**Cellular Physiology and Synaptic Pharmacology of the Rat's External Cortex
of the Inferior Colliculus Studied Using *In Vitro* Brain Slice Techniques**

by

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requirements for the degree of

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Abstract

The inferior colliculus (IC) is a principal structure of the auditory midbrain and serves as a significant integrative center within the central auditory pathway. In mammals the IC can be divided into three anatomical subdivisions: the central nucleus (ICc), the dorsal cortex (ICd) and the external cortex (ICx). Unlike the ICc, the ICx receives its primary inputs from the contralateral and ipsilateral ICc, auditory cerebral cortical areas and from regions of motor and other sensory systems. This wide array of primary projections from both auditory and non-auditory sources makes the ICx a unique structure within the auditory brainstem. The current study supports the notion that the ICx may serve as a center to integrate auditory and non-auditory information, which is modulated by behavioural state. In this study we examined intrinsic membrane properties and synaptic responses of ICx neurons in young rat's brain slice preparations.

Visual whole-cell patch clamp recordings were taken from ICx neurons (N=99) in coronal slices from rats between the ages of 8 to 11 days. The ICx neurons displayed various types of firing patterns in response to depolarizing and hyperpolarizing current injection, including *regular*, *adaptation*, *pauser* and *bursting* type. For distribution, the regular type cells constitute the majority (68%), followed by adaptation (17%), pauser (12%) and bursting cells (3%). In response to hyperpolarizing current injection, many ICx neurons illustrated a pronounced sag in the membrane potential, representing a hyperpolarization-activated current (I_h). Some neurons (25%) displayed a Ca^{2+} -dependent rebound depolarization following hyperpolarization of the membrane with negative current injection. In response to depolarizing current injection, 70% of ICx neurons displayed the presence of a Ca^{2+} -mediated potential expressed as Ca^{2+}

spikes/humps, uncovered when Na^+ and K^+ currents were removed. To study the synaptic responses, a stimulating electrode was placed in the ICc. The graded responses displayed a combination of excitatory and/or inhibitory potentials and pharmacological analyses showed the presence of glutamatergic, GABAergic and glycinergic transmission. Serotonin (5-HT) application showed modulation of the synaptic responses in the ICx. The modulatory effects of 5-HT were most pronounced on the glutamatergic NMDA and AMPA receptor mediated responses.

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List of Abbreviations

5-HT	serotonin or 5-hydroxytryptamine
AC	auditory cortex
ACSF	artificial cerebrospinal fluid
AGS	audiogenic seizures
AHP	after-hyperpolarization potential
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	action potential
APV	($\pm$)-2-amino-5-phosphonovaleric acid
BIC	brachium of the inferior colliculus
CCK	cholecystokinin
CN	cochlear nucleus
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CoIC	commissure of the inferior colliculus
CPP	($\pm$)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
DCN	dorsal cochlear nucleus
DNLL	dorsal nucleus of the lateral lemniscus
E_h	reversal potential
EPSP	excitatory post synaptic potential
GABA	γ -aminobutyric acid
GAD	glutamic acid decarboxylase
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
HRP	horseradish peroxidase
IC	inferior colliculus
ICc	central nucleus of the inferior colliculus
ICd	dorsal cortex of the inferior colliculus
ICx	external cortex of the inferior Colliculus
I_h	hyperpolarization-activated K^+ current
IID	interaural intensity differences
I_{ins}	instantaneous current
I_{max}	maximum current
IPSP	inhibitory post synaptic potential
I_{tail}	deactivating tail current
I_{ss}	steady-state level current
IV	current-voltage
k	slope factor, Boltzman equation
LL	lateral lemniscus
LSO	lateral superior olive
MGB	medial geniculate body
NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	<i>N</i> -methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase

Par	parietal area
PDS	paroxysmal depolarizing shifts
PENK	enkephalin
PHA-L	Phaseolus vulgaris-leucoagglutinin
PPT	preprotachykinin/substance P
R_{input}	input resistance
RIF	rate intensity function
RLF	rate level function
RMP	resting membrane potential
RPO	reticularis pontis oralis
SAM	sinusoidally amplitude modulated
SC	superior colliculus
SOC	superior olivary complex
SOM	somatostatin
SPN	superior olivary nucleus
τ	time constant
Te	temporal area
TEA	tetraethylammonium
TTX	tetrodotoxin
$V_{1/2}$	memb.pot. at which the current is half-maximally activated
VCN	ventral cochlear nucleus
VNLL	ventral nucleus of the lateral lemniscus

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Cellular Physiology and Synaptic Pharmacology of the Rat's External Cortex of the Inferior Colliculus Studied Using *In Vitro* Brain Slice Techniques

1. Introduction

The development in our understanding of how the auditory system processes sound has been gradual. Years of investigation have furthered our knowledge of the auditory brainstem revealing details about its anatomy, morphology and even some physiological properties. The greater part of this knowledge has been in our realization of the workings of the peripheral auditory system, and in the behavioural responses to auditory stimuli. In recent years, interest has been placed in elucidating the mechanisms behind the processing of sound stimuli by the auditory brainstem including signal integration, factors involved with synaptic transmission and neuronal firing patterns. One such structure within the auditory brainstem which has received notice is the inferior colliculus (IC). Several studies have investigated the physiological properties of IC neurons *in vivo*, primarily using extracellular recording techniques, and significant findings have been gathered. However, more recently *in vitro* techniques have been introduced to study the details of the physiology of IC neurons that would be difficult to acquire using *in vivo* methodology. Using the brain slice technique, the membrane properties, synaptic response properties, and neurotransmission of IC neurons can be examined.

1.1 *Inferior colliculus (IC)*

The IC is the principal component of the auditory midbrain serving as a major integrative center within the central auditory system, and is critical for processing auditory information. Located in the most dorsal and caudal regions of the midbrain, this nucleus receives synapses from virtually all ascending and descending auditory pathways. The IC displays remarkable homogeneity in cytoarchitecture and connectivity across species such as human (Geniec and Morest, 1971), monkey (Fitzpatrick, 1975), cat (Morest, 1966; Morest and Oliver, 1984; Rockel and Jones, 1973) and rat (Coleman and Clerici, 1987; Faye-Lund, 1985; Faye-Lund and Osen, 1985). There are however some minor species variations, as would be expected since there are known variations in the anatomy of other structures which project to the IC. Early interpretations of general cytoarchitecture by Cajal (1909) led to the partitioning of the IC into an assortment of nuclear groups, which has since been elaborated and modified by different researchers. In its simplest form the IC can be divided into two parts, the central nucleus and the pericentral nucleus. More complex schemes utilize this main framework, but generally subdivide one or both of these nuclei (*table 1*). Faye-Lund and Osen (1985) utilized Nissl-stained sections, Golgi impregnations and myelin stains to create the now most commonly used parcellation scheme of the IC, dividing it into three main subdivisions. There is the central nucleus of the inferior colliculus (*ICc*), the dorsal cortex of the inferior colliculus (*ICd*) and the external cortex of the inferior colliculus (*ICx*). The two cortices together almost completely encapsulate the central nucleus (see figure 1,5).

1.2 *Central nucleus of the inferior colliculus (ICc)*

1.2.1 *Afferent inputs*

The ICc is a densely celled, laminated region which is tonotopically organized. It is the ventrolateral region of the IC which receives the principal relay afferent fibers of the ascending auditory system. In the rat, the ICc receives bilateral (~81-87% contralateral) input from neurons of the dorsal, anteroventral, and posteroventral cochlear nuclei (Coleman and Clerici, 1987; Faye-Lund, 1986). This includes the ascending auditory projections from the dorsal (*DCN*) and ventral cochlear nuclei (*VCN*), superior olivary complex (*SOC*), and the nuclei of the lateral lemniscus (*LL*). The five nuclear groups within the SOC contributing afferent projections to the ICc include the lateral superior olivary nucleus and the lateral nucleus of the trapezoid body, which project bilaterally. The medial superior olivary nucleus, superior paraolivary nucleus, and the ventral nucleus of the trapezoid body display predominantly ipsilateral projections. The dorsal nucleus of the lateral lemniscus (*DNLL*) contributes bilateral input to the ICc, while the ventral nucleus delivers ipsilateral input. This clearly illustrates the extent of convergence from auditory brainstem nuclei to the ICc, supporting the notion that this structure is important for signal integration and processing. Furthermore, due to the source of these afferent projections, the ICc is labeled as being part of the lemniscal (tonotopic) pathway (Li, *et al.*, 1998). In this pathway, frequencies represented along the length of the cochlear partition are mapped in a precise fashion onto auditory nuclei, and the projection of fibers from one nucleus to the next up the auditory pathway, maintains its tonotopic configuration (Brunso-Bechtold, *et al.*, 1981; Druga and Syka, 1984;

Henkel and Spangler, 1983; Oliver, 1984; Ryugo, *et al.*, 1981; Zook and Casseday, 1982).

1.2.2 Efferent projections

Ascending projections of the ICc include major ipsilateral input to the medial and ventral divisions of the medial geniculate body (*MGB*), with some minor input to the equivalent contralateral MGB structures (Andersen, *et al.*, 1980; Frisina, *et al.*, 1997; Morest, 1966; Morest and Winer, 1986; Oliver, 1984). There are also some minor inputs from the ICc to the ipsilateral superior colliculus (SC) (Coleman and Clerici, 1987; Covey, *et al.*, 1987; Frisina, *et al.*, 1989; Frisina, *et al.*, 1997). The ICc also provides contralateral input to the opposing ICc via the commissure of IC (CoIC), particularly to corresponding areas of tonotopy, along with some minor projections to the contralateral ICd and ICx (Beyerl, 1978; Bruno-Bechtold, *et al.*, 1981; Druga and Syka, 1984; Frisina, *et al.*, 1997; Moore and Goldberg, 1963; Moore and Goldberg, 1966; Shneiderman, *et al.*, 1988; Woollard and Harpman, 1940). Using small horseradish peroxidase (HRP) and Phaseolus vulgaris-leucoagglutinin (*PHA-L*) injections into the ICc of rat, cat, bat and mouse, researchers have shown labeled neurons in the contralateral ICc at a position homogeneous to the “reciprocal” injection site (Adams, 1980; Coleman and Clerici, 1987; Druga and Syka, 1984; Saldaña and Merchán, 1992; Zook and Casseday, 1982).

