiments revealed clearly labeled cells including large Purkinje cells (Figure 11). The neuropil regions surrounding these cells were also labeled. These results suggest that the antibodies used in our study were effective.

Negative control experiments were conducted by using cerebellar tissue from one pair (i.e. one case) of animals. These experiments were conducted by omitting either the primary or secondary antibody for probing the GABA$_B$R1 or GABA$_B$R2 subunit while all the other experimental procedures were kept exactly the same as those described in the “immunohistochemical (IHC) experiments” section. The experiments revealed very faint labeling of cell bodies and surrounding neuropil regions, suggesting that the antibodies used in the present study was specific in labeling the GABA$_B$R1 or GABA$_B$R2 subunits (Figure 11).

The level of labeling in the molecular layer of the cerebellum was quantified for both the GABA$_B$R1 and GABA$_B$R2 subunits in six cases. Results revealed that SS did not affect the level of either of the GABA$_B$ receptor subunits (Figure 12). The quantification showed no significant difference between the two treatments (two-tail T-test; 0.158; 6). These results agree with that revealed by a previous study from our lab (Butt et al., 2016). The lack of the effect of SS on the level of receptor subunits in the molecular layer of the cerebellum suggest that any changes observed in the amygdala and hippocampus were specific to these limbic structures.

3.2 Effect of SS on the level of GABA$_B$ receptor subunits in the amygdala
Figure 12: No change in molecular layer of cerebellum in SS-treated animals. (A) low magnification images of cerebellum from PS- and SS-treated animals probed with GABA\(_B\)R1 and GABA\(_B\)R2. (B) group results showing little change between the molecular layers of PS- and SS-treated in both subunits. Results are from six pairs of animals. Change is not statistically significant (two-tail T-test (5); t = 0.8296; p = 0.158). Scale bar: 100\(\mu\)m.
3.2.1 Normative immunoreactivities against GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 in the amygdala

Results from six PS-treated animals were examined to understand the normative immunoreactivities against GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2. For both subunits, an immunoreactivity was observed throughout the entire structure. The level of labeling was reduced from the rostral to the caudal part of the structure. A closer examination revealed a similar level of labeling in BLA, BMA, and LA, and a higher level of labeling in the CeN. This was consistent throughout the rostrocaudal extent of the structure.

3.2.2 Effect of SS on the overall level of receptor subunits in the amygdala

The effect of SS on the level of GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 in amygdala was studied in six pairs of PS- and SS-treated animals. Results were quantified in all six cases. For each animal, ten tissue sections containing the amygdala and one section containing the cerebellum were used in IHC experiments.

As shown by an example in Figure 13, levels of immunoreactivity were similar between cerebellum tissues obtained from the PS- and SS-treated animals for both the GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 (Figure 13 top row). In contrast, the level of labeling in the amygdala was higher in the SS-treated than the PS-treated animal for both subunits (Figure 13 middle and bottom rows).

The level of labeling was compared between PS- and SS-treated animals over the rostrocaudal extent of the amygdala. For both the GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits, SS resulted in an overall increase in immunolabeling throughout the entire extent (Figure 14 and Figure 15). The increase tended to be larger in the caudal than the rostral part of the structure.
Figure 13: Change in overall mean OD of SS-treated animals in the amygdala. (A) shows immunolabeling against GABA_βR1 subunit and (B) shows the immunolabeling against the GABA_βR2 subunit in the cerebellum (top panel) and amygdala (bottom panel) in both PS- and SS-treated animals. Scale bar: 300µm.
Figure 14: Immunolabeling against GABA\textsubscript{B}R1 in the rostrocaudal slices of the amygdala. Major subdivisions are outlined. Scale bar: 300\,\mu m.
Figure 15: Immunolabeling against GABA<sub>B</sub>R2 in the rostrocaudal slices of the amygdala. Major subdivisions are outlined. Scale bar: 300µm.
Figure 16: Group results from IHC experiments showing overall changes in normalized OD values of the amygdala in both GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits. Results were combined from the OD of the major subdivisions and then normalized the cerebellum from the specific IHC experiment. Results are from six pairs of animals. The change is statistically significant for both GABA\textsubscript{B}R1 (two-tail T-test (22); t=3.5524 p<0.005) and GABA\textsubscript{B}R2 (two-tail T-test (22); t=4.8263; p<0.001).
A weighted average OD was used to evaluate the overall level of
immunoreactivity against a receptor subunit (GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2) in the amygdala
of an animal. Results from 3 pairs of animals revealed that SS produced an overall
increase in level of both receptor subunits (Figure 16), with the increase being
statistically significant for both GABA\textsubscript{B}R1 (two-tail T-test; p<0.005; n=6) and
GABA\textsubscript{B}R2 (two-tail T-test; p<0.001; n=6).

3.2.3 Effects of SS in major amygdaloid subdivisions

Six cases were analyzed to study the effect of SS on the GABA\textsubscript{B}R1 and
GABA\textsubscript{B}R2 subunits in the four major subdivisions of the amygdala. A weighted average
OD was obtained for each subdivision of the structure. For the GABA\textsubscript{B}R1 subunit, results
from 6 pairs of PS- and SS-treated animals indicate that the increase was similar among
the subdivisions including the BLA, BMA, and CeN, and slightly larger in LaN (Figure
17 and Figure 19a). The rostral slices became more area specific, showing a larger
increase in labeling in the LaN, compared to other subdivisions. The increase in all the
subdivisions was statistically significant (two-way ANOVA; p<0.001; n=6).

