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# The effect of sodium salicylate on the level of GABAB receptor subunits in the rat's central auditory system

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**THE EFFECT OF SODIUM SALICYLATE ON THE LEVEL OF GABA<sub>B</sub> RECEPTOR  
SUBUNITS IN THE RAT'S CENTRAL AUDITORY SYSTEM**

By

**Sehrish Butt**

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

2014

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June 18, 2014

### **Author's Declaration of Originality**

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## ABSTRACT

Tinnitus is a phantom sensation of sound in the absence of acoustic stimulus and can be induced by ototoxic drugs such as sodium salicylate (SS). Tinnitus is likely related to hyperactivity in central auditory structures. In the central nervous system, activity is dependent on excitatory/inhibitory neurotransmission. Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter. It can activate the type-B GABAergic receptor (GABA<sub>B</sub> receptor), which can control the level of excitation/inhibition through regulating the release of neurotransmitters and lowering the cell membrane potential. I hypothesized that SS affects hearing by changing the level and distribution of the GABA<sub>B</sub> receptor in central auditory structures. My results from immunohistochemical and western blotting experiments revealed that SS reduced the level of GABA<sub>B</sub> receptors in all major auditory structures. The reduction was observed in both neuronal cell bodies regions and areas containing axonal and dendritic fibers of neurons.

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## LIST OF ABBREVIATIONS

AC- auditory cortex

AMPA-  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANF- auditory nerve fibers

AUD- dorsal belt area of the auditory cortex

AUV- ventral belt area of the auditory cortex

AVCN- anterior ventral cochlear nucleus

CAS- central auditory system

CB- cerebellum

CN- cochlear nucleus

DAB- 3'3 diaminobenzidine tetrahydrochloride

DCN- dorsal cochlear nucleus

DNLL- dorsal nucleus of the lateral lemniscus

EPSP- excitatory post-synaptic potential

ER- endoplasmic reticulum

GABA- gamma-aminobutyric acid

GAD- glutamatic acid decarboxylase

GDC- granule cell domain

HD- heptahelical domain

IC- inferior colliculus

ICc- central nucleus of the inferior colliculus

ICd- dorsal cortex of the inferior colliculus

ICx- external cortex of the inferior colliculus

IHC- immunohistochemistry

IPSP- inhibitory post synaptic potentials

LNTB- lateral nucleus of the trapezoidal body

LSO- lateral superior olivary nucleus

MG- medial geniculate nucleus

MGd- dorsal division of the medial geniculate nucleus

MGm- medial division of the medial geniculate nucleus

MGv- ventral division of the medial geniculate nucleus

MNTB- medial nucleus of the trapezoidal body

MSO- medial superior olivary nucleus

NDS- normal donkey serum

NLL- nucleus of the lateral lemniscus

NMDA- N-methyl-D-aspartic acid

OHC- outer hair cells

PB- phosphate buffer

PBS- phosphate buffer saline

PFA- paraformaldehyde

PVCN- posterior ventral cochlear nucleus

SOC- superior olivary complex

SPN- superior paraolivary nucleus

SS- sodium salicylate

Te1/AU1- primary auditory cortex

TBST- tris-buffered saline tween

VCN- central cochlear nucleus

VFT- venus flytrap

VNLL- ventral nucleus of the lateral lemniscus

VNTB- ventral nucleus of the trapezoidal body

# **1 Introduction**

## **1.1 Audition**

Audition or hearing is the ability of an organism to sense sound. It is one of the five basic senses and is important for an organism's biological fitness. Hearing is involved in many behaviours such as prey catching, predator avoidance, mating, and social communication. Hearing impairment can have detrimental consequences on an organism's survival (Capranica, 1965). In vertebrates including human, hearing is dependent on the auditory system, which can be divided into the peripheral and the central auditory system (Pickles, 2012).

## **1.2 Peripheral auditory system**

The peripheral auditory system consists of the outer, middle, and inner ear. The outer ear contains the external ear, also known as pinna, and the ear canal. The middle ear includes the three ossicles, i.e., the malleus, incus, and stapes. The inner ear includes the cochlea, a spiral shaped structure. The outer and the middle ears are separated by the tympanic membrane, also called the eardrum. The middle and inner ears are separated by the oval window of cochlea (Silverthorn, 2007). Sound waves are transmitted by the pinna to the ear canal where they hit the tympanic membrane, causing the membrane to vibrate. The vibrations of the tympanic membrane are amplified by the three ossicles and transferred via the oval window to the Organ of Corti which is the sensory portion of the cochlea.

A cochlear duct divides the cochlea into three fluid-filled compartments called scalae vestibuli, scalae tympani, and scalae media. Scalae vestibuli and tympani contain a fluid known as perilymph. This fluid has a high concentration of  $\text{Na}^+$  ions and a low concentration of  $\text{K}^+$  ions (Johnstone and Sellick, 1972). Scalae media contains endolymph which has a high concentration

of  $K^+$  ions and a low concentration of  $Na^+$  ions. A thin sheet of connective tissue, called the basilar membrane, prevents the endolymph and perilymph from mixing (Bosher and Warren, 1968).

The Organ of Corti is a sensory epithelium that sits upon the basilar membrane. It is composed of two types of sensory cells: inner hair cells (IHC) and outer hair cells (OHC). OHCs are able to change their length in response to cell membrane depolarization/hyperpolarization, and this property is referred to as electromotility (Bronwell, 1990). OHCs contact another membrane called the tectorial membrane which overlays the hair cells. IHCs and OHCs contain stereocilia at their tips or free ends. The stereocilia of hair cells are connected through tip-links. Sound vibrations cause the basilar membrane and the tectorial membrane to move, which in turn moves the hair cells. This movement displaces the stereocilia, causing mechanosensitive cation channels located near the tips of stereocilia to open, resulting in the influx of  $K^+$  ions from the endolymph into a hair cell and depolarization of the cells (Hudspeth, 1982). Thus mechanical sound vibrations are converted into electrical potentials, which travel via the cochlear nerve to the central auditory system (Silverthorn, 2007).

### **1.3 Central auditory system (CAS)**

In mammals including humans, the central auditory system is composed of six major neural structures including the cochlear nucleus (CN), superior olivary complex (SOC), nucleus of lateral lemniscus (NLL), inferior colliculus (IC), medial geniculate nucleus (MG or MGN), and the auditory cortex (AC) (See Figure 1). CN, SOC and NLL make up the auditory brainstem, whereas IC makes up the midbrain. MG and AC constitute the auditory forebrain. The six major auditory structures form a hierarchy with the CN at the bottom receiving inputs from the inner

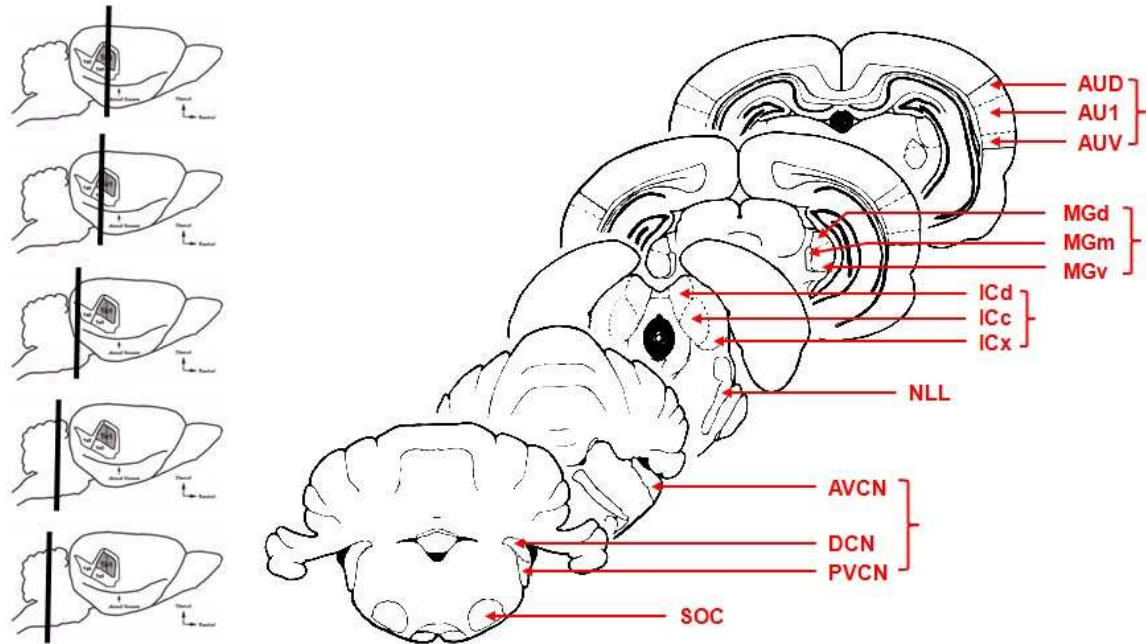


ear while the AC is at the top. The six central auditory structures and their subdivisions can be seen in Figure 1.

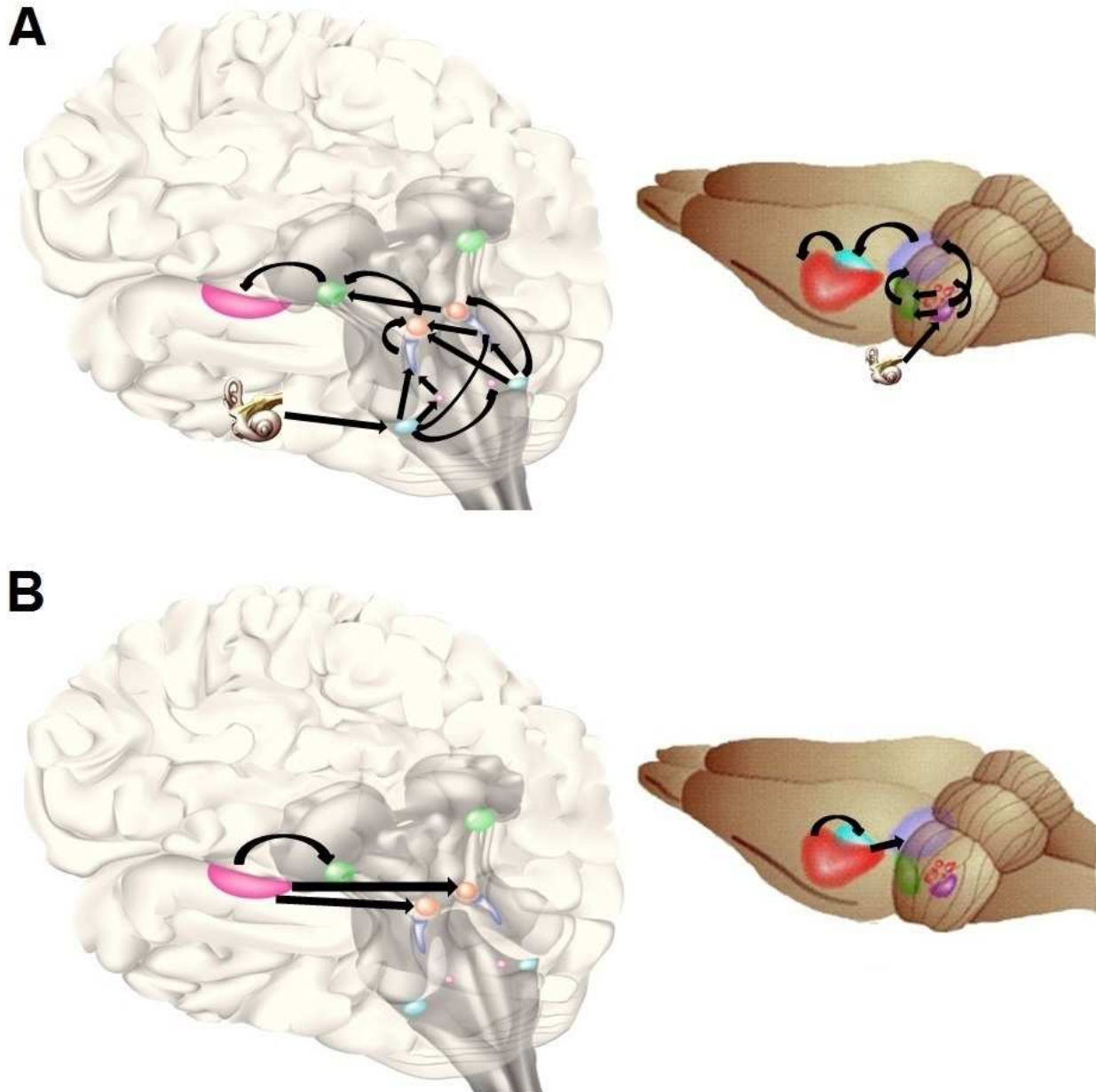
### **1.3.1 Neural connections in CAS**

Projections in the CAS can be defined as ascending or descending. Ascending projections refer to projections from lower structures i.e. structures located near the sensory organ, to higher structures i.e. structures located near the cerebral cortex (Figure 2a). Descending projections refer to projections from higher structures to lower structures (Figure 2b). Ascending projections connect CN to SOC, NLL, and IC (Schofield and Coomes, 2005). SOC sends ascending projections to NLL (Grothe and Koch, 2011), and NLL send projections to IC (Zhao and Wu, 2001). The IC sends ascending projections to MG on both sides of the brain (Malmierca and Merchan, 2005). MG sends ascending projections exclusively to the ipsilateral AC (Winer and Schreiner, 2005).

The most important descending projections include those from the AC to the MG and the IC. The IC also sends descending projections to the periolivary medial olivocochlear cells which innervate the OHCs. Thus, a pathway from the AC to the sensory OHCs is formed (Faye-lund, 1986; Caicedo and Herbert, 1993; Vetter et al., 1993, Saldana, 1993). It is thought that the descending projections from the AC to the MG modulate the processing of auditory neural signals carried by ascending projections to this thalamic structure (Winer and Larue, 1987; Roger and Arnault, 1989; Arnault and Roger, 1990, Shi and Cassel, 1997). Descending projections also modulate processing in the IC (Faye-Lund, 1985; Coleman and Clerici, 1987; Games and Winer, 1988; Roger and Arnault, 1989), the SOC (Feliciano et al., 1995), and the CN (Feliciano et al., 1995; Weedman et al., 1996a, 1996b).



**Figure 1:** Schematic representation of coronal sections of the rat brain containing central auditory structures. Left: diagrams of a rat brain with the black bar indicating the location of its respective coronal slice. Right: Coronal sections containing central auditory structures in ascending order going from the bottom to top slice. The first 2 slices contain SOC, CN and its three subdivisions: DCN, PVCN, and AVCN. The third slice contains NLL and IC which is divided into its three subdivisions: ICd, ICx, and ICc. The fourth slice contains the three subdivisions of the MG: MGd, MGv, and MGm. The top slice contains the three subdivisions of the AC: AU1, AUD, and AUV.



**Figure 2.** Diagrams of a human brain (left) and a rat brain (right) showing projections between auditory structures. A: Major ascending projections between auditory structures from cochlea to the auditory cortex. Intrinsic and commissural projections are not shown for the purpose of clarity. B: Major descending projections between central auditory structures.

There are also intrinsic projections, i.e., projections confined into an individual nucleus, and commissural projections, i.e., projections crossing the midline of the brain and connecting the structures on both sides (mouse: Gonzalez-Hernandez et al., 1986; rat: Saldana and Merchan, 1992).

### **1.3.2 Cochlear nucleus (CN)**

The CN is located in the hindbrain and is the first neural structure along the ascending auditory pathway. It receives direct projections from the spiral ganglion cells in the peripheral auditory system. CN sends projections to the contralateral SOC, IC, NLL, and MG. CN also receives descending projections from the AC, IC, SOC, and ventral NLL (Schofield and Coomes, 2005). CN can be divided into two major subdivisions: the dorsal CN (DCN) and the ventral CN (VCN). The VCN can be further subdivided into anterior VCN (AVCN) and posterior VCN (PVCN). These subdivisions can be seen in Figure 1.

#### *VCN*

VCN is divided into its posterior and anterior subdivision by the cochlear root nerve. AVCN receives input from the ascending branch of the cochlear root nerve, whereas PVCN receives input from the descending branch. Cells in the VCN also receive descending inputs from the DCN and AC. Cells in PVCN receive a large number of afferents from auditory nerve fibers (ANF) (McGinley and Oertel, 2006), and send projections to the contralateral NLL, the dorsal region of ipsilateral SOC (Winer and Schreiner, 2005), and the ventral region of the contralateral SOC (Warr, 1982). Cells in the AVCN project to the ipsilateral DCN, contralateral and ipsilateral ventral SOC, ventral IC, and contralateral CN (Merchan et al., 1988). The innervation of VCN by ANFs is in a tonotopic manner i.e., ANF fibres with lower frequencies

innervate the ventral region of VCN, while fibres with high frequencies innervate the dorsal region of VCN (Manis, 1989; Zhang and Oertel, 1994).

Cells in PVCN fire strongly at the onset of a stimulus (McGinley and Oertel, 2006). They also respond to the transient component of an ongoing stimulus, and are specialized to detect temporal fluctuations in complex sounds (Cao and Oertel, 2010). Other cells in PVCN encode complex sounds in a noisy background, and convey fluctuations in ongoing stimuli (Cao and Oertel, 2010).

Cells in the AVCN fire whenever ANFs fire, and are able to send precise information about the temporal properties of acoustic stimuli to the SOC. This information is used by the SOC in sound localization (Young et al., 1988a).

### *DCN*

The DCN can be divided into four layers and a granule cell domain (GCD). The most superficial layer, called molecular layer, contains granule cells and small interneurons (Oertel and Young, 2004). The second layer contains pyramidal cells, which are the major cell type of DCN. These pyramidal cells receive projections from VCN (Mugnaini et al., 1980a, 1980b), and send projections to the contralateral IC and the medial division of MG (Schofield and Coomes, 2005). The third and fourth layers, also called the deep layers, contain giant and vertical cells. Neurons in these layers are contacted by axons of auditory nerve fibers. There is a tonotopic organization in the DCN. While the lateral and ventral part of the structure is innervated by ANFs with low frequencies, the dorsal and medial part of DCN is innervated by fibers with high frequencies (Manis, 1989; Zhang and Oertel, 1994). Thus cells in DCN form isofrequency sheets, with similar frequency neurons grouping together (Wickesberg and Oertel, 1988). Deep

layer neurons also receive local innervations from interneurons in the neighbouring superficial layers, and send projections to the contralateral IC and the MG (Osen et al., 1990).

The GCD is continuous with the first layer of DCN, and is made up of small cells. These small cells receive ascending projections from VCN and the cochlear nerve (Mugnaini et al., 1980a, 1980b). The cells in the GCD project to the pyramidal cells in the superficial layers of DCN, the IC, medial MG, and SOC (Malmierca et al., 2002).

DCN is involved in many complex functions. It is affected by somatosensory inputs and may help suppress sounds generated by movements or from self-vocalization (Kanold et al., 2011; Keohler et al., 2011).

### **1.3.3 Superior Olivary Complex (SOC)**

Like the CN, the SOC is also located in the hindbrain. This structure is made up of a group of small subnuclei including: 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 projections from the CN, and ipsilateral IC. It sends projections to the CN, NLL, and IC. SOC plays a role in sound localization (van Adel and Kelly, 1998), and has also been shown to play a role in inhibiting responses to echoes of sounds (Malmierca and Merchan, 2005). In spite of the fact that the SOC is an important structure in central auditory processing, it wasn't a part of my thesis research as existing results indicate that this structure does not play an important role in the generation of tinnitus and it has a relatively low level of the type-B gamma-aminobutyric acid receptor (GABA<sub>B</sub> receptor) (see section

1.4.2.2.2). The level of the GABA<sub>B</sub> receptor and the effect of a tinnitus inducer sodium salicylate on the level and distribution of the receptor was the focus of the present study.

### **1.3.4 Nucleus of the Lateral Lemniscus (NLL)**

The NLL is a fibrous structure located beneath the IC (Figure 1). It can be divided into two subdivisions: the dorsal NLL (DNLL) and the ventral NLL (VNLL). VNLL receives inputs driven by the contralateral ear, and is involved in detecting temporal features of auditory stimuli (Aitkin et al., 1970). DNLL receives inputs driven by both ears, and is thought to play an important role in sound localization (Aitkin et al., 1970; Bruggae et al., 1970; Bajo et al., 1998). In spite of the fact that the NLL is an important structure in central auditory processing, it wasn't a part of my thesis research due to the low level of the GABA<sub>B</sub> receptor and the lack of evidence showing the involvement of this structure in the generation of tinnitus.

### **1.3.5 Inferior Colliculus (IC)**

The IC is a midbrain structure and is a major auditory processing centre. It receives input from all the brainstem structures and higher structures, and is the primary source of input to the MG, which in turn projects to the AC. The IC can be divided into three subdivisions: dorsal cortex of the inferior colliculus (ICd), central nucleus of the inferior colliculus (ICc) and the external cortex of the inferior colliculus (ICx) (Figure 1). These subdivisions differ in their cytoarchitecture, neural connections, and functions. ICc is considered an auditory nucleus, and is also referred to as the "lemniscal" part of the auditory midbrain because it receives heavy inputs from the nucleus of lateral lemniscus. ICx and ICd are called "non-lemniscal" or "extra-lemniscal" nuclei. They form a multisensory auditory pathway and surround the specific auditory pathway formed by ICc (Morest, 1964a).

### *The ICc*

The ICc primarily receives inputs from the ipsilateral VNLL and MSO, contralateral CN, and ipsilateral and contralateral DNLL and LSO. Inputs from MSO and LSO are sent to the ventral part of the ICc, whereas inputs from CN and DNLL end up in the dorsal region of the ICc (Malmierca et al., 1999a; 1999b). ICc also receives inputs from the ipsilateral and contralateral ICx and ICd (Winer, 2005). There are some inputs to the ICc from higher structures, such as weak projections from the AC (Schofield, 2009). ICc projects strongly to the ventral region of the MG on both sides of the brain, and sends weak projections to the medial and dorsal regions of the MG. It also sends some projections to the contralateral IC (Malmierca and Merchan, 2005).

Neurons in the ICc are classified into “flat” and “less flat” categories based on the shape of their dendritic fields (Malmierca et al., 1993). Afferent lemniscal fibers and neurons with flattened dendritic fields in the ICc form “fibrodendritic laminae” (Morest, 1964a). Less flat neurons populate the space between laminae. These fibrodendritic laminae are thought to be responsible for processing sounds with different frequencies, with those in the dorsal region responsible for low frequencies and those in the ventral region responsible for high frequencies. Thus the fibrodendritic laminae form a structural basis of a tonotopic map. Physiological studies have shown differences in the best frequency for different laminae, supporting that each lamina covers a small range of frequencies so that all frequencies may be represented (Schreiner and Langner, 1997).

Physiological studies have also shown that cells in ICc possess directional selectivity. Inputs driven by the contralateral ear generate higher/greater responses than inputs driven by the



ipsilateral ear (Kuwada et al., 2011). Because IC receives projections from higher and lower auditory structures, it plays an important role in auditory processing.

### *The ICx*

The ICx is located on the lateral side of the IC and can be divided into three layers. It receives the majority of its inputs from the ipsilateral MG, neighbouring ICc and ICd, and ipsilateral and contralateral AC (Malmierca et al., 1999a; 1999b). It also receives weak projections from the NLL. ICx also receives somatosensory input from the trigeminal nuclei. It projects to the medial and dorsal regions of the ipsilateral and contralateral MG (Faye-Lund and Osen, 1985; Huffman and Henson, 1990).

Neurons in ICx respond to binaural input i.e. input from both ears, and have been shown to habituate rapidly. These neurons are excited by auditory stimuli and inhibited by somatosensory stimuli (Aitkin et al., 1978). The exact function of ICx has not been established but researchers believe that this structure might be an integrative area for auditory and somatosensory inputs (Jain and Shore, 2006).

### *The ICd*

The ICd can be divided into four layers. Main sources of input to this structure arise from the ipsilateral and contralateral AC (Bajo et al., 2007). It also receives inputs from the ipsilateral ICx and ICc, the contralateral IC, DNLL, SPN, and CN. ICd projects to the dorsal region of the MG and ICc (Malmierca and Merchan, 2005). The function of this subdivision has not been established, though it has been shown to play a part in sensory systems other than auditory system (Malmierca and Merchan, 2005).

Neurons in the ICd respond to both monaural and binaural stimuli and habituate rapidly (Aitkin et al., 1975). These neurons show a strong response at the onset of stimuli, and to changes in stimuli, suggesting that ICd might be involved in detecting novel sounds (Lumani and Zhang, 2010).

### **1.3.6 Medial Geniculate Nucleus (MG)**

The MG is a thalamic structure, and is the last major structure before the AC in the ascending auditory pathway. It receives projections from the IC and sends ipsilateral projections to the AC. It also receives heavy inputs from the AC. MG can be divided into three subdivisions: dorsal division of the medial geniculate nucleus (MGd), medial division of the medial geniculate nucleus (MGm), and ventral division of the medial geniculate nucleus (MGv) (Figure 1).

#### *The MGv*

MGv is part of the "lemniscal" pathway and is considered a specific auditory relay centre. It receives inputs from the ipsilateral IC, and weak projections from the contralateral ICc (Gonzalez-Hernandez et al, 1991). It also receives descending inputs from the AC (Malmierca and Merchan, 2005). MGv is a major source of input to the AC in the ascending pathway, and has major targets in the ipsilateral AC (Winer and Schreiner, 2005). MGv is known to be mainly involved in auditory processing, whereas MGm and MGd are known to play a role in integration of inputs from auditory and other sensory systems (Bordi and LeDoux, 1994).

MGv has a laminar structure, with the laminae running parallel to the isofrequency planes. These isofrequency regions form a tonotopic organization, with the dorsal region of MGv responsible for low frequencies and the ventral region responsible for high frequencies (Cetas et al., 2003). Physiological studies have shown that the best frequency changes between the planes,

suggesting that patches of neurons might form functional groups, each representing a range of frequencies (Cetas et al., 2003). Neurons in MGv respond to binaural stimuli. Behavioural studies have shown that this structure does not play a major role in sound localization (Kelly and Judge, 1985).

### *The MGm*

This is the smallest of the three subdivisions of MG, and is composed of a thin layer in the medial part of the MG. This subdivision receives ascending inputs from a wide range of auditory structures, including the ICx, CN, SOC, and VNLL (LeDoux et al., 1987). MGm also receives descending inputs from the AC and reticular thalamic nucleus (Winer, 2005). Besides auditory inputs, MGm receives somatosensory and visual inputs (Malmierca et al., 2002). MGm projects to the ICd, ICx, and the AC (LeDoux et al., 1987; Winer, 2005). It also projects to non-auditory areas including the somatosensory cortex (Spreafico et al., 1981), and the amygdala (Otterson and Ben-Ari, 1979; Otterson, 1981; LeDoux, 1985b, 1987).

MGm is not tonotopically organized. However, the ventral region of MGm contains higher frequency neurons. Because this subdivision projects to auditory and somatosensory regions, it might respond to both types of sensory stimuli (Bordi and LeDoux, 1994b). Its projections to the amygdala suggest that MGm is involved in processing the emotional significance of auditory stimuli (LeDoux et al., 1984, 1985b).

### *The MGd*

The MGd receives ascending projections primarily from the non-lemniscal parts of the IC i.e. the ICd, and ICx. It receives descending projections from the AC (Winer, 2005). MGd also receives projections from the somatosensory system (Winer, 1985). As MGd sends projections to

the ICx, a feedback loop may exist between the two structures (Winer, 1992). The main target of MGd projections is the non-primary auditory cortex i.e. secondary auditory cortical areas (Malmiera and Merchan, 2004).

MGd is not tonotopically organized and its neurons do not respond strongly to acoustic stimuli. These neurons have been shown to prefer complex sounds over pure tone stimuli, suggesting that this subdivision might be involved in discriminating sound patterns (Bordi and LeDoux, 1994a, 1994b).

### **1.3.7 Auditory Cortex (AC)**

The AC is part of the temporal cortex, and is found on the dorsolateral surface of the brain. It plays an important role in auditory sensation, perception, and cognition (Gaese and Ostwald, 1995). The AC in the rat can be subdivided in two ways. The first way is based on thalamocortical inputs to the structures as well as physiological responses. In this way the AC is subdivided into three regions; a central core which is called AU1 or Te1, and surrounding belt regions located dorsally and ventrally, called Te2 and Te3 (Figure 1). Te1 or AU1 is considered to be the primary auditory cortex (Games and Winer, 1987; Roger and Arnault, 1989; Herbert et al., 1991; Saldana et al., 1996) whereas Te2 and Te3 are considered to be secondary auditory cortices (Arnault and Roger, 1990). The AC can also be divided into six layers based on neuronal architecture. These layers are labelled layer I to VI, and are anatomically and physiologically distinct from each other.

#### *Te1(AU1)*

Te1 is connected with MGv in a tonotopic manner. It also receives some projections from MGd and MGm (Roger and Arnault, 1989), and projects to Te2 and Te3 (Romanski and

LeDoux, 1993b). Studies involving ablation of Te1 have shown that it is not involved in sound localization (Kelly, 1980). However, it is important for recognition of complex acoustic signals (Gaese and Ostwald, 1995).

### *Te2 and Te3*

Te2 is connected to MGd and MGm, whereas Te3 is only connected to Mgm (Arnault and Roger, 1990; Winer et al., 1999c). Te2 and Te3 also send projections to the amygdala (Romanski and LeDoux, 1993a; Shi and Cassel, 1997). Te3 has been shown to play an important role in discriminating novel complex sounds (Wan et al., 2001).

### *Layers I-IV*

Layer I is the most superficial layer of the AC and contains a very small number of neurons. Its primary sources of input are from MGm and cortical cells from within (Winer, 1992; Winer and Schreiner, 2005). Layer II lies below Layer I, and contains many pyramidal and non-pyramidal shaped neurons (Winer, 1992). This layer projects to the neighbouring layers III and IV. Like Layer II, Layer III also has many pyramidal and non-pyramidal neurons. This layer receives intrinsic input from the neighbouring ipsilateral Layer II, contralateral AC, and MGv. Layer IV is the thinnest among all the six layers of the AC and is densely packed with non-pyramidal small neurons (Games and Winer, 1988). This layer receives direct projections from MGv and Layer II (Winer and Schreiner, 2005).

### *Layer V*

This is the thickest layer in the AC and contains many pyramidal neurons with long apical dendrites that project perpendicular to the surface of the brain. This layer receives inputs

from ipsilateral Layers III and IV, and from MG. Output from this layer varies according to the neuron type that is projecting. Large neurons with long apical dendrites, located deep within the layer, project to the ICd and ICx on both sides of the brain. Small neurons, located in the superficial region of the layer, project to the contralateral AC. Medium sized neurons project to the AC and IC (Games and Winer, 1988). Neurons in the most superficial and the deepest regions of this layer project to the ipsilateral MGv and MGd (Winer and Prieto, 2001).