Another very interesting set of outputs from the ICc are those that are intracollicular in nature. The ICc projects to the ipsilateral ICd and has dense, direct projections to the ipsilateral ICx (Adams, 1980; Aitkin, *et al.*, 1981; Andersen, *et al.*, 1980; Coleman and Clerici, 1987; Faye-Lund and Osen, 1985; Saldaña and Merchán,

1992). Saldaña and Merchán (1992) were able to show that in rats the projection from the ICc to the ICx displayed a topographic arrangement. Previous anatomical studies had failed to provide clear experimental evidence of topography and the possibility of tonotopy in the ICx was still under debate (Aitkin, *et al.*, 1981; Andersen, *et al.*, 1980; Kudo and Niimi, 1980; Stiebler and Ehret, 1985). The topographic assembly of the ICc-ICx projections suggests that the ICx could have a topographic organization of its own, which is distinct from that of the ICc. Furthermore, studies were able to demonstrate that label injection in the ICc resulted in labeling of neurons in ipsilateral ICx and ICd neurons along with their contralateral counterparts in the same corresponding position within their respective nuclei (Coleman and Clerici, 1987; Druga and Syka, 1984). This further supports the notion of a tonotopic organization within the cortices of the IC. It is also interesting to note that the ICc does not have any direct outputs or does not receive any major direct projections from the auditory cortex (AC) (Faye-Lund, 1985; Huffman and Henson, 1990; Syka, *et al.*, 1984) (figure 2). It may however have some indirect cortical influence via its projections from the SC, ICd and ICx. The lack of neocortical input to the ICc has been shown with degeneration and anterograde transport studies in cat (Andersen, *et al.*, 1980; Morest and Oliver, 1984), rhesus monkey (Kuypers and Lawrence, 1967) and rat (Coleman, *et al.*, 1984; Faye-Lund, 1985). The results obtained from retrograde transport studies did not agree with the impression that there was a lack of neocortical input to the ICc. Such studies in rat, reported some level of labeling of neocortical neurons from the ICc, but associated the findings to contamination of the surrounding cortical regions of the IC (Beyerl, 1978; Druga and Syka, 1984). In spite of

the various results, it is generally accepted that the ICc does not receive any major direct neocortical input.

1.2.3 Neuronal organization

The ICc has an obvious laminar organization, assembled with small, densely packed neurons with flattened, disc-shaped dendritic fields (Huffman and Henson, 1990). These dendritic arbors are arranged in fibrodendritic sheets that run in a ventrolateral-dorsomedial plane, stacked in parallel giving rise to the ICc's laminated appearance (Faye-Lund and Osen, 1985; Oliver and Morest, 1984). Neurons of the ICc display well-defined, stable frequency-specific responses to acoustic stimuli, and frequency-threshold tuning curves are narrow in comparison to the neurons of the ICd and ICx. As mentioned above, neurons of the ICc are tonotopically organized with the isofrequency contours oriented in parallel planes reiterating the laminar organization of the ICc. The lamination of the ICc and the projection patterns of the auditory brainstem nuclei are the key features for the formation of spatial representations of sound parameters and neuronal response parameters. All these factors considered, it seems quite evident that the ICc is a crucial subnuclei involved in various aspects of auditory processing including sound localization, modulation and pitch coding (Aitkin, *et al.*, 1975), and is an obligatory site of convergence for most ascending and some descending auditory information from the brainstem and forebrain.

1.2.4 Functional role

The ICc is a major synaptic integration center in the central auditory pathway. It receives ascending inputs from essentially every lower auditory brainstem structure. The binaural rate/intensity functions (RIFs) of many ICc units have been classified, and they show that many cells receive both excitatory and inhibitory inputs from each ear (see Irvine, 1986 *for review*). Most ICc units respond differentially to a combination of interaural intensity difference (IID) and average binaural intensity (Irvine and Gago, 1990; Semple and Kitzes, 1987). It is thought that the IID sensitivity of ICc units is vital to azimuthal sound localization (Aitkin *et al.*, 1975). Many units in the ICc are sensitive to time-varying stimuli such as amplitude or frequency modulated tones, features found in complex communication signals (Burger and Pollak, 1998; Koch and Grothe, 1998). The ICc has been shown to possess distinct laminae which have been associated with certain sound frequencies. The spectral resolution of sound seems to emerge from the structure of these isofrequency laminae. Topographical representations of frequency, tone-response latency, tone-response threshold, sharpness of excitatory tuning, azimuth angle, and best-modulation frequency for amplitude-modulated sounds have been shown to be associated with the ICc (see Ehret, 1997 *for review*). Thus, it is clear that the ICc is a major integrative center and is integral in processing various aspects of sound.

1.2.5 Neurotransmission

Previous studies in a number of species have utilized a range of techniques to examine neurotransmitters, neuromodulators and their receptors in various parts of the auditory brainstem. It seems that in the majority of lower auditory brainstem structures,

transmission is dictated by a central group of candidates. Most transmission is modulated by the excitatory amino acid glutamate, and the inhibitory amino acids γ -aminobutyric acid (*GABA*) and glycine. The various inputs to the IC use a variety of neurotransmitters in modulating the firing of these neurons. Particularly high levels of GABA have been found in the ICc and this seems reasonable since it receives significant GABAergic input from the contralateral DNLL, ipsilateral ventral nucleus of the lateral lemniscus (*VNLL*), superior paraolivary nucleus (*SPN*), and MGB (Adams and Mugnaini, 1984; Faingold, *et al.*, 1993; Glendenning, *et al.*, 1992; Shneiderman, *et al.*, 1993). In iontophoretic studies, GABA action was shown to be mediated by GABA_A (a ligand gated Cl⁻ channel) receptors and to a lesser extent the GABA_B (a GTP binding protein linked receptor) (Faingold, *et al.*, 1989; Kuriyama, *et al.*, 1989; Ma, *et al.*, 2002a). Klug, *et al.*, (1995) examined the influence of inhibition on binaural processing in the IC of the mustache bat and found similar results. First, recordings were made in binaural cells from the IC followed by the iontophoretic application of the glycine antagonist, strychnine. Then, the effects of the GABA_A antagonist, bicuculline were tested on these same cells. The results indicated that once the GABAergic and glycinergic inhibition were eliminated by application of the antagonists, the response of the IC neurons changed. Thus, both types of inhibition (GABA, glycine) were present in the IC. However, strychnine and bicuculline had different effects on the magnitude of the spike counts evoked by stimulation. The results showed that on average strychnine had relatively small effects on response magnitude, while bicuculline caused a marked increase in spike count. This suggests, that the in the IC GABAergic inhibition is the major contributor while glycine is a secondary source of inhibition. Unfortunately, like

most studies of the IC, the researchers failed to discriminate between the subnuclei of the structure, with emphasis almost always placed on the ICc. This seems logical because it is the major subnuclei of the IC and it receives the most significant auditory input from lower auditory brainstem structures.

In vivo and *in vitro* pharmacological studies have shown that synaptic excitation in the ICc is mediated by glutamate through α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (Faingold, *et al.*, 1989, 1991; Feldman and Knudsen, 1994; Kelly and Zhang, 2002; Ma, *et al.*, 2002b; Wu, *et al.*, 2002; Zhang and Kelly, 2001). *In vivo* single unit recordings from cells in the ICc clearly indicate the involvement of the excitatory amino acid, glutamate (Faingold, *et al.*, 1989, 1991). They demonstrated that iontophoretic application of glutamate or NMDA increased acoustically evoked firing. Furthermore, application of a glutamate antagonist can lead to the suppression of excitatory responses, (Burger and Pollack, 1998; Faingold, *et al.*, 1989, 1991; Zhang and Kelly, 2001). Several *in vitro* brain slice studies have shown analogous results where glutamate and its receptors (AMPA, NMDA) contribute to excitation in this structure (Li, *et al.*, 1998; Ma, *et al.*, 2002b; Wagner, 1996; Wu, *et al.*, 2002). In these studies, the synaptic responses elicited in the ICc neurons were a combination of both excitation and inhibition. The relative strength of excitation and inhibition varied from cell to cell and were determined by stimulation location and magnitude. When the glutamatergic excitation was examined more closely, it revealed the presence of two separate components that may be distinguished on the basis of their time course and pharmacology. The earlier component of the synaptic responses in the ICc were mediated by the AMPA receptors, while the

later component by the NMDA receptor (Ma, *et al.*, 2002b). Pharmacological analysis supports this notion since the early component was effectively blocked with the application of the AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), while the latter component was blocked by the NMDA antagonists ($\pm$)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) or ($\pm$)-2-amino-5-phosphonovaleric acid (APV). This presence of these components for glutamatergically driven synaptic responses has been shown in auditory structures as well (Hestrin, *et al.*, 1990; Keller, *et al.*, 1991; Ozawa, *et al.*, 1991; Wu and Kelly, 1996).

1.3 Dorsal cortex of the inferior colliculus (ICd)

1.3.1 Afferent-efferent projections

The ICd has input patterns which are quite distinct from the other two subnuclei of the IC. The ICd receives very limited ascending input from the lower auditory brainstem nuclei, coming primarily from the DNLL and the DCN (Coleman and Clerici, 1987; Huffman and Henson, 1990). The ICd does receive significant input from the ipsilateral ICc and the contralateral ICc via the CoIC (Aitkin and Phillips, 1984; Druga and Syka, 1984; Druga, *et al.*, 1997; Saldaña and Merchán, 1992.). These contralateral projections are tonotopically organized, such that the terminal fields of the fibers in the ICd are associated with an isofrequency contour that complements the tonotopic origin of the efferent fibers from the opposite ICc. Unlike the ICc, the ICd receives significant sets of afferents from the AC.