The effect of SS on the GABA\textsubscript{B}R2 subunit was also evaluated in the four major
amygdaloid subdivisions. Results indicate that the level of the receptor subunit was
increased in all the subdivisions. The SS-induced increase was similar among the BLA,
BMA, and CeN, but somehow larger in the LaN (Figure 18 and Figure 19b). The
increase in all the subdivisions were statistically significant (two-way ANOVA; p<0.05;
n=6).

3.2.4 The effect of SS on immunoreactivities in cell bodies
Figure 17: High magnification images of the major subdivisions of the amygdala labeled against GABA_B R1. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300µm. High magnification image scale bar: 25µm.
Figure 18: High magnification images of the major subdivisions of the amygdala labeled against GABA\textsubscript{R2}. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300\textmu m. High magnification image scale bar: 25\textmu m.
Figure 19: Group results from IHC experiments showing overall changes in normalized OD values of the major subdivisions of the amygdala in both GABA_{B}R1 and GABA_{B}R2 subunits. Results were normalized against the cerebellum from the specific IHC experiment. Results are statistically significant for both GABA_{B}R1 two-way ANOVA (22); p<0.001; n=6) and GABA_{B}R2 (two-way ANOVA (22); p<0.05; n=6).
Figure 20: High magnification images showing immunolabeled cells against $\text{GABA}_B\text{R1}$ and $\text{GABA}_B\text{R2}$ in the four major subdivisions of the amygdala. Both PS- and SS-treated labeled against $\text{GABA}_B\text{R1}$ and $\text{GABA}_B\text{R2}$ in all subdivisions including the (A) BLA, (B) BMA, (C) CeN, and (D) LaN. Scale bar: 10 $\mu$m.
For both the GABABR1 and GABABR1 subunits, the effect of SS on the level of immunoreactivity was studied in cellular regions in six cases. High magnification images indicate that the overall increase in the level of immunoreactivity against the receptor subunits was highly dependent on the increase in cell body regions. Further observations indicated a difference in the pattern of labeling between the GABABR1 and GABABR2 subunit (Figure 20). While the labeling by the GABABR1 antibody was generally stronger in the periphery than the center of a cell body, the labeling by the GABABR2 antibody was typically stronger in the center than the periphery of a cell body. This was consistent in both PS- and SS-treated animals.

A mean OD value was obtained for each cell body (See Section 2.3 for method). The number of immunoreactive cell bodies and the distribution of OD values of immunoreactive cell bodies were compared in six pairs of PS- and SS-treated animals. For GABABR1 subunit, there was a significant increase in the mean OD of the SS-treated animals in all subdivisions (two-way ANOVA; p<0.01; n=6). Compared with that of a PS-treated animal, the distribution of the normalized OD of an SS-treated animal was shifted rightward, indicating an increase in the level of labeling in the cell bodies of the amygdaloidal cells (Figure 21 and Figure 22a). This change was observed in all four subdivisions, with the largest change being in the LaN. The density of labeled cells, measured as number per square millimeter, was also increased in the SS-treated animal (Figure 22b). This increase was significant (two-way ANOVA; p<0.05; n=6)

The effect of SS on GABABR2-immunoreactive cell bodies was similar that on GABABR1-immunoreactive cell bodies. The drug caused a rightward shift to the distribution of the OD of labeled cell bodies, indicating the drug increased the level of
Figure 21: Histograms showing the number of neurons within a specific normalized mean OD in GABA<sub>β</sub>R1 immunolabeled cells in the amygdala. Black bars represent PS-treated animals and white bars represent SS-treated animals.
Figure 22: Group results from IHC experiments showing changes in (A) normalized OD values of the cells bodies in each subunit and (B) cell density of GABA_B R1 immunolabeled cells in the amygdala. (A) Results were normalized against the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals and are statistically significant (two-way ANOVA (22); p<0.01) (B) Results were measured as cells per square millimeter. Results were obtained from six pairs of animals and are statistically significant (two-way ANOVA (22); p<0.05).
Figure 23: Histograms showing the number of neurons within a specific normalized mean OD in GABA<sub>B</sub>R2 immunolabeled cells in the amygdala. Black bars represent PS-treated animals and white bars represent SS-treated animals.
Figure 24: Group results from IHC experiments showing changes in (A) normalized OD values of the cells bodies in each subunit and (B) cell density of $\text{GABA}_B\text{R2}$ immunolabeled cells in the amygdala. (A) Results were normalized against the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals and are statistically significant (two-way ANOVA (22); $p<0.001$). (B) Results were measured as cells per square millimeter. Results were obtained from six pairs of animals and are statistically significant (two-way ANOVA (22); $p<0.05$).
GABA\textsubscript{B}R2 subunit in all the amygdaloid subdivisions (Figure 23). The increase was the highest in the LaN. Group results revealed a significant increase in all the mean OD of all subdivisions (two-way ANOVA; p<0.001; n=6), along with a significant increase in the number of labeled cells per square millimeter (two-way ANOVA; p<0.05; n=6) (Figure 24).