#### *Layer VI*

This layer contains pyramidal and non-pyramidal neurons which are small in size and very densely packed (Schofield, 2009). Input to this layer comes from MGm (Winer and Schreiner, 2005). Output from this layer is exclusive to the ipsilateral structures including MGd, MGm, ICd, and ICx. It also weakly projects to the neighbouring Layer V, the SOC, and CN (Schofield, 2009).

### **1.4 Neurotransmission in the CAS**

Neural processing of acoustic information is dependent on the transmission of signals among auditory neurons. This transmission is dependent on molecules called neurotransmitters. In the central auditory system, major neurotransmitters leading to an excitatory effect is glutamate while major neurotransmitter leading to an inhibitory effect include glycine and gamma-aminobutyric acid (GABA). Binding of a neurotransmitter to its specific receptor can lead to either excitation or inhibition in a neuron on which a receptor is located. The receptors can be of two types: ionotropic or metabotropic.

Ionotropic receptors are linked directly to ion channels. Their extracellular domain has a site for ligand binding. Their membrane-spanning domain is an ion channel. Upon

neurotransmitter binding, the ion channel is opened, allowing for flow of ions. Ionotropic receptors are also called ligand-gated ion channels. Because these receptors can open ion channels directly after neurotransmitter binding, they mediate rapid post-synaptic effects. They can be activated within a millisecond of action potential arrival, and only remain activated for a few tens of milliseconds (Purves et al., 2001).

Metabotropic receptors are not directly linked to ion channels. Instead, they modulate ion channels through an intracellular signalling cascade. Metabotropic receptors are linked to molecules called G-proteins. The extracellular domain of metabotropic receptors has a binding site for a neurotransmitter. The intracellular domain binds to G-proteins. Binding of the neurotransmitter causes the activation and subsequent dissociation of G-proteins from the receptor. The dissociated G-proteins can directly interact with an ion channel, or can lead to the production of intracellular second messengers and activate a cascade of events and eventually modify ion channels. Because their mode of action involves multiple steps and proteins, they produce slower responses ranging from hundreds of milliseconds to minutes or longer. Metabotropic receptors are also called G-protein-coupled receptors (Purves et al., 2001).

#### **1.4.1 Glutamatergic neurotransmission**

Glutamate is the most abundant neurotransmitter in the central nervous system (Silverthorn, 2007). The neurotransmitter is stored in vesicles in the terminal of an axon of a neuron, and is released upon the depolarization of the cell membrane of the terminal by an action potential. The binding of glutamate with its post-synaptic receptors can lead to either excitatory or inhibitory effect, depending on the specific subtypes of the receptors. Glutamate is usually considered an excitatory neurotransmitter, as in most cases its effect is associated with depolarization of the cell membrane. In the central nervous system, there are two major

categories of glutamatergic receptors, namely the ionotropic glutamatergic receptors and the metabotropic glutamatergic receptors.

Major types of glutamatergic receptors in the central auditory system are ionotropic. They include the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and the N-methyl-D-aspartic acid (NMDA) receptors (Silverthorn, 2007).

#### **1.4.1.1 AMPA receptors**

These ionotropic receptors are gated ion channels that are permeable to sodium and potassium ions. Upon binding by glutamate, the channel opens and allows sodium ions to flow into the cell and potassium ions to flow out of the cell, resulting in a net effect of depolarization of the cell membrane and production of excitatory post-synaptic potentials or EPSPs. These receptors have a fast time course of activation, and are important for processing information associated with changes, e.g. the acoustic onset of a sound (Zhang and Kelly, 2001).

#### **1.4.1.2 NMDA receptors**

Similar to the AMPA receptors, NMDA receptors are gated ion channels that are permeable to sodium and potassium ions. In addition to those ions, NMDA channels are also permeable to calcium ions. Under normal resting potentials, the channel pore of an NMDA receptor is blocked by magnesium ions. This blockage prevents fluxes of ions. The blockage can be removed by a slight depolarization of the membrane, which is provided by the activation of nearby AMPA receptors. When the magnesium ions are removed, cations including calcium ions can move through the channel, causing further depolarization of the membrane. In contrast to the AMPA receptors, the NMDA receptors are important for mediating responses to sustained sound stimulation (e.g., the plateau of a tone burst) (Zhang and Kelly, 2001; Kelly and Zhang 2002). Calcium ions can also act as second messengers which can activate an intracellular signalling



cascade, ultimately resulting in increased excitability of the neuron. The effect of calcium on the intracellular signalling cascade last for hours or longer and results in enhanced excitatory responses in a postsynaptic cell. This enhancement of excitatory responses is referred to as long-term potentiation (Silverthorn, 2007).

### **1.4.2 GABAergic neurotransmission**

GABA is the major inhibitory neurotransmitter in the brain. Neurons producing GABA or responding to GABA are referred to as GABAergic. Approximately 40% of all synapses in the brain are GABAergic, alluding to its importance in the central nervous system (Malcangio et al., 1996). GABA is a neutral amino acid synthesized from glutamate by the action of the enzyme glutamic acid decarboxylase (Fagg and Foster, 1983; Ribak et al., 1979). GABA neurotransmitter is stored in vesicles by a sodium-independent, ATP-dependant transport system. Upon depolarization, GABA is released into the synaptic cleft where it can interact with post-synaptic GABA receptors. GABA is inactivated by diffusion, and by a high-affinity sodium-dependant transport system that transports GABA into synaptic terminals and glial cells (Fykse and Fonnum, 1988; Hell et al., 1988).

Upon release, GABA can activate three different types of receptors; GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> (Enna, 2001). GABA<sub>A</sub> and GABA<sub>B</sub> receptors exist in the central nervous system of the rat and other animals, and are present at high concentrations in the hippocampus, thalamus, cortex, and cerebellum (Chu et al., 1990; Wamsley et al., 1986; McCabe and Wamsley, 1986; Zezula et al., 1988). GABA<sub>C</sub> receptors have been found mostly in the retina of vertebrae (Bormann and Feigesnspan, 1995). GABA<sub>A</sub> receptors are present at high levels in the frontal cortex, the hippocampus, the granule cell layer of cerebellum, the thalamic medial geniculate nucleus, and the olfactory bulb. GABA<sub>B</sub> receptors are present at high levels in the molecular

layer of cerebellum, interpeduncular nucleus, frontal cortex, anterior olfactory nucleus, and thalamic nuclei (Bowery et al., 1987; Young and Chu, 1990). Studies have also found a high level of expression of GABA<sub>B</sub> receptors in auditory structures of rats (Bowery et al., 1987; Charles et al., 2001; Jamal et al., 2011, 2012; Milbrandt et al., 1994). GABAergic receptors are very important for the functions of the brain. These receptors are involved in numerous processes such as appetite regulation (Morley, 1980; Hansen and Ferreira, 1986; Kelly and Grossman, 1979; Lenin Kamatachi et al., 1984, 1986), cardiovascular regulation (Chahl and Walker, 1980; Persson and Henning, 1980; Robinson et al., 1986), depression (Berretini et al., 1983; Gerner and Hare, 1981; Gold et al., 1980; Lloyd et al., 1983; Petty and Sherman, 1981, 1984), seizures (Lloyd et al., 1986; Manyam et al., 1980; Meldrum, 1975; Schmidt and Loscher, 1981; Tower, 1960; Wood and Peesket, 1973; Wood et al., 1979; Worms and Lloyd, 1981), anxiety (Iverson, 1983; Macdonald et al., 1986; Olsen and Snowman, 1982; Study and Barker, 1982), and stress (Biggio et al., 1981; Otero-Losada, 1988; Schwartz et al., 1987; Skerit et al., 1981).

#### **1.4.2.1 GABA<sub>A</sub> receptors**

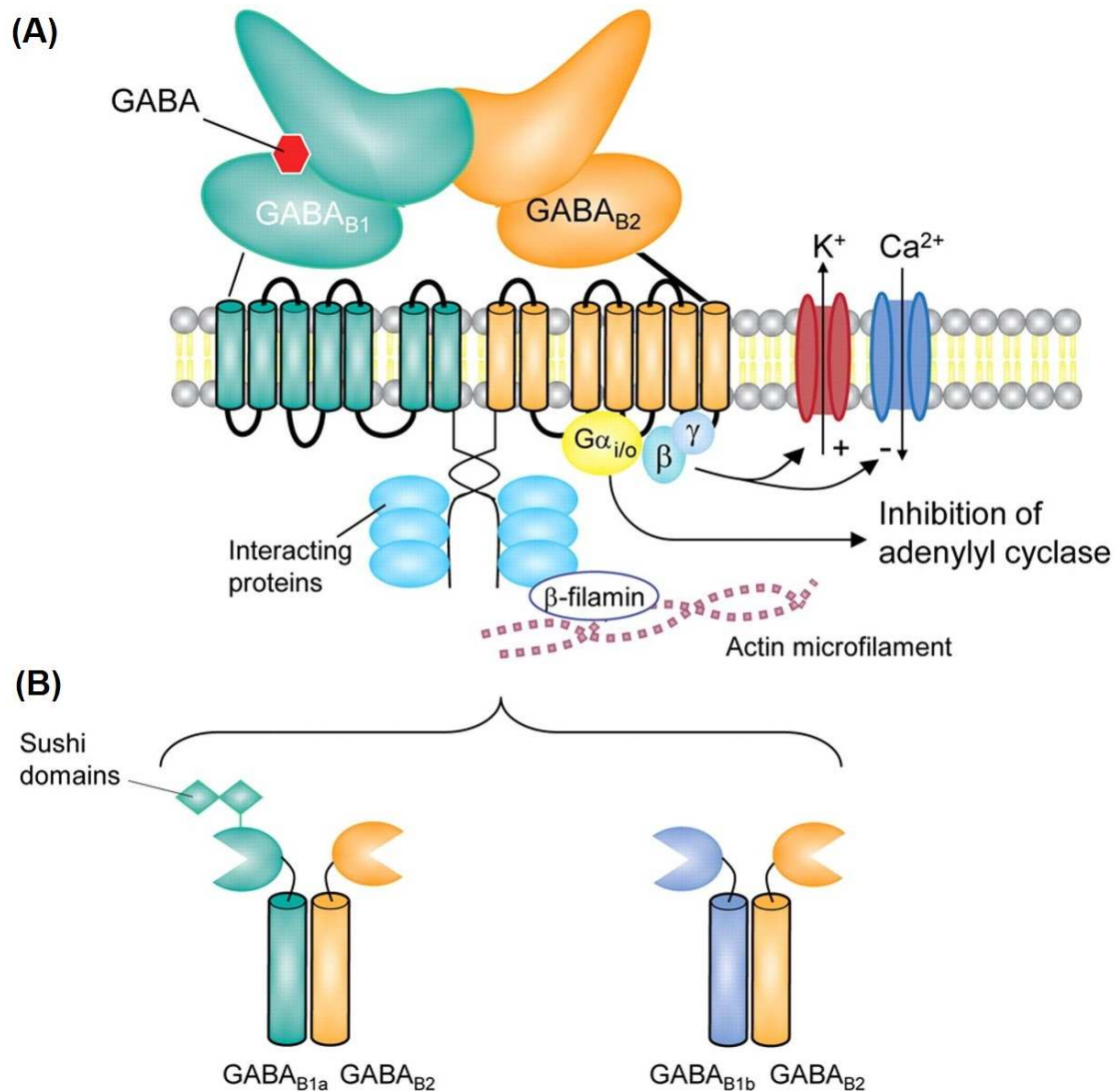
GABA<sub>A</sub> receptors are ionotropic receptors. These receptors can be modulated by benzodiazepines and barbiturates, and are sensitive to the alkaloid convulsant bicuculline. These receptors are membrane associated, ligand-gated chloride channels that allow influx of chloride ions upon opening. Activation of a GABA<sub>A</sub> receptor leads to hyperpolarisation of the cell membrane and a fast inhibitory response (Huang, 2006). GABA<sub>A</sub> receptors outnumber GABA<sub>B</sub> receptors in most areas of the brain (Chu et al., 1990).

#### **1.4.2.2 GABA<sub>B</sub> receptors**

GABA<sub>B</sub> receptors are metabotropic receptors that are linked to G-proteins and indirectly affect Ca<sup>2+</sup> and K<sup>+</sup> channel conductance in the membrane (Figure 3a). GABA<sub>B</sub> receptors are

located either pre-synaptically or post-synaptically. Pre-synaptic GABA<sub>B</sub> receptors inhibit the release of GABA or glutamate neurotransmitters (Kornau, 2006; Ma et al., 2002), thereby regulating either inhibitory or excitatory neurotransmission. For presynaptic GABA<sub>B</sub> receptors, binding by GABA causes the  $\beta\gamma$ -subunit of the G-protein to dissociate from the  $\alpha$ -subunit, leading to modification of voltage-dependant Ca<sup>2+</sup> channels. This modification reduces Ca<sup>2+</sup> influx into a cell and subsequently decreases neurotransmitter release from a pre-synaptic terminal (Ikeda, 1996; Herlitze et al., 1996).

Activation of post-synaptic GABA<sub>B</sub> receptors leads to prolonged inhibition through the action of the  $\beta\gamma$ -subunits dissociated from the G-protein. The dissociated  $\beta\gamma$ -subunits activates inward rectifier K<sup>+</sup> channels (GIRK), thereby causes hyperpolarization of a postsynaptic neuron (Luscher et al., 1997; Ulrich and Bettler, 2007). The  $\beta\gamma$ -subunit also activates phospholipase C which in turn activates protein kinase C, thereby triggering a signalling cascade that can modulate neuronal function (Blank et al., 1992; Selbie and Hill, 1998). The  $\alpha$ -subunit of the G-protein can modulate cAMP production, initiating a signalling cascade that involves many intracellular proteins and events. This way, GABA<sub>B</sub> receptors can have long-term effects on neuronal activity (Bowery et al., 2002).



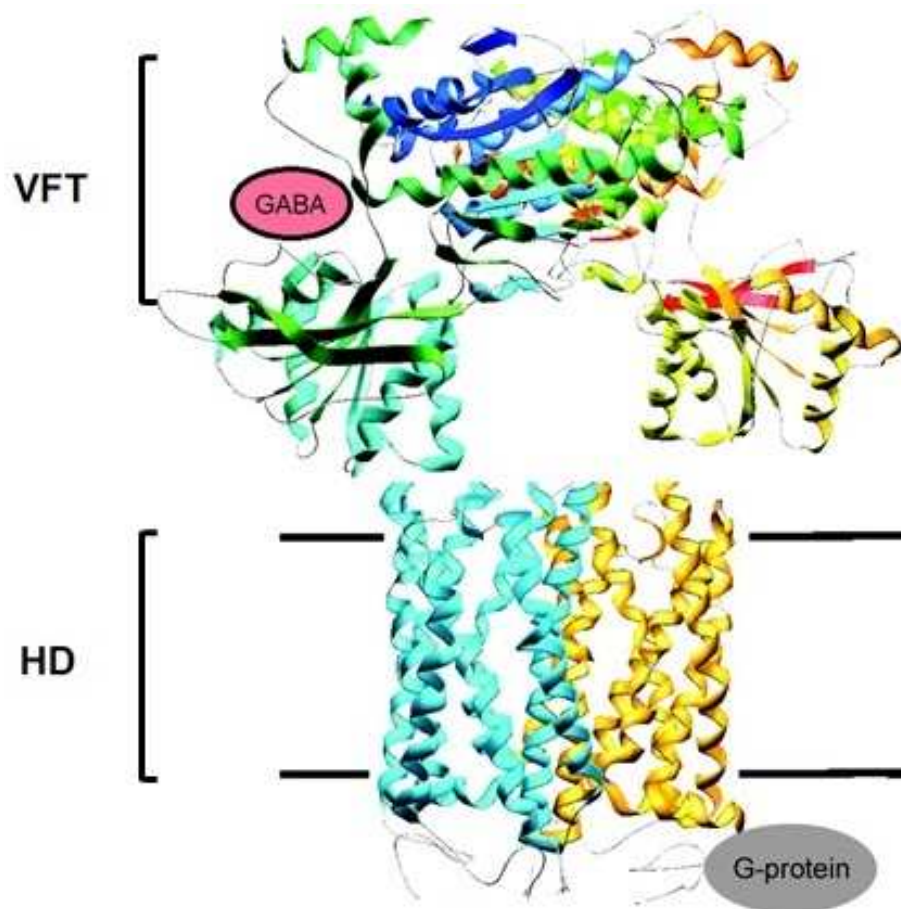
**Figure 3.** General structure and mechanism of action of metabotropic GABA<sub>B</sub> receptor. (A) The receptor is coupled with a G-protein which dissociates upon receptor activation by GABA neurotransmitter. The dissociated G-protein subunits can directly affect ion channels and can modulate adenylyl cyclase activity. (B) The two known isoforms of the GABA<sub>B</sub>R1 subunit. The GABA<sub>B</sub>1a subunit has two sushi domains on its N-terminus.

#### 1.4.2.2.1 GABA<sub>B</sub> receptor subunits

A functional GABA<sub>B</sub> receptor is a heterodimer, consisting of a GABA<sub>B</sub>R1 and a GABA<sub>B</sub>R2 subunit (Figure 4). These subunits interact through their coiled-coil domains and form a functional GABA<sub>B</sub> receptor. Both subunits are very similar in conformation, with each containing a heptahelical domain (HD) and a venus flytrap (VFT) module (Pin et al., 2004).

GABA<sub>B</sub>R1 subunit has two splice variants. These are the GABA<sub>B</sub>R1 $\alpha$  and GABA<sub>B</sub>R1 $\beta$  subunits. The two splice variants are identical in structure except for the presence of repeating sequences on the N-terminus of the GABA<sub>B</sub>R1 $\alpha$  splice variant (Figure 3b). These repeating sequences are known as sushi domains and are evolutionarily conserved (Huang, 2006). The exact function of the sushi domains on the GABA<sub>B</sub>R1 $\alpha$  subunit is not known, but it is believed that they serve to work as axonal targeting signals that guide the subunit towards pre-synaptic terminals to regulate the release of glutamate. GABA<sub>B</sub>R1 $\beta$  subunit binds to potassium channels and allows K<sup>+</sup> ions to flow, and is important for functioning post-synaptic GABA<sub>B</sub> receptors. GABA<sub>B</sub>R2 subunit may have isoforms as well, but they have not been characterized (Chalifoux and Carter, 2011).

Both subunits and their variants are formed in the endoplasmic reticulum (ER) (Ige et al., 2000). The GABA<sub>B</sub>R1 subunit has a retention signal and remains in the ER after production (Restituito et al., 2005). The GABA<sub>B</sub>R2 subunit is needed for the release and subsequent trafficking of the GABA<sub>B</sub>R1 subunit from ER to its destination. Upon binding between the two subunits, the GABA<sub>B</sub>R2 subunit masks the retention signal on GABA<sub>B</sub>R1, thus allowing the heterodimer to leave the ER and reach the plasma membrane (Pin et al., 2004; Restituito et al., 2005).



**Figure 4.** Binding structure of a functional GABA<sub>B</sub> receptor. The GABA<sub>B</sub>R1 subunit is on the left in blue. The GABA<sub>B</sub>R2 subunit is on the right in yellow. Each subunit is composed of a venus flytrap (VFT) module and a heptahelical domain (HD). The VFT of GABA<sub>B</sub>R1 binds GABA neurotransmitter. The HD of GABA<sub>B</sub>R2 is associated with a G-protein which is activated when GABA<sub>B</sub>R1 binds GABA (Pin et al., 2004).

The presence of both subunits is needed to form a functional GABA<sub>B</sub> receptor. Studies have shown that on their own, both subunits have no activity. GABA<sub>B</sub>R2 is able to leave the ER and go to the plasma membrane but is unable to have any physiological effect on the membrane. Without the GABA<sub>B</sub>R2 subunit, GABA<sub>B</sub>R1 subunit can be trafficked to the plasma membrane when the retention signal is blocked due to mutation or removal. However, the subunit is non-functional by itself (Pin et al., 2004). This is because each subunit has a specific function in the GABA<sub>B</sub> heterodimer. GABA<sub>B</sub>R1 subunit has the binding domain for the neurotransmitter GABA on its VTF module. GABA<sub>B</sub>R2 subunit is associated with the G-protein via its HD domain (Enna, 2001). When the two subunits interact, the affinity of GABA<sub>B</sub>R1 for GABA neurotransmitter is increased so that when it binds GABA, the G-protein associated with the GABA<sub>B</sub>R2 subunit is activated and can have various downstream effects (Pin et al., 2004; Enna, 2001).

#### **1.4.2.2.2 Distribution of GABA<sub>B</sub> receptors in the CAS**

Autoradiographical and immunohistochemical studies have revealed a high level of expression of GABA<sub>B</sub> receptors in most auditory structures. Using the autoradiographic technique, Fubara et al (1996) studied the expression of GABA<sub>B</sub> receptors in the big brown bat's central auditory system and found that the expression of the receptor to be high in AC, MG, and the dorsal nucleus of IC. The expression in ventral IC, NLL, SOC, and CN was relatively low. Similar results were reported by other researchers based on research using the same technique conducted in specific structures of other species, including guinea pig's CN (Juiz et al., 1994), and the rat's IC (Mibrandt et al., 1994).

The autoradiography results were supported by immunohistochemistry results that examined the expression of the GABA<sub>B</sub> receptor in the entire auditory system (Charles et al.,

2001, Jamal et al., 2011). These results reported a high level of the receptor in the AC and MG, intermediate level in the IC, and low levels in the NLL, SOC, and CN. Other immunohistochemistry studies found GABA<sub>B</sub> expression in the dorsal CN, and medial SOC (Lujan et al., 2004; Hassfurth et al., 2010).

Immunohistochemistry studies also gave information about the regional as well as cellular localizations of GABA<sub>B</sub> receptors in central auditory structures. Qualitative results from Jamal et al., (2011) showed that there were regional differences in the level of the receptor in a few structures. AC and MG showed no significant differences in receptor labelling across their subdivisions. In the IC, receptor expression was the highest in the ICd and intermediate in the ICc and ICx. In the CN, receptor expression was high in the DCN compared to VCN. Jamal et al., (2012) quantified receptor expression in the IC and reported a high level of expression in the ICd and an intermediate level in ICc and ICx. The study also found that receptor expression was similarly high in cell bodies across all the IC subdivisions. The differences in receptor expression were seen in the neuropil i.e. the region surrounding the cell bodies. Neuropil contains the axons and dendrites of cell bodies. While the level of labeling in the neuropil is higher in the ICd, the level is lower in the ICc and ICx.

#### **1.4.2.2.3 Functions of the GABA<sub>B</sub> receptors in the CAS**

Physiological studies have supported results from the autoradiography and immunohistochemistry studies showing the existence of GABA<sub>B</sub> receptors in the CN (Lim et al., 2000), SOC (Isaacson, 1998; Sakaba and Neher, 2003; Yamauchi et al., 2000), IC (Faingold et al., 1989; Ma et al., 2002; Sun et al., 2006; Sun and Wu, 2009; Vaughan et al; 1996; Zhang and Wu, 2000), MG (Peruzzi et al., 1997; Bartlett and Smith, 1999), and AC (Buonomano and Merzenich, 1998; Metherate and Ashe, 1994; Brandowski et al, 2001).



In the CN, using antagonists of GABA<sub>B</sub> receptors decreased the amplitude of evoked IPSCs, leading researchers to believe that activation of these receptors can reduce neurotransmitter release (Lim et al., 2000). *In vivo* extracellular recordings from IC showed that activation of GABA<sub>B</sub> receptors reduced responses to sounds, whereas antagonizing these receptors increased responses to tones and sinusoidally amplitude modulated sounds (Faingold et al., 1989; Vaughan et al., 1996; Szczepanial and Moller, 1996). Yamauchi and colleagues (1989) recorded the activity of neurons in the IC and reported that blocking GABA<sub>B</sub> receptors in the IC caused epileptiform burst discharge of action potentials. In the MG, GABA<sub>B</sub> receptors were shown to be important for regulating the firing patterns of neurons (Peruzzi et al., 1997). In the AC, GABA<sub>B</sub> receptors were found to be important for regulating synaptic activity mediated by NMDA receptors (Metherate and Ashe, 1994). GABA release also improved temporal processing in the AC (Jones, 1993; Schulze and Langner, 1999; Wang et al., 2000). These studies indicate that GABA<sub>B</sub> receptors are highly involved in auditory processing.

### **1.5 Normal auditory functioning requires a balance between excitation and inhibition**

The function of the central auditory system is dependent not only on excitation but also on inhibition. A balance between excitatory and inhibitory neurotransmission is important for the function. A disruption of this balance can manifest itself as various auditory disorders or hearing impairments.

Loss of this inhibition can have pathological behavioural consequences (Bauer et al., 1999; Gerken et al., 1984). At the physiological level, a reduction in inhibition can lead to hyperactivity in the CAS (Salvi et al., 1990; 2000). The hyperactivity was demonstrated by an increase in the spontaneous firing rate of neurons in the IC (Jastreboff and Sasaki, 1986; Chen and Jastreboff, 1995) and the AC (Kenmochi and Eggermont, 1997; Eggermont and Kenmochi,

1998). Decreased inhibition also causes synchronous firing of groups of neurons, which can change auditory neural processing (Ochi and Eggermont, 1997). Decrease in inhibition can also alters gene expression in the AC, causing aberrant plastic changes in the AC, and subsequently alter auditory perception (Panford-Walsh et al., 2008). Reduction in inhibitory neurotransmission can also lead to an increase in excitation (Gong et al., 2008), which can have various detrimental effects on the CAS. Increased excitatory neurotransmission has been shown to produce epileptiform firing in the auditory nerve fibers and alter auditory perception (Guitton et al., 2003; Guitton and Puel, 2004). Increased excitation can cause swelling of the ANF dendrites and lead to membrane disruption (Puel, 1995). Prolonged excitation in the AC can cause shifts in the receptive fields of neurons (Scholl and Wehr, 2008). Among hearing disorders, tinnitus may also be related to an imbalance between excitation and inhibition.

## **1.6 Tinnitus**

Tinnitus is a phantom sensation of sound in the absence of an external sound stimulus. Such sensation can be experienced as constant buzzing, hissing, or whistling. Tinnitus can interfere with an individual's ability to sleep, hear, and work. Almost all adults in the general population experience transient tinnitus at some point in their life. However, 6-20% of adult North American population suffers from chronic tinnitus, which can be devastating to a person's quality of life (Heller, 2003). Only twenty-five percent of people suffering from chronic tinnitus seeks medical treatment. Tinnitus is detrimental to a person's quality of life because it is often accompanied by maladies such as migraines, dizziness, hearing loss, and decreased sound tolerance. Forty percent of patients suffering from tinnitus also suffer from hyperacusis, i.e., over-amplification of environmental sounds, and phonophobia, i.e., fear of environmental sounds. Tinnitus is more common in aging population and among females. Furthermore, its

prevalence is significantly higher in pregnant females than non-pregnant females (Axelsson and Ringdahl, 1989; Lockwood et al., 2002). There are two types of tinnitus, called objective tinnitus and subjective tinnitus.

### **1.6.1 Objective vs. subjective tinnitus**

Objective tinnitus is relatively rare. It is defined by the presence of an actual sound from inside the body, which leads to the perception of the sound through normal hearing mechanisms. The actual sound can be generated by a pulsing blood vessel near the middle or inner ear (Weissman and Hirsch, 2000; Liyanage et al., 2006; Sonmez et al., 2007), involuntary muscle contractions in the middle ear (Abdul-Baqi, 2004; Howsam et al., 2005), or inner ear hair cell stimulation (Chandler, 1983). As such, objective tinnitus is the normal perception of an abnormal sound in the body, and is believed to originate in the peripheral auditory system.

Subjective tinnitus is a common form of tinnitus. It differs from objective tinnitus in that there is no actual, overt sound present. Many researchers believe that subjective tinnitus is associated with changes in the peripheral and/or central auditory system (Moller, 2003; Lockwood et al., 2002; Jastreboff, 1990). Since many patients reported tinnitus in one but not both ears, it was believed for many years that tinnitus may originate from a peripheral source such as the cochlea. However, clinical observations have showed that this cannot account for all forms of subjective tinnitus. Studies found that blocking auditory inputs to the brain did not diminish tinnitus, indicating that central mechanisms must be involved in tinnitus (Berliner et al., 1992; Barrs and Brackman, 1984; House and Brackman, 1981; Kaltenbach et al., 2005).

### **1.6.2 Inducers of tinnitus**

There are many inducers of tinnitus in the human population. These inducers can be divided into non-medical and medical ones. The most common non-medical cause of tinnitus is loud noise. Individuals who are exposed to loud noises on regular basis e.g. working at a construction site, or if they are exposed to it once e.g. at a concert, can develop tinnitus.

Medical inducers of tinnitus include various over-the-counter and prescription drugs. Many antibiotics, anti-depressants, anti-malarial drugs, anti-inflammatory drugs, diuretics, and certain cancer drugs have been shown to induce tinnitus in humans. Of the above medications, anti-inflammatory drugs are the most prevalent inducers of tinnitus in humans. Aspirin is an example of such a drug. It is one of the most consumed drugs in the world (PharminfoNet, 1994, 1999; Vane et al., 1998). One hundred and twenty billion pills of Aspirin are prescribed in North America each year (Warner and Mitchell, 2002).

Aspirin is an odorless, white, powdery substance which is highly soluble in lipids and slightly soluble in water. Upon exposure to moisture, it rapidly hydrolyzes into salicylic acids and acetic acids, with 68% of oral dose reaching systemic circulation (Rowland et al., 1972). Peak plasma levels of salicylic acid are detected 1-2 hours after aspirin ingestion. Salicylic acid is widely distributed to all the tissues in the body including CNS, liver, renal cortex, heart, and lungs, and has a half-life of 6 hours. Salicylic acid is excreted in the urine in a pH dependant manner. Renal excretion increases from 5% to 80% as urinary pH rises above 6.5 (Bayer Corp. USA).

Aspirin is used for treating a variety of maladies e.g. ischemic stroke, acute myocardial infarction, angina pectoris, and rheumatoid diseases such as arthritis. Aspirin acts by inhibiting the synthesis of prostaglandins, and prevents platelet aggregation by inactivating cyclo-

oxygenase enzyme. Aspirin has also been shown to inhibit inflammatory mediators by non-specifically suppressing cyclo-oxygenase activity in peripheral tissue, causing gastric irritation as a side effect. Other side effects of aspirin include coagulation abnormalities due to inhibition of platelet function, stomach pain, nausea, vomiting, and gross gastrointestinal bleeding, and auditory sensorineural alterations such as tinnitus and hyperacusis. A detailed description of the side effects of Aspirin can be seen in Table 1 (Bayer Corp. USA).