Studies in several species, using anterograde and retrograde labeling techniques have shown that the ICd is the major target for descending auditory information to the

IC, most heavily concentrated medially and caudally (Andersen, *et al.*, 1980; Coleman and Clerici, 1987; Druga and Syka, 1984; Morest and Oliver, 1984). Faye-Lund (1985) demonstrated the presence of three distinct projections originating from the temporal neocortex of the rat. They found a partly bilateral projection from area 41 to the deep layers of the ICd, along with a second ipsilateral projection from area 36 to the superficial layer. Therefore, their results showed that the central parts of the AC seemed to project to the ICd. The data presented by Coleman and Clerici (1987) using retrograde HRP labeling described projections from areas 41, 20 and 36 of the AC to the ICd, consistent with Faye-Lund's findings (1985) (*figure 2*). Recent studies used anterograde and retrograde transport of HRP and HRP conjugated with lectin to determine if any secondary auditory cortical areas projected to the various subnuclei of the IC. The results showed weak anterograde labeling in the ICd from injections into the laminar distribution of corticocollicular neurons in the temporal area Te 1 (central part of the AC). However, injections into neurons in temporal areas Te 2 (caudoventral part of AC) and Te 3 (rostroventral part of AC) led to significant anterograde labeling in the ICd (Arnault and Roger, 1990; Druga, *et al.*, 1997; Roger and Arnault, 1989; Vaudano, *et al.*, 1991). The corticocollicular projections from the AC in rat appear to be mostly ipsilateral in nature with a weaker contralateral component (Druga, *et al.*, 1997; Herbert, *et al.*, 1991). The ICd sends projections to the dorsal division of the medial geniculate body, which in turn sends some ascending projections to cortical area 36 and to the basal ganglia (Coleman and Clerici, 1987; Faye-Lund, 1985; Huffman and Henson, 1990).

1.3.2 Neuronal organization

The dorsal cortex of the IC is a laminated structure composed of three layers and is situated dorsocaudally and dorsomedially to the ICc (Faye-Lund and Osen, 1985). Layer 1 is superficial, thin ($\sim 7 \mu\text{m}$) and the cell density is relatively sparse, with many fibers streaming near the surface of the IC. Below this is layer 2, which is slightly thicker and consists of small and medium-sized multipolar cells. Together, layer 1 and 2 account for approximately one third the maximal thickness of the ICd. Layer 3 narrows considerably towards the circumference of the ICd, containing many small and medium-sized, multipolar neurons. There is also an obvious change in neuronal orientation at the border between the ICd and the ICc. In humans and cats, the ICd possess a fourth layer, thought to be the site of convergence for cortical and collicular terminals (Geniec and Morest, 1971).

1.3.3 Functional role

Unlike the ICc, the functional role of the ICd is not well understood. The neurons of the ICd have response properties similar to those in the ICc. However, neurons in the superficial layers are poorly driven by acoustic stimuli and tend to habituate rapidly, responding primarily to stimulus onset and have relatively broad tuning curves (Aitkin, *et al.*, 1975; Merzenich and Reid, 1974). The response properties of these ICd neurons and their monaural input suggest that they don't play a significant role on auditory signal processing. One theory is that since the ICd has projections to the ICc, that it may in some part contribute to "auditory attention", modulating the subjects' attention towards auditory stimuli (Aitkin, *et al.*, 1975). Lesion studies have shown that damage to the ICd

can lead to deficits in attention to auditory stimuli (Jane, *et al.*, 1965). In this respect, the ICd would act as a modulator of auditory processing being performed in the ICc, where all lower auditory brainstem stimuli is converging.

1.4 External cortex of the inferior colliculus (ICx)

1.4.1 Afferent inputs

The third subdivision of the inferior colliculus is the ICx, found around the ventral part of the central nucleus laterally, ventrally, rostrally and ventrocaudally. The ICx is much like its dorsal cortical counterpart, in that it receives very limited ascending lower auditory brainstem projections. There are minor projections from the cochlear nucleus (CN), SOC, and some limited ipsilateral projections from the DNLL. The ICx receives its most significant lower auditory brainstem projection through a local connection from the ipsilateral ICc and also receives some minor input from the contralateral ICc through the CoIC (Adams, 1980; Aitkin, *et al.*, 1975, 1978; Moore and Goldberg, 1963; Willard and Ryugo, 1979). This binaurally influenced nucleus is unique from the ICc and ICd since it possess multisensory connections, and for its role in acousticomotor responses. Studies using anterograde and retrograde transport of HRP have illustrated that the ICx receives input from somatosensory structures including the contralateral cuneate and gracile nuclei, and the contralateral spinal trigeminal nucleus (Aitkin, *et al.*, 1981; Feldman and Kruger, 1980; Tokunaga, *et al.*, 1984). Inputs from the deep superior colliculus, parabrachial nucleus, and substantia nigra par lateralis, globus pallidus and the posterior hypothalamus have also been noted (Aitkin, *et al.*, 1981; Coleman and Clerici, 1987; Inagaki, *et al.*, 1990; Morest and Oliver, 1984; Tokunaga, *et*

al., 1984; Willard and Ryugo, 1979; Yasui, *et al.*, 1990). Faye-Lund (1985) used anterograde transport to show that the ICx (predominantly layer 2 and 3) receives ipsilateral input from area 22 of the AC, which is situated between the primary AC and the somatosensory and visual cortices. Coleman and Clerici (1987) used anterograde and retrograde transport of HRP to illustrate the presence of a projection from area 39 of the AC, just rostral to area 41 (figure 2,3). More recent studies have examined the projections from non-primary auditory cortices and have found that the ICx receives mainly ipsilateral projections from auditory cortical areas Te 1, Te 2, Te3 and from the parietal area Par 2 (Druga and Syka, 1997). Due to the source of its inputs, both the ICd and the ICx are said to function in the extralemniscal or “diffuse” pathway, unlike the ICc which receives direct auditory input from the lemniscal pathway.

1.4.2 Efferent projections

The ICx projects fibers to the ICc, to the medial division of the MGB, the superior colliculus, the cerebellum and the pontine gray matter (Edwards, *et al.*, 1979; Hashikawa, 1983; Hashikawa and Kawamura, 1983). This lends support to the notion that the ICx is a multisensory integrative nuclei since these acousticomotor centers to which it projects (medial MGB, superior colliculus and cerebellum) are themselves multisensory integrative centers. Interestingly, these structures go on to project fibers to motor areas which control orientation of the eyes, pinnae and head (Casseday, *et al.*, 1979; Edwards and Henkel, 1978).

1.4.3 Neuronal organization

Like the ICd, the ICx has a cortical cytoarchitecture of three layers. The most superficial stratum, layer 1 has scattered small neurons and increases in size ventrolaterally. It is continuous with the adjacent structures, brachium of IC (BIC), the sagulum, and the ICd forming a fibrocellular capsule on the surface of the IC laterally and dorsally. Layer 2 is a thin, myelin-dense cortex of clusters of small cells with highly interwoven dendrites. In Golgi sections the cells appear multipolar or bipolar with thin, curved dendrites. Layer 3 is the deepest and major portion of the ICx, composed of scattered small, medium and large multipolar cells. The largest cells of the IC are found ventrolaterally close to the entrance of the LL, here in the ICx. These large cells also help form the distinct boundary between the ICx and the small, densely packed cells of the ICc (Faye-Lund and Osen, 1985).

1.4.4. Functional role

The functional role of the ICx has been debated over the years. Some researchers believe that this subnucleus of the IC plays no significant role in auditory processing or integration and is simply a somatosensory driven center. Others believe that due to its significant projections from the ICc, which is a clear contributor to auditory processing receiving major inputs from the lower auditory brainstem, the ICx is a purely auditory driven center. It seems most likely that the ICx is a combination of the two, acting as a multisensory nucleus. Aitkin, *et al.*, (1978) were able to show from *in vivo* recordings in anesthetized cats, that roughly half of the recorded ICx cells responded to both somatosensory stimuli (*via the dorsal column*) and to acoustic stimuli (*pure tone bursts*).

The remaining portion of neurons recorded were only influenced by one type of stimulation, either auditory or somatosensory. They also found that neurons of the ICx had large receptive fields to skin stimulation. Those neurons that were activated by both modes of stimuli were excited by acoustic stimuli and inhibited by tactile stimuli. The neurons of the ICx were quite similar to those found in the ICd, since they were poorly driven by acoustic stimuli, displayed broad irregular tuning curves, and habituated rapidly to presented tones. After 2-5 repetitions of a tone, most units did not respond until a novel frequency was presented (Aitkin, *et al.*, 1978). The bimodality of input from both somatosensory and auditory centers and a lack of clear tonotopy suggest that the ICx may be involved in general sensory signaling, certain aspects of motor coordination associated with auditory function and even the integration of both auditory and somatic information. Thus, it seems quite improbable that these neurons are involved strictly in auditory or somatosensory processing.

Several researchers believe that the ICx is involved in multisensory reflex behaviour, which includes control of the auditory receptor organ (*ear, head, pinnae*) (Aitkin, *et al.*, 1978, 1981; Faye-Lund, 1985) and perhaps even modification of the position of the subject towards the incoming auditory stimulus. This notion is supported by the projections of the ICx to structures such as the pontine nuclei and cerebellum, normally associated with motor function. The ICx is also believed to contribute to the visceral or emotional responses to acoustic stimuli (Iwada, *et al.*, 1986; LeDoux, *et al.*, 1988), endorsed by its direct projections from the globus pallidus and posterior hypothalamus (Inagaki, *et al.*, 1990; Yasui, *et al.*, 1990). The ICx may therefore play an

integrative function which is imperative for coordinated movements of the head, eyes and ears toward a sound source, which may be modulated by emotion.

1.4.5 Neurotransmission

When neurotransmission in the ICx is examined, the data available is not as thorough as that for the ICc. As mentioned earlier, most studies focus their energies on the central nucleus since it is the most prominent area of the IC and it does receive the most direct projections from the lower auditory brainstem. Still, the ICx receives its leading ascending auditory projection from the ipsilateral ICc, and much significant descending information from both the auditory and somatosensory cortices. The question remains however, what neurotransmitters and neuromodulators are associated with the ICx. Since the ICx receives major projections from the ICc, it is reasonable to believe that the two subnuclei share many of the same neurotransmitters.

1.4.5.1 Classical neurotransmitters

A select few have used *in vitro* brain slice studies to examine the synaptic response of ICx neurons (Li, *et al.*, 1998, 1999; Smith, 1992). These studies stimulated the CoIC while recording from ICx neurons and found the presence of excitatory responses that were mediated by both AMPA and NMDA receptors and inhibitory responses that were mediated by GABA_A receptors. Synaptic responses were generally observed as a complex mix of inhibitory post synaptic potentials (*IPSPs*) and excitatory post synaptic potentials (*EPSPs*). The magnitude and evidence of each type of synaptic response was dependent upon the relative intensity of stimulation of the CoIC.