3.3 Effect of SS on the level of GABA\textsubscript{B} receptor subunits in the hippocampus

3.3.1 Normative immunoreactivities against GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 in the hippocampus

Results from six PS-treated animals were examined to understand the normative immunoreactivities against GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2. For both subunits, an immunoreactivity was observed throughout the entire structure. The subdivisions were not found consistently throughout the structure: the SUB appeared only in caudal slices, while all other subdivisions were found throughout the whole rostrocaudal extent. The level of labeling was reduced from the rostral to the caudal part of the structure. A closer examination of the subdivisions showed a similar level of labeling in CA1-CA3, DG, and SUBd and a higher level of labeling in the SUBv. In caudal slices, the DG showed an area difference: the dorsal portion showed a higher labeling than the ventral portion.

3.3.2 Effect of SS on the overall level of receptor subunits in the hippocampus

IHC experiments were conducted using tissues from the hippocampus in six pairs of PS- and SS-treated animals. Overall level of labeling (i.e., all the cell bodies and surrounding neuropil regions combined) in the entire hippocampus were quantified in these six cases.
Figure 25: Change in overall mean OD of SS-treated animals in the hippocampus. (A) shows immunolabeling against GABA\(_{\beta}\)R1 subunit and (B) shows the immunolabeling against the GABA\(_{\beta}\)R2 subunit in the cerebellum (top panel) and hippocampus (bottom panel) in both PS- and SS-treated animals. Scale bar: 300 µm.
As shown by an example in Figure 25, there was no difference in the level of labeling between the cerebellar sections from PS- and SS-treated animals for both subunits (Figure 25). In contrast, a change in the level of labeling was observed in certain areas of the hippocampus for both the GABA_BR1 and GABA_BR2 subunits (Figure 25). The two pairs of hippocampal sections in Figure 25 show immunoreactivities against GABA_BR1 and GABA_BR2, respectively. Each pair of sections was from a pair of PS- and SS-treated animals at the mid portion of the rostrocaudal extent of the hippocampus. Overall, the level of the receptor subunits was increased in the section from the SS-treated animal.

The effect of SS on the level of the GABA_BR1 and GABA_BR2 subunits was examined over the entire rostrocaudal extent of the hippocampus (Figure 26 and Figure 27). Overall, an increase in immunoreactivity against the GABA_BR1 subunit was observed in caudal sections in SS-treated animals. In contrast, a reduction in immunoreactivity against GABA_BR1 subunit was observed in rostral sections in SS-treated animals.

The GABA_BR2 subunit showed a very similar spatial pattern of change to that of change in GABA_BR1 throughout the rostrocaudal extent of the hippocampus (Figure 27). Overall, an increase in the immunoreactivity against GABA_BR2 was observed in caudal sections and a reduction in immunoreactivity was observed in rostral sections in SS-treated animals.

For each receptor subunit, a weighted average OD was calculated for each animal by using the measurements from all the hippocampal regions in the entire set of 12 sections (Figure 28). Group results from six pairs of PS- and SS-treated animals
Figure 26: Immunolabeling against GABA_{B}R1 in the rostrocaudal slices of the hippocampus. Hippocampal region is outlined. Scale bar: 300µm.
Figure 27: Immunolabeling against GABA<sub>B</sub>R2 in the rostrocaudal slices of the hippocampus. Hippocampal regions is outlined. Scale bar: 300µm.
Figure 28: Group results from IHC experiments showing overall changes in normalized OD values of the hippocampus in both $\text{GABA}_B \text{R1}$ and $\text{GABA}_B \text{R2}$ subunits. Results were combined from the OD of the major subdivisions and then normalized the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals and statistically significant in both $\text{GABA}_B \text{R1}$ (two-tail T-test (35); $t=3.1781$; $p<0.05$) and $\text{GABA}_B \text{R2}$ (two-tail T-test (35); $t=3.6048$; $p<0.05$).
Figure 29A: High magnification images of the CA1-CA3 subdivisions of the hippocampus labeled against GABA_BR1. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300μm. High magnification image scale bar: 25μm.
Figure 29B: High magnification images of the DG, SUBv, and SUBd subdivisions of the hippocampus labeled against GABA$_B$R1. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300µm. High magnification image scale bar: 25µm.
indicate that SS increased the level of receptor subunits in the hippocampus (Figure 28). This increase was statistically significant in both GABA_{B}R1 (two-tail T-test; p<0.05; n=6) and GABA_{B}R2 (two-tail T-test; p<0.05; n=6).

### 3.3.3 The effect of SS in major hippocampal subdivisions

Levels of immunoreactivities against the GABA_{B}R1 and GABA_{B}R2 subunits in 6 main subdivisions of the hippocampus were quantified in three cases. A weighted average OD was obtained for each subdivision for both GABA_{B}R1 and GABA_{B}R2 subunits. For GABA_{B}R1 the largest increase was observed in the CA1-CA2 regions. Similar changes were observed in CA3, DG, SUBd, and SUBv (Figure 29 and Figure 31a). For the CA3 and DG subdivisions, there exist a rostrocaudal area difference in which an increase in OD is observed in the caudal slices and a decrease is observed in the rostral slices. Overall, the increase of mean OD in all subdivisions was not statistically significant (two-way ANOVA; 0.11541; n=6).

The effect of SS on GABA_{B}R2 was also quantified in six cases examining all six subdivisions. Results indicated an increase in level of labeling in all subdivisions. Similar increase was observed in CA2, DG, and SUBd, but larger in CA1, CA3, and SUBv (Figure 30 and Figure 31b). Similar to the GABA_{B}R1 subunit, CA1 and DG showed an increase in mean OD in caudal slices and a decrease in rostral slices in SS-treated animals. Overall the increase was not statistically significant in all subdivisions (two-way ANOVA; p<0.05; n=6).