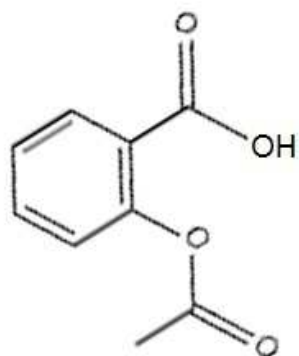
### **1.6.3 Sodium salicylate (SS)**

Salicylate is one of the metabolites of Aspirin (Acetylsalicylic acid) (Figure 5), and has been shown to cause auditory abnormalities in patients. Early reports of salicylate ototoxicity were given by Muller (1877) who observed the adverse ototoxic effects of salicylate in patients being treated for rheumatoid arthritis. Since then, many studies have been conducted in humans and animals to study the effects of Aspirin and salicylate.

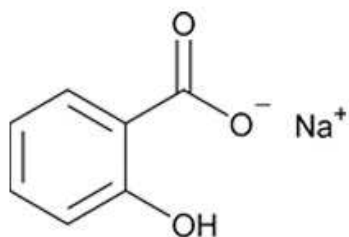
#### **1.6.3.1 Effects of salicylic drugs on hearing: Studies conducted with human patients**

Many studies were conducted with patients taking either aspirin or salicylate to determine whether tinnitus was induced, and the plasma salicylate level at which the phantom auditory sensation is induced. These studies found that there was individual variability amongst subjects. Jagar and Alway (1946) found that tinnitus appeared in 34 out of 38 patients being treated with salicylate injections for several weeks. Tinnitus was seen at blood salicylate levels as low as 200 mg/L. Similar findings were reported by Graham and Parker (1948) who studied oral salicylate therapy in 40 rheumatic patients and 30 non-rheumatic patients. They found that most subjects experienced deafness at plasma salicylate levels averaging 250 mg/L. Further studies in human subjects receiving oral salicylate treatment (Myers et al., 1965, McCabe and Day, 1965; Mongan

Acetylsalicylic acid



Sodium salicylate



**Figure 5.** Molecular structure of aspirin (acetylsalicylic acid) and sodium salicylate.

**Table 1: Adverse reactions to Aspirin/salicylate ingestion**

Body as a whole	Fever, hypothermia, thirst
Cardiovascular	Dysrhythmia, hypotension, tachycardia
Central Nervous System	Agitation, cerebral edema, coma, confusion, dizziness, headaches, subdural or intracranial hemorrhage, lethargy, and seizures
Fluid and electrolyte	Dehydration, hyperkalemia, metabolic acidosis, respiratory alkalosis
Gastrointestinal	Dyspepsia, GI tract bleeding, ulceration and perforation, nausea, vomiting, hepatitis, pancreatitis, Reye's Syndrome, transient elevation of hepatic enzymes
Hematological	Prolongation of prothrombin time, disseminated intravascular coagulation, coagulopathy, thrombocytopenia
Hypersensitivity	Acute anaphylaxis, angioedema, asthma, bronchospasm, laryngeal edema, urticaria
Musculoskeletal	Rhabdomyolysis
Metabolism	Hypoglycemia in children, hyperglycemia in adults
Reproductive	Prolonged pregnancy and labor, stillbirths, lower birth weight infants, antepartum and postpartum bleeding
Respiratory	Hyperpnea, pulmonary edema, tachypnea
Special senses	Hearing loss, tinnitus
Urogenital	Interstitial nephritis, papillary necrosis, proteinuria, renal insufficiency and failure

et al., 1973; Halla and Hardin, 1988; Halla et al., 1991; Hicks and Bacon, 1999; and Janssen et al., 2000) have confirmed that patients experience tinnitus at average blood salicylate plasma levels of 200 mg/L - 250 mg/L. These studies also reported that the minimum plasma salicylate levels that induced tinnitus were almost always below 100 mg/L (Jager and Alway, 1946; Graham and Parker, 1948). For salicylate-induced tinnitus, the pitch reported by patients was always in the high frequency range, and showed a linear increase with plasma salicylate levels (Alway, 1946; Graham and Parker, 1948).

#### **1.6.3.2 Effects of salicylic drugs on hearing: Studies conducted with animals**

Animal models of salicylate-induced tinnitus have been developed and behavioural paradigms as well as neurophysiological methods have been used to study the neurobiological bases of the auditory phantom sensation. Jastreboff and his colleagues (Jastreboff et al, 1988a, 1988b; Jastreboff and Sasaki, 1994) modified the operant conditioning procedure used by Estes and Skinner (1941) and used it in the study of tinnitus. Jastreboff et al. (1988a, 1988b) used a combination of water-deprivation and electric foot shocks to establish a behavioral paradigm for evaluating the effect of salicylate on rat's hearing. Experimental subjects were trained to suppress licking of a licking spout in the presence of silence. These animals were tested to find how systemic application of salicylate affect the suppression of licking during the silent period. Using this behavioural method, the researchers found that subcutaneous injections of 300mg/kg/day sodium salicylate (SS) could reliably induce a tinnitus-like behavior in rats. The pitch of tinnitus sensation was about 10 to 11 kHz, values consistent with those from human subjects with high pitch tinnitus.

Jastreboff and his colleagues also studied the dependence of loudness of tinnitus on the



dose of sodium salicylate. A strong correlation was found between the dose and the loudness of tinnitus when the drug was applied at doses of 50-300mg/kg. The researchers also measured blood salicylate levels of the animals and found that the data fit extremely well with the human data (Jastreboff et al., 1988b).

Recently, other researchers developed other behavioural paradigms to assess the presence and pitch of tinnitus (Lobarinas et al., 2004; Yang et al., 2007; Turner and Parrish, 2008; Ralli et al., 2010, and Kizawa et al. 2010). Their results basically confirmed findings by Jastreboff and his colleagues and have approximated the pitch of tinnitus to 16 kHz.

### **1.7 Effect of Sodium Salicylate (SS) on peripheral and central auditory systems**

As indicated in section 1.6.3.2, many studies have shown that application of SS can induce tinnitus-like behaviour in animals (Jastreboff et al, 1988a; 1988b; Jastreboff and Sasaki, 1994). The tinnitus-like behaviour persisted even when the cochlea or the auditory nerve were disconnected from the brain (Berliner et al., 1992), leading researchers to believe that tinnitus involved changes in both the peripheral and the central auditory systems.

#### **1.7.1 Effect of SS on peripheral auditory system**

Researchers studied the effect of SS by recording compound action potentials (CAP) of auditory nerve fibers (ANFs). Results indicate that SS application results in a reduction in the output from cochlea (Cazals, 2000). Muller and colleagues (2003) measured changes in the activity in the cochlea and auditory nerve fibers after systemic SS injection. They found that after the application of 250mg/kg of SS, the output from peripheral structures was significantly decreased. Muller et al., (2009) directly applied SS to cochlea and found that sound-evoked activity in the cochlea was decreased. Studies by other researchers (Shehata et al., 1991, Oliver

et al., 2000; Chen et al., 2010) showed that SS application decreased the electromotility of the outer hair cells (OHC) in the ear, thus decreasing the signal output from the ear to the central auditory system. This supplemented the point that tinnitus was not a result of increased activity in the ear, and instead had neural correlates in the central auditory system.

## **1.7.2 Effect of SS on the Central Auditory System**

### **1.7.2.1 Effect of SS on CN**

CN is the first structure in the ascending auditory pathway and receives direct inputs from the peripheral auditory system. Previous studies have suggested that CN might play a key role in the generation of salicylate-induced tinnitus (Brozoski et al., 2002; Kaltenbach and Godfrey, 2008; Shore et al., 2007). Basta et al. (2008) conducted experiments using brain slice preparations. They perfused the tissue containing CN with salicylate solution and used electrodes to record the activity of neurons. They found increased activity in the CN after SS application (Basta et al., 2008). Similar brain slice experiments were conducted by Wei et al. (2010), who also reported an increase in spontaneous firing rate of neurons in the CN after SS application. Holt and colleagues (2010) used manganese enhanced functional magnetic resonance imaging (MEMRI) to map the activity in auditory structures after systemic salicylate injections in rats. They found a significant increase in activity in the dorsal cochlear nucleus (DCN) but no change in the ventral cochlear nucleus (VCN). All the results above support the involvement of the CN in salicylate induced tinnitus.

### **1.7.2.2 Effect of SS on IC**

IC is a mid-brain structure, and is a major auditory processing centre. It is the target of ascending inputs from lower auditory structures as well as descending inputs from higher

auditory structures. Because of its importance in auditory processing, it has been a major focus in the study of neural mechanisms responsible for tinnitus (Manabe et al., 1997; Moller, 1995; Bauer et al. 2000). Wu and colleagues (2003) found that after SS application, c-fos expression in IC was increased, implying increased activity. Basta and Ernst (2004) worked with brain slices perfused with salicylate-containing solution, and found that the spontaneous activity of neurons in the IC was significantly increased after SS application. Increases in spontaneous activity were also found in studies involving *in vivo* animal preparations (Jastreboff and Sasaki, 1986); Chen and Jastreboff, 1995); Manabe et al., 1997). In these studies, SS was injected systemically and an increase in the spontaneous firing rate was found in neurons in the ICc and ICx after drug application. Holt et al. (2010) used the MEMRI technique to measure the activity in IC after systemic salicylate injections and found a significant increase in the ICd. These studies support that the IC serves as a contributor to the phenomenon of tinnitus.

#### **1.7.2.3 Effect of SS on MG**

Limited research has been conducted to study the effect of SS on the MG. Basta and colleagues (2008) conducted a brain slice study with MG and found a change in the spontaneous firing rate of neurons. These researchers perfused the brain slice with salicylate perfusate and recorded the spontaneous activity of neurons in the dorsal and ventral parts of the MG, and found that neurons increased their spontaneous firing rate after local application of SS. This change in firing rate was correlated with the concentration of SS, with higher concentrations eliciting a greater percent change in the firing rate of neurons (Basta et al., 2008).

#### **1.7.2.4 Effect of SS on AC**

AC has been a target of many studies in the tinnitus research field, with most researchers reporting a change in the neuronal spontaneous activity after salicylate application. Basta and

colleagues (2008) found that salicylate caused an increase in spontaneous firing rate in neurons in the AC. This increase was more apparent at higher concentrations of SS. Yang et al. (2007) recorded the activity of neurons in a live rat after systemic salicylate application, and found that sound-evoked neural activity in the AC was increased after SS application. Lu et al. (2011) measured the activity of AC neurons after systemic and local application of SS. They reported that after either application, the amplitude of sound-evoked neural activity was increased in the AC neurons, but the spontaneous activity was reduced.

### **1.8 Current understanding of neural mechanisms responsible for tinnitus**

Available results related to the effect of SS on neural activity in auditory structures suggest that the tinnitus sensation may be due to a tip of balance between excitation and inhibition i.e. either an increase in excitatory neural activity or a decrease in inhibitory neural activity. Tinnitus may also be related to an increase in spontaneous firing of auditory neurons, or an enhancement in synchronization of activity among different auditory neurons. The latter two might also be a result of an imbalance between excitation and inhibition.

The hypothesis that dysregulation between excitatory and inhibitory activity might be a cause of tinnitus is very commonly invoked, and there are many studies to support it. Holt and colleagues (2010) used manganese-enhanced MRI to measure the activity of neurons in CN and IC after tinnitus induction using SS. They found an increase in neural activity in the DCN and ICd in rats that displayed tinnitus-like behaviour. *In vitro* physiological studies examining the immediate effect of SS showed that SS application reduced GABA-mediated inhibition in CAS, leading to increased hyperexcitability (Wang et al., 2008; Gong et al., 2008). A reduction in GABAergic inhibition was reported by Sun et al., (2009) and Lu et al. (2011) as the cause of increase activity in the AC. Zou and Shang (2012) found a significant decrease in the levels of

GABA<sub>A</sub> receptor and the enzyme GAD in CAS after salicylate application. Since GAD catalyzes the production of GABA neurotransmitter, a reduction in GAD levels could translate as a reduction in the level of GABA (Zou and Shang, 2012). Similar decreases in GABA<sub>A</sub> receptor expression and GAD levels have also been reported by another study (Basta et al., 2000). A significant decrease in the number of GABA<sub>A</sub> receptor binding sites has been found in the IC after SS application. It has been suggested that these changes result in decrease in inhibition in the CAS and manifest as perception of tinnitus (Basta et al., 2000).

Decreased inhibition can also lead to increases in spontaneous firing of neurons in the CAS. Increased spontaneous activity following salicylate treatment has been observed in various structures including the auditory nerve, DCN (Kaltenback et al., 1998, 2000, 2002; Brozoski et al., 2002; Chang et al., 2002), IC, and secondary auditory cortex (Mulheran and Evans, 1999; Manabe et al., 1997; Jastreboff and Sasaki, 1986; Eggermont and Kenmochi, 1998). These increases in spontaneous activity were measured using physiological methods. While sound-driven activity are responsible for normal hearing (Clark, 2008; Colletti et al., 2009), an increases in spontaneous activity after SS application might lead to a phantom sensation of sound, i.e., tinnitus.

Decreased inhibition can also enhance neural synchrony i.e. synchronous firing of groups of neurons. Synchronous discharges have the potential to drive post-synaptic targets at higher auditory structures (Roberts et al., 2008; 2010). Evidence of increased neural synchrony and increased bursting activity has been found following systemic application of SS. Chronic increases in bursting activity was observed in the IC after salicylate treatment (Chen and Jastreboff, 1995; Kwon et al., 1999). Increased synchrony of discharge across neural populations was seen in the auditory nerve after salicylate treatment (Cazals et al., 1998). In human patients

suffering from tinnitus, electroencephalography and magnetoencephalography results showed abnormal synchronous activity between cortical areas of tinnitus patients (Weisz et al., 2005; Schlee et al., 2009). It is believed that increased synchronous activity and burst finding might magnify the perceptual weight of tinnitus sensation.

SS can cross the blood-brain barrier and affect neurons directly (Silverstein et al., 1967). As discussed above, effects of the drug can be immediate and associated with modifications of physiological processes such as neurotransmission and/or opening and closing of ion channels. In addition to these changes, SS might also affect other cellular mechanisms such as the expression of proteins. Zou and Shang (2012) reported that SS could reduce the level of mRNA of the GABA<sub>A</sub>α1 subunit as well as that of the enzyme GAD67. It has been found that SS can increase the level of mRNA of brain-derived neurotrophic factor in the cochlea and decrease the expression of Arg3.1, an activity dependant cytoskeletal protein, in the AC (Panford-Walsh et al., 2008). An increase in the level of mRNA of tumor necrosis factor-α and that of NMDA receptor subunit 2A has been found in the CN of rats after SS administration (Hu et al., 2014). Existing results therefore indicate that SS can cause changes in the expression of proteins, including those related to inhibitory neurotransmission mediated by the GABA<sub>A</sub> receptors and those related to excitatory neurotransmission mediated by the NMDA receptors. Most of these changes are seen after a period of 2-3 hours and may reflect homeostatic regulation of the level of activity by auditory neurons (Panford-Walsh et al., 2008; Hu et al., 2014).

It has yet to be found whether the level of the GABA<sub>B</sub> receptor can be affected by SS. The GABA<sub>B</sub> receptor has a relatively high level of expression in the structures possibly responsible for the induction/generation of tinnitus. The receptor can mediate slow inhibitory post-synaptic potentials as well as modulate the release of both inhibitory and excitatory

neurotransmitters. Therefore, it might play a regulatory role in maintaining a proper level of activity in the auditory system, similar to what it does in other nervous systems (Malcangio and Bowery, 1996). Behavioural studies have shown that the peak effect of SS occurs 3 hours after systemic SS application. This time course coincides with the time frame associated with change in the expression of many proteins. Therefore a possibility exists that the central auditory system uses the alteration of the level and distribution of the GABA<sub>B</sub> receptor as a compensatory mechanisms for rebalancing the reduction in the input from the auditory peripheral to the central nervous system.

### **1.9 Research Objectives and Hypothesis**

The objective of my research project was to examine changes in the level of GABA<sub>B</sub> receptors following salicylate treatment. Specifically, I wanted to find out whether SS could change the level of GABA<sub>B</sub> receptors in four major central auditory structures i.e. CN, IC, MG, and AC. All these major auditory structures have a relatively high level of the GABA<sub>B</sub> receptor and there is evidence showing an involvement of these structures in the generation of tinnitus. CN is considered by many as a trigger centre for tinnitus (Brozoski et al., 2002; Kaltenback and Godfrey, 2008; Shore et al., 2007). The IC is an obligatory auditory processing centre and receives convergent ascending and descending inputs from all major auditory structures (Anderson et al., 1980; Druga and Syka, 1984; Druga et al., 1997; Faye-Lund, 1985). MG is a major source of input to the AC in the ascending pathway, and has major targets in the ipsilateral AC (Winer and Schreiner, 2005). AC plays an important role in auditory sensation, perception, and cognition (Gaese and Ostwald, 1995). I wanted to determine if salicylate-induced changes in receptor levels had any specific cellular and/or regional localization. My hypothesis was that SS would reduce the level of GABA<sub>B</sub> receptor subunits in all the major structures.

## **2 Experimental methods**

The level of the GABA<sub>B</sub> receptors in auditory structures was examined using western blotting and immunohistochemical methods. These methods have been well established in our laboratory and have been used to measure the expression of the GABA<sub>B</sub>R1 and the GABA<sub>B</sub>R2 subunits in auditory structures (Jamal et al, 2011 and 2012).

### **2.1 Animal Preparation**

Experiments were conducted using male Wistar rats (*Rattus norvegicus*), weighing between 250-350g. The rats were obtained from Charles River Laboratories Inc. (St. Constant, QC). The animals were housed in the animal care facility at the University of Windsor for at least one week before being used for experiments. The noise level in the animal care facility was maintained at 55-60dB SPL. All experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care.

### **2.2 Immunohistochemistry (IHC) Experiments**

A pair of animals was used for each experiment, with each pair named as one case elsewhere in the present document. In the pair, one animal was injected with physiological saline (as control) and the other animal was injected with SS (250mg/kg). SS and physiological saline were injected intraperitoneally (i.p.). After the injection, the animals were kept in the animal care facility for three hours before euthanasia. I used this time frame because behavioural studies showed that SS applied at 250mg/kg resulted in a tinnitus-like behavior with the peak effect appearing three hours after the drug application (Jastreboff and Sasaki, 1986, 1988).

#### **2.2.1 Tissue collection**



Animals were euthanized by an overdose of sodium pentobarbital (120mg/kg, i.p.). They were then transcardially perfused with 200 ml of 0.1M phosphate buffer saline (PBS) and 200 ml of 4% paraformaldehyde (PFA). The fixed brain was extracted and placed in 30% sucrose for cryoprotection. After the brain had sunk, it was sectioned in the coronal plane at 30µm using a CM1050 S cryostat (Leica Microsystems, Heidelberg, Germany). The sections containing auditory structures were collected on SuperFrost Plus glass slides (Fisher Scientific, Pittsburg, PA, USA). Sections with the same neural structures from the two animals treated with saline and salicylate-treated were collected on the same slide besides each other. Sixteen sections were collected for each auditory structure and were placed on 4 slides. Each slide also contained a piece of cerebellar tissue serving as a positive control and for the purpose of normalizing the level of immunoreactivity (see section 3.1). A set of 4 slides containing tissue from one auditory structure was used for each IHC experiment. The arrangement of the tissue on the slide can be seen in Figure 6.

### **2.2.2 IHC protocol**

Slides with tissue of auditory structures were incubated overnight in the primary antibody against the GABA<sub>B</sub>R1 or GABA<sub>B</sub>R2 subunit (1:1000) in 0.1M PBS containing 0.05% Triton and 5% normal donkey serum (NDS). After the incubation, the slides were rinsed with 0.1M PBS three times, 10 minute each time, to wash away excess unbound primary antibody. The slides were then incubated in a 0.1M PBS solution containing biotinylated secondary antibody (1:400) and 2% NDS for 2.0 hours at room temperature. The slides were washed again with 0.1M PBS three times, 10 minute each time, and then incubated in a solution containing ExtrAvidin-



**Figure 6.** Schematic of a slide showing the mounted location of brain slices used in immunohistochemistry experiments. SAL CB represents the cerebellum section from saline-treated animal whereas SS CB represents the cerebellum section from SS-treated animal. SAL represents auditory slices from saline-treated animal. SS represents auditory slices from SS-treated animal.

peroxidase (E2886, 1:400) (Sigma-Aldrich, Oakville, ON, Canada) in 0.1M PBS for 1.5 hours at room temperature. After another set of three rinses in 0.1M PBS, the tissue was stained using 3'3' Diaminobenzidine tetrahydrochloride (DAB) in 0.1M phosphate buffer (PB). The reaction was stopped by repeatedly pouring distilled water in and out of the keeper containing the slides. The tissue was dehydrated by using an ethanol gradient of 60%, 70%, 95%, 100%, and 100%, 5 minutes for each ethanol concentration. The tissue was then cleared using histosol two times (10 minute each time). Permount (SP-500) (Fisher Scientific) was used to coverslip the slides. The level of immunoreactivity was examined using bright field microscopy.

## **2.3 Western Blot experiments**

### **2.3.1 Tissue collection**

A pair of animals was used for each experiment and the pair was labeled as one case. After receiving an intraperitoneal injection of either saline or sodium salicylate (250mg/kg), the animals were given a 3 hours survival period. They were then euthanized by an overdose of sodium pentobarbital (120mg/kg, i.p.). The brain was extracted right away and placed in chilled artificial cerebrospinal fluid containing (in mM): 126 NaCl, 3 KCl, 1.4 KH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 4 glucose, 1.3 MgSO<sub>4</sub>, and 1.4 CaCl<sub>2</sub>. The brain was cut in the coronal plane into 1 mm sections, from which tissues of the CN, IC, MG, and AC were collected using a #11 scalpel blade, a Silgard dish, and a stereoscope (Olympus SZX7, Tokyo, Japan). “The Rat Brain in Stereotaxic Coordinates” (Paxinos and Watson, 2007) was used as a guide during tissue collection. Tissues of the cerebellum and the liver were also collected from the rats and used for positive and negative control experiments. Tissues were placed in eppendorf tubes with homogenization buffer consisting of 0.32 M sucrose in 5 mM Tris, pH 7.4. The homogenization buffer also

contained the following protease inhibitors: 3  $\mu$ M aprotinin, 10  $\mu$ M phenylmethul sulfonyl fluoride (PMSF), 1  $\mu$ M leupeptin, and 3  $\mu$ M pepstatin-A. This homogenization buffer was used in all the eppendorf tubes containing collected auditory, cerebellar, and level tissues. The tubes were kept on ice at all times. The tissue was manually homogenized using a plastic pestle, and the homogenate was centrifuged at 3400 rpm for 20 minutes at 4°C to remove cell debris and nuclei. The supernatant was transferred into new eppendorf tubes. The protein concentration of each sample was determined using a Bradford Assay (Sigma-Aldrich, Oakville, ON, Canada) and a Biomate5 spectrophotometer (Thermo Scientific, Surrey, UK).

### **2.3.2 Immunoblotting protocol**

Thirty microgram of protein from each sample was added to 4X sample buffer and subjected to electrophoresis using a 7.5% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) for 2 hours at 125V. The separated proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF)-Plus 0.45  $\mu$ m membrane (Osmonics Inc., Minnetonka, MN, USA) for 2 h at 30V. The membrane was blocked with 1% milk in Tris-buffered saline tween (TBST) for 1 hour at room temperature. The membrane was then incubated overnight at 4°C in the primary antibody (1:1000) against the specific GABA<sub>B</sub> receptor subunit and the loading controls. After primary antibody incubation, the membrane was rinsed in TBST three times (10 minutes each wash) to wash off excess antibody. The membrane was then incubated at room temperature in the secondary antibody (1:6000 for GABA<sub>B</sub> receptor subunit, 1:10000 for loading controls) for 2 hours. The membrane was rinsed in TBST for an additional three times after secondary antibody incubation. Protein bands were detected using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA). Images were acquired using an HD2 gel imaging system and AlphaEase digital analysis software (Alpha Innotech, CA, USA).

## 2.4 Antibodies

The primary antibody used for GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 was the same for western blot and immunohistochemical experiments. The primary antibody used against GABA<sub>B</sub>R1 subunit was rabbit polyclonal GABA<sub>B</sub>R1 antiserum (Santa Cruz Biotechnology Inc, R-300, 1:1000, Santa Cruz, CA, USA). The primary antibody used against GABA<sub>B</sub>R2 was affinity purified guinea-pig polyclonal GABA<sub>B</sub>R2 antiserum (Chemicon, AB5394, 1:1000, Temecula, CA, USA). The primary antibody used for Actin in western blot procedures was mouse monoclonal primary antibody (Chemicon, MAB1501, 1:1000), and the primary antibody used for  $\alpha$ -Tubulin was also mouse monoclonal primary antibody (Chemicon, 05-829, 1:1000).

The secondary antibodies used in western blot experiments were horseradish peroxidase (HRP)-conjugated. The secondary antibody for GABA<sub>B</sub>R1 was goat anti-rabbit IgG-HRP (sc-2000, 1:6000, Santa Cruz Biotechnology Inc.), and for GABA<sub>B</sub>R2 was goat anti-guinea pig IgG-HRP (AQ108, 1:6000, Chemicon). The secondary antibody for probing both Actin and  $\alpha$ -Tubulin was anti-mouse IgG (12-349, 1:10000, Santa Cruz Biotechnology Inc.). The secondary antibody used in immunohistochemistry experiments for GABA<sub>B</sub>R1 was biotinylated donkey anti-rabbit IgG (711-005-152, 1:400, Jackson ImmunoResearch Laboratories, Burlington, ON, Canada), and for GABA<sub>B</sub>R2 was biotinylated donkey anti-guinea pig IgG (706-065-148, 1:400, Jackson ImmunoResearch Laboratories, Burlington, ON, Canada).

## 3 Data analysis

Western blots were quantified using the FluorChem 9900 imaging system (ProteinSimple, California, USA). Densitometry values of bands for GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 were measured and normalized against the densitometry values of bands for  $\alpha$ -Tubulin.

Optic density (OD) was used to quantify the level of the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits following an immunohistochemical experiment. For this purpose, photomicrographic images of tissue sections containing auditory structures were taken using a CTR 6500 microscope and a DFC 425 digital camera (Leica Microsystems, Heidelberg, Germany). Fixed settings of light intensity and exposure were used in all photomicrographic imaging sessions. For this purpose, a piece of tissue containing a defined cerebellar area was used at the beginning of each imaging session for setting the intensity and exposure of the microscope light. Thus, ODs of target areas/cells imaged in different sessions could be compared quantitatively. The images taken by the microscope system were exported in tiff format for offline analysis.

Offline analysis of OD was conducted using the LAS V4.4 analysis software (Leica Microsystems). In this analysis, black colour was assigned a value of 0 and white colour was assigned a value of 255. The Rat Brain Atlas in Stereotaxic Coordinates (Paxinos and Watson, 2007) was used as a guide in defining auditory structures and their subdivisions. In four major auditory structures (i.e., the CN, IC, MG, and AC) including all their subdivisions, the level of labeling was analyzed for cell bodies and the neuropil in combination (i.e., the overall level), cell bodies, and the neuropil. Since each structure was collected over a course of 16 slices, the results from all 16 slices were combined for each structure.

For measuring the overall level of labeling, an auditory structure or a subdivision of a structure was defined in each tissue section by using anatomical features in a microscopic image and the Rat Brain Atlas in Stereotaxic Coordinates. The area, integrated grey (i.e., the sum of the OD values of all pixels in the defined area), and grey mean (i.e., the mean of the OD values of all pixels in the defined area) were measured.

For counting the number of labeled cell bodies in a target structure, an OD threshold was set for the structure. Criteria of inclusion as well as exclusion were also set based on the size of a cell body. For setting an OD threshold, ten small areas ( $5\mu\text{m} \times 5\mu\text{m}$ ) were randomly chosen in the neuropil of the target structure. The mean OD was measured for each of the ten small areas using ImageJ analysis software (National Institute of Health). The standard deviation of the ODs of all pixels in the small area was also obtained. Grand mean values were obtained for both the mean OD and the standard deviation of OD across the ten small areas to reflect the overall level and the fluctuation of labeling in the auditory structure/subdivision. A value equalled the grand mean OD minus 3 times of standard deviation of OD was set as a threshold OD in the identification of cell bodies in the structure/subdivision. This ensured that only objects darker than the neuropil by a certain level (defined by 3 times of standard deviation in OD across different pixels in an area) were measured by the LAS software as cell bodies. A threshold was determined for each auditory structure individually. For each pair of sections (control animal treated with saline vs. animal treated with SS ), a threshold was determined by using the section from the control animal. A resulting threshold value was used for tissues from both the control animal and the SS treated animal. In addition to OD, the length of an object (i.e. the longest axis of an object) was used to determine whether to exclude or include an object in the analysis. Only objects with the longest axis between  $6\mu\text{m}$  and  $25\mu\text{m}$  were considered as cell bodies and were measured. This criterion was the same for all the auditory structures and cases. In spite of the criteria, the software could include non-cell body objects in the analysis or fail to include cell bodies in the analysis. To avoid errors made by the software, human intervention was used in the identification of cell bodies. The parameters measured by the LAS software for each identified

cell body included: area, length, breadth, perimeter, roundness, derived orientation, integrated grey, grey mean, and grey variance.

The mean grey value was obtained for the neuropil to reflect the level of labeling outside cell body regions in a target structure. An integrated grey of the neuropil was obtained by subtracting the integrated grey of a structure by the integrated grey of all the cell bodies in the structure. The integrated grey for the neuropil was then divided by the area of the neuropil in the structure (difference between the area of the structure and the combined area of cell bodies) to obtain the mean grey of the neuropil.