Application of the AMPA antagonist, CNQX completely removed the excitation indicating that this was mediated by glutamatergic activity through AMPA receptors.

Further evidence of glutamatergic activity in the ICx was recently presented with AC lesion studies which monitored changes in nicotinamide adenine dinucleotide phosphate (*NADPH*)-diphorase/nitric oxide synthase (*NOS*) staining (Druga and Syka, 2001). NO is a free-radical gas involved in signal transduction acting as a diffusible intercellular signaling molecule. It is synthesized from L-arginine by the enzyme NOS, which has been reported to have *NADPH*-diphorase activity. *NADPH*-diphorase histochemistry and NOS immunoreactivity are consistently used to identify the presence of NO in neurons (Druga and Syka, 2001). Reduction in levels of *NADPH*-diphorase/*NOS* have been reported after monocular enucleation in the rat subcortical visual centers (Zhang, *et al.*, 1996). Any changes in NOS expression will be illustrated by changes in *NADPH*-diphorase activity, and thus may reflect changes in transmitter levels and production. The ICx is known to receive excitatory glutamatergic synapses and it has been suggested that NOS may be localized in postsynaptic neurons (Druga and Syka, 2001; Feliciano and Potashner, 1995). Druga and Syka (2001) found that unilateral lesion of the AC (Te 1-3, Par 2) produced a significant down-regulation of *NADPH*-diphorase staining in ICx neurons, more pronounced on the ipsilateral side. The authors equate this loss of immunoreactivity to the decrease in glutamate release from cortico-collicular terminals and also to reduced synthesis of glutamate in postsynaptic ICx cells. This direct and indirect evidence effectively illustrates the involvement of the excitatory amino acid glutamate and its receptors in neurotransmission in ICx neurons.

Examination of the inhibitory transmission revealed that application of the glycine antagonist strychnine had no effect, while application of the GABA_A receptor antagonist bicuculline blocked the inhibitory events elicited via stimulation of the CoIC. In some cases, removal of the inhibition revealed a large, long-lasting excitatory potential which was effectively blocked by the NMDA receptor antagonist APV. These excitatory potentials have been compared to synaptically generated paroxysmal depolarizing shifts (PDSs) that have been seen in cortex and the hippocampus when GABAergic inhibition has been compromised (Gutnick, *et al.*, 1982; Smith, 1992). GABA_B binding sites have also been shown to be present in the IC cortex, but their functional role remains unclear (Glendenning and Baker, 1988; Milbrandt, *et al.*, 1996). Some researchers believe that the GABA_B receptors of the IC cortex may exist presynaptically and modulate GABA release through negative feedback, as seen in other central nervous system (CNS) neurons (Li, *et al.*, 1999; Tanabe and Kaneko, 1996). Neurons in the ICx have also been shown to be immunoreactive for GABA and the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), while the terminals of these neurons possess the characteristic features of inhibitory synapses as seen in other areas of the CNS (i.e. flattened or pleomorphic vesicles) (Chakravarty and Faingold, 1996; Moore and Moore, 1987; Wynne, *et al.*, 1995). Wynne, *et al.*, (1995) attempted to study the activity of the neurotransmitter systems in the IC using in situ hybridization, covering more than the usual excitatory/inhibitory amino acids (i.e. glutamate, GABA, glycine). Their study indicated the presence of GAD mRNA in the ICx, along with the other subdivisions of the IC and the presence of label for the GABA_Aα1 receptor subunit. These factors

support those already mentioned and clearly indicate the presence of GABAergic inhibition in the ICx.

1.4.5.2 Neuropeptides

These researchers also employed the technique to examine the possible distribution of neuropeptides within the IC including somatostatin (*SOM*), enkephalin (*PENK*), cholecystokinin (*CCK*), and preprotachykinin/substance P (*PPT*). The analysis revealed moderate to high levels of labeling for *SOM*, *CCK*, *PPT* and lower levels of label of *PENK* in the ICx. The functional role each of these neuropeptides plays in the ICx remains to be elucidated, but their presence indicates that neurotransmission within this structure may be more complicated than the simple excitatory/inhibitory interactions between glutamate and GABA. Some have suggested that since the ICx receives non-auditory projections, some of these neuropeptides may be involved in transmission from those structures or even perhaps participate in short-range intrinsic connections within the IC itself (Saldaña and Merchán, 1992; Wynne, *et al.*, 1995).

1.4.5.3 Contribution of serotonin (5-HT)

Serotonin or 5-hydroxytryptamine (5-HT) is one of the neurotransmitters found throughout the brain in species from insects to humans, and has been thoroughly studied in the mammalian brain. 5-HT transmission forms a major modulatory network within sensory systems. This network influences various information-processing mechanisms and in particular, filtering of auditory information (Johnson *et al.*, 1998). Experimental data have shown that serotonergic fibers and terminal endings are present in most of the

auditory nuclei in the CNS, the CN, the IC, the nuclei of the LL (Thompson *et al.*, 1994) and superior olivary complex (Woods and Azeredo, 1999). The serotonergic innervation of the ICc has been shown to originate mainly from cells in the dorsal raphe nucleus, which also projects to other auditory nuclei as well as to other regions of the nervous system (Klepper and Herbert, 1991). Studies have illustrated that rather than evoke discharges in auditory neurons like most transmitter, 5-HT usually modulates activity either pre- or postsynaptically, thus modifying response features evoked by acoustical stimulation (Ebert and Ostwald, 1992; Faingold *et al.*, 1991; Fitzgerald and Sanes, 1999). Ionophoretically applied 5-HT has been shown to influence CN neurons by either facilitating or inhibiting activity in response to inputs from non-auditory inputs and has also been shown to inhibit action potential (AP)s within the amygdala (Stutzmann *et al.*, 1998). The possible modulatory effects of 5-HT have also been studied *in vivo* for the IC in bats. Hurley and Pollak (2001) investigated the modulatory effects of 5-HT on the tuning of ICc neurons in response to complex signals in bats. The findings showed that 5-HT exerted a gain control, facilitating or depressing the responses to all frequencies in their response areas. These researchers suggest that 5-HT functionally reconfigures the circuitry of the IC and may modulate the perception of acoustic signals under different levels of 5-HT in bats.

1.5 Disorders in ICx associated with neurotransmission

The functional significance of glutamatergic, GABAergic and glycinergic activity in IC neurons remains to be fully uncovered. Once again the greater part of the data available examines the contribution of the individual neurotransmitters in ICc function,

but very little is known about the ICx. Deficiencies or alterations in “normal” transmitter function in many cases may lead to pathologies or disorders. A number of studies have implicated the IC cortex as a critical site in the neuronal network for audiogenic seizures (AGS) (Chakravarty and Faingold, 1996; McCown, *et al.*, 1984; Ribak, *et al.*, 1994; Wada, *et al.*, 1970). These various studies utilized lesions or transactions, pharmacological blockade or stimulation, direct electrical stimulation and immunochemical techniques to demonstrate the participation of this structure in the initiation of AGS. An AGS can be induced by high-intensity sound stimulation in genetically susceptible mice and rats or with prior exposure of intense sound called acoustic priming in resistant mice and rats (Ishida, *et al.*, 2002; Mishra, *et al.*, 1988; Pierson and Swann, 1991; Reid and Collins, 1989). AGS are expressed as wild running, clonic and tonic convulsions. The first stage of AGS is illustrated by the wild running, seen as a burst of rapid running. This is followed by the next stage of convulsions, where the mice/rat falls down and starts to tremble (clonic) and begins to extend its limbs (tonic). Wild running may proceed to clonic and tonic seizures, but sometimes the subject may recover without proceeding to the seizure stage (Ross and Coleman, 2000). After priming, the IC has been shown to be hypersensitive to high-intensity auditory stimulation (Urban and Willott, 1979; Willott and Lu, 1982). These results suggest that the IC may play an important role in the priming effect for AGS (Ishida, *et al.*, 2002; Sakamoto and Niki, 2001).

Recent studies have tried to elucidate what role each specific subnuclei of the IC plays in the generation of AGS. Sakamoto and Niki (2001) utilized acoustic priming to examine what effect it had on the threshold for inducing AGS in the areas of the IC. The

results clearly demonstrate the contribution of the ICx and the ICc in eliciting AGS, and the lack of involvement from the ICd neurons. It has been proposed that the ICc may serve as the initiation site for AGS, and the ICx acts as the output site for the sensory-motor integration. Furthermore, the ICx then propagates this information to the deep layers of the superior colliculus, which has been noted as the primary generator for wild running (Faingold and Casebeer, 1999; Faingold and Randall, 1999; Faingold, *et al.*, 1998; Sakamoto and Niki, 2001). This notion seems to agree with earlier ideas that the ICx is a site of multisensory input that has integrative functions. It is believed that AGS susceptibility may be associated with a reduced efficacy of GABAergic transmission in conjunction with increased action of glutamate in the IC (Browning, *et al.*, 1989; Faingold, *et al.*, 1993, Li, *et al.*, 1998). In ICx neurons, a reduction in GABAergic activity results in epileptiform like activity in rat's genetically predisposed to seizures. As mentioned earlier, ICx neurons have illustrated the presence of PDSs when GABAergic inhibition has been removed. Prince (1985) suggests that PDSs are a cellular hallmark of focal epileptogenesis, and can generate the EEG abnormality known as interictal spikes, lending further support to the notion that the ICx is involved with AGS. It is still unclear how deficiencies in normal ICx activity, either via GABA receptor abnormalities, antagonist/agonist application, or genetic susceptibility, can initiate motor output expressed as convulsions. It is useful to note that the ICx is a multisensory structure that has projections to somatosensory and motor areas, and thus it is plausible that it may indirectly influence the initiation of convulsions. Some cells of the ICx form a projection to the pons and medulla, both areas that could mediate motor activity. Lesion and stimulation studies have shown that bilateral lesions of a region of the pons termed

the nucleus reticularis pontis oralis (*RPO*) significantly reduces audiogenic convulsions (Browning, *et al.*, 1985). However, the ICx neurons do not terminate directly at the RPO, but instead are associated with the periolivary nuclei (Smith, 1992). Therefore, our understanding of the involvement of the ICx and the initiation of audiogenic convulsion remains unclear.