### 3.3.4 The effect of SS on immunoreactivities in cell bodies

Both GABA_{B}R1 and GABA_{B}R2 subunits were studied to understand the effect of SS on the level of immunoreactivity in cellular regions in six cases. High magnification
Figure 30A: High magnification images of the CA1-CA3 subdivisions of the hippocampus labeled against GABA<sub>B</sub>R2. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300µm. High magnification image scale bar: 25µm.
Figure 30B: High magnification images of the DG, SUBv, and SUBd subdivisions of the hippocampus labeled against GABA$_B$R2. Low magnification images show the different subdivisions for the PS and SS-treated animals. Corresponding high magnification images are from the specific subdivisions. Low magnification image scale bar: 300µm. High magnification image scale bar: 25µm.
Figure 31: Group results from IHC experiments showing overall changes in normalized OD values of the major subdivisions of the hippocampus in both \( \text{GABA}_B \text{R1} \) and \( \text{GABA}_B \text{R2} \) subunits. Results were normalized against the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals. Results were not statistically significant for \( \text{GABA}_B \text{R1} \) (two-way ANOVA (35); 0.11541) and statistically significant for \( \text{GABA}_B \text{R2} \) (two-way ANOVA (35); p <0.05).
Figure 32: High magnification images showing immunolabeled cells against GABA$_B$R1 and GABA$_B$R2 in the six major subdivisions of the hippocampus. Both PS- and SS-treated animals were labeled against GABA$_B$R1 and GABA$_B$R2 in the (A) CA1, (B) CA2, (C) CA3, (D) DG, (E) SUBd, and (F) SUBv. Scale bar: 10µm.
Figure 33: Histograms showing the number of neurons within a specific normalized mean OD in GABA\textsubscript{B}R1 immunolabeled cells in the hippocampus. Black bars represent PS-treated animals and white bars represent SS-treated animals.
images revealed a difference in the cellular distributions of the GABA\(_B\)R1 and GABA\(_B\)R2 subunits in hippocampal cells (Figure 32). While immunoreactivity against GABA\(_B\)R1 subunit was typically stronger in the surrounding area than the center of a cell body, the immunoreactivity against the GABA\(_B\)R2 subunit was typically stronger in the center than the surrounding area of a cell. This difference was observed throughout the structures in both PS- and SS-treated animals.

In six pairs of PS- and SS-treated animals, the level of labeling was evaluated in GABA\(_B\)R1-immunoreactive cell bodies in the hippocampus using mean OD values (Figure 33). For each subdivision, there was a rightward shift in the distribution of mean OD in an SS-treated animal, indicating an overall increase in the level of GABA\(_B\)R1 in the SS-treated animals. Group results supports that the drug caused an increase in the level of labeling in the GABA\(_B\)R1 subunit. The increase was highest in SUBv subdivision (Figure 34a). This change for all subdivisions was statistically significant (two-way ANOVA; p<0.01; n=6). Group results also indicated a tendency of increase in the number of labeled cells per square millimeter (Figure 34b). However, this increase was not statistically significant in all subdivisions (two-way ANOVA; p=0.22588; n=6).

Similar analysis was conducted for cell bodies showing immunoreactivities against the GABA\(_B\)R2 subunit. The distribution of ODs of labeled cells was shifted rightward in the SS-treated animal of each of the 6 pairs of animals (Figure 35). Group results supports that the drug caused an increase in the mean OD of labeled cells in all subdivisions of the hippocampus, with the largest change being in the SUBv (Figure 36a). These results were statistically significant (two-way ANOVA; p<0.01; n=6). Group results also indicated a tendency of increase in the number of labeled cell bodies per
Figure 34: Group results from IHC experiments showing changes in (A) normalized OD values of the cell bodies in each subunit and (B) cell density of GABA<sub>B</sub>R1 immunolabeled cells in the hippocampus. (A) Results were normalized against the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals and were statistically significant (two-way ANOVA (35); p<0.01). (B) Results were measured as cells per square millimeter. Results were obtained from six pairs of animals and were not statistically significant (two-way ANOVA (35); p=0.22588).
Figure 35: Histograms showing the number of neurons within a specific normalized mean OD in GABA<sub>2</sub>R2 immunolabeled cells in the hippocampus. Black bars represent PS-treated animals and white bars represent SS-treated animals.
Figure 36: Group results from IHC experiments showing changes in (A) normalized OD values of the cells bodies in each subunit and (B) cell density of GABA_B R1 immunolabeled cells in the hippocampus. (A) Results were normalized against the cerebellum from the specific IHC experiment. Results were obtained from six pairs of animals and were statistically significant (two-way ANOVA (35); p<0.01). (B) Results were measured as cells per square millimeter. Results were obtained from six pairs of animals and were not statistically significant (two-way ANOVA (35); 0.08328).
square millimeter (Figure 36b). This increase was not statistically significant (two-way ANOVA; 0.08328; n=6).