### **3.1 Normalization of IHC results:**

OD values obtained from auditory structures were normalized using the OD in the molecular layer of the cerebellum (CB). Previous research has shown that the molecular layer of cerebellum (CB) is among the neural structures showing the highest level of GABA<sub>B</sub> receptor expression (Ige et al., 2000). No receptors are present in the white matter. Western blotting and IHC results from the present study showed that SS did not change the level of GABA<sub>B</sub> receptors in the CB (Figure 7). Thus, any changes seen in the CAS in SS-treated animals could be attributed to the effect of the drug. Due to the above reasons, OD values in the molecular layer and white matter of the CB were used as reference points and were used for normalizing the OD values from the auditory structures. Two CB sections, one from Saline-treated animal and one from SS-treated animal, were placed on each slide containing auditory structures. OD values from Saline- and SS-treated animals were used for normalizing OD values of auditory structures of corresponding animals.

$$\text{Normalized OD} = (\text{OD}_{\text{Aud}} - \text{OD}_{\text{WM}}) / (\text{OD}_{\text{Mol}} - \text{OD}_{\text{WM}})$$



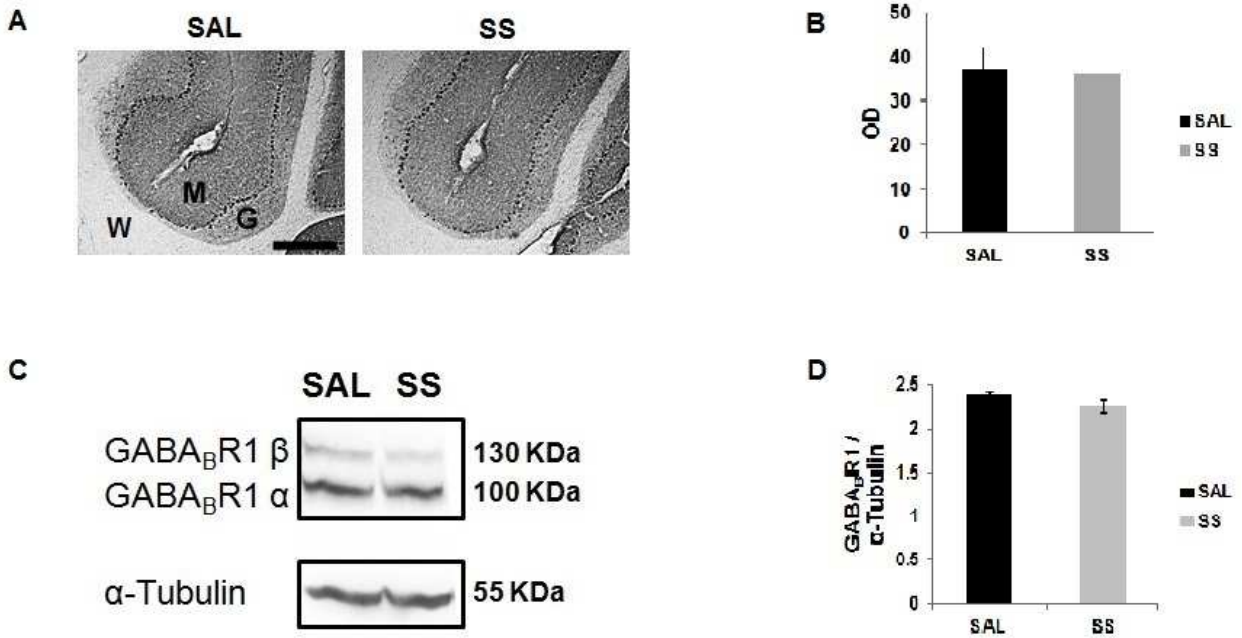
where  $OD_{Aud}$  is the OD or grey value of an auditory structure/neuron.  $OD_{WM}$  is the OD of the lightest area of cerebellum.  $OD_{Mol}$  is the OD of the darkest area in molecular layer of CB. This normalization allowed for reliable comparison of results from different structures and cases.

## **4 Results**

### **4.1 The effect of SS on level of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunit in the CB:**

Western blot and IHC experiments were conducted to study the effect of SS on CB. Neither type of experiment revealed an effect of SS on the level of GABA<sub>B</sub> subunits (Figure 7). Shown in Figure 7a and 7b are IHC results. The OD of molecular layer of CB in slices of saline-treated animal and SS-treated animal was measured for 7 cases. For each case, 5 CB sections were measured. OD was measured at ten randomly chosen spots from each of the 5 sections. A mean OD was obtained by using the 50 measurements from the CB of an animal. Group results from 7 cases indicated that the mean OD for GABA<sub>B</sub>R1 receptor subunit in the CB was 37.29 for saline-treated animals, and 36.27 for SS-treated animals (Figure 7b). No statistical difference was found in the level of labeling between the two groups of animals. Similar experiments were conducted to examine the effect of SS on the level of GABA<sub>B</sub>R2 receptor subunit in the CB. No statistically significant difference was found between control and SS-treated animals (date not included). This lack of difference between the two groups of animals allowed us to use the CB as a reference for normalizing the level of receptor subunits in auditory structures.

Shown in Figure 7c and 7d are results from western blotting experiments. Bands of immunoblots for GABA<sub>B</sub>R1 receptor subunits were normalized against those of  $\alpha$ -Tubulin (Figure 7c). The band for CB from saline animal gave a normalized value of 2.37 whereas the CB band from SS animal was at 2.26, indicating no significant difference (Figure



**Figure 7:** Immunohistochemistry and western blot results showing the effect of SS on GABA<sub>B</sub>R1 immunoreactivity in cerebellum. **(A)** Photomicrograph of a cerebellar section from saline-treated animal and a cerebellar section from SS-treated animal. The molecular layer in the cerebellar section of saline-treated animal is labeled with an "M". The granule cell layer is labeled with a "G" and white matter is labeled with a "W". Scale bar: 500 $\mu$ m. **(B)** Bar chart for group results from immunohistochemical experiments showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in the molecular layer of the cerebellum of saline-treated animal and the cerebellum of SS-treated animal. The group results are based on analyses from seven animals treated with saline and seven animals treated with SS. **(C)** Western blots showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in cerebellar tissue from saline-treated animal and cerebellar tissue from SS-treated animal. Top blot shows immunoreactivity against the two isoforms of GABA<sub>B</sub>R1 subunit. The bottom blot shows  $\alpha$ -Tubulin immunoreactivity. **(D)** Bar chart for group results from western blot experiments showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in the cerebellum. The ratio between the OD of the GABA<sub>B</sub>R1 (including GABA<sub>B</sub>R1 $\alpha$  and GABA<sub>B</sub>R1 $\beta$ ) band, and OD of  $\alpha$ -Tubulin band was obtained. The group results are based on ratios from three animals treated with saline and three animals treated with SS.

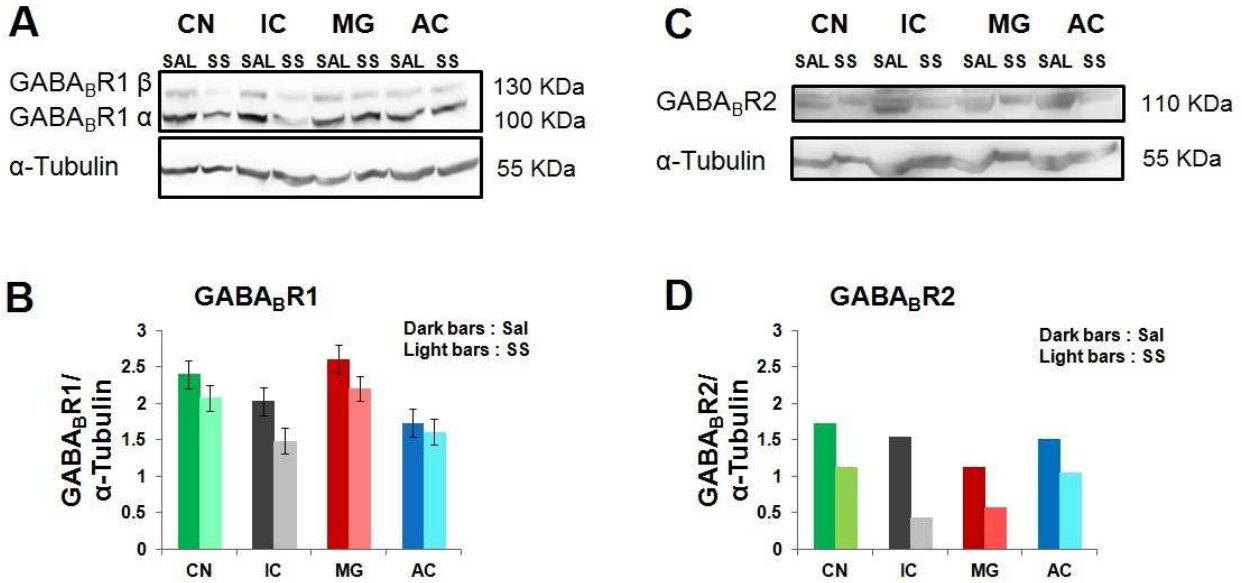
7d). Similar results were seen for the GABA<sub>B</sub>R2 receptor subunit levels in the CB (results not shown in figure). The band for CB from one saline-treated animal and CB from one SS-treated animal gave a normalized value of 1.04 and 1.10 respectively, indicating no significant difference.

#### **4.2 The effect of SS on overall levels of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits in auditory structures: Western Blotting**

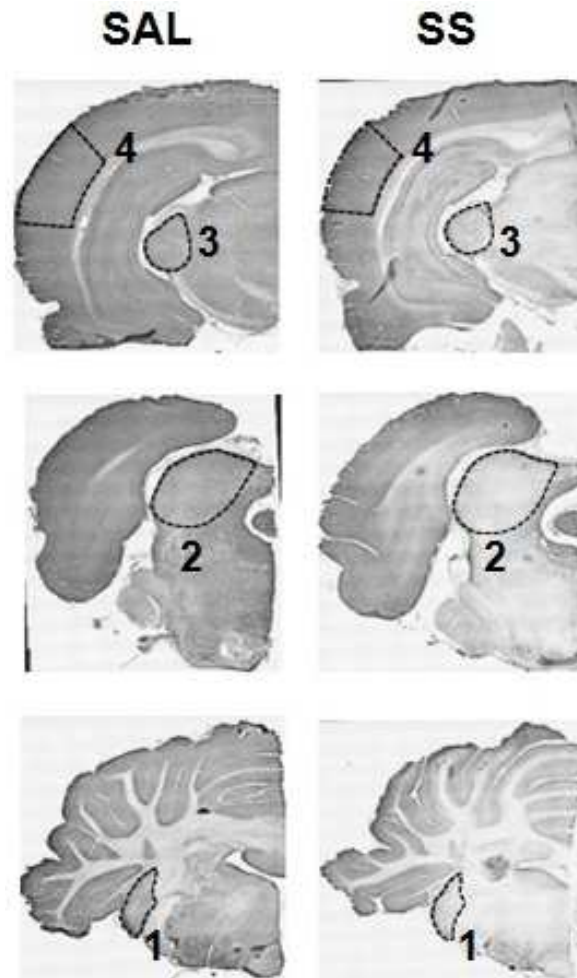
Western blotting experiments were conducted using tissues of auditory structures from three rats for GABA<sub>B</sub>R1 subunit and tissues from one rat for GABA<sub>B</sub>R2 subunit. Bands of immunoblots for GABA<sub>B</sub> receptor subunits were normalized against those of  $\alpha$ -Tubulin. Results revealed a trend of reduction in the level of the GABA<sub>B</sub>R1 subunit in the CN, IC, and MG 3 hours after the intraperitoneal injection of SS (Figure 8a). The reduction in the CN was 13.6%. The reduction in IC and MG was 26.8% and 15.5%, respectively (Figure 8b). The reduction in the level of subunit in the AC was minimal at 7.1%. The level of the GABA<sub>B</sub>R2 subunit also appeared to be reduced by SS in all the structures (Figure 8c). The reduction was at 35.4%, 71.7%, 49.3%, and 29.8% in the CN, IC, MG, and AC, respectively (Figure 8d).

#### **4.3 The effect of SS on overall levels of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits in auditory structures: Results based on low magnification IHC images**

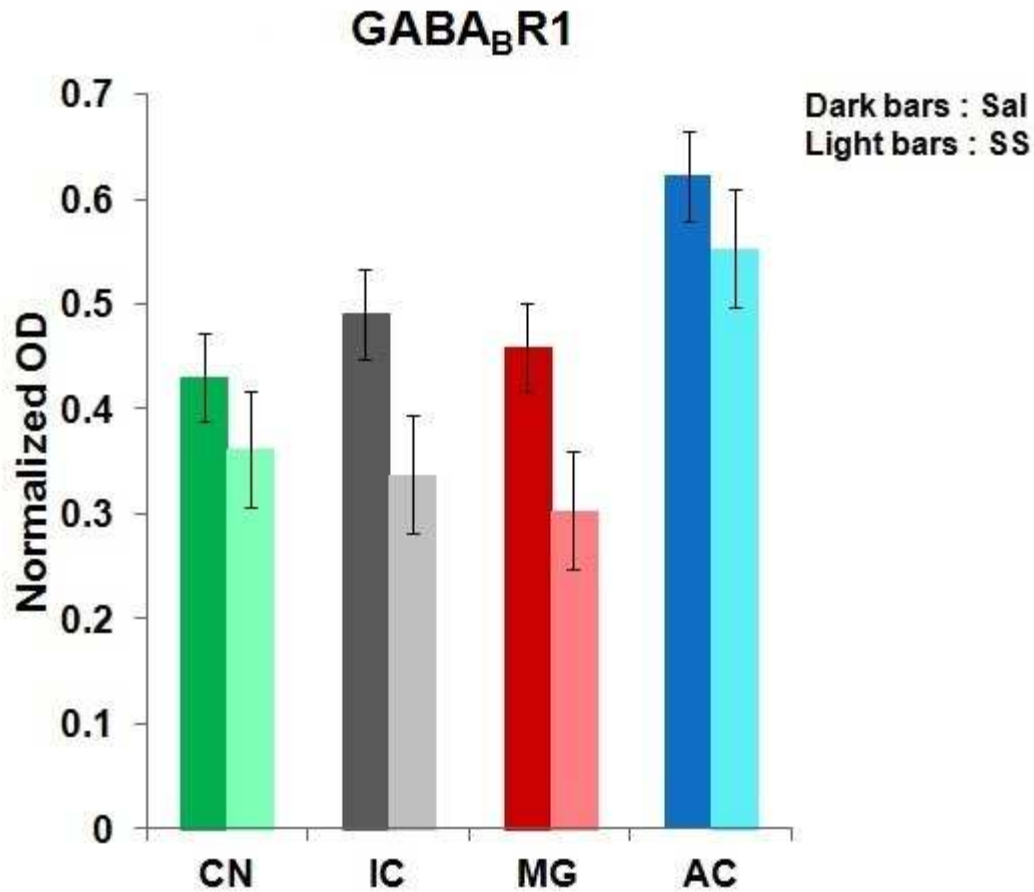
IHC experiments were conducted using five pairs of rats. In each pair, both subunits were probed. Three of the five cases or pairs were analyzed for overall level of labeling, as well as cell bodies and neuropil labeling. Low magnification images indicated that the level of the GABA<sub>B</sub>R1 subunit was decreased by SS in the CN, IC, MG, and AC (Figure 9a). For CN, IC,



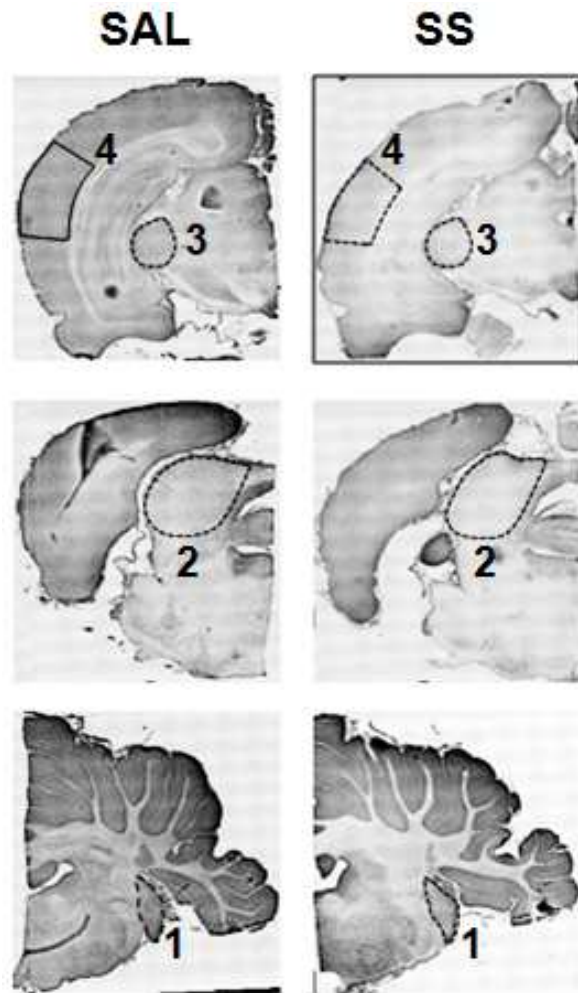
**Figure 8:** Western blots showing immunoreactivity against the GABA<sub>B</sub>R1 and the GABA<sub>B</sub>R2 subunit in the major auditory structures of saline-treated animals and SS-treated animals. **A:** Western blots showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in the major auditory structures. SAL represents tissue from saline treated animals. SS represents tissue from SS treated animals. Top blots show immunoreactivity against the GABA<sub>B</sub>R1α and GABA<sub>B</sub>R1β subunits. The bottom blot shows α-Tubulin immunoreactivity. **B:** Bar chart for group results from western blot experiments showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in major auditory structures. The ratio between the OD of the GABA<sub>B</sub>R1α and GABA<sub>B</sub>R1β band, and OD of α-Tubulin band was obtained. The group results are based on ratios from three animals. Error bars represent standard error. **C:** Western blots showing immunoreactivity against the GABA<sub>B</sub>R2 subunit in the major auditory structures. Top blots show immunoreactivity against the GABA<sub>B</sub>R2 subunit. The bottom blot shows α-Tubulin immunoreactivity. **D:** Bar chart for results from western blot experiment showing immunoreactivity against the GABA<sub>B</sub>R2 subunit in major auditory structures. The ratio between the OD of the GABA<sub>B</sub>R2 band and OD of α-Tubulin band was obtained. The results are based on ratios from one animal.



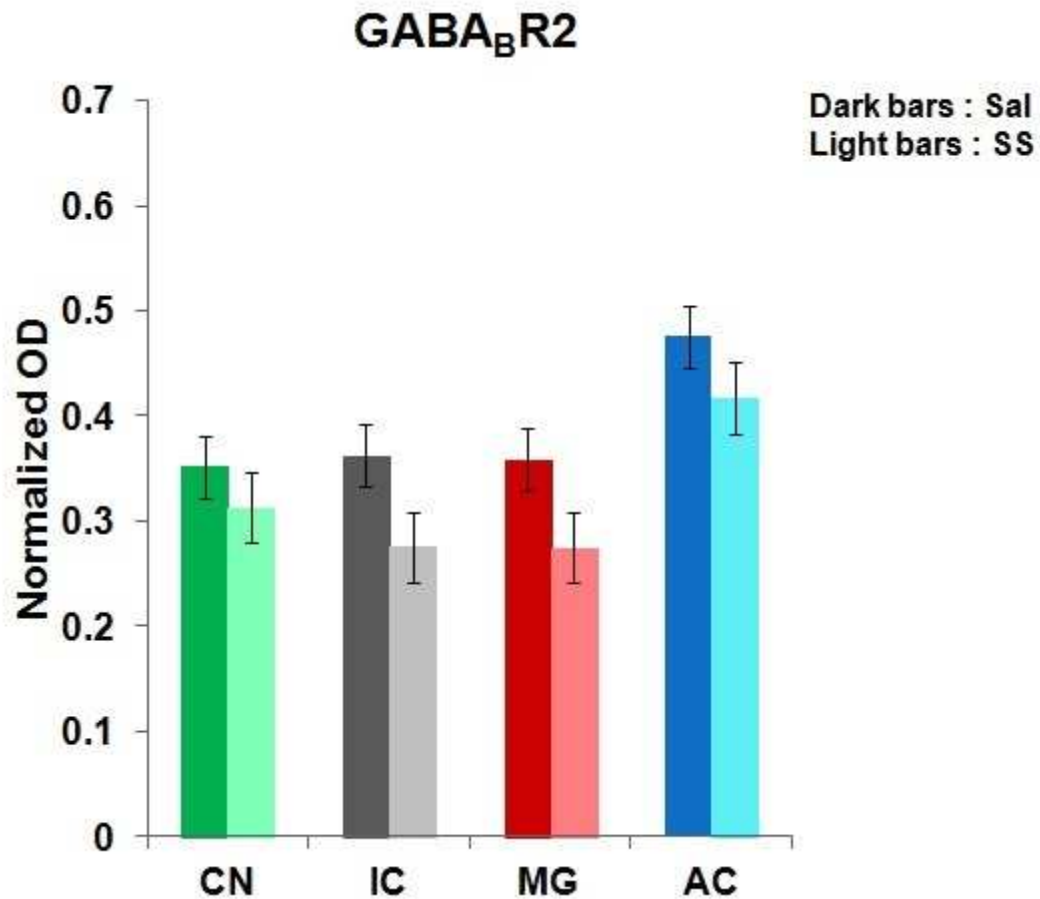
**Figure 9A.** Low magnification images showing immunoreactivity against the GABA<sub>B</sub>R1 subunit in the auditory structures. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. The bottom panel contains CN (1). The middle panel contains IC (2). The top panel contains MG(3) and AC (4).



**Figure 9B.** Group results from immunohistochemical experiments showing overall (neuropil and cell body combined) immunoreactivity against the GABA<sub>B</sub>R1 subunit in major auditory structures. The mean OD for each structure was obtained by combining the OD of all the subdivisions in the structure. The OD was normalized against GABA<sub>B</sub>R1 immunoreactivity in the cerebellum. Results are from analyses conducted using three animals treated with saline and three animals treated with SS.



**Figure 10A.** Low magnification images showing immunoreactivity against the GABA<sub>B</sub>R2 subunit in the auditory structures. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. The bottom panel contains CN (1). The middle panel contains IC (2). The top panel contains MG(3) and AC (4).



**Figure 10B.** Group results from immunohistochemical experiments showing overall (neuropil and cell body combined) immunoreactivity against the GABA<sub>B</sub>R2 subunit in major auditory structures. The mean OD for each structure was obtained by combining the OD of all the subdivisions in the structure. Results are from analyses conducted using three animals for saline and three animals for SS.



and MG, sections from SS-treated animal showed lighter labeling compared to those animals treated with saline control. In the AC, sections from Saline and SS treated animals showed a small difference in labeling. Overall labeling in each structure was quantified by using OD. Resulting values were normalized against those of the molecular layer and the white matter of the cerebellum (Figure 9b). For each structure of an individual animal, the integrated OD and the area were measured in each section with the structure and a weighted average was obtained for this structure. The quantified results showed a trend of reduction in all auditory structures. The labeling in saline CN was reduced by 16% by SS. In IC, the labeling was reduced by over 31% by SS. The labeling in the MG was reduced by close to 34% by SS. The labeling in the AC was reduced by 11%.

For the GABA<sub>B</sub>R2 subunit, low magnification images revealed a decrease in labeling in all the auditory structures (Figure 10a). The reduction was about 11%, 24%, 23%, and 12% in the CN, IC, MG, and AC, respectively (Figure 10b).

#### **4.4 The effect of SS on regional and cellular distributions of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 in auditory structures: : Results based on high magnification IHC images**

##### **4.4.1 The effect of SS on the CN**

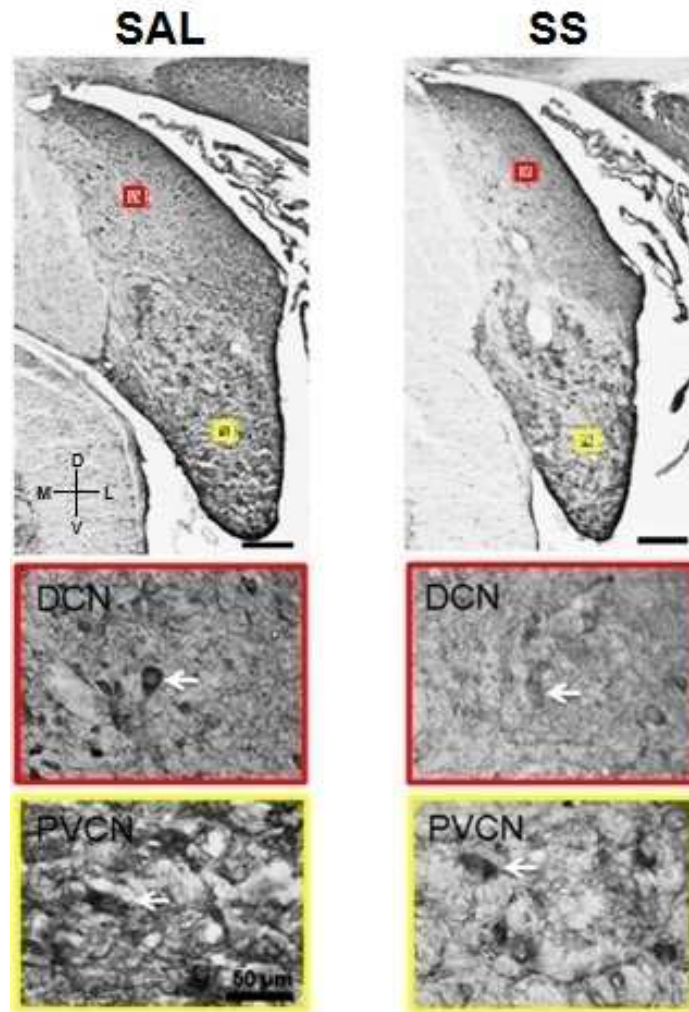
##### **4.4.1.1 The GABA<sub>B</sub>R1 subunit in the CN**

Regional differences exist in the level of immunoreactivity against the GABA<sub>B</sub>R1 subunit in the three subdivisions of the CN. Under both saline and SS conditions, GABA<sub>B</sub>R1 immunoreactivity was high in the DCN and PVCN compared to AVCN (Figure 11a,b). In both the saline treated animal and the SS treated animal, the superficial layers of DCN and the GCD showed highest level of immunoreactivity. In particular, the neuropil (region containing the

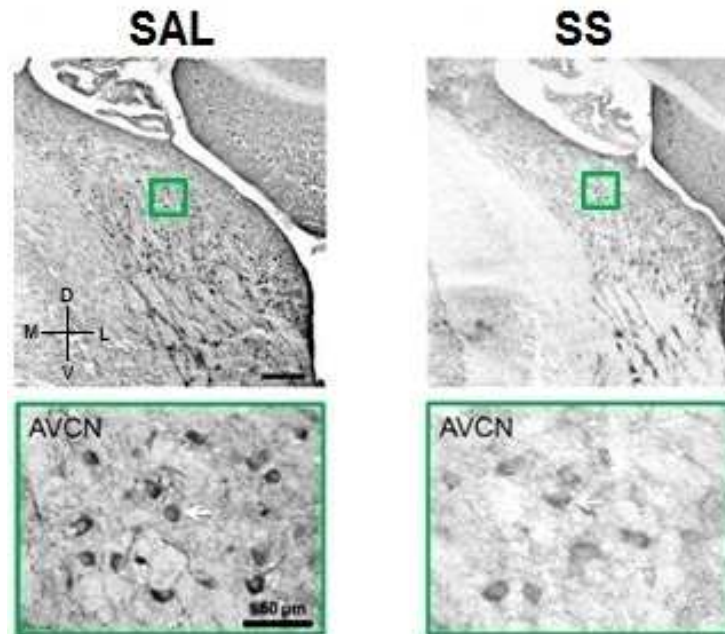
axons and dendrites of cell bodies) was darkly labeled with lots of visible puncta (i.e. dark spots or dots). In contrast, the neuropil labeling in the deep layers of DCN was light with fewer labeled puncta, indicating lower immunoreactivity. Cell body immunoreactivity was similar across all the layers of DCN and in GCD. Both PVCN and AVCN showed less punctate labeling in the neuropil compared to the superficial layers of DCN and GCD. Large cell bodies resembling globular bushy cells and octopus cells were darkly labeled in the PVCN. In the AVCN, moderately large labeled cell bodies, presumably globular bushy cells, were darkly labeled. There was no apparent difference in immunoreactivity across the rostral-caudal extent of the structure.

For SS treated animals, the percentage reduction in labeling was the greatest in the AVCN followed by PVCN. The reduction was the smallest in the DCN. Quantitative analysis revealed that the reduction in labeling by SS was about 35% in the AVCN, 28% in the PVCN, and 16% in the DCN (Figure 11c). The reduction was not statistically significant ( $p > 0.05$ ).