Another methodology which supports the proposed contribution of ICx neurons in AGS is changes in the expression of the Fos protein. Fos protein is the nuclear transcription product of the *c-fos* immediately early gene. Many studies have demonstrated that epileptiform events in the IC depend upon NMDA receptor activation and/or reduction in GABAergic activity (Faingold, *et al.*, 1989; Jensen, 1999). Ishida, *et al.*, (2002) found that after the occurrence of a generalized tonic-clonic seizure, there was a pronounced expression of Fos activation in cells immunopositive for NMDA receptors in all subnuclei of the IC. Genetically epilepsy-prone rat IC has been shown to be lacking in proper GABAergic inhibition in comparison to “normal” rats, and it could be this deficiency that is critical to the initiation of AGS susceptibility (Evans, *et al.*, 1994; Faingold, *et al.*, 1986; Li, *et al.*, 1998). Metabolic activity studies have shown that after seizures induced by electrical stimulation, the ICx exhibits the highest [^{14}C]-2-deoxyglucose uptake within the IC (McCowan, *et al.*, 1991). This supports the notion that the ICc and ICx are involved in the initiation and propagation of AGS and the phenomenon is in some part mediated by changes in GABA and NMDA receptor activation. Normal auditory function in the IC must be dictated by the precise balance between excitation and inhibition, and any deviation from this balance may lead to epileptiform firing patterns (Li, *et al.*, 1998).

Changes in GABAergic inhibition in the IC are also evident with increasing age (Casparry, *et al.*, 1999; Milbrandt, *et al.*, 1997; Palombi, *et al.*, 2001), and this can lead to the hearing disorder termed presbycusis. Presbycusis is age-related hearing loss involving functional deficits of the ear and brain, which for the most part is treated using hearing-aids. The use of hearing-aids may assist individuals suffering from presbycusis, but does not resolve the central nervous system components of the disorder. In terms of auditory perception, presbycusis is characterized by at least two perceptive difficulties; a high-pitch (high frequency) loss in sensitivity, and an inability to discriminate speech and other biologically relevant sounds in noisy acoustic environments (Frisina, *et al.*, 1997). Studies using *in vivo* preparations can examine responses of auditory units to sinusoidally amplitude modulated (SAM) stimuli, and how they change with age. Palombi, *et al.*, (2001) found age-related changes in SAM coding for both the ICc and the ICx, and attributed the change to diminished GABAergic inhibition resulting in imbalance between excitation and inhibition. It is also believed that loss of hair cells, and other cochlear changes may contribute to presbycusis (Frisina, *et al.*, 1997; Willott and Lu, 1982) and propagate further alterations in the auditory brainstem. These changes may ultimately manifest themselves as the deficiencies seen in the ICx and ICc.

1.6 *Feedback loops for the IC*

The projections discussed thus far have been kept fairly simplified in that flow of information was classified as either ascending or descending. Any particular structure, the ICx for example, has been described as receiving inputs and projecting outputs to neurons in other structures. Realistically, in an intact auditory system, the flow of

information is multidirectional with constant feedback within and between brainstem nuclei. Sound initially enters the outer ear and travels to the middle and then inner ear, and this auditory information eventually arrives at the cochlea. This auditory information is systematically processed by the various nuclei of the auditory brainstem, and does so in an ascending or bottom-up direction. However, for most nuclei in this ascending or lemniscal pathway, there are descending projections back to the lower auditory nuclei from which the auditory information was just received. This “feedback” can be beneficial in modulating the processing and acquisition of the continuously incoming auditory information. The subnuclei of the IC are no exception, elements of a descending (corticofugal) system which plays an important role in signal processing (*figure 2*). Previous studies have shown that this corticofugal system adjusts and improves the signal processing in subcortical auditory neurons in the frequency, time and spatial domains (Jen, *et al.*, 1998, 2001; Sun, *et al.*, 1996; Syka and Popelar, 1984; Zhang, *et al.*, 1997). Corticofugal stimulation has been shown to decrease the number of impulses, increase the minimum threshold, decrease the auditory spatial area, and even sharpen the excitatory frequency tuning curve of corticofugally inhibited neurons in the ICc. The opposite effects were seen in ICc neurons that were normally corticofugally excited (Jen, *et al.*, 1998, 2001; Sun, *et al.*, 1996). This presents a clear example of both positive and negative feedback to subcortical neurons, namely the ICc. The IC is a unique structure with the auditory system because it acts almost as a midpoint, an obligatory synaptic module where ascending and descending auditory pathways meet. The ICx is even more interesting because it also receives non-auditory (i.e. somatosensory) information, and integrates this with the ascending/descending auditory

information. As discussed earlier, the functional role of the ICx is yet to be fully understood, however, many have speculated that it may in part modulate signal processing in the ICc via feedback through descending from the AC. Jen, *et al.*, (2001) showed that stimulation of corticofugally excited ICx neurons inhibited auditory responses of neurons in the ICc. Furthermore, stimulation of corticofugally inhibited ICx neurons lead to inhibition of auditory responses of neurons in the ICc. These data support the presence of the AC-ICx-ICc descending pathway, that utilizes feedback to shape the processing of auditory information in the ICc. The descending projections from the AC to the ICx may act as feedback in itself perhaps facilitating auditory motor orientation, while feedback from the ICx to the ICc via the AC may improve signal processing (Jen, *et al.*, 2001). This polysynaptic pathway from the AC-ICx-ICc presents two feedback loops within itself, the first from the AC to the ICx and the second from the ICx to the ICc. Thus it is simple to see how the entire auditory system can have countless feedback loops which can interact with one another to create a network of multiple feedback loops.

1.7 Ionic basis for membrane conductance

Whenever the intrinsic membrane properties of neurons for any structure are studied, an analysis of the ionic basis behind these conductances is crucial. Generally, physiological analyses first examine the conductances thought to be mediated by ions from the classical Hodgkin & Huxley model. This is accomplished by using specific Na^+ and K^+ channel antagonists to verify the ionic basis behind the inward and outward currents, respectively. In some cases the removal of the Na^+/K^+ driven APs can reveal an

underlying “hump” or depolarization which has been shown to be mediated by Ca^{2+} (Li *et al.*, 1998; Sivaramakrishnan and Oliver, 2001; Smith, 1992). Many of these studies have also reported the occurrence of a Ca^{2+} -mediated rebound potential which occurs after the end of a negative current injection. This rebound has been shown to be distinct from a typical after hyperpolarizing potential or anode-break spike (Li *et al.*, 1998; Sivaramakrishnan and Oliver, 2001; Smith, 1992). Similar Ca^{2+} -mediated depolarizing humps have been shown in auditory neuronal structures. Disterhoft *et al.*, (1993) reported that nimodipine blockade of the Ca^{2+} -mediated hump in hippocampal slices enhanced neuronal firing and long-term potentiation. The movement of calcium is an important factor in influencing membrane and intracellular events.

Another conductance which has been described as a depolarizing “sag” has been shown in response to hyperpolarizing current injection. This sag has been described in many central neurons as an increased conductance on hyperpolarization or an *inward rectification* (see review, Pape, 1996). There are two general types of inward rectifying currents. The first is a classical inward rectifier discovered in muscle cells by Katz. This conductance is mediated purely by K^+ and is activated at values negative to the K^+ equilibrium potential. This conductance has faster kinetics and a much greater sensitivity to barium than cesium, with the rectification arising from a voltage-dependent block by polyamines and Mg^{2+} (Kubo *et al.*, 1993; Nichols and Lopatin, 1997). The second type of inward rectifier is called I_h , seen as a slowly developing inward current activated on hyperpolarization of the membrane beyond the resting potential. This I_h current is composed of a mixed K^+ and Na^+ current and behaves in a way expected of a time- and voltage dependent membrane conductance. The voltage dependence of the activation of

I_h can be regulated by neurotransmitters and metabolic stimuli via the intracellular adenosine 3',5'-cyclic monophosphate (cAMP) pathway (Pape, 1996). A general pharmacological feature of I_h is its sensitivity to low concentration extracellular cesium and its relative insensitivity to barium (Fu *et al.*, 1997). I_h current has been recorded in many cells including primary auditory neurons (Chen, 1997), brain stem auditory neurons (Banks *et al.*, 1993; Fu *et al.*, 1997), and non-auditory neurons (Doan and Kunze, 1999; Pape, 1996; Vargas and Lucero, 1999).

2. Purpose of the study

In the majority of papers cited above, researchers examined the neurophysiology of neurons either in the whole IC irrespective of individual subnuclei, or placed their focus on the ICc. Only a select few studies have attempted to systematically compare the various properties of the neurons within these various nuclei, specifically the ICx (Aitkin, *et al.*, 1975, 1984; Li, *et al.*, 1998, 1999; Smith, 1992; Syka, *et al.*, 2000). The purpose of this study is to complement the very limited data about the neurophysiology of the ICx, particularly the membrane properties and synaptic response properties, along with examination of the neurotransmitters involved in these neurons.

Several differences in these experiments will set it apart from those previously conducted in the ICx. One of the most significant distinctions lies in the stimulation source, the ICc. To my knowledge, no other study has examined the prominent projections from the ICc to the ICx. This is the chief auditory input for the ICx, receiving all of its lower auditory brainstem input indirectly via the ICc. Previous studies have focused their analyses of the ICx through stimulation of the CoIC (Li, *et al.*, 1998, 1999; Smith, 1992). Stimulation of this CoIC activates several sources of input to the ICx, including the contralateral ICc, ICd and to a lesser degree the contralateral ICx (Coleman and Clerici, 1987; Druga and Syka, 1984; Faye-Lund and Osen, 1985). These subnuclei of the IC which are projecting to the ICx of interest, are themselves receiving input from the lower auditory brainstem as well as descending inputs from the AC and somatosensory cortex. Thus, it seems that this contralateral input eventually reaching the ICx of interest has already undergone significant processing, and has traveled across yet another polysynaptic pathway via the CoIC. On the other hand, by stimulating the

projections from the ICc to the ICx we are evoking activity that would under normal conditions be the natural input of auditory information that these neurons would receive. Secondly, stimulation of this pathway examines almost exclusively the auditory input to the ICx while leaving out the somatosensory input, normally derived from descending input from the somatosensory cortex, and the auditory input indirectly from the contralateral IC via CoIC. The possibility of any descending non-auditory inputs to the ICx have been removed and even auditory inputs from the AC are limited since the brain slice is cut in such a way as to exclude these structures. This allows for a focused, localized study of lower auditory brainstem inputs to the ICx via the ICc.