4 Discussion

My results revealed that a systemic application of SS caused an increase in the level of GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits in the amygdala and the hippocampus. The increase was generally larger in the amygdala than in the hippocampus. A regional difference in the level of increase was seen in the amygdala. For both subunits, the percent increase was the greatest in the LaN and smallest in the BMA. A moderate increase was seen in the BLA and CeN. Apparent area differences existed in the hippocampus. Overall, for GABA\textsubscript{B}R1 subunit showed a larger percentage increase in the weighted average OD of CA1-CA2 regions, whereas a moderate increase was observed in CA3, DG, SUBd, and SUBv. The GABA\textsubscript{B}R2 subunit showed a larger percentage increase in the weighted average OD of CA1, CA3, and SUBv, whereas a moderate increase was observed in the CA2, DG, and SUBd.

4.1 Technical considerations

Both positive and negative control experiments were conducted to ensure the effectiveness of experimental procedures and reliability of results from the limbic structures (Figure 11). Each of the immunohistochemical experiments done to probe GABA\textsubscript{B}R1 or GABA\textsubscript{B}R2 subunit in a pair of PS- and SS-treated animals consisted of a pair of cerebellar sections from the same pair of animals. While the experiments revealed an increase in the level of immunolabeling in the limbic structures, they did not reveal a change in the cerebellum. This indicates that changes in the level of GABA\textsubscript{B}R1 and
GABA_b R2 subunits observed in the limbic structures were due to the drug and not other possible factors.

An SS injection of 250mg/kg was used to reliably induce tinnitus in the SS-treated animals. This amount is relatively high in comparison to the amount humans are regularly exposed to. Therefore, a direct comparison of tinnitus between humans and experimental animals cannot be made. The amount was used to ensure that tinnitus had been subjected in the animals, so that neurochemical changes could be studied. Understanding of such changes can lead to possible future treatments.

The current study examined GABA_b R1 and GABA_b R2 subunits separately. Results can’t be used to make a definite conclusion about the effect of SS on functional GABA_b receptors. However, parallel changes in the level of immunoreactivity between the two receptor subunits strongly suggest that the level of functional GABA_b receptors were changed by the drug. Existing results indicate that GABA_b R1 is synthesized in the endoplasmic reticulum and contains a retention signal that does not allow it to travel to the plasma membrane. GABA_b R2 is required to bind to the GABA_b R1 subunit, which leads to masking of the retention signal and consequently a functional GABA_b receptor can be formed in the plasma membrane (Pin et al., 2004). Immunofluorescent double labeling experiments should be conducted to make a definite conclusion regarding the effect of SS on the level functional GABA_b receptors in limbic structures.

4.2 Possible neurophysiological consequences of an increase in the level of GABA_b R1 and GABA_b R2

An increase in the level of GABA_b receptors can lead to different neurophysiological consequences depending on whether these receptors are distributed
pre- or postsynaptically. Upon activation of postsynaptic GABA<sub>B</sub> receptors a prolonged inhibition occurs. After GABA binds to the receptor, it leads to the dissociation of the βγ-subunit from the α-subunit in the G-protein. This subunit activates GIRK, which is activated and allowed K<sup>+</sup> to leave the cell, thereby hyperpolarizing the cell membrane (Luscher et al., 1997; Ulrich and Bettler, 2007). By increasing the level of postsynaptic GABA<sub>B</sub> receptors, it is possible to decrease the neural activity due to an increase in inhibitory neurotransmission.

Presynaptic GABA<sub>B</sub> receptors function by inhibiting the release of GABA or glutamate neurotransmitters (Ma et al., 2002; Kornau, 2006). Upon GABA binding, the βγ-subunit dissociates from the α-subunit. It leads to the modification of voltage-gated Ca<sup>2+</sup> channels. This causes a reduction in Ca<sup>2+</sup> influx, which leads to a decrease in neurotransmitter release (Herlitze et al., 1996; Ikeda, 1996). By increasing the level of presynaptic GABA<sub>B</sub> receptors, there is possibility of either an increase or decrease in neural activity depending on which neurotransmitter is regulated. Regulation of glutamate can lead to a decrease in neural activity as this means a reduction in excitatory neurotransmission. Regulation of GABA can lead to an increase in neural activity as this means a reduction in inhibitory neurotransmission.

4.3 The effect of SS on the amygdala and hippocampus

4.3.1 Neural activity vs. the level of GABA<sub>B</sub> receptors

Previous studies have indicated that SS can lead to an increase in neural activity in the amygdala and hippocampus (Chen et al., 2014). The present study focused on neurochemical changes following a systemic application of SS and revealed an increase in the level of GABA<sub>B</sub> receptors in the amygdala and hippocampus. It is highly likely that
it was presynaptic GABA_B receptors that were increased in the present study. Such
presynaptic GABA_B receptors serve as auto-receptors and regulate the release of GABA.
This regulation can certainly enhance neural responses as revealed by the previous
physiological study conducted by Chen and colleagues. It should be noted that
GABAergic auto-receptors have been found in both the amygdala and hippocampus
(Yamada et al., 1999; Ariwodola and Weiner, 2004).

4.3.2 Area dependence in the change of level of GABA_B receptors

The amygdala showed a larger change in the level of GABA_B receptors than the
hippocampus. There is a logical explanations for such findings. The amygdala has direct
connections from the auditory structures, i.e. projections from the MGN, whereas the
hippocampus is indirectly connected to the auditory structures by projections to and from
the amygdala (Kraus and Canlon, 2012). The MGN sends direct projections to the LaN of
the amygdala (Graybiel, 1972; Aitkin, 1986) and the AC sends indirect projections to the
LaN (Kraus and Canlon, 2012). The LaN then sends projections to the BLA, which
sends direct projections to the CA1, CA3 and indirect connections to the SUB (Kraus and
Canlon, 2012).