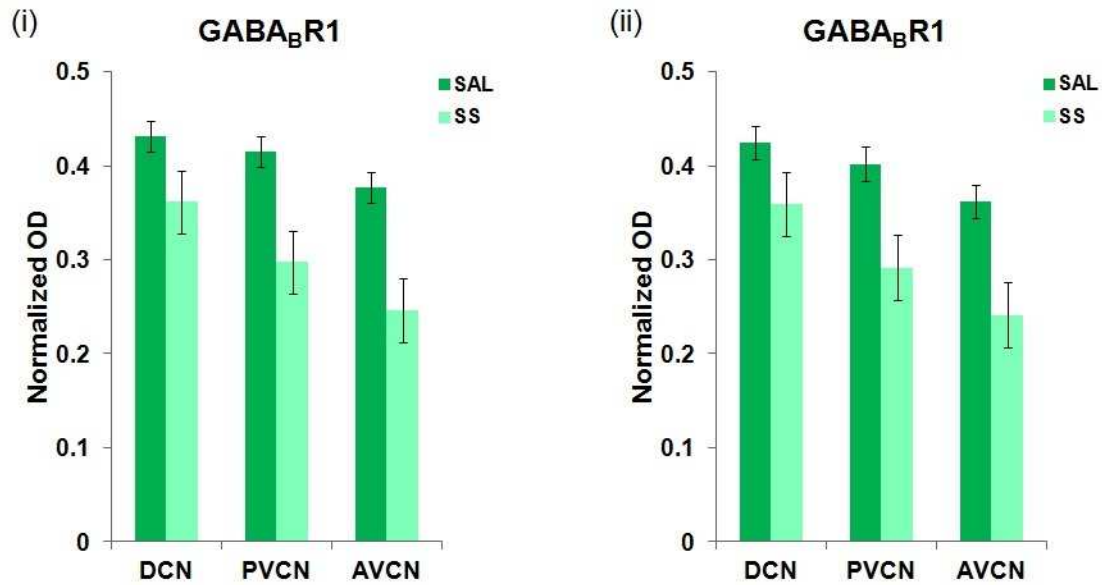
High magnification images showed that the reduction in the overall level of the GABA<sub>B</sub>R1 subunit was due to changes in the level of labeling in both cell body and neuropil regions (Figure 11a,b). The neuropil is the region outside cell bodies and it contains the axons and dendrites of neurons. For sections from SS-treated animals, both diffused and punctate labeling in the neuropil was reduced. In the DCN, the deep layers showed the greatest decrease in immunolabeling amongst all the layers. Both diffused and punctate labeling was reduced in the neuropil. In the PVCN and AVCN, diffused and punctate labeling was reduced without any regional specificity. Cell body labeling was also equally reduced across the entire subdivision. Quantification of the labeling in the neuropil showed that the reduction was greatest in the



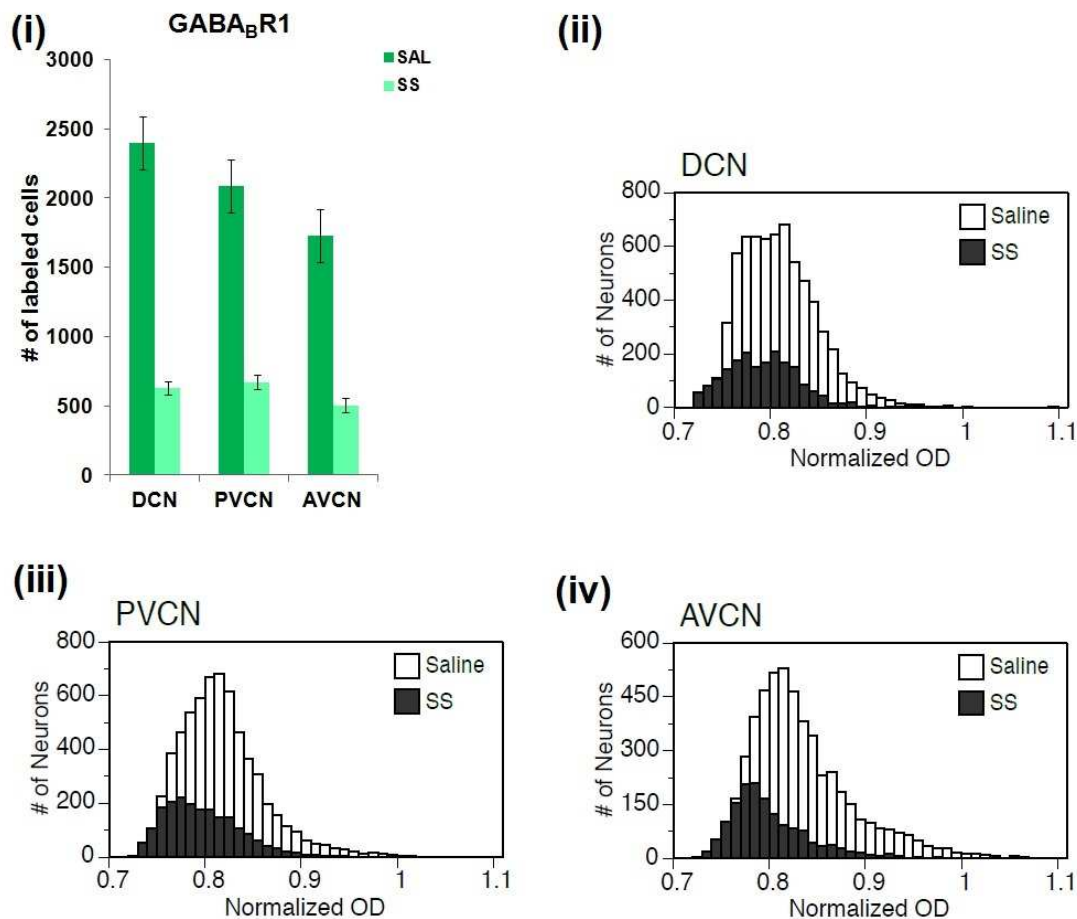
**Figure 11A.** Immunoreactivity to the GABA<sub>B</sub>R1 subunit in the CN. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body combined) immunolabeling in the DCN and PVCN. High magnification images show neuropil and cell body labeling. White arrows point towards a labeled cell bodies. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.



**Figure 11B.** Immunoreactivity to the GABA<sub>B</sub>R1 subunit in the AVCN. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body in combination) immunolabeling. High magnification images show neuropil and cell body labeling. White arrows point towards labeled cell bodies. Scale bar: 200μm in low magnification images; 50 μm in high magnification image.



**Figure 11C.** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R1 subunit in the subdivisions of CN. Analyses were conducted using three animals treated with saline and three animals treated with SS. The OD of neuropil for each subdivision was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.



**Figure 11D.** (i) Bar chart showing the total number of GABA<sub>B</sub>R1 immunoreactive cell bodies in each subdivision of CN. Error bars indicate standard error. (ii-iv) Histograms showing cgroup results for normalized OD distribution of GABA<sub>B</sub>R1 immunoreactive cell bodies in the subdivisions of CN. Results are from analyses conducted using three animals treated with saline and three animals treated with SS.

AVCN (33%), followed by PVCN (27%) and then DCN (15%) (Figure 11c). The reduction was not statistically significant ( $p > 0.05$ ).

SS also caused a decrease in the labeling in cell bodies in all three subdivisions (Figure 11d, i, ii, iii). The decrease was reflected in the ODs of labeled cell bodies and the total number of labeled cell bodies. As indicated in Figure 11d (i), (ii), (iii), the distribution of the OD values of labeled cell bodies in SS-treated animals was shifted leftward in comparison to that in saline-treated animals. For both animals treated with saline and SS, the total number of labeled cell bodies was highest in DCN, followed by PVCN and AVCN (Figure 11d). The cell bodies seemed more densely packed in the DCN than in the other two subdivisions. The SS-induced decrease in the number of labeled cell bodies was highest in the DCN, followed by AVCN, and then PVCN. The reduction in the number of labeled cell bodies was about 74% in the DCN, 71% in the AVCN, and about 68% in the PVCN. This reduction in cell body number was statistically significant ( $p < 0.0001$ ).

#### **4.4.1.2 The GABA<sub>B</sub>R2 subunit in the CN**

For both animals treated with saline and SS, DCN and PVCN showed the highest overall immunoreactivity, followed AVCN (Figure 12a, b). Similar to the GABA<sub>B</sub>R1 subunit distribution, the GABA<sub>B</sub>R2 immunoreactivity was the highest in the superficial layers of the DCN and GCD compared to the deep layers. Both diffused and punctate labeling was relatively high in the superficial layers. Cell body labeling was similar across all the layers of the DCN and in the GCD. PVCN and AVCN had lower levels of diffused and punctate labeling compared to the DCN. There were no regional differences in neuropil labeling or cell body labeling between

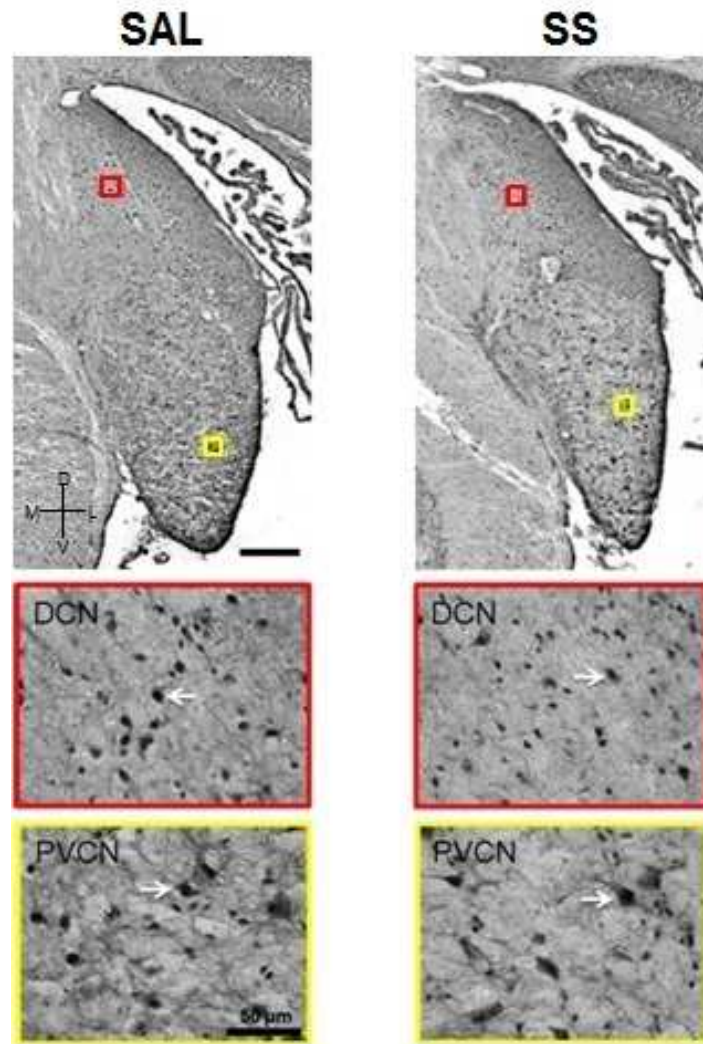
PVCN and AVCN. Qualitative observations revealed that the CN subdivisions were similar in labeling across the rostral-caudal extent of the structure.

After treatment with SS, there was a trend of reduction in the overall level of the GABA<sub>B</sub>R2 subunit in all the three subdivisions of the CN (Figure 12a,b). The reduction in the overall level of labeling was the highest in the PVCN at 22% (Figure 12c). DCN showed an intermediate reduction at 11%, and AVCN showed the smallest reduction at 7%. This reduction was not statistically significant ( $p > 0.05$ ).

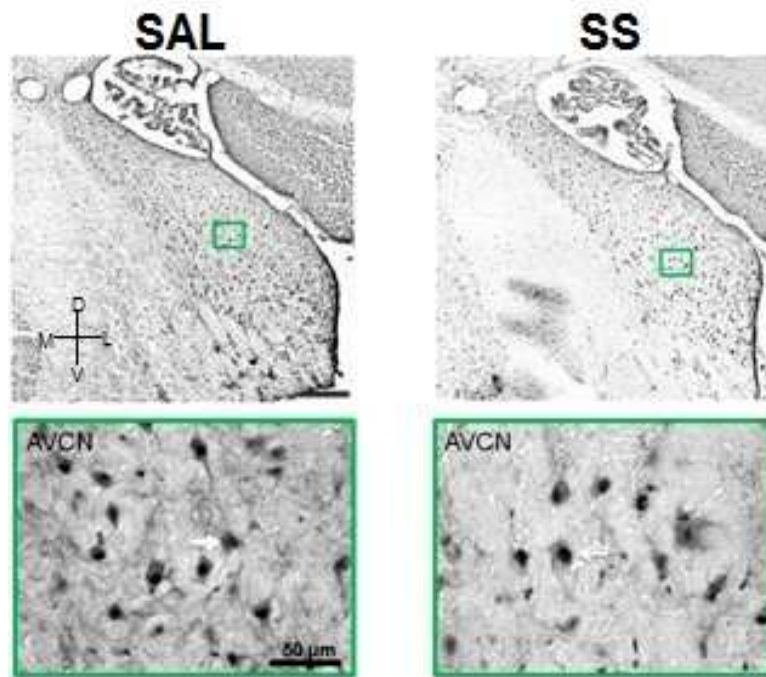
High magnification images showed that both diffused and punctate labeling was reduced in the neuropil of CN in sections of SS treated animals (Figure 12a,b). This reduction was greater in the deep layers of DCN than the superficial layers. The reduction in neuropil immunolabeling was similar across the entire PVCN and AVCN. OD values revealed that SS-induced decrease in neuropil immunoreactivity was the greatest in the PVCN (22%). DCN and AVCN showed smaller decreases, at 10% and 8% respectively (Figure 12c). The reduction was not statistically significant ( $p > 0.05$ ).

High magnification images showed that for all three subdivisions there weren't large differences in the optic density of labeled cell bodies between saline- and SS-treated animals (Figure 12a,b). The cell bodies appeared equally dark in all subdivisions for animals in both groups. However, SS-treatment resulted in a reduction in the number of labeled cell bodies (Figure 12d). Similar to GABA<sub>B</sub>R1, the number of GABA<sub>B</sub>R2-immunoreactive cell bodies was the highest in the DCN, followed by PVCN and AVCN. The percentage reduction in the number of cell bodies was the highest in AVCN, followed by PVCN, and then DCN. Qualitative examination revealed that in the DCN, the decrease was greatest in the deep layers. The

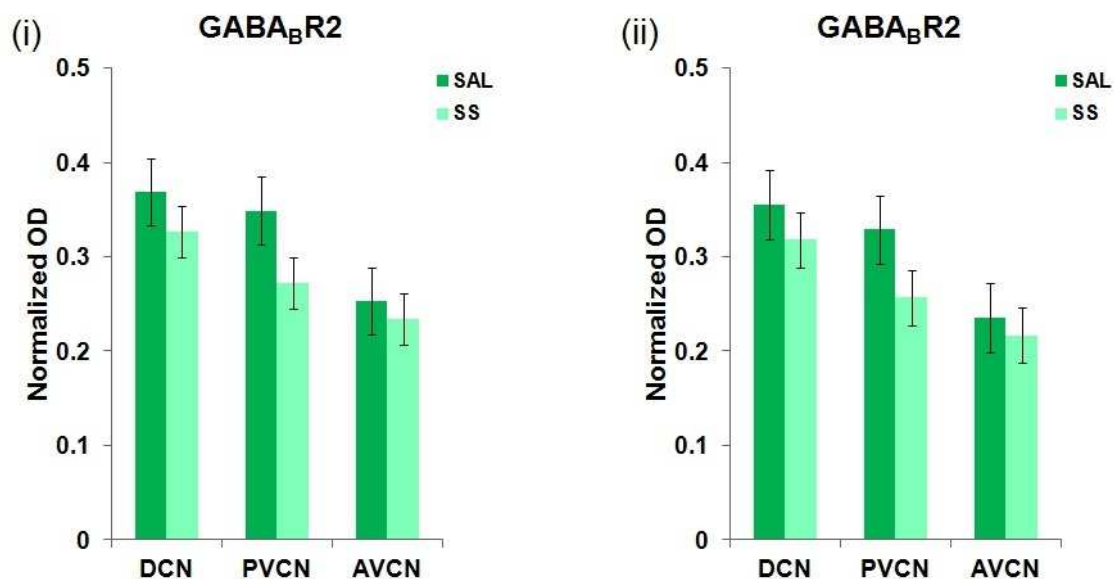




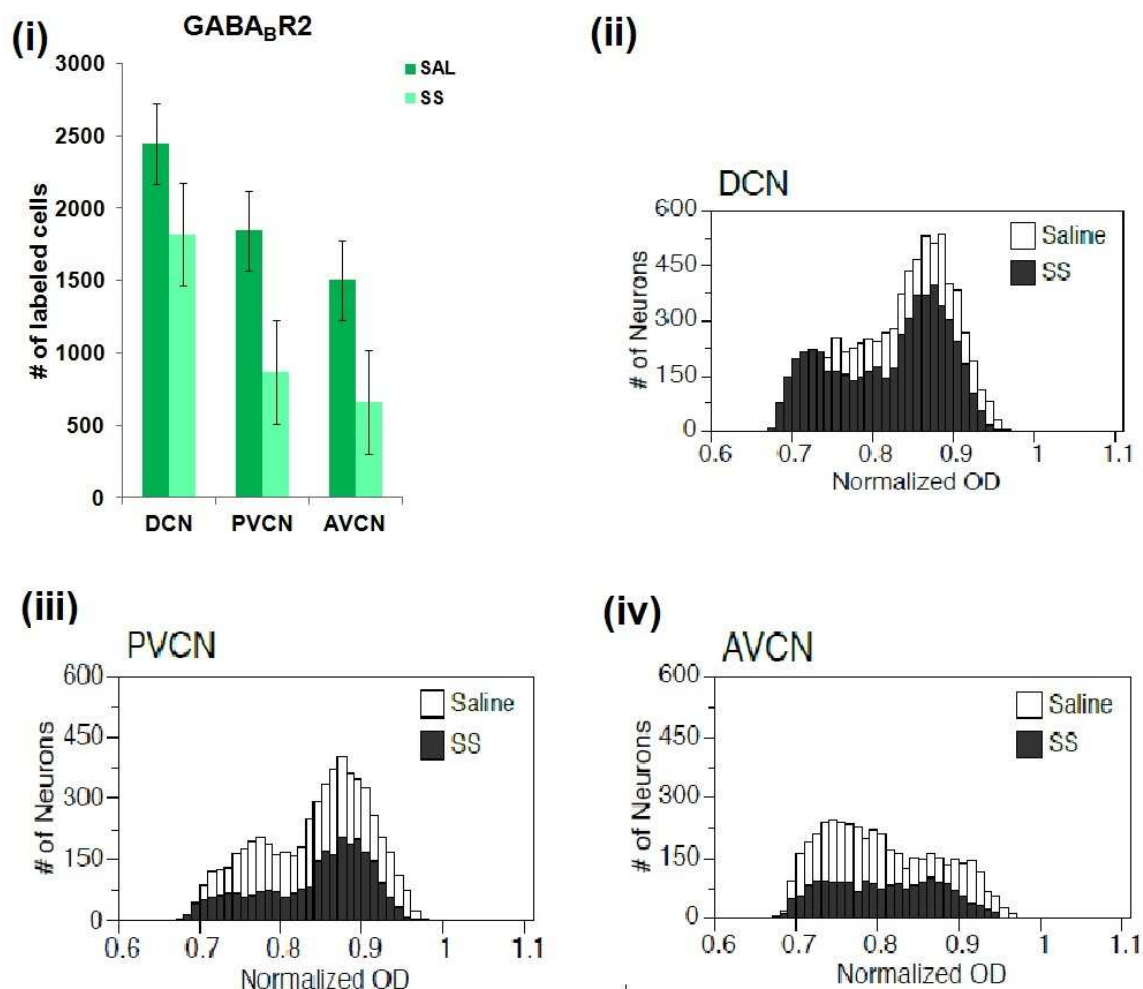
**Figure 12A:** Immunoreactivity to the GABA<sub>B</sub>R2 subunit in the CN. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body combined) immunolabeling in the DCN and PVCN. High magnification images show neuropil and cell body labeling in the DCN and PVCN. White arrows point towards a labeled cell body. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.



**Figure 12B:** Immunoreactivity to the GABA<sub>B</sub>R2 subunit in the AVCN. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body in combination) immunolabeling. High magnification images show neuropil and cell body labeling. White arrows point towards a labeled cell body. Scale bar: 200μm in low magnification images; 50 μm in high magnification image.



**Figure 12C:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body in combination) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R2 subunit in the subdivisions of the CN. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.



**Figure 12D:** (i) Bar chart showing the total number of GABA<sub>B</sub>R2 immunoreactive cell bodies in each subdivision. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R2 immunoreactive cell bodies in the subdivisions of CN. Results are from analyses conducted using three animals for saline and three animals for SS.

percentage reduction in the number of labeled cell bodies in the AVCN of SS-treated animals was 44%. The percentage reduction in the number of labeled cell bodies in the PVCN and DCN of SS-treated animals was at 47% and 74% respectively. The reduction in number of labeled cell bodies was statistically significant ( $p < 0.0001$ ). The labeling in cell bodies was also reduced, as indicated by the leftward shift of the distribution of the OD values of labeled cell bodies in SS-treated animals in comparison to that in saline-treated animals.

#### **4.4.2 The effect of SS on the IC**

##### **4.4.2.1 The GABA<sub>B</sub>R1 subunit in the IC**

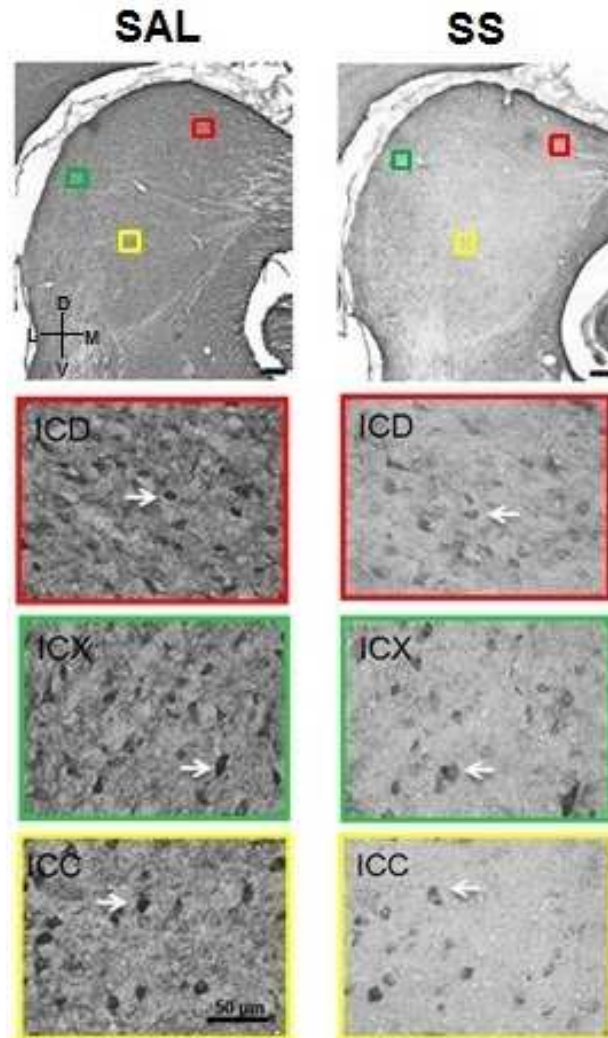
Low magnification images showed that there were differences in the overall immunoreactivity against the GABA<sub>B</sub>R1 subunit among the three subdivisions of the IC (Figure 13a). For both sections from animals treated with saline and SS, ICd had the highest level of labeling, followed by ICc and ICx. The neuropil in the ICd had a high level of diffused and punctate labeling. The immunolabeling gradually reduced going from dorsal to medial side of the ICd. ICx had a moderate level of diffused and punctate labeling. The labeling on the medial side of the ICx was somewhat lighter than the lateral side of the structure. ICc had low levels of diffused and punctate labeling. The labeling was darker along the borders with ICd and ICx and gradually reduced along the dorsoventral-ventromedial axis. The cell body labeling was high in the ICd and ICx. In the ICc, the cell bodies were only slightly lighter. There were differences in overall labeling amongst the subdivisions of the IC across the rostral-caudal extent, with the caudal slices showing higher immunoreactivity in each subdivision.

A treatment with SS resulted in a decrease in the overall level of labeling in all three subdivisions (Figure 13b). The percentage reduction was the greatest in the ICc (a 47%

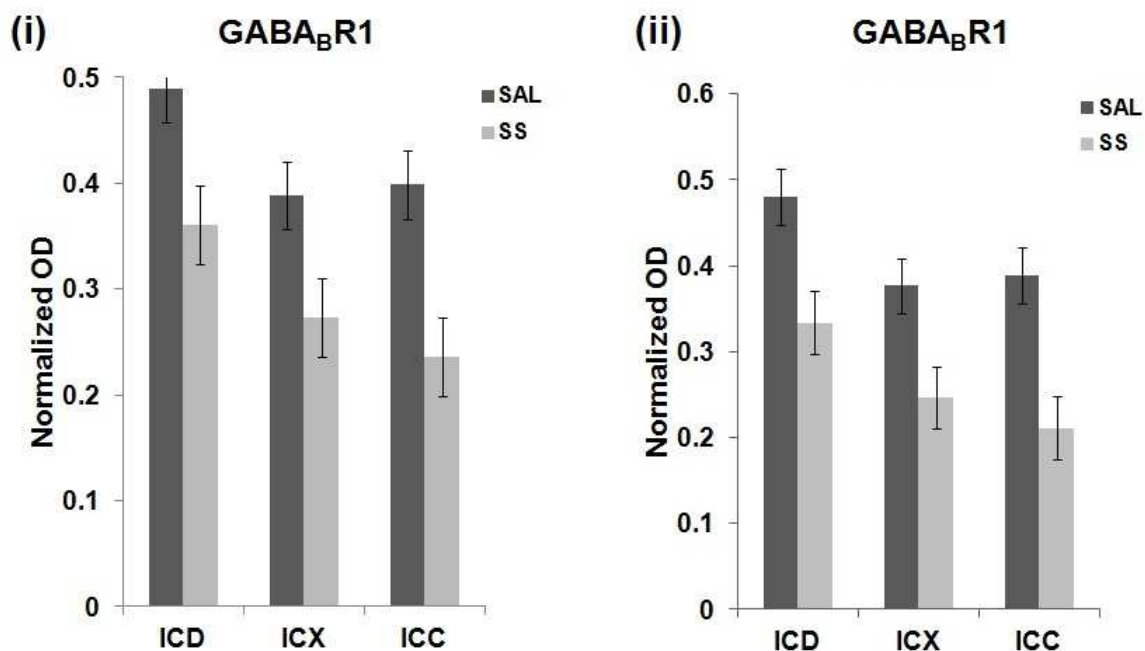
reduction), followed by the ICx (a 36% reduction) and ICd (a 31% reduction). The reduction was similar across the rostral-caudal extent of the structure, and was not statistically significant ( $p > 0.05$ ).

High magnification images showed that the decrease in the immunoreactivity against the GABA<sub>B</sub>R1 antibody occurred in both the neuropil and cell bodies (Figure 13a). The neuropil of the IC showed a gradient of labeling, with the ICd showing the highest level of immunoreactivity, followed by ICc and ICx. In the neuropil of all the IC subdivisions of saline treated animals, there was a high level of punctate labeling. Both diffused and punctate labeling was greatly reduced in all IC subdivisions of SS treated animals. Quantitative results revealed that the immunoreactivity against GABA<sub>B</sub>R1 in the neuropil was reduced by 46% in the ICc, by 34% in the ICx, and by 30% in the ICd (Figure 13b). The reduction was not statistically significant ( $p > 0.05$ ).

Differences in cell body labeling were also observed in all the subdivisions of the IC between saline and SS treated animals (Figure 13c). Cell bodies in sections from saline-treated animals were darker compared those in sections from SS-treated animals (Figure 13a). There were fewer labeled cell bodies in the IC of SS-treated animals compared to that in the same structure of saline-treated animals. The total number of labeled cell bodies was highest in the ICx, followed by ICc and ICd in both saline and SS. The reduction in the number of labeled cell bodies was highest in the ICc at 96%, intermediate in ICx at 81%, and lowest in ICd at 70% (Figure 13c). The decrease in number of labeled cell bodies was statistically significant ( $p < 0.0001$ ). Histograms of OD values of cell bodies indicated that the cell body labeling was decreased after treatment with SS, indicated by a leftward shift of the distribution.

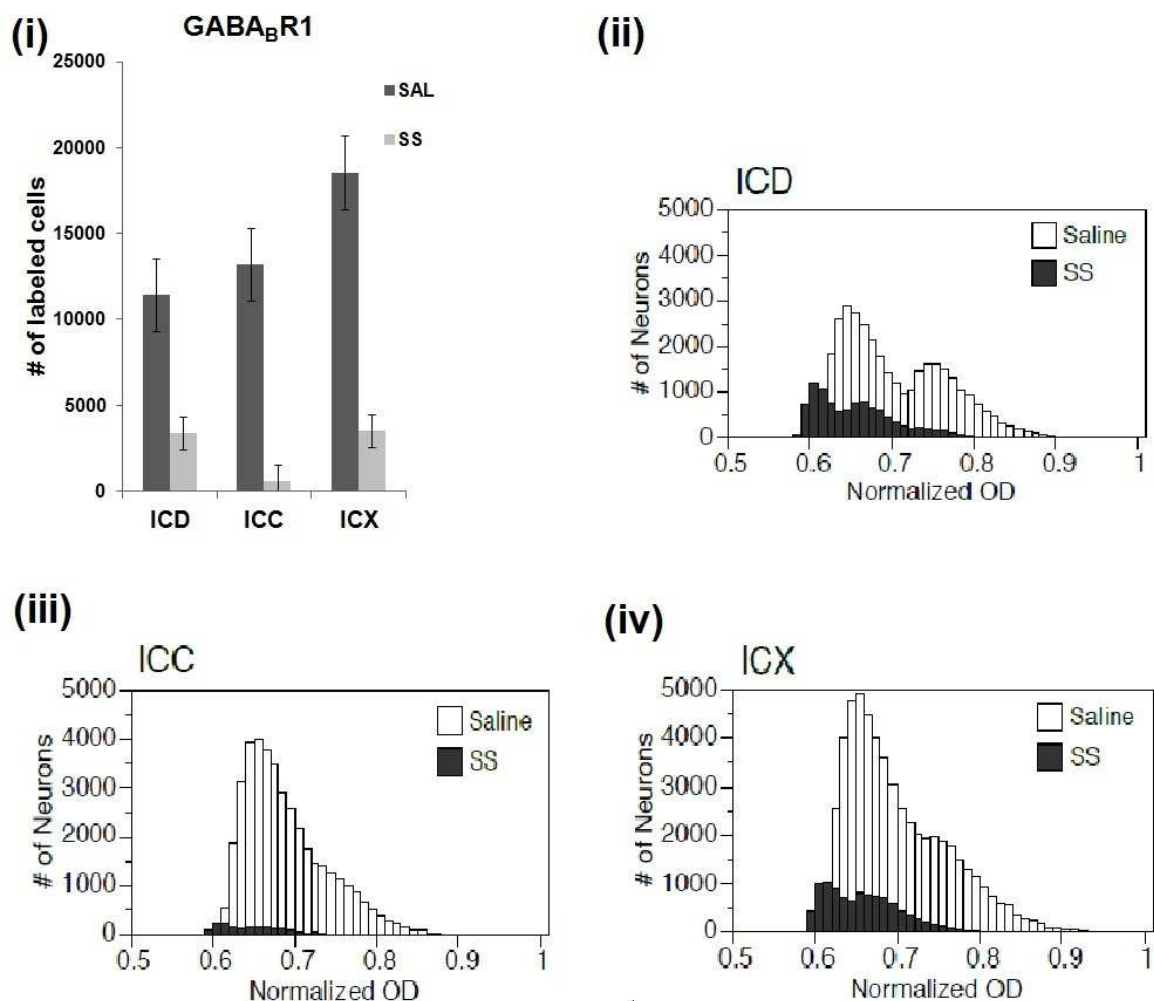


**Figure 13A:** Immunoreactivity to the GABA<sub>B</sub>R1 subunit in the IC. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body combined) immunolabeling in the IC. High magnification images show neuropil and cell body labeling in the ICd, ICx, and ICC. White arrows point towards a labeled cell body. Scale bar: 200μm in low magnification images; 50 μm in high magnification image.



**Figure 13B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R1 subunit in the subdivisions of the IC. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.





**Figure 13C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R1 immunoreactive cell bodies in each subdivision of the IC. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R1 immunoreactive cell bodies in the subdivisions of IC. Results are from analyses conducted using three animals for saline and three animals for SS.