Another distinction within this study is the methodology employed, visual whole-cell patch clamp recordings from *in vitro* brain slice. The majority of previous studies on the ICx were done in *in vivo* preparations or if brain slices were used intracellular recordings were employed. By using the visual patch clamp technique, the location and “health” of the ICx neurons can be confirmed by the experimenter visually. Also, by utilizing *in vitro* brain slice, electrophysiological recordings and pharmacological analyses can be made possible, which would be difficult in an *in vivo* setup.

2.1 Purpose 1: Intrinsic membrane properties

The first purpose of this study was to examine the basic membrane properties of ICx neurons. The analyses were mainly in response to both hyperpolarizing and depolarizing current injection. These analyses included measures such as current-voltage relationships of neurons, membrane input resistance and time constant, and AP properties. Also, the firing pattern of ICx neurons was examined to determine if any

different cell types existed within this subnucleus. These findings were then compared with those already uncovered within the ICc (Sivaramakrishnan and Oliver; 2001) and evaluated on the limited results found in the ICx (Li, *et al.*, 1998).

Also, the characteristics of the hyperpolarization-activated inward current I_h , were examined, including analysis of voltage-current relationships, reversal potential and voltage-dependent activation.

2.2 Purpose 2: Contribution of Ca^{2+} to neurons excitability

In response to depolarizing current injection, some neurons have shown an underlying Ca^{2+} -mediated conductance when the Na^+/K^+ components are pharmacologically removed. ICx neurons were studied to see if such an underlying conductance was present and its properties were characterized. In response to hyperpolarizing current injection, studies have shown a Ca^{2+} -mediated after-hyperpolarization hump or “rebound”. The presence of this rebound hump was examined using ionic manipulations of the ACSF.

2.3 Purpose 3: Analysis of synaptic responses

Synaptic responses elicited in the ICx via stimulation of fibers from the ipsilateral ICc were examined. First, responses were studied in reaction to varying levels of electrical stimulation. The resultant responses were qualitatively categorized as an excitatory, inhibitory or mixed response.

Next, pharmacological analyses were performed to chemically dissect the synaptic responses. This pharmacological methodology included the examination of the

“classical” neurotransmitters (i.e. glutamate, glycine, GABA) along with 5-HT. These drugs were used to examine the contribution of AMPA, NMDA, and GABA_A receptors to synaptic transmission and to the overall synaptic response. The possible modulatory role of 5-HT was examined on synaptic currents with no other pharmacological agents and on pharmacologically isolated currents. This diversity in transmitter analysis should support the multi-sensory role that the ICx plays, confirming the various inputs (auditory/non-auditory) that it receives.

3. Materials and Methods

3.1 Brain slice preparation

Brain slices were obtained from young albino rats (*Wistar, Charles River Co., Quebec, Canada*) between 8 and 11 days of age. The animals were euthanized by decapitation, and their brains were removed (*see figure 4*). Once removed, the brain was placed in heated oxygenated artificial cerebrospinal fluid (*ACSF*) at a temperature range of 31°C-33°C. The auditory midbrain was blocked and mounted on the stage of a tissue slicer using cyanoacrylate adhesive. Brain slices were cut at 200 µm in the frontal plane through the auditory midbrain with a Vibratome tissue slicer. An intact slice containing the structure(s) of ICx, ICc, and ICd was selected on the basis of structural clarity (*figure 1,5*), and placed in a small recording chamber (*figure 4*). The remaining tissue slices were then transferred to a beaker containing oxygenated saline at room temperature for subsequent recordings.

An oxygenated saline solution circulated continuously through the recording chamber and around the brain slice during the course of the experiment. The slice was held in the chamber with two pieces of nylon mesh and two magnetic clips, completely submerged in the saline (*figure 3, 4*). The volume of the chamber was ~1.5 ml and the flow rate of the saline was between 6-8 ml/min. The temperature of the saline was monitored and maintained at between 30°C - 32°C. The saline solution consisted of (*in mM*) 129 NaCl, 3 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, 3 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10 glucose in de-ionized water collected using a Milli-O synthesis water filtration unit (*Millipore, France*), saturated with 95% O₂-5% CO₂ before it was circulated through the brain slice chamber.

The pH was tested prior to each experiment and was adjusted with concentrated HCl or NaOH to 7.4 after complete saturation with O₂-CO₂ mixture. The osmolarity of the ACSF was tested using a Vapro vapor pressure osmometer 5520 (*Viescor Instruments, Utah*) regularly between experiments and had a range of 310-330 mOsm/kg. The brain slice was housed in the recording chamber for approximately fifteen minutes before any recordings were made.

3.2 *Visual whole-cell patch clamp recording*

The brain slice chamber was illuminated from below by light passed through a Hoffman modulation contrast system into a Zeiss Axioskop microscope, so that the structures of the auditory brainstem were clearly visible. The outlines of the subdivisions of the IC (*ICx, ICc, and ICd*) were first identified using a low power (10x) objective lens (*figure 1,4*). A bipolar tungsten stimulating electrode was placed in the ICc in close proximity (~1 mm lateral medial) to the intended recording site in the ICx (*figure 5*). Then under high power microscopy (40x objective lens), the neurons of the ICx were examined to reveal neurons that appear “healthy” based on the clarity of their surface membrane, proportionality of their morphology and overall visibility. The chosen neuron was also in close proximity to the stimulation site (~1 mm lateral medial).

The patch pipettes were produced using a Narishige PP-830 vertical puller (*Narishige, Japan*), from 1.1 mm OD and 0.8 mm ID thin-walled glass tubing (*Kimax-51, Kimble*). The glass microelectrodes were filled with an internal solution of the following composition (*in mM*): 130 K gluconate, 2 MgCl₂, 5 KCl, 0.3 GTP, 2 ATP, 0.6 EGTA, 10 HEPES. The internal solution had a pH range of 7.20-7.25 and an osmolarity of 303 mOsm/kg. The impedance of the electrodes was between 4-6 MΩ. The visual

whole-cell patch configuration was achieved utilizing the “blow and suck” procedure done in voltage clamp mode. While in voltage clamp mode, small voltage steps were passed and the changes in current responses were observed throughout the recording. The patch clamp method will be discussed in further detail later. First, under high power (40x objective lens) the recording electrode was brought into focus in the center of the field of view and its impedance was verified and the shape (i.e. slope and straightness) of its tip was assessed. In ideal situations the impedance of the electrode was approximately 5 M Ω and the tip was clean with no debris attached. If these factors were within acceptable limits, the electrode was positioned above a pre-selected neuron and then lowered with a fine three-dimensional manipulator (*Newport, 423 series*) towards its surface. While being lowered, positive pressure (“*blow*”) was applied by mouth through the electrode holder to prevent the electrode from being clogged by tissue or debris (*figure 6.1*). After the electrode had touched the tissue there was an increase in the impedance of the electrode (*figure 6.2*). Once the tip of the electrode had hit the neuronal membrane, suction was applied creating a gigaohm seal (*figure 6.4*). Waiting for approximately one minute after the gigaohm seal had been reached, sometimes helped in further stabilization of the seal. A whole-cell patch clamp recording was achieved by further application of sudden negative pressure by mouth through the electrode holder, tearing the cell membrane (*figure 6.5*). The changes in the square wave, as seen in figure 6, were used as an indicator apprising the experimenter about the proximity of the electrode to a cell and when a gigaohm seal had been made. An EPC 8 patch clamp amplifier (*HEKA, Germany*) was used for the whole-cell patch-clamp recordings. The data were initially filtered by the amplifier at 5 kHz, and were then digitized and analyzed at a

sample rate of 10 kHz using pClamp 6.0.5 software (*Axon Instruments Inc., USA*) on an IBM-compatible PC.

3.3 *Whole-cell patch clamp methodology*

In voltage clamp mode, the holding potential was set to the expected membrane potential of the neuron since most cells survive best near the resting potential (set at -60 mV). Once the whole-cell configuration had been achieved, the capacitance transients at the start and end of the voltage pulse appeared because the cells capacitance was added to the pipette capacitance (*figure 6.5*). Throughout the duration of the recording, the series resistance was maximally compensated with the following protocol.

In whole-cell voltage clamp recording, the membrane potential of the cell was controlled by the voltage applied to the pipette electrode. This control of potential was not perfect, but depended on the size of the access resistance between the pipette and the cell interior, and on the size of the currents that must flow through this resistance. This access resistance is called *series resistance*, named so because it constitutes a resistance in series with the pipette. The series resistance refers to the pipette resistance and another major part that arises from the residual resistance of the broken patch membrane. Low values of the series resistance are desirable; approximately two times the resistance of the pipette alone. When the command voltage was stepped, a large amount of current flowed into the pipette capacitance during the transition from one potential to the next. This transient can be minimized if the fast transient associated with the pipette capacitance is attenuated. Minimization of the fast capacitance transients also allows for stable series-resistance compensation. Uncompensated pipette capacitance has a

detrimental effect upon the stability of the series-resistance correction circuitry. In an ideal experiment, the resistance of the whole-cell patch electrode would be zero but this is very seldom the case. Series resistance compensation uses positive feedback in an attempt to electronically achieve this ideal situation.

Once a gigaohm seal had been formed, the oscilloscope displayed a pair of capacitance transients of opposite polarity. These transients were attributed to the pipette capacitance, and were compensated using the *c-slow* and *g-series* control dials on the amplifier. The *c-slow* control was used to cancel the capacitance transient, while the *g-series* control adjusts the conductance in series with the capacitance. These two controls were used interactively until the transient had been minimized. Eventually, with continued adjustment it was possible to have the transients sink into the background noise and achieve a stable baseline. Finally, the series resistance compensation was adjusted using *%-COMP* controls to a maximal value with a lag of 20 μ s. If the compensation was set too high (>95%), oscillations would destabilize the seal. The amount of compensation that was possible before oscillations occurred was related to two factors. First, as the level of compensation approached 100%, the increase in the command potential approached infinity, causing the electronic circuits to saturate. Secondly, since this system ran on positive feedback, the stability of the circuit was degraded by the feedback and at high levels of compensation the circuit became an oscillator (Sherman-Gold, 1993). All of the above mentioned compensations were readjusted throughout the recording to keep the transients minimized. The series resistance was recorded for each cell and had a range of 10 – 20 M Ω , and were compensated by at least 75%. Also, the series resistance was monitored throughout the

recording and when there were any significant changes in the series resistance the recording was terminated.