Projections towards the auditory system include the CA1 and SUB, which send
projections to the LaN, BMA, and BLA. The BLA then sends direct projections to the IC
in the auditory pathway (Sah et al., 2003; LeDoux, 2007; Kraus and Canlon, 2012).

The current study found an area difference in which the largest increase in
GABA_B receptors was found in the LaN in the amygdala and the SUBv in the
hippocampus. The involvement of LaN and SUBv in receiving and sending information
from/to the CAS, respectively, is quite important. LaN receives projections from the
MGN, while SUB sends information towards the amygdala to relay it back to the CAS (Kraus and Canlon, 2012). However, further studies are required to understand the neural activity in each subdivision and how it relates to GABA$_B$ receptors. This would allow to further speculating as to what activity changes would be caused in the CAS due to limbic structures.

4.4. Effects of SS on the level of GABA$_B$ receptors in limbic structures vs. major auditory structures

Previous studies have indicated that systemic application causes a change in neural activity in both the central auditory system (House and Brackman, 1981; Barrs and Brackman, 1984; Berliner et al., 1992; Kaltenbach et al., 2005) and the limbic system (Mahlke and Wallhausser-Franke, 2004; Gong et al., 2008; Chen et al., 2014). Specifically, both these systems show an increase in neural activity following the application of the drug.

A neurochemical study (Butt et al., 2016) revealed that SS reduced the level of GABA$_B$ receptors in an auditory structure, the IC. In contrast, the present study found that the drug caused an increase in the level of GABA$_B$ receptors in limbic structures. Therefore, a seeming discrepancy exists between the drug effects on the level of GABA$_B$ receptors in the IC and limbic structures, inspite of similar increases in the physiological activity in these structures.

It is speculated that in the IC SS reduced postsynaptic GABA$_B$ receptors and/or presynaptic GABA$_B$ receptors on glutamatergic axon terminals. A reduction in these GABA$_B$ receptors would enhance the release of glutamate from presynaptic terminal or reduce the inhibitory postsynaptic potentials mediated by the postsynaptic GABAergic
neurons. Consequently, this could enhance the overall activity in the IC. In the limbic structures, it is possible that the drug increase presynaptic GABA\textsubscript{B} receptors on GABAergic axon terminals. Such an increase could reduce the release of GABA, leading to a decrease in inhibition and an increase in activity.

4.5 SS-induced change in the level of GABA\textsubscript{B} receptors: Implications for higher brain functions

4.5.1 Tinnitus

SS-induced tinnitus leads to changes in neural activity in the limbic structures (Chen et al., 2012) as well as central auditory structures (House and Brackman, 1981; Barrs and Brackman, 1984; Berliner et al., 1992). Previous studies have suggested that SS can affect neurotransmitter receptors and ion channels (Basta et al., 2000; Basta and Ernst, 2004). The present study viewed the neurochemical changes in GABA\textsubscript{B} receptors caused by SS in limbic structures. The drug showed to cause an increase in the GABA\textsubscript{B} receptors, which can lead to a change in neural activity, thereby tipping the balance of excitation and inhibition in the limbic structures.

Previous studies have found an increase in neural activity in the limbic structures following SS-induced tinnitus (Chen et al., 2012). It is not fully understood what changes could cause this gain. The present study showed an increase in the level of GABA\textsubscript{B} receptors in both the amygdala and hippocampus. The increase in activity can be due to the increase in GABA\textsubscript{B} receptors, specifically presynaptic GABA\textsubscript{B} receptors (see section 4.2 for details).

The limbic structures are connected to CAS, forming a neural circuit. The CAS forms a neural circuit as well in which they are also connected to each other. Change in
neural activity in one structure an affect others found in the neural circuit. Hyperactivity in the limbic structures can cause an enhancement in the CAS. It is likely that such an increase can lead to an enhanced synchronization and an epileptic form of firing responses. Such responses can be speculated to be responsible for tinnitus sensation.

4.5.2 Other functions (e.g., emotion, memory etc.)

The main roles of the amygdala and hippocampus are in emotion and memory, respectively. Regulation of neural activity is required throughout the CNS for appropriate higher order functioning (Gerken et al., 1984; Bauer et al., 1999). Therefore, it is highly possible that SS-induced tinnitus can affect these functions. An enhanced level of GABA receptors as observed in the present study can lead to a change in neural activity and consequently a change in higher order functions.

A study found strong correlations between tinnitus and stress, anxiety, and depression. Individuals with tinnitus have reported to be stressed due to the condition (Langguth, 2011). More research is required on understanding how memory is affected due to tinnitus. It is highly probable that there may be a decrease in memory formation. Many subjects that report tinnitus, explain a hard time concentrating. Since concentration is required during memory formation, it may become more difficult and require a longer period of time for those with chronic tinnitus to consolidate their memory.