#### 4.4.2.2 The GABA<sub>B</sub>R2 subunit in the IC

For both sections from animals treated with saline and SS, ICd showed the highest level of immunoreactivity against the GABA<sub>B</sub>R2 among the three subdivisions. Both diffused and punctate labeling was high in ICd (Figure 14a). There was a gradient of labeling with the immunolabeling decreasing going from dorsal to the ventral part of ICd. The level of immunolabeling was moderate in the ICx. Diffused labeling was lighter compared to ICd and there was less punctate labeling. There was a reduction in the level of immunolabeling along an axis, from the lateral to the medial part of ICx. ICd and ICx also had darkly labeled cell bodies packed close together, indicating high cell packing density. The level of immunoreactivity was low in the ICc for both diffused and punctate labeling. The labeling was darker along the borders with ICd and ICx. There were also differences in labeling along the rostral-caudal extent of the IC with the caudal slices showing higher immunolabeling.

Differences were observed in the IC in overall immunoreactivity against the GABA<sub>B</sub>R2 subunit between saline and SS-treated animals. SS-induced reduction in overall level of immunoreactivity was similar among the three subdivisions of the IC (28%, 27%, and 24% in the ICc, ICx, and ICd, respectively) (Figure 14b).

High magnification images showed that a reduction in the immunoreactivity against the GABA<sub>B</sub>R2 receptor subunit existed in both neuropil and cell body regions (Figure 14a). For tissues from SS-treated animals, diffused and punctate labeling in the neuropil was reduced in all the subdivisions. The level of reduction was similar across the three collicular subdivisions (28% in the ICc, 26% in the ICx, and 23% in the ICd) (Figure 14b). This reduction was not statistically significant ( $p > 0.05$ ).

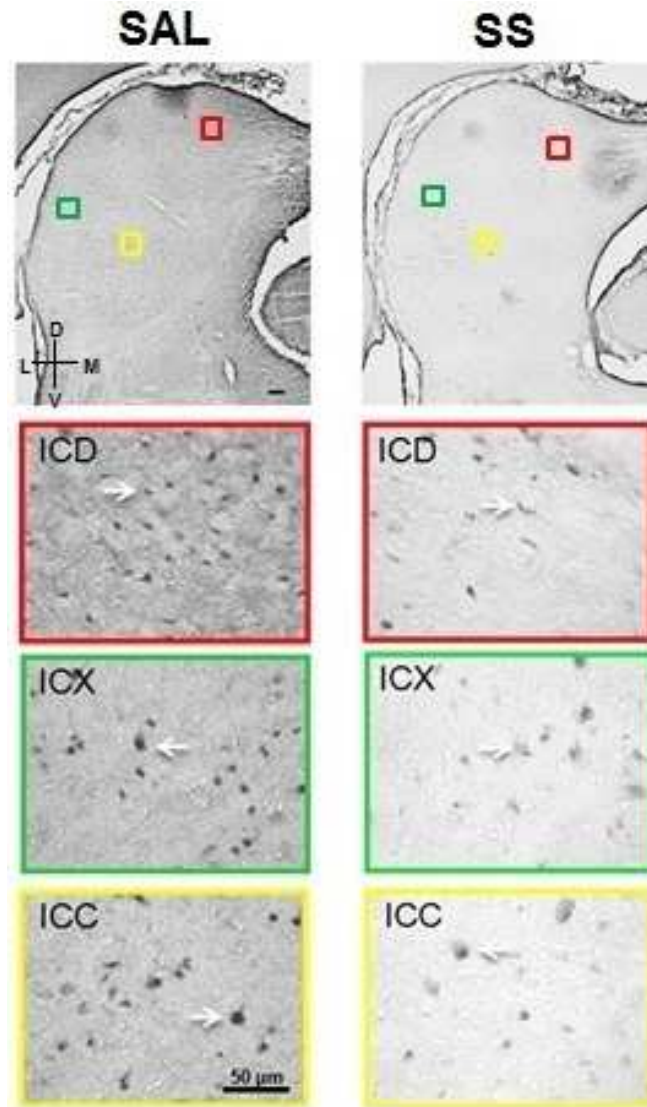
Differences also existed in cell body labeling between tissues from control animals and SS-treated animals (Figure 14a). The total number of labeled cell bodies in each subdivision also decreased after SS-treatment. Quantitative results revealed that the ICc had an 83% reduction in the number of labeled cell bodies, which was the highest among those in the three subdivisions (Figure 14c). The reduction in the number of labeled cell bodies was 73% in the ICx and 62% in the ICd. This decrease was statistically significant ( $p < 0.0001$ ). In addition to the reduction in the number of labeled cell bodies, the ODs of labeled cell bodies also decreased after SS treatment. This decrease was observed in all the three subdivisions of the IC, as indicated by the leftward shift of the histogram of OD values (Figure 14c).

#### **4.4.3 The effect of SS on the MG**

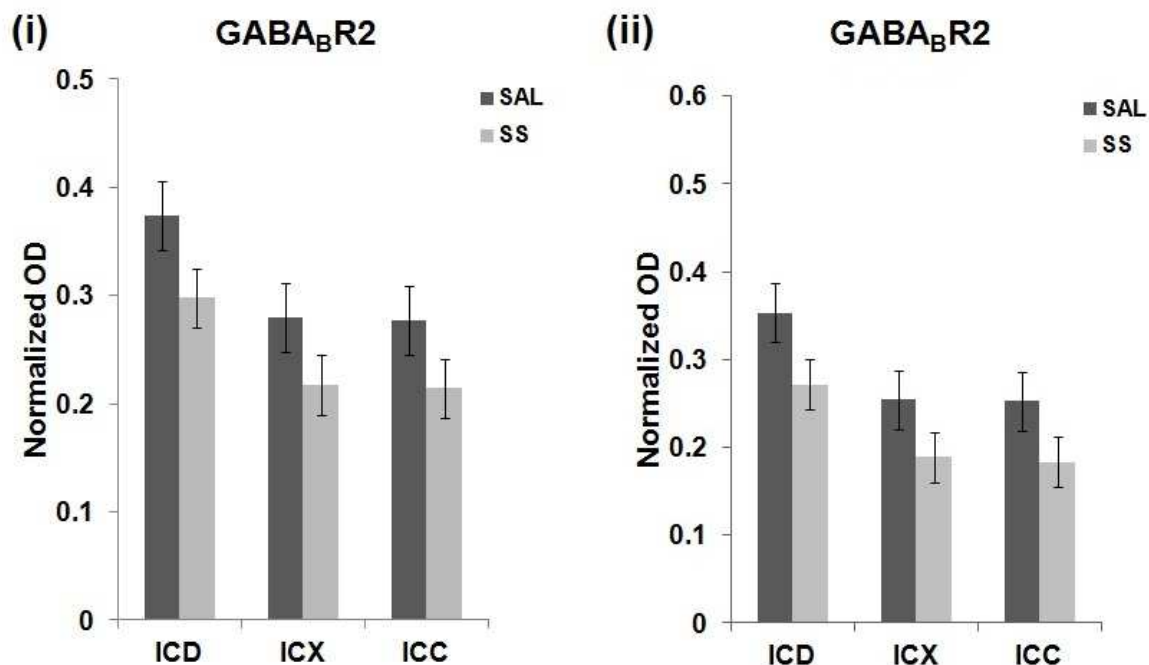
##### **4.4.3.1 The GABA<sub>B</sub>R1 subunit in the MG**

For both saline and SS treated animals, all subdivisions showed the similar levels of immunoreactivity against the GABA<sub>B</sub>R1 subunit. The neuropil of the MG showed a high level of punctate labeling along with diffused labeling. Cell body labeling was similar across the three subdivisions of the MG. The labeling was also similar across the rostral-caudal extent of the MG as indicated by qualitative examination of tissue.

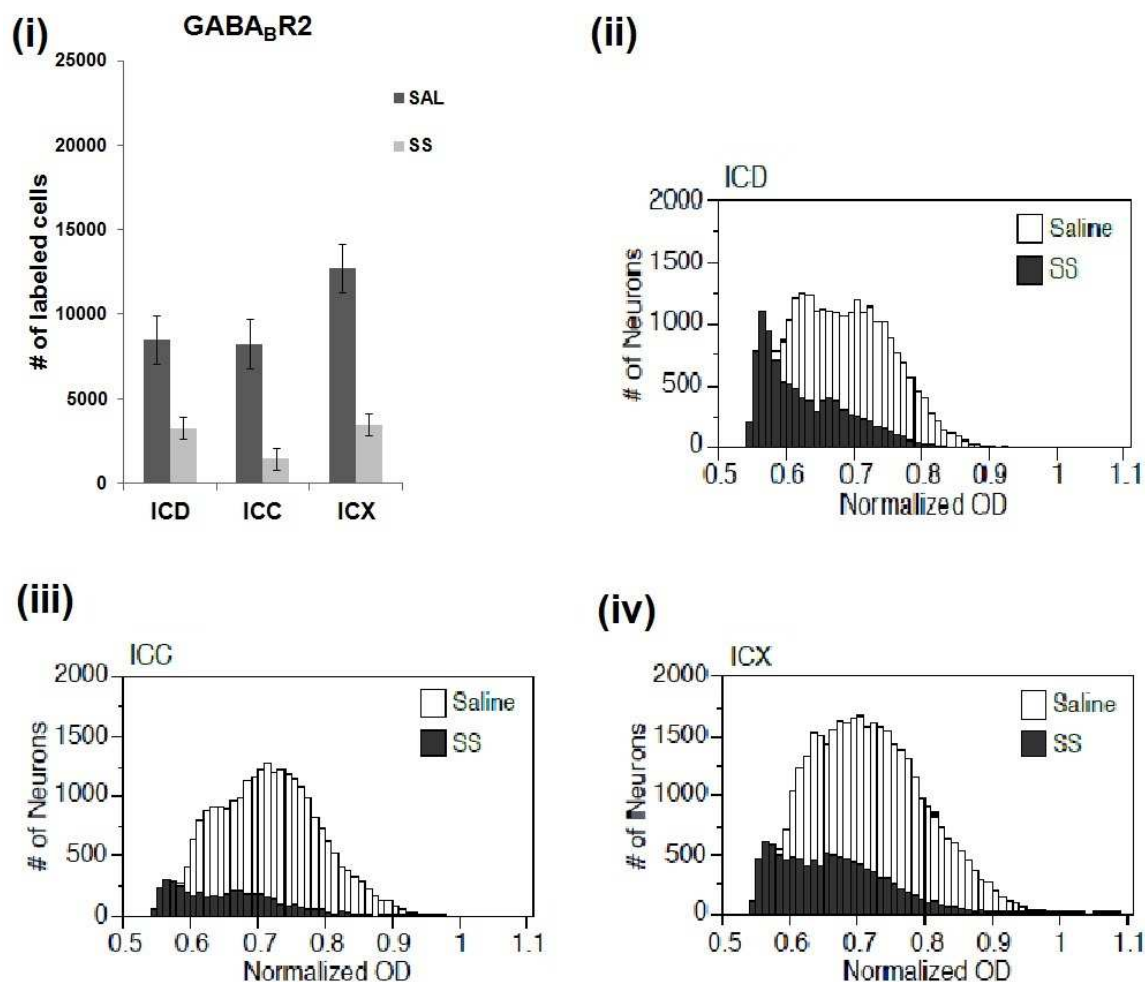
The overall level of immunoreactivity was different between animals treated with saline and SS. Low magnification images showed that SS reduced the overall level of immunoreactivity in all three subdivisions (Figure 15a). The MGm showed the greatest percentage reduction among the three subdivisions (Figure 15b). The percentage reduction was 47%, 42%, and 36% respectively in the MGm, MGv, and MGd, respectively. This reduction was not statistically significant ( $p > 0.05$ ), and was similar across the rostral-caudal extent of the MG.



**Figure 14A:** Immunoreactivity to the GABA<sub>B</sub>R2 subunit in the IC. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body combined) immunolabeling in the IC. High magnification images show neuropil and cell body labeling in the ICd, ICx, and ICc. White arrows point towards a labeled cell body. Scale bar: 200μm in low magnification images; 50 μm in high magnification image.



**Figure 14B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R2 subunit in the subdivisions of the IC. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.



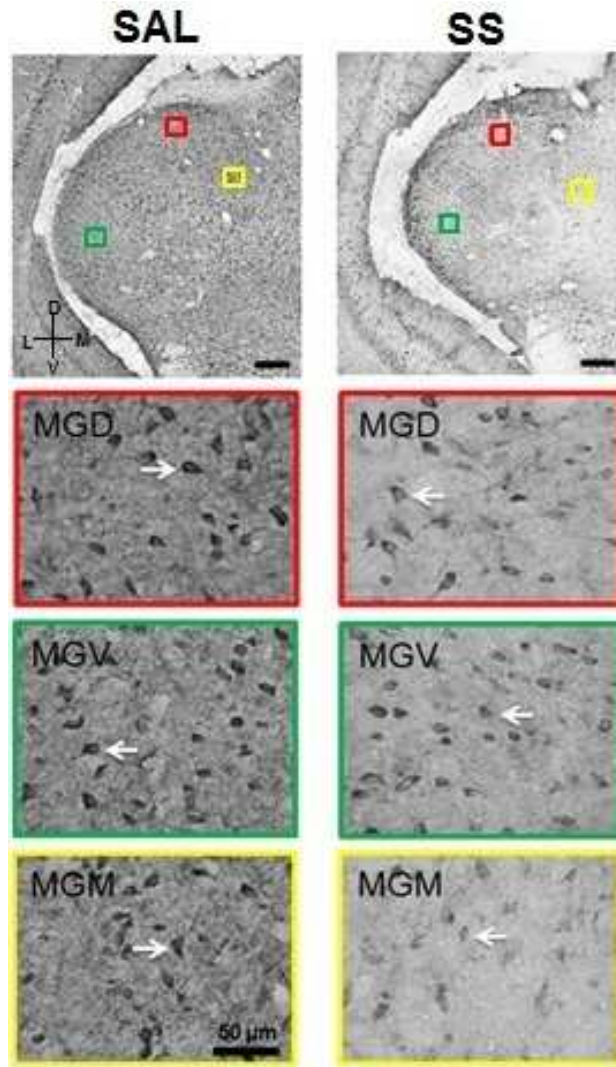
**Figure 14C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R2 immunoreactive cell bodies in each subdivision of the IC. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R2 immunoreactive cell bodies in the subdivisions of IC. Results are from analyses conducted using three animals for saline and three animals for SS

High magnification images showed that SS reduced the level of immunoreactivity against the GABA<sub>B</sub>R1 in both the neuropil and cell body regions (Figure 15a). After SS treatment, the diffused and punctate labeling in the neuropil was reduced in all the subdivisions. This reduction was the greatest in MGm (46% decrease), followed by the MGv (a 40% decrease) and MGd (a 34% decrease) (Figure 15b). The reduction was similar across the rostral-caudal extent of the structure. However, this reduction was not statistically significant ( $p > 0.05$ ).

High magnification images showed that after treatment with SS, there was reduction in the number of labeled cell bodies (Figure 15a). Cell bodies in sections from saline treated animals were darkly labelled in all the subdivisions of MG. After SS-treatment, the ODs of cell bodies in all the subdivisions of MG in SS-treated animals were not significantly changed. Quantitative results showed that there was a decrease in the total number of labeled cell bodies after SS treatment (Figure 15c). The reduction was the greatest in the MGm (an 89% reduction), followed by the MGv (an 80% decrease) and MGd (a 67% decrease). This reduction was statistically significant ( $p < 0.0001$ ). Histograms of cell body labeling indicated that the labeling in cell bodies was not different (Figure 15c). The distributions of labeling in cell bodies for saline-treated animals and SS-treated animals was the same.

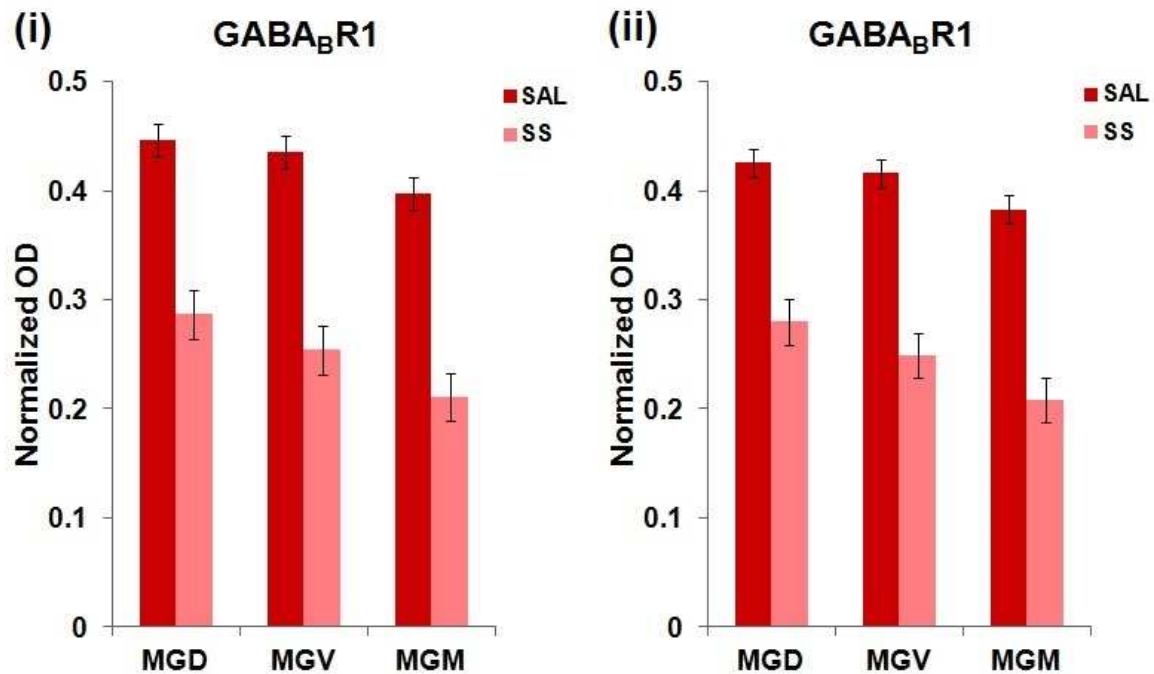
#### **4.4.3.2 The GABA<sub>B</sub>R2 subunit in the MG**

For saline-treated animals, there were no differences in the overall level of labeling amongst the three subdivisions of the MG (Figure 16a). Neuropil and cell body labeling was similar across all three subdivisions. Qualitative examination of tissue revealed that there were no differences in labeling across the rostral-caudal extent of the structure.

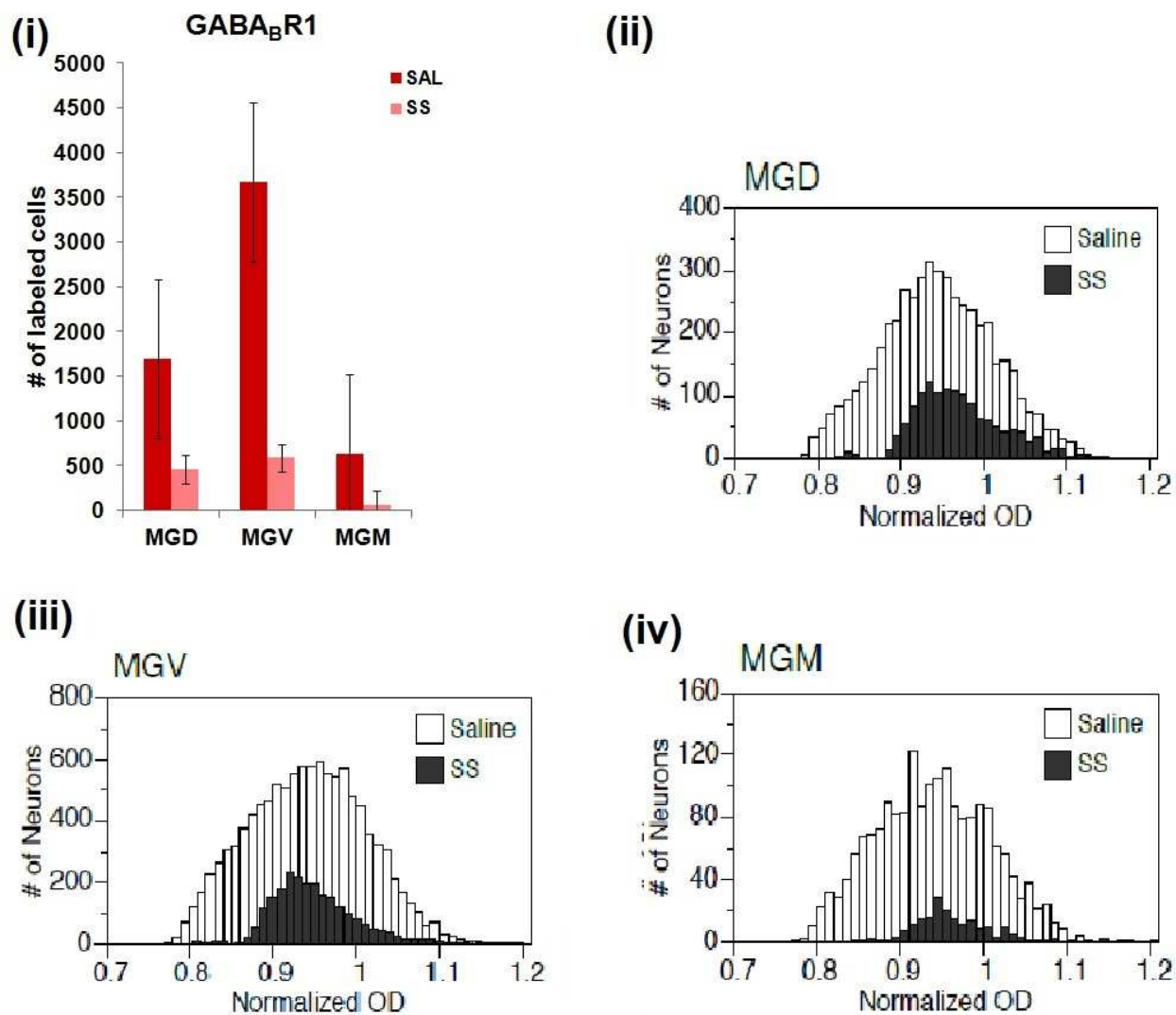


**Figure 15A:** Immunoreactivity to the GABA<sub>B</sub>R1 subunit in the MG. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body in combination) immunolabeling in the MG. High magnification images show neuropil and cell body labeling in the MGd, MGv, and MGM. White arrows point towards a labeled cell body. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.





**Figure 15B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R1 subunit in the subdivisions of the MG. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.

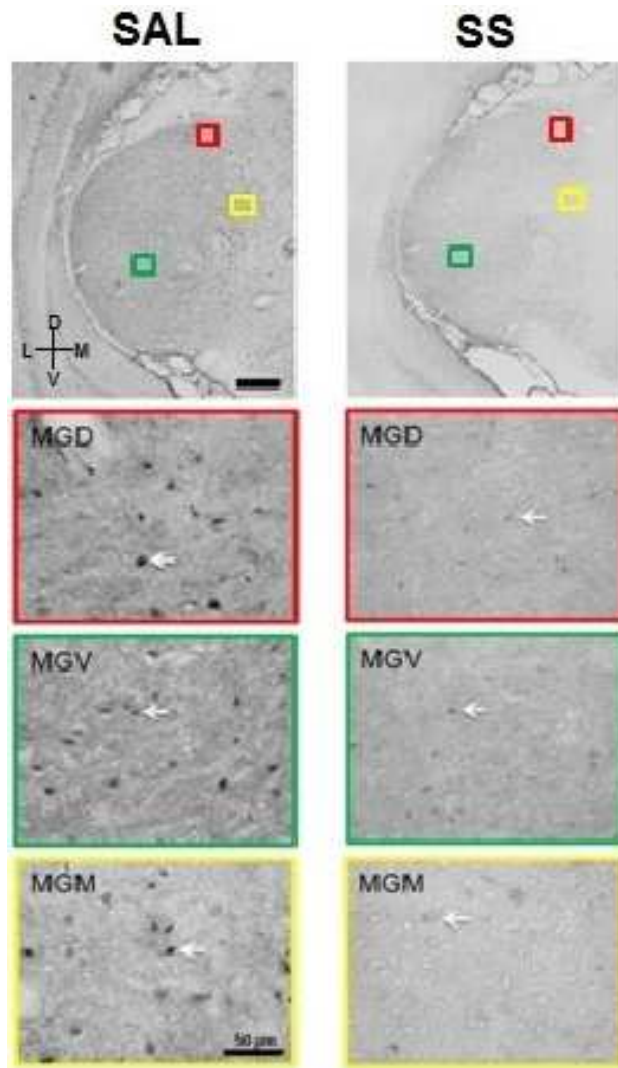


**Figure 15C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R1 immunoreactive cell bodies in each subdivision of the MG. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R1 immunoreactive cell bodies in the subdivisions of MG. Results are from analyses conducted using three animals for saline and three animals for SS.

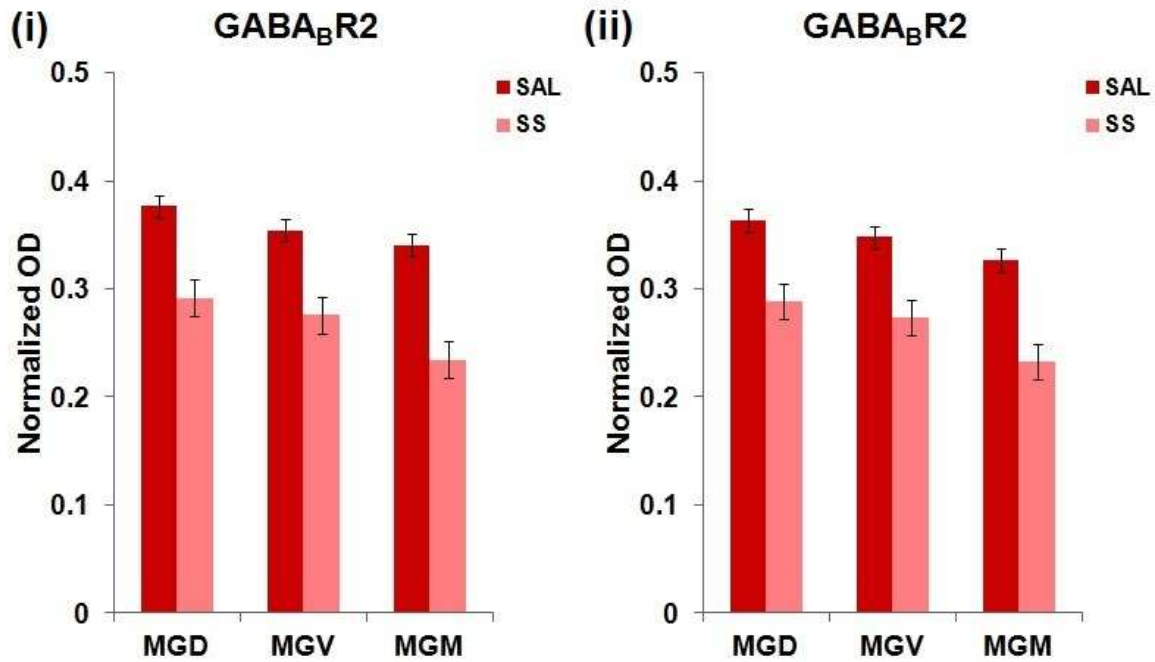
After treatment with SS, the MGd and MGv showed similar levels of reduction. The MGm was less darkly labeled compared to MGd and MGv in SS-treated animals. Low magnification images showed a decrease in the overall immunoreactivity against the GABA<sub>B</sub>R2 subunit after SS treatment (Figure 16a). The MG of SS treated animals was lighter in labeling compared to the MG of saline-treated animals. The reduction in the overall level of labeling was highest in MGm (31%), followed by MGd (22%) and MGv (22%) (Figure 16b). This reduction was not statistically significant ( $p > 0.05$ ).

There were not big differences in the level of labeling in the neuropil among different MG subdivisions for saline treated animals. While the neuropil in the MGd and MGv was equally dark, that in the MGm was slightly lighter. There was a lot of punctate labeling in the neuropil across the MGd and MGv, and moderate level of punctate labeling in the MGm. High magnification images showed that after SS treatment, the reduction in immunoreactivity against the GABA<sub>B</sub>R2 existed in both the neuropil and cell bodies (Figure 16a). The neuropil of all the three MG subdivisions was lighter with reduced diffused and punctate labeling in animals treated with SS. The reduction in immunoreactivity in the neuropil was 29% in the MGm, 21% in the MGd, and 21% in the MGv (Figure 16b). This reduction was not statistically significant ( $p > 0.05$ ).