When a whole cell patch was achieved, some criterion were used to determine whether an acceptable recording had been accomplished and further analysis should be done. If the neuron displayed a marked change in input resistance or a noticeable alteration in either shape or amplitude of the synaptic response at a repeated stimulus intensity, then the data was discarded. Also, the “test seal” function (*pClamp 6.0.5*) was constantly monitored to ensure that the seal and its transients were relatively stable with little fluctuation during the period of recording. Furthermore, if the resting membrane potential (*RMP*) of the cell was at any point during the recording -45 mV or less negative, the recording procedure was aborted. If the *RMP* of the neuron seem unstable, fluctuating ± 10 mV during the recording, the cell was discarded. If synaptic responses were to be studied, the cell had to undergo a series resistance compensation of at least 75%.

3.4 Membrane properties

After the above manipulations were completed, the amplifier was set to operate in current clamp mode, seen as CC_{fast} , to examine the membrane properties and synaptic responses. However, synaptic responses were also recorded in voltage clamp mode since the holding potential was kept stable, further supporting the results seen in current clamp mode. Synaptic potentials or currents underlying the synaptic events were elicited via electrical stimulation of the corresponding afferent pathway from the ICc or the ICx. The bipolar stimulating electrode was constructed from paired tungsten wires with a tip

separation of 50 μm . Electrical stimuli were either positive or negative square waves, 100 μs in duration, obtained from a Grass S-8800 stimulator and stimulus isolator unit.

The current-voltage (IV) relationships and firing patterns were examined for each of the neurons in response to current injection over the range of -300 to 1000 pA, applied in 10 pA and 20 pA increments, with a duration range of 100 to 300 ms. To study the possible diversity of firing patterns, neurons were also subjected to a prehyperpolarizing current injection of -200 pA for a duration of 250 ms prior to a depolarizing current injection range of 0 to 200 pA, applied in increments of 10 pA for 300 ms. A second prehyperpolarizing protocol was also employed which first applied a prehyperpolarizing current injection range of -200 to 0 pA, in 10 pA increments for a duration of 250 ms followed by a single depolarizing current injection set at an intensity which consistently produced several APs (4 or more spikes) for that individual neuron, for a duration of 300 ms.

If the neuron displayed a hyperpolarization activated inward current (I_h) in response to hyperpolarizing current injection, the amplifier was switched to voltage clamp mode and the series resistance of the cell was compensated with %COMP to 75% or higher. Several protocols were administered, which clamped the neuron at various depolarizing and hyperpolarizing voltage steps from a resting level of -60 mV to examine the effects upon I_h . One protocol clamped the neuron from a resting potential of -60 mV and subjected the cell to hyperpolarizing voltage steps to -150 mV. The 300 ms and 500 ms voltage steps caused a jump in current termed instantaneous current (I_{ins}). At the end of the 300/500 ms voltage step the inward current then increased slowly to a steady-state level (I_{ss}). Voltage-current plots could be plotted from this data, measuring the current at

the varying voltage steps for both I_{ins} and I_{ss} . The difference between I_{ins} and I_{ss} was representative of the hyperpolarization activated current, I_h . The methods described by Bayliss *et al.* (1994) and Mayer and Westbrook (1983) were used to estimate the reversal potential of I_h in the ICx. To assess the reversal potential (E_h), ICx neurons which displayed I_h were subjected to depolarizing voltage step to -60 mV and to hyperpolarizing voltage steps reaching -150 mV from a holding potential of -130 mV. This data was used to create voltage-current plots with both the I_{ins} from this data and the I_{ins} from the data derived from the previous voltage-current plot (responses to voltage steps from a holding potential of -60 mV to -150 mV). The slopes of the voltage-current relationships represented the membrane conductance at each holding potential. These points were then fitted with linear regression in order to extrapolate the points to determine their point of intersection. The voltage at the intersection of the two lines was an estimate of E_h .

To examine the voltage dependence of I_h in ICx neurons, the methods described by Kamondi and Reiner (1991) were employed. The membrane potential was held at -60 mV and then stepped towards -150 mV for 500 ms. This conditioning hyperpolarizing step was employed to activate I_h . Next, the neuron was clamped at a hyperpolarizing test voltage step of -150 mV, where I_h appeared to be fully activated (I_{-150}). The currents observed during this pulse represented the proportion of I_h that was not fully activated during the conditioning hyperpolarizing step. The voltage steps elicited the slowly deactivating tail currents (I_{tail}). I_{max} represents the maximum current that was recorded at -150 mV after the conditioning hyperpolarizing step. I was the difference between I_{max} and I_{tail} , representing the activation portion of the amplitude of the current recorded at I_h .

The activation curve of I_h was derived by plotting the normalized current (I/I_{max}) against the membrane potential. The data derived was fitted by a Boltzman function

$$I/I_{max} = 1 / \{1 + \exp[k(V - V_{1/2})]\} \quad (1)$$

$V_{1/2}$ is the membrane potential at which the current is half-maximally activated and k is the slope factor.

3.5 Firing patterns

When a neuron was encountered and it was deemed acceptable for further analysis, it was classified based on firing pattern in response to depolarizing current injection. The cell was subjected to a range of positive current injection from 0 to 300 pA for 200 ms. When a train of APs was elicited at a sufficient level of current injection, the neuron was categorized based on the appearance of its firing pattern as *regular-type*, *adaptation-type*, *pauser-type* or *bursting-type*. The criteria used to determine the cells firing pattern was as follows; in regular cells the interspike interval is uniform or equal throughout the spike train. In adaptation neurons the first interspike interval has a shorter duration than the subsequent interspike intervals. Furthermore, the last interspike interval must be at least 50% longer than the first interspike interval of that spike train. For pauser neurons the first interspike interval has a longer duration than the subsequent interspike interval. In these pauser cells, an initial “pause” should be followed by a sustained train of APs with a shorter, more regular interspike interval. The classification of pauser-type cell is to verified by subjecting the neuron to a series of prehyperpolarizing protocols which delivers varying levels of hyperpolarizing current injection (0 to -200 pA) before administering levels of depolarizing current sufficient

enough to elicit a spike train (0 to 200 pA). If the “pause” is still clearly evident in these traces, the cell will be categorized as pauser-type. A cell is to be classified as bursting if it displays “bursts” of activity seen as several (i.e. 4-5) APs arising from a depolarizing hump, followed by a period of silence. If the depolarizing current injection is sufficient, a second or third period of bursting activity should be seen. The firing pattern of the neuron was verified at varying levels of depolarizing current injection.

3.6 *Synaptic response measures*

When neural responses were encountered, the stimulus intensity was adjusted to produce reliable postsynaptic responses. The graded postsynaptic responses were examined by the systematic increase of stimulus intensity with a stimulus duration of 0.2 ms. This led to a stepwise or *graded* increase in the amplitude of the synaptic response, and in some cases led to a suprathreshold response. Synaptic responses were recorded first in current clamp mode, then the same stimulus intensities were used to examine the synaptic responses in voltage clamp mode. If a graded response was observed, and the synaptic currents were clearly evident, the cell was subjected to pharmacological analysis, also in voltage clamp mode.

3.7 *Pharmacology*

Once a postsynaptic response had been elicited, pharmacological manipulations were conducted to elucidate the nature of transmission in the ICx. First, responses were identified qualitatively as either excitatory, inhibitory or a combination of the two (*mixed*). The glutamatergic nature of the excitatory responses were examined using the

AMPA receptor antagonist, CNQX (10 μ M) and the NMDA receptor antagonist, APV (25 μ M). The inhibitory responses were tested for GABA_A and glycinergic activity with application of the antagonists bicuculline (20 μ M) and strychnine (0.5 μ M) respectively. Furthermore, the possibility of an underlying Ca²⁺ ionic component in APs was considered by removal of the Na⁺ and K⁺ components using their respective antagonists TTX (1 μ M) and TEA (5 mM). If an underlying Ca²⁺ conductance was realized, its identity was substantiated by bathing the cell in a 0 Ca²⁺-ACSF. Finally, to examine the interaction of serotonin (5-HT) with ICx neurons, 20 μ M 5-HT was administered when a reliable synaptic response was achieved both with and without the above mentioned pharmacological agents. Concentrated stock solutions of the above mentioned drugs were prepared in de-ionized water, aliquoted, and stored at -15°C. When required, small amounts of the stock solutions were diluted into heated, oxygenated ACSF until the desired concentration had been attained, and was then delivered through the recording chamber. Once the effect or non-effect of the pharmacological agent(s) had been seen, delivery was stopped and the brain slice was perfused in normal ACSF. On average, significant effects of the drug were observed within approximately 7-10 minutes or perfusion was terminated. If no effect was seen within this time period, the pharmacological manipulation was deemed as having “no effect”. When an array of antagonists was to be added, they were applied in succession. Each was administered after the previous drug actions had stabilized or showed no significant changes for at least 2-3 minutes. Following this the next antagonist was introduced in addition to the preexisting pharmacological agent(s). After the entire schedule of antagonists had been

employed the neuron was washed in normal saline until a response similar to the control returned, usually in 15-20 minutes.

3.8 Data Analysis

The change in membrane potential elicited via intracellular injection of negative current and subthreshold positive current was obtained by measuring between the baseline membrane potential and the peak hyperpolarization or depolarization respectively. These values were then used to plot the current-voltage (IV) relationships. From these curves, the slope was calculated from a range of values which were approximately linear. This slope represented input resistance (R_{input}) of the cell membrane, measured in $M\Omega$. The time constant (τ) of the cell membrane from these neurons was determined by measuring the time took the membrane potential to move 63% of the way towards its final value in response to a hyperpolarizing current pulse.

All AP analyses including amplitude, half-width duration, and frequency were done using responses at levels of current injection which consistently achieved firing threshold. The threshold current for firing was the level of positive current injection that was required to elicit at least one AP. Amplitude measurements were taken from the base of the spike to the peak, and not from the beginning of the depolarization. The AP duration measurements were taken at 50% amplitude of the spike. Some ICx neurons displayed an *after-hyperpolarization potential* (AHP) in response to positive current injection. If the neuron displayed an obvious AHP, at the threshold current for firing the amplitude and duration of the undershoot was determined. The properties of the *single spike AHP* were derived using the RMP of the cell as baseline. The amplitude was

calculated by measuring the maximum deviation of the AHP from the AP threshold to baseline. The duration was calculated by measuring the duration of the AHP once the falling phase of the AP had passed the threshold for firing, until the undershoot returned to this level. Furthermore, in response to 300 pA of depolarizing current injection some neurons displayed an AHP after the spike train. This undershoot was termed *post-depolarization AHP* and the amplitude was measured from the end of the current step at baseline to the maximum deviation from baseline.