Not much evidence exists for the changes tinnitus can cause to emotion and memory. Future research should focus on how SS can affect these functions. Understanding this can lead to possible clinical methods designed to help patients battling with emotional distress or who have trouble forming memories.
4.6 Possible cellular mechanisms underlying changes in the level of the GABA<sub>B</sub> receptors

As noted in this study, there was an increase in the level of GABA<sub>B</sub> receptors, following a systemic application of SS. Many proteins are required to allow this increase to happen. Previous studies have examined other proteins and their changes to proteins due to SS-induced tinnitus. In the amygdala, two immediate early genes were studied: arg3.1 and c-fos (Mahlke and Wallhausser-Franke, 2004). Following SS-induced tinnitus, the genes showed an increase in expression. These genes are required in many biological functions and its possible that they aid in synthesis of GABA/GABA<sub>B</sub> receptors. In another study, an increase in GAD1 levels was observed in the hippocampus after SS-administration (Im et al., 2007). The enzyme GAD1 is required to synthesize GABA; therefore an increase in GAD1 is an indirect indication of an increase in GABA. An increase in the neurotransmitter could indicate that it is upregulated because it is required to bind functional GABA<sub>B</sub> receptors.

4.7 Conclusions and Future directions

A systemic application of SS can lead to an increase in the level of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits in the amygdala and hippocampus three hours later. The drug causes a larger change in the amygdala than the hippocampus. These findings can help us understand the induction/manifestation of SS-induced tinnitus. Furthermore, they strongly suggest that further research should be conducted to examine how SS can cause changes in other functions of the brain such as emotion and memory.

The current study focused on how SS changed the level of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits. Further experiments should be conducted by using techniques such
as immunofluorescence double labeling and ligand binding. Double labeling can give insight to if the two receptor subunits are co-localized and forming heterodimers, which can give a firm conclusion about SS changes the level of functional receptors. Ligand binding can also be used to examine the effect of SS on functional receptors in the limbic structures.

Other experiments should be conducted to examine whether SS primarily affect pre- or postsynaptic GABA_B receptors. From the current study it was inferred that the drug-induced increase maybe due to presynaptic auto-receptors. To confirm these findings, experiments should be designed to label both GABA_B receptors and other pre- or postsynaptic proteins in PS- and SS-treated animals. In this way, a more definite answer as to whether pre- or postsynaptic GABA_B receptors are mainly affected by a systemic application of SS can be obtained. Furthermore, patch-clamp recordings can be used to examine whether blocking of GABA_B receptors leads to the change in the neurotransmitter release in PS- and SS-treated animals. All the experiments described above will all help enhance our understanding of the effect of SS on the level and function of the GABA_B receptor in limbic structures.
TABLES

**Table 1:** Summary results of OD values of the molecular layer of the cerebellum.

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<td>SS-treated</td>
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**Table 2:** Summary results of normalized OD values of overall changes in the amygdala

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**Table 3:** Summary results of normalized OD values of overall changes in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R1

<table>
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<th>BMA</th>
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<tbody>
<tr>
<td>PS-treated</td>
<td>0.2733</td>
<td>0.3982</td>
<td>0.3412</td>
<td>0.2418</td>
</tr>
<tr>
<td>SS-treated</td>
<td>0.5159</td>
<td>0.5238</td>
<td>0.6314</td>
<td>0.5112</td>
</tr>
</tbody>
</table>

**Table 4:** Summary results of normalized OD values of overall changes in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th></th>
<th>BLA</th>
<th>BMA</th>
<th>CeN</th>
<th>LaN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.2907</td>
<td>0.3272</td>
<td>0.2325</td>
<td>0.2543</td>
</tr>
<tr>
<td>SS-treated</td>
<td>0.4698</td>
<td>0.5005</td>
<td>0.4149</td>
<td>0.5171</td>
</tr>
</tbody>
</table>
Table 5: Summary results of normalized OD values of cell bodies in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R1

<table>
<thead>
<tr>
<th></th>
<th>BLA</th>
<th>BMA</th>
<th>CeN</th>
<th>LaN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.8167</td>
<td>0.9500</td>
<td>0.9500</td>
<td>0.7250</td>
</tr>
<tr>
<td>SS-treated</td>
<td>1.0917</td>
<td>1.1833</td>
<td>1.2700</td>
<td>1.0167</td>
</tr>
</tbody>
</table>

Table 6: Summary results of cell density (in cell bodies per square millimeter) in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R1

<table>
<thead>
<tr>
<th></th>
<th>BLA</th>
<th>BMA</th>
<th>CeN</th>
<th>LaN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>25</td>
<td>41</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td>SS-treated</td>
<td>36</td>
<td>46</td>
<td>51</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 7: Summary results of normalized OD values of cell bodies in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th></th>
<th>BLA</th>
<th>BMA</th>
<th>CeN</th>
<th>LaN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.8667</td>
<td>0.9750</td>
<td>0.7700</td>
<td>0.7500</td>
</tr>
<tr>
<td>SS-treated</td>
<td>1.3500</td>
<td>1.4417</td>
<td>1.2800</td>
<td>1.2100</td>
</tr>
</tbody>
</table>

Table 8: Summary results of cell density (in cell bodies per square millimeter) in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th></th>
<th>BLA</th>
<th>BMA</th>
<th>CeN</th>
<th>LaN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>38</td>
<td>38</td>
<td>48</td>
<td>34</td>
</tr>
<tr>
<td>SS-treated</td>
<td>46</td>
<td>45</td>
<td>57</td>
<td>48</td>
</tr>
</tbody>
</table>
### Table 9: Summary results of normalized OD values of overall changes in the hippocampus

<table>
<thead>
<tr>
<th></th>
<th>GABA_BR1</th>
<th>GABA_BR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.3768</td>
<td>0.4976</td>
</tr>
<tr>
<td>SS-treated</td>
<td>0.4555</td>
<td>0.5977</td>
</tr>
</tbody>
</table>