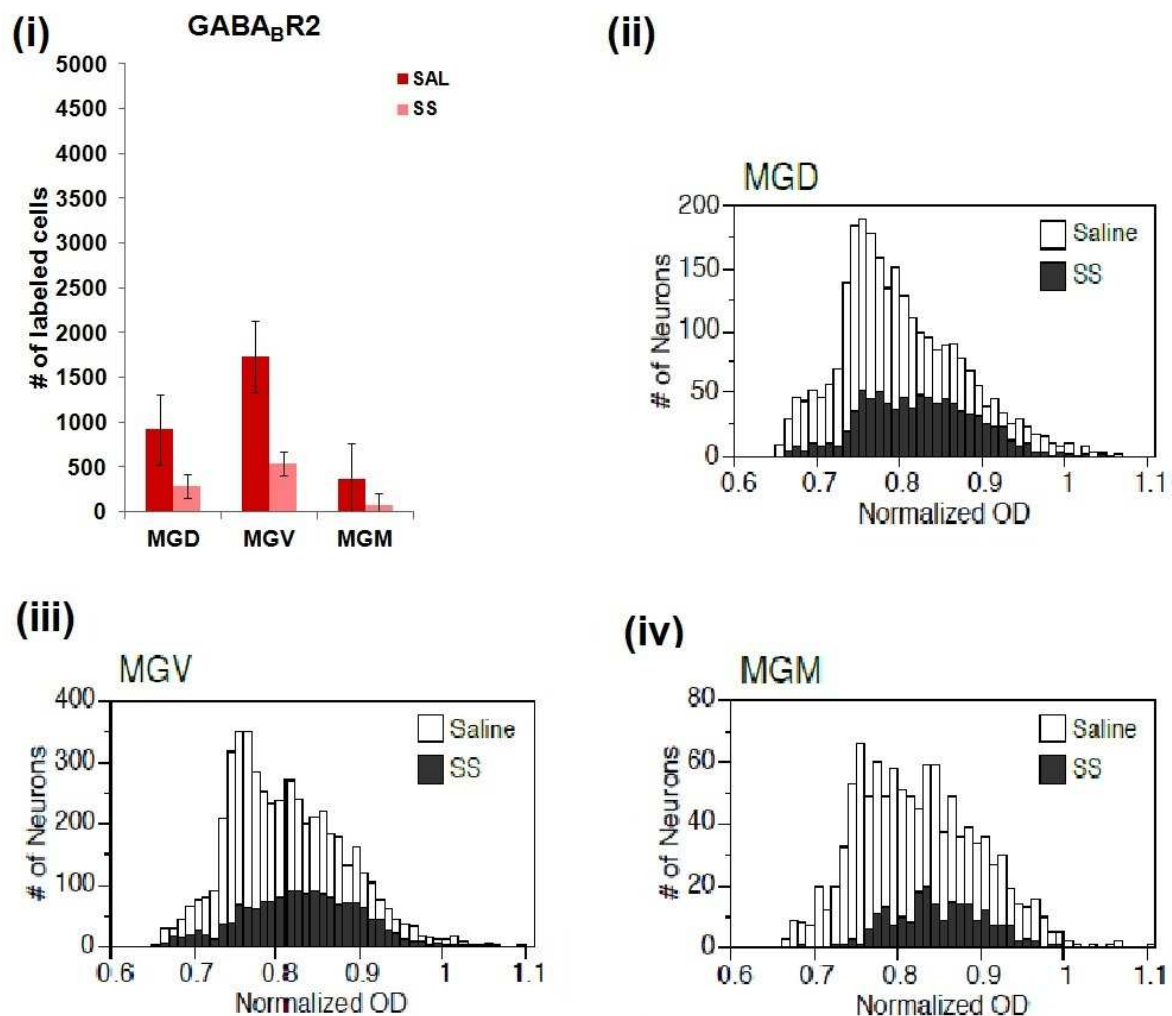
The level of labeling in cell bodies was different between animals treated with saline and SS (Figure 16a). For the MG of saline treated animals, there were no differences in cell packing density amongst the three subdivisions. Cell bodies on all the subdivisions appeared equally dark. In contrast, there were very few labeled cell bodies after SS treatment. Quantitative results showed that MGm had the highest decrease in the number of labeled cell bodies (Figure 16c). It showed a 79% decrease. MGd showed a 66% decrease and MGv showed a 65% decrease. This



**Figure 16A:** Immunoreactivity to the GABA<sub>B</sub>R2 subunit in the MG. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body in combination) immunolabeling in the MG. High magnification images show neuropil and cell body labeling in the MGd, MGv, and MGm. White arrows point towards a labeled cell body. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.



**Figure 16B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R2 subunit in the subdivisions of MG. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.



**Figure 16C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R2 immunoreactive cell bodies in each subdivision of the MG. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R2 immunoreactive cell bodies in the subdivisions of MG. Results are from analyses conducted using three animals for saline and three animals for SS.

decrease was statistically significant ( $p < 0.0001$ ). The distributions of labeling in cell bodies for saline-treated animals and SS-treated animals was the same, indicating equally dark cell body labeling.

#### **4.4.4 The effect of SS on the AC**

##### **4.4.4.1 The GABA<sub>B</sub>R1 subunit in the AC**

There was a small difference in immunoreactivity against GABA<sub>B</sub>R1 subunit between the AC of saline and SS treated animals (Figure 17a). For saline treated animals, there were no differences in labeling between the three subdivisions of AC i.e. AU1, AUD, and AUV. All three subdivisions showed a high level of diffused and punctate labeling. The cell bodies were also equally dark amongst the three subdivisions. Qualitative examination revealed that there were differences in labeling across the six layers of the AC. The most superficial layers i.e. Layers I-III showed darker diffused and punctate immunolabeling compared to the deep layers i.e. layers IV-VI. There were more labeled puncta in layers II and III compared to layer I. Layers IV and V displayed intermediate diffused labeling with fewer labeled puncta whereas layer VI has the lowest level of diffused labeling with very few puncta.

Qualitative examination of the layers showed that cell body labeling and packing density varied between the six layers. Layer I had few darkly labeled cell bodies interspersed between the neuropil. Layers II and III had a large number of darkly labeled pyramidal and non-pyramidal neurons. These neurons were tightly packed together, indicating high cell packing density. Layers IV and V had large pyramidal neurons which were darkly labeled. Labeled fibers running from the medial to the lateral extent of the AC were also seen in these layers. Layer VI had a large number of small pyramidal neurons packed close together, indicating high cell packing

density. However, the cell bodies were lighter in labeling compared to the cell bodies in the other five layers. There were no differences in neuropil or cell body labeling in the AC subdivisions and amongst the layers across the rostral-caudal extent of the structure.

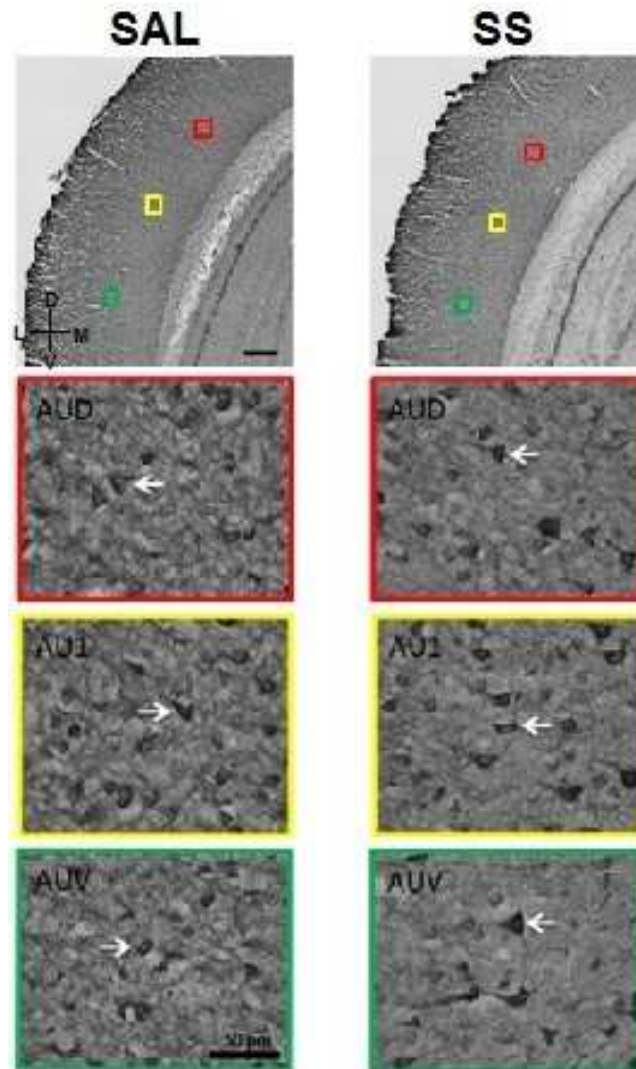
Low magnification images showed that after treatment with SS, there was a trend of decrease in the overall immunoreactivity across all the subdivisions of AC and across all the layers. The reduction in the overall level of labeling was highest in AUV (15%), followed by AUD (13%) and AU1 (11%) (Figure 17b). This reduction was not statistically significant ( $p > 0.05$ ).

There was no difference in neuropil labeling amongst the three AC subdivisions in saline treated animal (Figure 17a). All three subdivisions displayed a high level of diffused and punctate labeling. High magnification images showed that after treatment with SS, a small reduction in GABA<sub>B</sub>R1 immunoreactivity existed in the neuropil and cell bodies across the three subdivisions. The neuropil of SS treated animals showed a decrease in diffused and punctate labeling across all three subdivisions. The reduction in immunoreactivity in the neuropil was 14% in the AUV, 13% in the AUD, and 11% in the AU1 (Figure 17b). This reduction was not statistically significant ( $p > 0.05$ ).

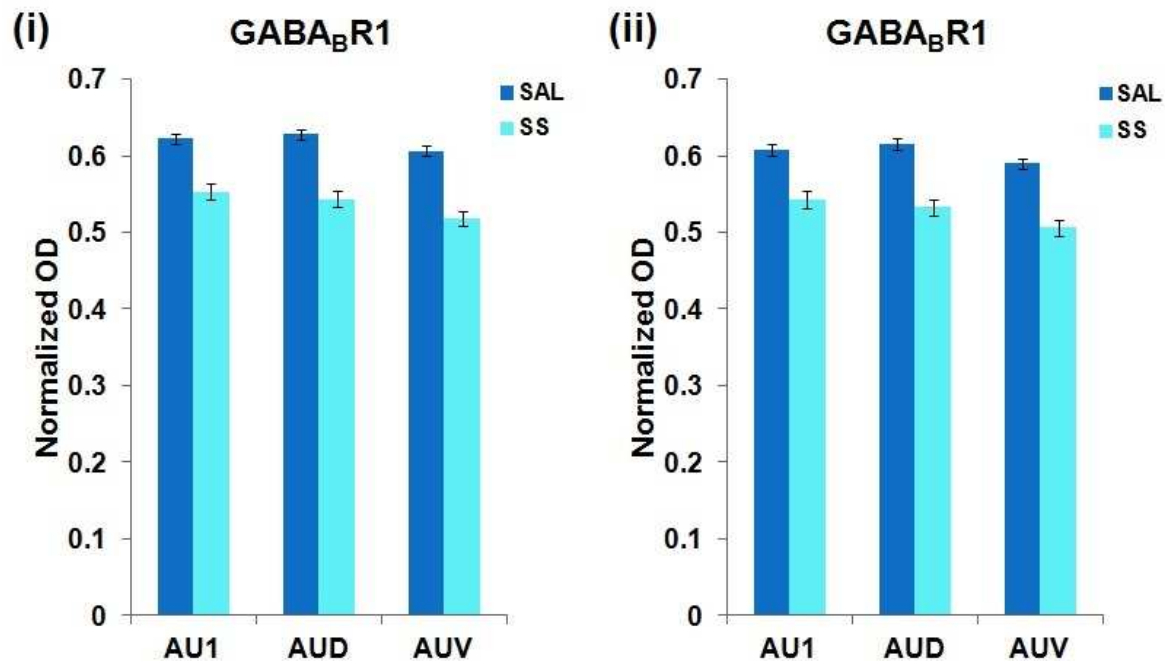
Diffused and punctate labeling was decreased across all the layers of the AC, with the largest decrease observed in layer VI. Layer IV and IV showed intermediate decrease in neuropil labeling and layers I-III showed the smallest decrease. This decrease in labeling was observed qualitatively.

There was a difference in cell body labeling between animals treated with saline and animals treated with SS (Figure 17a). The AC of saline treated animals showed no difference in

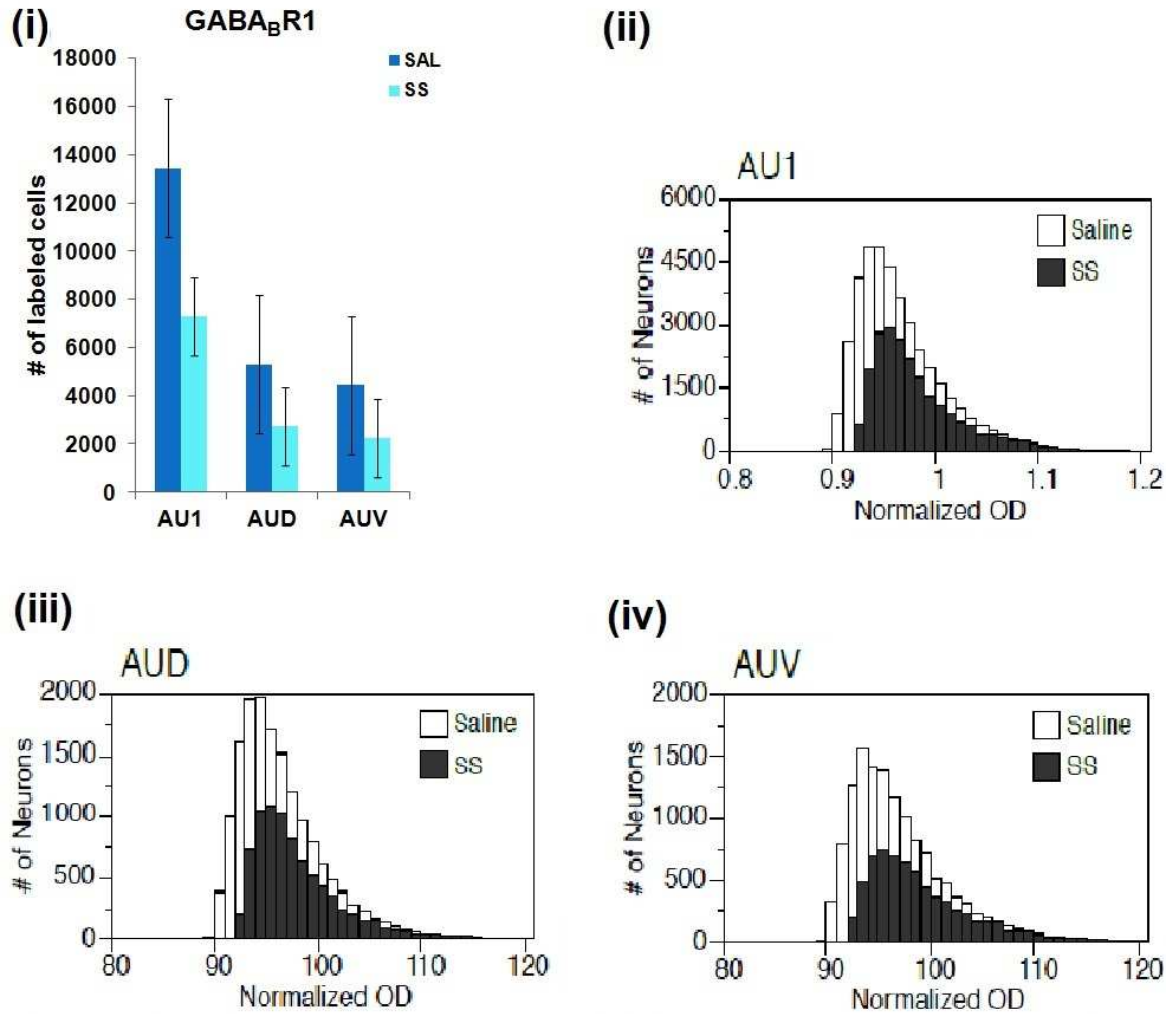




**Figure 17A:** Immunoreactivity to the GABA<sub>B</sub>R1 subunit in the AC. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body in combination) immunolabeling in the AC. High magnification images show neuropil and cell body labeling in the AU1, AUD, and AUV. White arrows point towards a labeled cell body. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.



**Figure 17B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body combined) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R1 subunit in the subdivisions of the AC. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.



**Figure 17C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R1 immunoreactive cell bodies in each subdivision of the AC. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R1 immunoreactive cell bodies in the subdivisions of AC. Results are from analyses conducted using three animals for saline and three animals for SS.

cell packing density or cell body labeling amongst the three subdivisions. After SS treatment, the number of labeled cell bodies decreased (Figure 17c). The decrease was similar across the three subdivisions with 54% decrease in the AU1, 51% decrease in the AUD, and a 50% decrease in the AUV. This decrease was statistically significant ( $p < 0.0001$ ). The distribution of the OD of cell bodies revealed no significant difference in the labeling between the saline-treated animals and SS-treated animals.

Qualitative observation of cell body labeling and packing density in the layers of AC revealed a decrease in the number of labeled cell bodies and in cell body labeling in all the layers. The largest decrease was seen in layer VI, followed by layer IV, layers I-III, and layer V.

#### **4.4.4.2 The GABA<sub>B</sub>R2 subunit in the AC**

Similar to the GABA<sub>B</sub>R1 subunit, the GABA<sub>B</sub>R2 subunit immunoreactivity was decreased in the AC after SS treatment (Figure 18a). There were no differences in labeling among the three subdivisions of AC in the saline treated animal. The level of diffused and punctate labeling was high in all the subdivisions. There were immunolabeling differences among the six layers, with layers I-III showing the highest level of diffused and punctate labeling, followed by layers IV and V which showed intermediate level. Layer VI had the lowest level of diffused and punctate immunolabeling amongst the six layers. Cell body labeling and packing density for GABA<sub>B</sub>R2 subunit immunoreactive cell bodies was similar to GABA<sub>B</sub>R1 subunit immunoreactive cell bodies. Layers I-III had darkly labeled, densely packed, small pyramidal and non-pyramidal neurons. Layers IV and V had large pyramidal neurons which were darkly labeled. The cell packing density in layers IV and V was low compared to that in

layers I-III. Layer VI had small neurons which were densely packed, but they were lighter in labeling compared to neurons in the other layers.

The labeling was decreased across all three subdivisions after SS treatment. The reduction in immunoreactivity was highest in the AUV (14%, followed by AUD (13%) and AU1 (12%) (Figure 18b). This reduction was not statistically significant ( $p > 0.05$ ).

Qualitative observations revealed that there were differences in immunoreactivity reduction among the six layers after treatment with SS. Layer VI showed the largest decrease in labeling, followed by layers IV and V, and layers I-III.

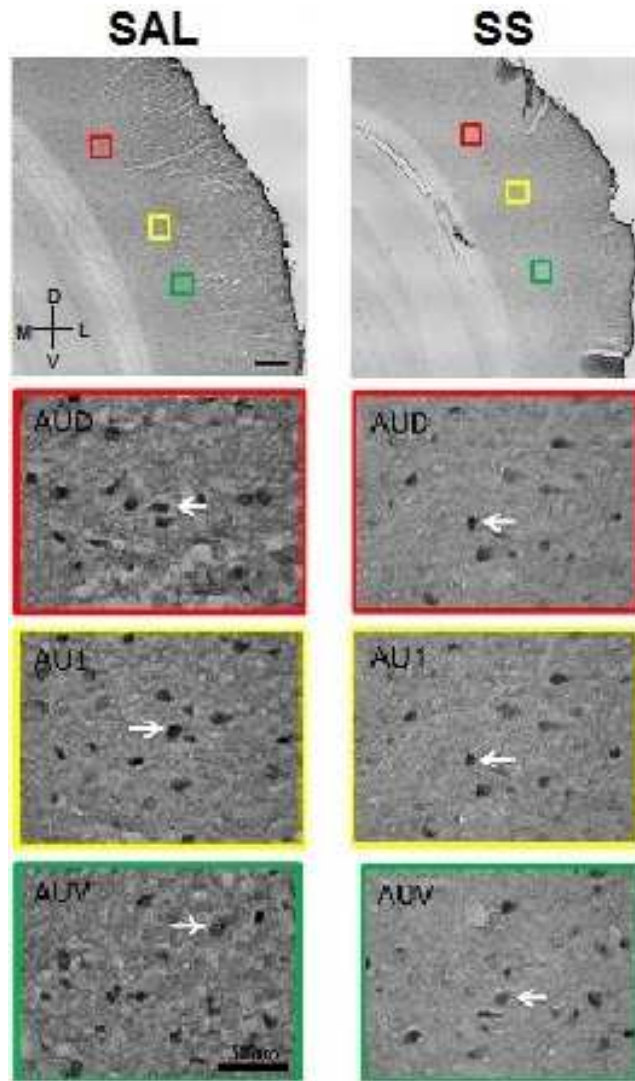
The neuropil of AC in saline treated animal was similarly labeled (Figure 18a). Each subdivision showed a high level of diffused and punctate labeling. The level of diffused and punctate labeling was decreased after SS treatment. This decrease was similar across the three subdivisions with AUV and AUD at 13% each. AU1 showed a 12% reduction in neuropil labeling (Figure 18b). This reduction was not statistically significant ( $p > 0.05$ ).

Qualitative observations revealed that there were differences in reduction in the neuropil among the six layers after treatment with SS. Layer VI showed the largest decrease in labeling, for both diffused and punctate labeling, followed by layers IV and V, and layers I-III.

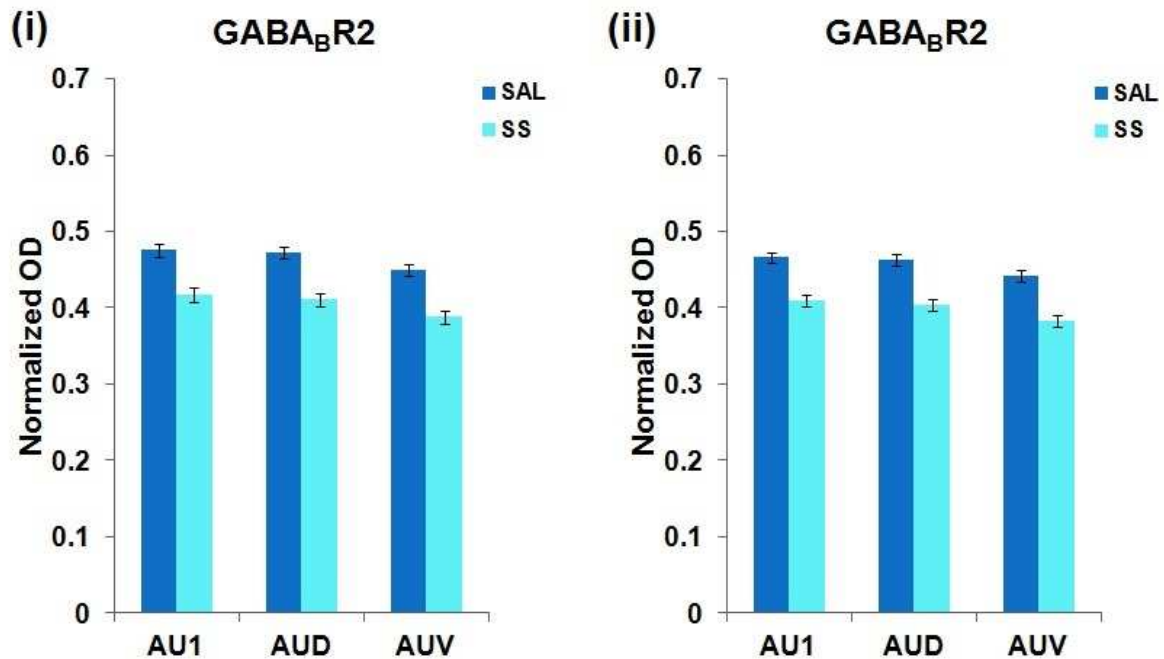
Cell body labeling in the AC was different between saline treated animal's AC and SS treated animal's AC (Figure 18a). The AC of saline treated animal showed no differences in cell packing density or cell body labeling among the three subdivisions. The cell bodies were densely packed and equally dark. After SS treatment, the number of labeled cell bodies decreased across all three subdivisions (Figure 18c). The reduction in labeled cell bodies was highest in the AU1

(68%). AUD showed a 66% reduction in the number of labeled cell bodies whereas AUV showed a 61% reduction. This reduction was statistically significant ( $p < 0.0001$ ).

There were differences in cell body labeling among the layers after SS treatment. Layer VI showed the largest decrease in the number of labeled cell bodies, as well as labeling in the cell bodies, followed by layers I-III, layer IV, and layer V.

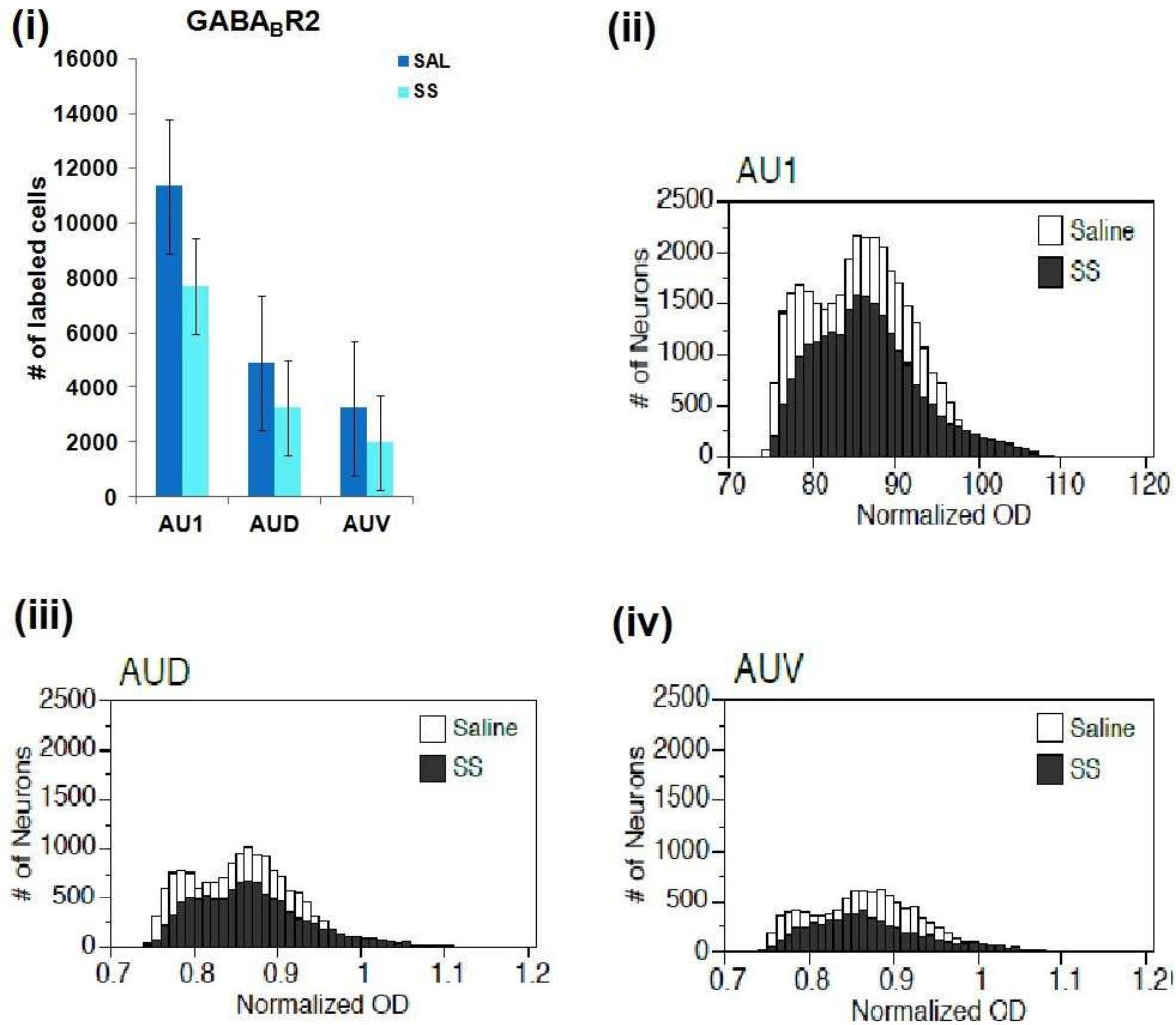


**Figure 18A:** Immunoreactivity to the GABA<sub>B</sub>R2 subunit in the AC. Left panel contains coronal sections from saline-treated animal. Right panel contains coronal sections from SS-treated animal. Low magnification images show overall (neuropil and cell body combined) immunolabeling in the AC. High magnification images show neuropil and cell body labeling in the AU1, AUD, and AUV. White arrows point towards a labeled cell body. Scale bar: 200µm in low magnification images; 50 µm in high magnification image.

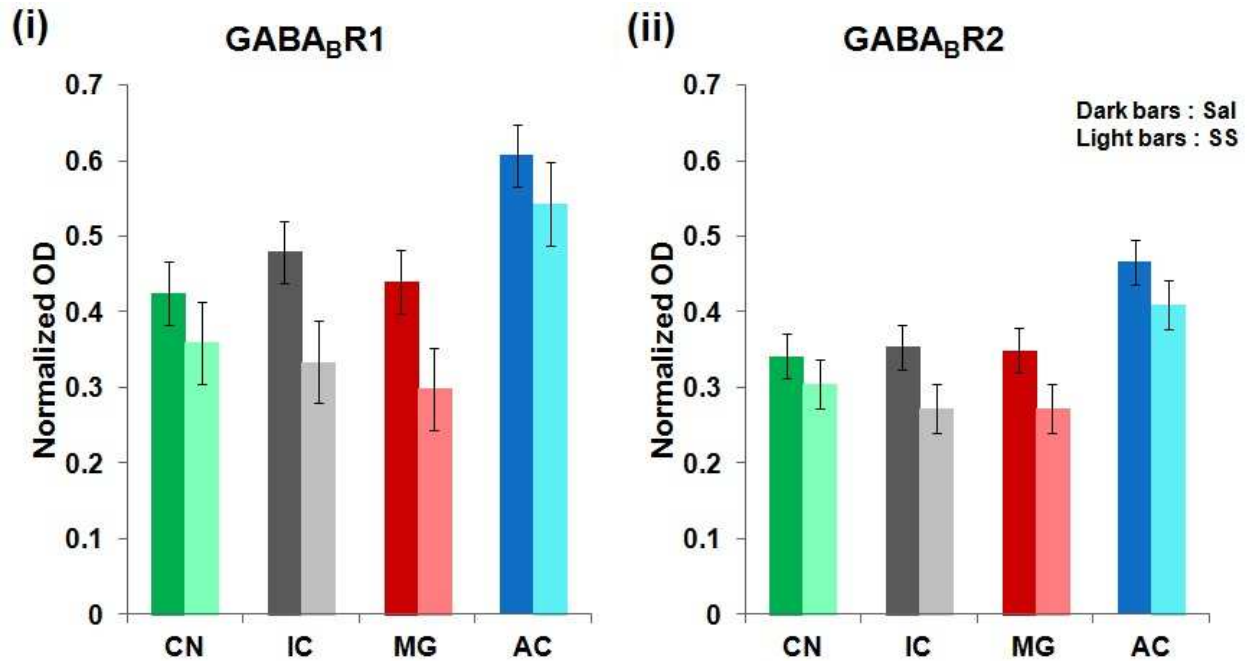


**Figure 18B:** Group results from immunohistochemical experiments showing (i) overall (neuropil and cell body in combination) and (ii) neuropil immunoreactivity against the GABA<sub>B</sub>R2 subunit in the subdivisions of the AC. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by subtracting the combined integrated grey of all the cell bodies in a subdivision from the overall integrated grey of the subdivision. Error bars indicate standard error.