### Table 10: Summary results of normalized OD values of overall changes in the major subdivisions of the amygdala labeled against GABA_BR1

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.3516</td>
<td>0.4090</td>
<td>0.4312</td>
<td>0.3520</td>
<td>0.2975</td>
<td>0.4195</td>
</tr>
<tr>
<td>SS-treated</td>
<td>0.4697</td>
<td>0.5483</td>
<td>0.5026</td>
<td>0.3842</td>
<td>0.3743</td>
<td>0.4540</td>
</tr>
</tbody>
</table>

### Table 11: Summary results of normalized OD values of overall changes in the major subdivisions of the amygdala labeled against GABA_BR2

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.4741</td>
<td>0.5717</td>
<td>0.4892</td>
<td>0.4567</td>
<td>0.4386</td>
<td>0.5551</td>
</tr>
<tr>
<td>SS-treated</td>
<td>0.5958</td>
<td>0.6248</td>
<td>0.6049</td>
<td>0.5447</td>
<td>0.5235</td>
<td>0.6925</td>
</tr>
</tbody>
</table>

### Table 12: Summary results of normalized OD values of cell bodies in the major subdivisions of the amygdala labeled against GABA_BR1

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>0.9750</td>
<td>0.9517</td>
<td>1.0200</td>
<td>1.0333</td>
<td>0.9083</td>
<td>0.9500</td>
</tr>
<tr>
<td>SS-treated</td>
<td>1.1750</td>
<td>1.1417</td>
<td>1.2500</td>
<td>1.2333</td>
<td>1.1167</td>
<td>1.2000</td>
</tr>
</tbody>
</table>
**Table 13:** Summary results of cell density (in cell bodies per square millimeter) in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R1

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>28</td>
<td>58</td>
<td>44</td>
<td>19</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>SS-treated</td>
<td>33</td>
<td>71</td>
<td>44</td>
<td>21</td>
<td>47</td>
<td>39</td>
</tr>
</tbody>
</table>

**Table 14:** Summary results of normalized OD values of cell bodies in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>1.10</td>
<td>1.11</td>
<td>1.16</td>
<td>1.08</td>
<td>1.04</td>
<td>1.15</td>
</tr>
<tr>
<td>SS-treated</td>
<td>1.42</td>
<td>1.43</td>
<td>1.53</td>
<td>1.41</td>
<td>1.43</td>
<td>1.55</td>
</tr>
</tbody>
</table>

**Table 15:** Summary results of cell density (in cell bodies per square millimeter) in the major subdivisions of the amygdala labeled against GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>DG</th>
<th>SUBd</th>
<th>SUBv</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-treated</td>
<td>27</td>
<td>42</td>
<td>36</td>
<td>32</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>SS-treated</td>
<td>29</td>
<td>53</td>
<td>43</td>
<td>34</td>
<td>46</td>
<td>44</td>
</tr>
</tbody>
</table>
REFERENCES


autoradiographic localization of individual components. *Alan R. Liss, Inc.* 299-313.


APPENDICES

Solutions needed for immunohistochemistry experiments:

**0.5M Sodium Phosphate Dibasic**

For 1L:

- 70.98g sodium phosphate dibasic powder (NaH$_2$PO$_4$$\cdot$H$_2$O)
- 1L of diH$_2$O

**0.5M Sodium Phosphate Monobasic**

For 1L:

- 68.99g anhydrous sodium phosphate monobasic (NaH$_2$PO$_4$)
- 1L diH$_2$O

**0.4M Sodium Phosphate Buffer (PB) (pH=7.2)**

For 1L:

- 600mL 0.5M sodium dibasic solution
- 200mL 0.5M sodium phosphate monobasic solution
- 200mL diH$_2$O

**0.1M Sodium Phosphate Buffer (PB) (pH=7.2)**

For 1L:

- 250mL 0.4M PB
- 750mL diH$_2$O

**0.1M Phosphate Buffer Saline (PBS) (pH=7.2)**

For 1L:

- 250mL 0.4M PB
- 750mL diH$_2$O
4% Paraformaldehyde (PFA) in 0.1PB (pH=7.2)

For 1L:

- 40g PFA powder
- 1L 0.1M PB

PFA can be dissolved into the PB by heating on a stir plate at a low heat setting. pH can be adjusted by adding HCl or NaOH.

Cyroprotectant solution

For 100mL:

- 30g sucrose
- 100mL PB

0.05% Triton in 0.1M PBS (TPBS)

For 1L:

- 0.05mL Triton X-100
- 1L 0.1M PBS

3,3’ diaminobenzidine tetrahydrochloride (DAB)-nickel solution

For 20mL:

- 5mL 0.4M PB
- 200µL 0.4% NH₄Cl in 0.1M PB
- 200µL glucose
- 13.6mL diH₂O
- 200µL 50mg/ml DAB in 0.1M PB
- 0.8mL 1% Nickel sulfate in diH₂O
- 20µL glucose oxidase
VITA AUCTORIS

NAME: Fareeha Ashraf
PLACE OF BIRTH: Gujrat, Pakistan
YEAR OF BIRTH: 1992
EDUCATION: Catholic Central High School, Windsor, ON, 2010
University of Windsor, B.Sc., Windsor, ON, 2014
University of Windsor, M.Sc., Windsor, ON, 2016