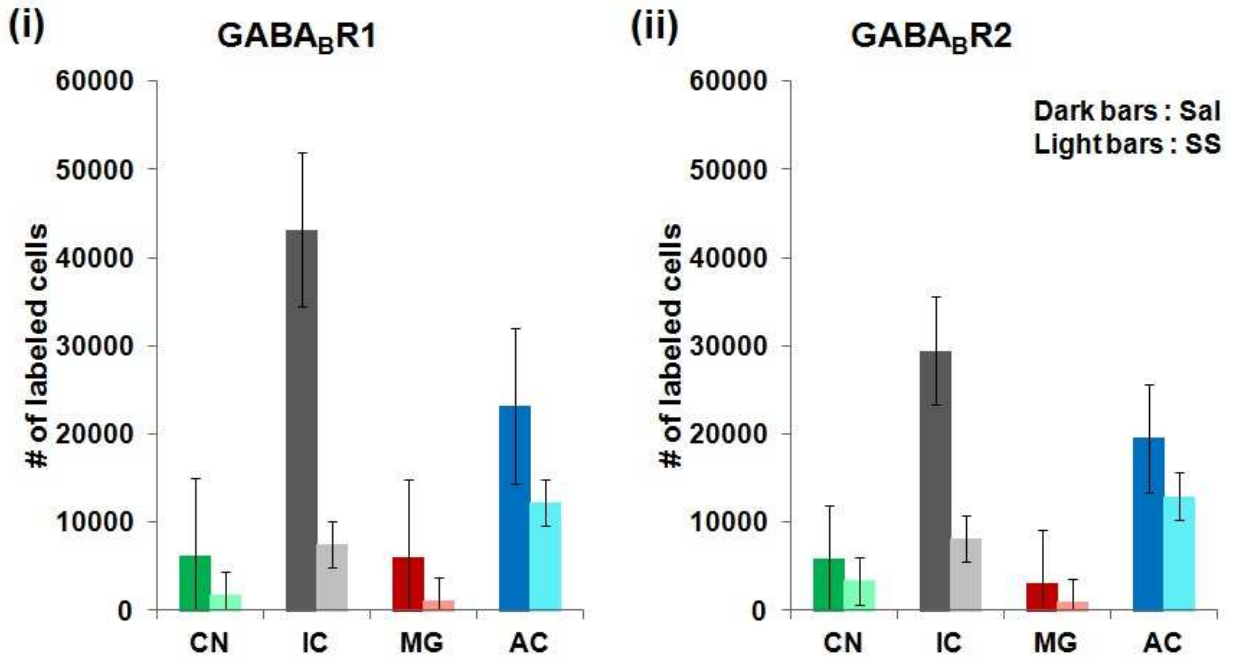




**Figure 18C:** (i) Bar chart showing the total number of GABA<sub>B</sub>R2 immunoreactive cell bodies in each subdivision of the AC. (ii-iv) Histograms showing group results for normalized OD distribution of GABA<sub>B</sub>R2 immunoreactive cell bodies in the subdivisions of AC. Results are from analyses conducted using three animals for saline and three animals for SS.



**Figure 19:** Bar charts showing neuropil immunoreactivity against the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits in the major auditory structures. Analyses were conducted using three animals for saline and three animals for SS. The OD of neuropil was obtained by combining the weighted averages of neuropil OD from each subdivision in a structure.



**Figure 20:** Bar charts showing the total number of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 immunoreactive cell bodies in the major auditory structures. Analyses were conducted using three animals for saline and three animals for SS. The number of immunoreactive cell bodies in the subdivisions was combined to obtain the total cell body number for each structure.

## **5 Discussion**

Results from present research indicated that intraperitoneal injection of sodium salicylate reduced the protein levels of the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor subunits in the four major auditory structures (i.e., the CN, IC, MG, and AC) 3 hours after the drug application. The change, presented as a percent difference between SS and saline, was the greatest in the IC and MG, intermediate in the CN, and minor in the AC. In the IC, MG, and CN, area differences exist in the degree of reduction among different subdivisions. The reduction in the level of receptor subunits was observed in both cell body (as revealed by the number of labelled cell bodies and the optic density reflecting the strength of immunoreactivity in a cell body) and the neuropil areas. Between the two receptor subunits, degrees of reduction were generally parallel across different auditory structures.

### **5.1 The relationship between a reduction in the level of the GABA<sub>B</sub> receptor and SS-induced tinnitus**

SS-induced tinnitus is accompanied by changes in the neural activity in the central auditory system (Berliner et al., 1992; Barrs and Brackman, 1984; House and Brackman, 1981; Kaltenbach et al., 2005). Previous neurophysiological/neuropharmacological experiments have indicated that SS can directly affect neurotransmitter receptors and ion channels (Basta et al. 2000, 2008; Basta and Ernst, 2004). There was evidence showing that the drug could change the level of certain neurotransmitter receptors in the CAS (Bauer et al., 2000). The current study provides a direct evidence showing that SS can change the level of the GABA<sub>B</sub> receptor in the CAS. Along with the effect of the drug on other neurotransmitter receptors, the drug effect on the

GABA<sub>B</sub> receptor can affect the balance between excitation and inhibition. This may consequently affect the level of neural activity in the CAS.

In contrast to a reduction in the output from the cochlea, there is an enhancement in neural activity in the AC after SS application (Sun et al., 2009). This difference suggest that the “gain” was enhanced in signal transmission along the ascending auditory pathway. It was unknown which auditory structure contributed to this increase in the gain. Results from the present study show that the level of the GABA<sub>B</sub> receptor was reduced in all the four major auditory structures. This reduction can very likely cause a reduction in inhibition in every of the four structures, suggesting that there is no single origin of SS-induced tinnitus. The activity in each auditory structure can be compromised due to decreased GABA<sub>B</sub> receptor subunit levels. This suggests that all the four structures may contribute to the increase in the gain, leading to hyperactivity in neurons in the AC.

All the major auditory structures are connected to each other, forming a neural circuit. An reduction in the level of the GABA<sub>B</sub> receptor and subsequent enhancement in neural activity in each of the structures can lead to a change in the way how a neural circuit works. It's very likely that such an increase in the level of activity in all the components of a network can lead to synchronized activation/resonance in the network and an epileptic form of physiological responses. These types of physiological responses may be responsible for the sensation of hyperacusis and tinnitus.

## **5.2 Change in GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor levels in the CN**

Results from the present study showed that intraperitoneal injection of a single dose of SS at the dosage of 250 mg/kg caused a decrease in the level of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor

subunits in the CN 3 hours after the drug application. This change was seen in all the subdivisions of the CN. CN is considered the trigger centre of tinnitus with many studies reporting hyperactivity in the DCN after SS treatment (Brozoski et al., 2002; Kaltenbach and Godfrey, 2008; Shore et al., 2007). Not many studies have been conducted on the VCN. Holt et al., (2010) used manganese enhanced-MRI to image neural activity after chronic SS treatment and found an increase in activity in the DCN but no change in the VCN. Wallhauser-Franke et al., (2003) also reported increase in DCN activity after chronic SS treatment but no change in the VCN. My results show a larger reduction in the GABA<sub>B</sub> subunit levels in the VCN subdivisions than in the DCN, indicating a greater decrease in inhibition in the VCN. This should translate to hyperactivity in the VCN. This difference in results might be due to a difference in experimental procedures. Holt and colleagues, and Wallhauser-Franke and colleagues used chronic SS-treatment whereas I used acute SS treatment. It is possible that in the case of chronic treatment, the nervous system was able to homeostatically regulate inhibition in the VCN but not in the DCN, resulting in persistent hyperactivity in the DCN which was measured.

VCN has low levels of GABA<sub>B</sub> receptor (Juiz et al., 1994). However, studies using neurochemicals have shown the entire VCN to contain numerous GAD and GABA immunoreactive endings and cell bodies (Godfrey et al., 1977, 1978, 1988). The majority of these GABA immunoreactive endings and cell bodies most likely contain GABA<sub>A</sub> receptors since GABA<sub>A</sub> receptors outnumber GABA<sub>B</sub> receptors in the CN. The levels of GABA<sub>A</sub> receptors in the VCN are amongst the highest in the brain (Chu et al., 1990). GABA<sub>B</sub> receptors regulate the release of GABA which could activate GABA<sub>A</sub> receptors, so a decrease in GABA<sub>B</sub> receptors could lead to a dysregulation of inhibitory activity in the VCN. GABA<sub>B</sub> receptors also regulate the release of glutamate, the excitatory neurotransmitter and regulate excitatory activity. It is

possible that in the VCN, reduction in GABA<sub>B</sub> receptor levels affects the excitatory and inhibitory neurotransmission in a similar way so that the net effect is either an insignificant change or no change in neural activity.

Studies using neurochemicals have shown that the superficial layers of DCN contains numerous GAD and GABA immunoreactive endings and cell bodies (Godfrey et al., 1977, 1978, 1988). Both GABA<sub>A</sub> and GABA<sub>B</sub> receptors are present in the DCN. GABA<sub>A</sub> receptors are present at high levels in all the layers of DCN whereas the molecular layer of DCN has the highest level of GABA<sub>B</sub> receptors in the entire CN (Juiz et al., 1994). The GABA<sub>B</sub> receptors in DCN might be more involved in regulation of inhibition than excitation. Therefore, the decrease in GABA<sub>B</sub> receptor levels in the DCN after SS treatment might cause a greater decrease in inhibition, leading to hyperactivity in the structure.

DCN receives projections from ANFs and projects to the IC and MG (Schofield and Coomes. 2005). It is an important structure in auditory processing, and has been shown to play a role in suppressing sounds generated by movement or self-vocalization (Kanold et al., 2011; Keohler et al., 2011). A disruption of neural activity in the DCN can not only interfere with its roles in sound suppression but these disruptions can be transmitted to IC and MG, leading to improper auditory processing, and tinnitus.

### **5.3 Changes in GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor levels in the IC**

Of the four central auditory structures analyzed, IC showed the greatest change in receptor subunit levels after SS treatment. This is in agreement with neuroimaging results which reported greater levels of hyperactivity in the IC compared to other auditory structures after chronic SS treatment (Holt et al., 2010). The increase in activity was attributed to a decrease in

inhibition. Many studies using systemic SS application have found a decrease in GABAergic inhibition after SS application (Bauer et al., 2000; Zou and Shang, 2012). My results show that the decrease in GABAergic inhibition in IC might partially be due to a decrease in GABA<sub>B</sub> receptor levels.

Among the three subdivisions of the IC, ICc had the highest reduction in GABA<sub>B</sub> subunit levels followed by ICx and then ICd. Chronic SS application studies have reported the greatest increase in activity in the ICd followed by ICc and then ICx (Holt et al., 2010, Wallhauser-Franke et al., 2003). The difference between their results and mine might be due to several reasons. Firstly, Holt and colleagues as well as Wallhauser-Franke and colleagues used chronic SS treatment, whereas I used acute SS treatment. Secondly, the difference in receptor subunit levels reported in this study is a percentage difference. It is measured relative to the labeling in each subdivision and does not reflect absolute change in receptor levels. The three subdivisions of IC have differential expression of GABA<sub>B</sub> receptors, with the ICd showing the highest level of receptor subunit levels and the ICc showing the lowest level. A small absolute change in ICc can translate to a large percent reduction whereas a similar or even greater absolute change in ICd might not reach the same percent reduction. This can be seen in Table X where absolute change and percentage change is listed for each subdivision. Therefore, a moderate change in ICd and ICx can have more serious consequences on auditory processing than the relatively large change in the ICc.

The hyperactivity reported by previously mentioned studies might be partially because of the decrease in GABA<sub>B</sub> receptor levels seen in this study. However, the reported hyperactivity can not entirely be attributed to a decrease in GABA<sub>B</sub> receptor levels. It must be noted that ICd and ICx have an abundance of excitatory NMDA receptors compared to ICc (Ohishi et al.,



1993). Therefore, it is possible that a slight decrease in inhibition i.e. a decrease in GABA<sub>B</sub> receptor level, can tip the balance between excitation and inhibition in these subdivisions via decreased regulation of the excitatory NMDA receptors. This decreased regulation leads to the hyperactivity measured by the physiological and neuroimaging studies.

A higher reduction in ICc compared to ICd can affect auditory processing. ICc projects mainly to the MGv which, like ICc, is part of the lemniscal pathway. MG is the last centre for auditory processing before inputs are sent to the AC. A decrease in inhibition in the ICc could mean that more excitatory inputs are sent to the MG which passes them on to the AC, thus leading to improper auditory processing and tinnitus.

The role of ICd and ICx in auditory processing is not known. They have been shown to contain neurons that habituate rapidly and respond to novel sounds. Habituation is a process by which a neuron decreases its response to a stimulus over time, and is thought to involve GABAergic inhibition. Failure to habituate can lead to audiogenic seizures (Chakravarty and Faingold, 1996). A decrease in GABA<sub>B</sub> receptor subunit levels in these subdivisions can cause the transmittance of noise that the neurons usually habituated to, thus producing hyperacusis. A decrease in GABA<sub>B</sub>-mediated inhibition can also lead to audiogenic seizures where neurons fire and transmit information to the higher structures even though no stimulus is present. This can lead to perception of phantom sounds i.e. tinnitus. Therefore, a decrease in GABA<sub>B</sub> receptor subunits and GABA<sub>B</sub> regulation in ICd and ICx can have more severe consequences on auditory functioning. These two subdivisions might be more involved in the sensation of tinnitus than ICc.

#### **5.4 Changes in GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor levels in the MG**

After IC, MG showed the greatest reduction in GABA<sub>B</sub> subunit levels. Not many studies have been conducted on salicylate's effect on MG. The conducted studies show conflicting results. Holt and colleagues (2010) found no change in activity in MG after chronic application of SS. Wallhauser-Franke and colleagues (1997, 2003) found a slight increase in c-fos expression, a marker for activity in neurons, in MGd but not in MGv after chronic SS application. My results show a large decrease in GABA<sub>B</sub> receptor subunit levels in all the subdivisions of the MG. The difference in findings might be due to the difference in experimental procedures.

MGm showed the largest reduction, followed by MGv and MGd. MGv and MGd have a higher level of GABA<sub>B</sub> receptors than MGm. Therefore, the percent reduction shown here might be different than the absolute reduction as the different subdivisions have differential levels of GABA<sub>B</sub> receptors. The three subdivisions also have different neural connections in the auditory system. MGv and MGd project to the auditory cortex whereas MGm projects to non-auditory areas. Thus a change in MGv and MGd might have more serious consequences on the function of MG as a thalamic auditory relay structure than MGm.

MGv is considered a specific auditory relay centre (Gonzalez-Hernandez et al, 1991). It receives ascending projections from ICc and descending projections from the AC (Malmierca and Merchan, 2005). It is a major source of input to the AC (Winer and Schreiner, 2005). Thus MGv is very important in auditory processing. A decrease in GABA<sub>B</sub> receptors in MGv can affect the processing of ascending neural connections from the ICc. As it is the last relay structure before inputs reach the AC, dysregulated neural inputs can be transmitted directly to the

AC which processes all the incoming information. This leads to faulty neural processing and can lead to hyperactivity in the AC, resulting in tinnitus and hyperacusis. Further research is needed to be conducted on the MG to study the effects of SS on neural processing in the MGv, and how this impacts neural processing in the primary auditory cortex that MGv projects to.

MGd receives ascending projections from ICd and ICx. It also sends descending projections to the ICx, forming a feedback loop. MGd sends ascending projections to secondary auditory cortices. A decrease in GABA<sub>B</sub> receptors in MGd not only leads to improper neural processing of incoming information to the MG, but also in the ICx through faulty management of the feedback loop. This can greatly impact the auditory information sent to the secondary auditory cortices that MGd projects to, and can lead to tinnitus and hyperacusis. Further studies are needed to be conducted to study the role of MG in induction of tinnitus as this structure is greatly affected by SS application in terms of GABA<sub>B</sub> receptor subunit levels.

### **5.5 Changes in GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 receptor levels in the AC**

Previous physiological studies have reported an increase in activity of AC neurons after systemic SS injection (Sun et al., 2009). This hyperactivity has been attributed to a decrease in GABAergic inhibition (Sun et al., 2009; Lu et al., 2011). My IHC results indicate a reduction in GABA<sub>B</sub> subunit immunoreactivity in the AC, and are in agreement with previous physiological findings. It is believed that all cell types in the rat's primary auditory cortex are GABAergic (Otterson and Strom-Mathisen, 1984; Winer and Larue, 1989; Winer, 1992). Thus GABAergic inhibition plays a major role in neural processing in the AC, and even a small decrease in GABA<sub>B</sub> receptor levels might lead to significant increases in neural activity. Since AC is

involved in auditory perception, the increase in neural activity can manifest as an auditory disorder e.g. tinnitus.

There were no significant differences in GABA<sub>B</sub> receptor subunit levels amongst the three subdivisions of the AC. However, the different layers of the AC showed differential affect of the drug. The deep layers i.e. layers IV-VI of the AC showed a greater qualitative reduction in GABA<sub>B</sub> receptor levels than the superficial layers. The deep layers of AC receive ascending inputs from IC and MG. These layers also send descending projections to those structures, forming feedback loops to regulate neural processing (Winer and Larue, 1987; Roger and Arnault, 1989; Arnault and Roger, 1990). A decrease in the levels of GABA<sub>B</sub> receptor not only causes faulty processing of incoming information, but also of descending inputs. The improper regulation through the descending inputs can cause inaccurate neural processing in the lower structures which in turn send inaccurate auditory information to the AC, thus forming a positive feedback loop. This can lead to information which is usually filtered being sent to the AC. These layers receive and process the gain of the auditory system due to changes in lower auditory, and in turn, display hyperactivity which leads to the sensation of tinnitus. Therefore, the deep layers of AC might play a greater role in tinnitus sensation than the superficial layers.

## **5.6 Cellular distributions of reduction in the level of the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits:**

In all the auditory structures analyzed, the reduction in the level of the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits was greater in cell bodies than in the neuropil,. The number of labeled cell bodies and the OD of labeled cell bodies decreased after SS treatment. Cell bodies contain the machinery to make proteins. Both the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunit are made in the ER

(Pin et al., 2004; Restituto et al., 2005). The subunits can join together to traffic to the cell membrane to form a functional receptor. In photomicrographic images, the cell membrane can be visualized in either cell body areas or the neuropil, which contains the axons and dendrites. A reduction in cell body receptor subunit levels could reflect a reduction in the level of functional GABA<sub>B</sub> receptors or a decrease in the levels of individual subunits available to make the functional receptor. Recent studies have shown that SS can modify mRNA levels of various proteins in the auditory structure 2-3 hours after systemic application of the drug (Panford-Walsh et al., 2008; Zou and Shang, 2012; Hu et al., 2014). The time frame in which these changes happen coincides with that in which levels of GABA<sub>B</sub> receptor subunits were reduced.

The levels of GABA<sub>B</sub> receptor subunits in the neuropil were reduced in all the auditory structures after application of SS. The subunits in the neuropil are most likely associated with functional receptors as neuropil contains the axons and dendrites of neuron cell bodies. The decrease in neuropil labeling is probably due to a reduction in receptor subunits available for making functional receptors and not due to the effect of SS on receptor stability. To date, no studies have been conducted to study the effect of SS on receptor stability. Studies looking at the early or immediate effect have shown that SS can inhibit GABA receptors and GABAergic inhibition, indicating that SS can interact with the GABA receptors. The possibility that SS might cause a change in receptor stability by destabilizing the receptor and might cause a reduction in receptor levels in the neuropil cannot be ruled out.

The decrease in subunit levels may or may not be related to receptor turnover. GABA<sub>B</sub> receptors constantly traffic in and out of the cell membrane. Studies monitoring GABA<sub>B</sub> receptor trafficking have found that the receptors are dynamically regulated, with an internalization rate of  $40 \pm 4$  mins. The rate of insertion is faster at  $7.8 \pm 2$  mins (Wilkins et al., 2008). The receptors

are constantly replenished to maintain proper neural function. SS might affect the cellular mechanisms involved in regulating receptor turnover, and may either enhance the internalization of receptors or prevent the reinsertion of receptors, thereby reducing the receptors expressed on the cell membrane.

The rate of internalization of GABA<sub>B</sub> receptors is affected by agonist and antagonist binding. Binding of agonists increases the rate of internalization whereas antagonist binding decreases the rate of internalization (Wilkins et al., 2008). Physiological studies on the direct effect of SS on neural activity report that SS decreases the inhibitory post-synaptic potentials caused by GABA<sub>B</sub> receptor activation, indicating that SS has an antagonistic effect on the GABA<sub>B</sub> receptors (Lu et al., 2011). Thus the reduction seen in this study cannot be attributed to receptor internalization since the antagonistic action of SS would decrease the rate of internalization and cause more receptors to be expressed on the cell membrane. However, the possibility that SS causes changes in receptor stability cannot be ruled out. Further research is needed to be conducted to address the effect of SS on receptor stability.

## **7 Conclusions and future directions:**

Intraperitoneal injection of SS causes a reduction in the level of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits in all the major auditory structures. This reduction is different for different structures. The IC and MG are affected the most by the effect of SS treatment. The drug had a greater affect on the levels of subunits in the cell bodies than in the neuropil..

My research provides a basis for future tinnitus research to focus on GABA<sub>B</sub> receptors. I studied the effect of SS, a tinnitus inducer, on the level of GABA<sub>B</sub> receptors in the auditory structures. However, tinnitus is also induced by exposure to loud noises. In fact, loud noise is the

cause of approximately 23% of reported tinnitus cases (Tinnitus archive, Oregon Health and Science University). Future studies can examine the possible involvement of GABA<sub>B</sub> receptors in noise-induced tinnitus by pairing behavioural studies with immunohistochemical studies. Researchers can study changes in GABA<sub>B</sub> receptor levels in each auditory structure after exposure to loud noise to determine if the receptor levels are affected in a manner similar to that seen after treatment with SS.

Physiological studies monitoring the activity of GABA<sub>B</sub> receptors during tinnitus can be conducted to decipher the functional role of these receptors in tinnitus. Since my results showed a decrease in GABA<sub>B</sub> receptor levels in all auditory structures, the activity of GABA<sub>B</sub> receptors in each auditory structure can be studied to determine the relative contribution of each structure to the tinnitus sensation. Pharmacological manipulation using agonists and antagonists can be paired with behavioural studies. The receptors can be blocked using antagonists or activated using agonists and the strength of tinnitus sensation can be measured using behavioural paradigms to fully decipher the involvement of these receptors in tinnitus sensation.

Future research can study the effect of SS on cellular mechanisms responsible for synthesizing GABA<sub>B</sub> receptor subunits. The higher reduction in the cell bodies indicates that SS may be affecting cellular mechanisms. Future studies can monitor effect of SS on transcriptional and translational machinery to determine the cellular targets of SS as the drug affects more than just GABA<sub>B</sub> receptors. The proteins involved in transcription or translation can be knocked out in a systematic manner and change in receptor levels monitored after SS application to determine the involvement of the protein in the effect of SS application. The transcription and translation of GABA<sub>B</sub> receptor subunits can also be monitored to see which stage is more affected by SS. The two subunits are needed to make a functional receptor, and in the absence of one, the other

subunit is degraded and recycled. Further research is needed to determine if SS affects one subunit's production or stability more than the other, resulting in the downregulation of the other subunit. The effect of SS on receptor stability can also be studied. Florescent tags can be used to tag the GABA<sub>B</sub> receptors and monitor their stability during the 3 hour time frame of SS's effect to determine if SS can destabilize the functional receptor or a subunit and cause changes in receptor levels.



## TABLES

TABLE 2: Summary results regarding the effect of SS on the GABA<sub>B</sub>R1 subunit in four auditory structures

% change in GABA <sub>B</sub> R1 labeling				
	CN	IC	MG	AC
OVERALL	16.1	31.2	34.0	11.1
CELL BODY	70.9	82.7	81.6	47.1
NEUROPIL	15.4	30.4	32.3	10.6

TABLE 3: Summary results regarding the effect of SS on the GABA<sub>B</sub>R1 subunit in the subdivisions of four auditory structures

GABA <sub>B</sub> R1 levels										
		overall			Cell body			neuropil		
		OD	OD	%	Total	Total	%	OD	OD	%
		SAL	SS	reduction	# SAL	# SS	reduction	SAL	SS	reduction
CN	DCN	90.01	100.1	16.1	2395	630	73.7	90.8	100.3	15.3
	PVCN	92.6	111.3	28.4	2084	672	67.8	94.3	111.8	27.4
	AVCN	101.5	114.1	34.6	6210	1806	70.9	103.2	114.3	33.3
IC	ICd	87.0	103.5	31.2	11435	3376	70.5	88.2	104.0	30.4
	ICx	97.9	113.4	36.1	18553	3522	81.0	99.4	113.7	34.5
	ICc	96.8	117.5	47.0	13202	564	95.7	98.0	117.6	45.7
MG	MGd	102.7	118.5	35.8	1688	456	73.0	104.6	119.1	34.3
	MGv	103.7	121.4	41.7	3674	585	84.1	105.4	121.8	40.1
	MGm	107.1	125.2	47.0	634	61	90.3	108.4	125.4	45.5
AC	AU1	85.4	94.9	11.1	13446	7310	45.6	86.9	95.8	10.6
	AUD	84.5	95.8	13.5	5291	2722	48.6	86.1	96.7	13.3
	AUV	86.9	98.2	14.5	4443	2227	49.9	88.5	99.4	14.2

TABLE 4: Summary results regarding the effect of SS on GABA<sub>B</sub>R2 subunit in four major auditory structures.

% change in GABA <sub>B</sub> R2 labeling				
	CN	IC	MG	AC
OVERALL	11.0	24.0	23.3	12.3
CELL BODY	42.2	72.3	70.0	33.7
NEUROPIL	10.4	23.1	21.9	12.2

TABLE 5: Summary results regarding the effect of SS on the GABA<sub>B</sub>R1 subunit in the subdivisions of four auditory structures.

GABA <sub>B</sub> R2 levels										
		overall			Cell body			neuopil		
		OD SAL	OD SS	% reduction	Total # SAL	Total # SS	% reduction	OD SAL	OD SS	% reduction
CN	DCN	87.3	93.5	11.4	2444	1819	25.6	88.8	94.4	10.4
	PVCN	89.5	99.5	21.9	1845	868	53.0	91.8	101.2	21.9
	AVCN	99.9	103.4	7.5	1502	661	56.0	101.9	105.3	7.8
IC	ICd	106.7	116.4	24.0	8491	3246	61.8	107.6	116.8	23.1
	ICx	117.0	125.3	27.5	12718	3473	72.7	118.0	125.6	25.7
	ICc	117.2	125.7	27.9	8234	1436	82.6	118.2	126.3	27.6
MG	MGd	106.9	115.8	22.5	918	291	68.3	108.2	116.1	20.7
	MGv	109.2	117.2	22.1	1729	539	68.8	109.8	117.4	21.3
	MGm	110.3	121.1	31.2	366	73	20.0	111.8	121.3	28.8
AC	AU1	100.8	106.9	12.3	11349	7699	32.1	101.8	107.7	12.2
	AUD	101.2	107.6	13.0	4902	3258	33.5	102	108.3	12.7
	AUV	103.3	109.7	13.6	3242	1975	39.1	104	110.2	13.4

TABLE 6: Densitometry values of GABA<sub>B</sub>R1 $\alpha$  and GABA<sub>B</sub>R1 $\beta$  subunits.

	CB		CN		IC		MG		AC	
	SAL	SS	SAL	SS	SAL	SS	SAL	SS	SAL	SS
GABABR1 $\alpha$	10512	9681	8611	6940	12306	8218	13576	13355	12363	10722
GABABR1 $\beta$	6634	7154	6017	5116	7366	5144	8935	7726	10506	8670

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## APPENDICES

### **Solutions used in Immunohistochemistry procedures:**

#### **0.5M Sodium Phosphate Dibasic**

For 1L

70.98g sodium phosphate dibasic powder ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )

1L of dH<sub>2</sub>O

#### **0.5M Sodium Phosphate Monobasic**

For 1L

68.99g anhydrous sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )

1L dH<sub>2</sub>O

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

For 1L

600ml 0.5M sodium phosphate dibasic solution

200ml 0.5M sodium phosphate monobasic solution

200ml dH<sub>2</sub>O

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

For 1L

250ml 0.4M PB

750ml dH<sub>2</sub>O

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

For 1L

250ml 0.4M PB

750ml dH<sub>2</sub>O

#### **4% Paraformaldehyde (PFA) in 0.1M PB (pH = 7.2)**

For 1L

40g PFA powder

1L 0.1M PB

Dissolve PFA powder into PB by heating on a stir plate at low heat settings

Adjust pH with either HCl or NaOH

### **Cryoprotectant 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 solution

5ml 0.4M PB

200µl 0.4% NH<sub>4</sub>Cl in 0.1M PB solution

200µl Glucose

13.6ml dH<sub>2</sub>O

200µl of 50mg/ml DAB (D5637, Sigma-Aldrich, Oakville, ON, Canada) in 0.1M PB

0.8ml 1% Nickel sulphate in dH<sub>2</sub>O

20µl of Glucose oxidase (G3660, Sigma-Aldrich)

### **Solutions used in Western blotting procedures:**

#### **Homogenization buffer:**

For 10ml solution:

25µl 1M Tris-HCL (pH = 8.0)

225µl 1M NaCl

20µl 0.5mM EDTA

100µl Triton x-100

**Protease inhibitors:**

0.5µl/ml aprotinin

10µl/ml PMSF (phenylmethanesulfonyl fluoride)

1µl/m/ leupeptin

1µg/ml pepstatin A

**Electrophoresis Sample Buffer (4X):**

For 100ml

40ml 87% Glycerol

10ml 2-β-mercaptoethanol

40ml 10% SDS (sodium dodecyl sulphate)

100ml 0.5M Tris-HCL

4ml 1% Bromophenol blue

6ml dH<sub>2</sub>O

**Lower Gel Buffer (pH = 6.8):**

For 1L

182g Tris base

4g SDS

Bring volume up to 1L with dH<sub>2</sub>O

**7.5% Lower gel:**

For approximately 30ml solution

16.875ml dH<sub>2</sub>O

7.5ml lower gel buffer

5.625ml acrylamide

112.5 $\mu$ l 10% APS (ammonium persulphate)

33.75 $\mu$ l TEMED (N,N,N,N'-tetramethylenediamine)

**Upper Gel Buffer (pH = 6.8):**

For 200ml

12.1g Tris base

0.8g SDS

Bring volume up to 200ml with dH<sub>2</sub>O

**Stacking Gel:**

For approximately 10ml solution

6.4ml dH<sub>2</sub>O

2.5ml upper gel buffer

1.125ml acrylamide

30 $\mu$ l 10% APS

20 $\mu$ l TEMED

**5X Running Buffer (pH = 8.2)**

For approximately 1L

15.1g Tris base

94g Glycine

25ml 10% SDS

**Transfer Buffer (pH = 9.2)**

TBS-Tween (TBST) Buffer:

For 1L

5ml 10% Tween

995ml TBS buffer

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