When neurons displayed consistent AP trains in response to increasing levels of positive current injection, the firing frequency or rate-level function (RLF) was calculated. The RLF was derived from a current injection range of 0 to 1000 pA for a duration of 200 ms. The firing frequency was determined by observing the number of APs that were elicited at a certain current injection level, divided by the duration of the injection (200 ms).

The data for this study acquired using the HEKA EPC 8 amplifier was stored on a PC and analyzed using the Clampfit module in pClamp 6.0.5 and pClamp 8.0. The analytical cursors within the program were used to measure the various analyses mentioned above. Statistical analyses were conducted with SPSS 8.0 and Microsoft Excel, using paired *t*-tests, and one-way ANOVA. Statistical tests were performed at a significance level of $\alpha = 0.01$ and 0.05 (*as indicated for each measure*) and was deemed non-significant if the probability was greater than 0.10 ($p > 0.10$). Averaged data were presented as mean $\pm$ SE, unless noted otherwise.

The Animal Care Committee of Carleton University has approved all procedures involving the use of the animals reported in this study, in accordance with the guidelines of the Canadian Council on Animal Care.

4. Results

The data presented here are based upon patch-clamp recordings from 99 ICx neurons. The analysis of intrinsic membrane properties were grouped based on the cells observed firing pattern in response to positive current injection (i.e. *Regular*, *Adaptation*, *Pauser*).

Each ICx neuron encountered was subjected to intracellular current injections ranging from 300 to -300 pA, and the data derived from these measures was used to calculate the current-voltage relationship for that particular cell. From a randomly chosen sample of these cells, measurements were made of, resting membrane potential (RMP), input resistance (R_{input}), time constant (τ), AP properties and AHP analysis (*see figure 14B for summary*).

4.1 Intrinsic membrane properties

4.1.1 Firing pattern: regular-type

Figure 7A shows traces from a representative *regular-type* neuron in response to both positive and negative current injection. The top two traces display the cells firing pattern in response to positive current of 60 and 110 pA for 200 ms. The data showed that the interspike intervals are uniform or “regular” throughout the spike train. The bottom two traces are in response to negative current injection at -80 and -200 pA. Figure 7B illustrates the linear current-voltage relationship for the representative regular-type cell, derived from its response to various levels of current injection. The slope from linear portion of the current-voltage relationship was used to calculate the R_{input} . The

mean R_{input} for the regular ICx neurons was $315.0 \pm 15.8 \text{ M}\Omega$ ($n = 48$), the mean RMP was $-57.7 \pm 2.9 \text{ mV}$ ($n = 48$) and τ was $18.3 \pm 0.9 \text{ ms}$ ($n = 48$).

4.1.2 Firing pattern: adaptation-type

Adaptation-type was another firing pattern which was evident in ICx neurons. Figure 8A illustrates traces from a representative adaptation-type neuron in response to both positive and negative current injection. The top two traces display the cells firing pattern in response to positive current of 190 and 260 pA for 200 ms. The data showed that the first interspike interval had a shorter duration than the subsequent interspike intervals. According to the criteria for defining an adaptation cell, the last interspike interval must be at least 50% longer than the first interspike interval of that spike train. This absolute difference in interspike interval duration was more pronounced at lower levels of positive current injection (190 pA vs. 260 pA) as can be seen in figure 8A. The neuron seemed to “adapt” to the sustained current injection and showed a longer interspike duration at the end of the spike train. The bottom two traces are in response to negative current injection at -80 and -160 pA.

Figure 8B illustrates the linear current-voltage relationship for the representative adaptation-type cell, derived from its response to various levels of current injection. The mean R_{input} for the adaptation ICx neurons was $241.7 \pm 12.1 \text{ M}\Omega$ ($n = 14$), the mean RMP was $-57.9 \pm 2.9 \text{ mV}$ ($n = 14$) and τ was $12.0 \pm 0.6 \text{ ms}$ ($n = 13$).

4.1.3 Firing pattern: pauser-type

Another type of ICx neuron encountered was the *pauser-type* cells. Figure 9A illustrates traces from a representative pauser-type neuron in response to both positive and negative current injection. The top two traces display the cells firing pattern in response to positive current of 100 and 130 pA for 200 ms. The data shows that the first interspike interval had a longer duration than the subsequent interspike interval. In these pauser cells, an initial “pause” was followed by a sustained train of APs with a shorter, more regular interspike interval. This difference in interspike interval duration was more pronounced at lower levels of positive current injection (100 pA vs. 130 pA) as can be seen in figure 9A. The neuron expressed a “pause” in the first interspike interval. The bottom two traces are in response to negative current injection at -40 and -100 pA.

Figure 8B illustrates the linear current-voltage relationship for the representative pauser-type cell, derived from its response to various levels of current injection. The mean R_{input} for the pauser ICx neurons was $276.9 \pm 13.8 \text{ M}\Omega$ ($n = 9$), the mean RMP was $-58.1 \pm 2.9 \text{ mV}$ ($n = 9$) and τ was $12.7 \pm 0.6 \text{ ms}$ ($n = 7$).

In order to substantiate the classification that pauser neurons were given, the cells also underwent a protocol which delivered a prehyperpolarizing current injection before the normal depolarizing current injection. This was done first with a fixed prehyperpolarizing current injection followed by varying levels of depolarizing current injection. Figure 10A illustrates three traces from a representative pauser ICx neuron at varying levels of depolarizing current injection for 300 ms after receiving a prehyperpolarizing current injection of -200 pA for a duration of 250 ms. The bottom two traces clearly showed a longer duration of the first interspike interval or “pause”. As

the level of positive current injection was increased, the pause duration became less pronounced as illustrated in the top trace. Figure 10B depicts this negative linear relationship between level of positive current injection and the duration of the first interspike interval or pause. The pause duration at the threshold level of current injection was used as the baseline or control value with which all other durations were normalized against. The chart shows that as the current injection level was increased the normalized duration of the first interspike interval decreased.

Each pauser neuron also underwent a protocol with varying levels of prehyperpolarizing current injection followed by a fixed depolarizing current injection. Figure 11A illustrates three traces from the same pauser ICx neuron as figure 10, after receiving a prehyperpolarizing current injection from -190 to -50 pA for a duration of 250 ms followed by a depolarizing current injection of 100 pA for 300 ms. The depolarizing current injection level was chosen at a level that elicited a spike train. The bottom two traces clearly show the longer duration of the first interspike interval or “pause”, evident at more negative levels of prehyperpolarizing current injection. As the level of prehyperpolarizing current injection became less negative, the pause duration was less pronounced as illustrated in the top trace. Figure 11B depicts this positive linear relationship between level of negative prehyperpolarizing current injection and the duration of the first interspike interval or pause. The pause duration at the most negative level of prehyperpolarizing current injection (200 pA) was used as the baseline or control value with which all other durations were normalized against. The chart shows that as the prehyperpolarizing current injection level became more negative the normalized duration of the first interspike interval increased.

4.1.4 Firing pattern: bursting-type

The only remaining firing pattern that was observed in the ICx neurons was the *bursting-type* cell. Figure 12A illustrates traces from a representative bursting-type neuron in response to both positive and negative current injection. The top two traces display the cells firing pattern in response to positive current of 100 and 210 pA for 200 ms. The bottom two traces are in response to negative current injection at -40 and -100 pA. The data showed that these neurons displayed “bursts” of activity seen as depolarizing humps comprised of several (i.e. 4-5) APs followed by a period of silence. If the depolarizing current injection was sufficient, a second or third period of bursting activity could be seen. This observation is illustrated in figure 13, which outlines five traces from a bursting-type ICx neuron at various levels of depolarizing current injection. Notice the long undershoot visible after the AP, seen at the threshold current injection level (*bottom trace*, 90 pA). As the level of current injection was increased, the number of spikes increased but the undershoot persisted. The APs became grouped together and created a burst of activity at lower levels of current injection. At higher levels of current injection (*top trace*, 220 pA) the long undershoot persisted, influencing the occurrence of the following bursting episode. Figure 12B illustrates the linear current-voltage relationship for the representative bursting-type cell, derived from its response to various levels of current injection. A comprehensive analysis of the intrinsic membrane properties was not conducted upon this subpopulation of ICx neurons because of their low occurrence (*see figure 14A*).

4.1.5 Cell type distribution, R_{input} and τ

The distribution of the various firing patterns observed in the ICx neurons is outlined in figure 14. Figure 14A shows that the ICx neurons predominantly displayed the regular-type firing pattern at 68% ($n = 67$), followed by the adaptation-type with 17% ($n = 17$), and pauser-type with 12% ($n = 12$). The bursting-type represented only a very small proportion of the ICx neurons with only 3% ($n = 3$), and so this firing pattern was not included in any of the quantitative analysis. This firing pattern was only included to illustrate its presence in the ICx and to demonstrate its limited contribution to the overall population of ICx neurons. All of the intrinsic membrane properties for the three main firing groups are outlined in figure 14B, including the n number, mean value for each group and the standard error for each parameter analyzed. This summary chart can be used in reference to the figures (*figures 14-16, 20-21*), which individually examine the trends of each of those intrinsic membrane measures.

The R_{input} for each firing pattern has been presented, however figure 15 examined this measure across the three groups. The regular-type ICx neurons ($n = 48$) showed a significantly higher mean R_{input} in comparison to the adaptation cells ($n = 14$) ($p < 0.02$)(●), but there was no statistical significance between regular and pauser-type cells ($n = 9$) and adaptation and pauser cells ($p > 0.10$). Analysis of τ between the groups illustrated in figure 16 showed that there was some significant difference. The regular cells ($n = 48$) had a statistically significant longer τ than the adaptation cells ($n = 13$) ($p < 0.02$) (●), and the pauser cells ($n = 7$) ($p < 0.10$) (▲). There was no statistically significant difference of the τ between the adaptation and pauser-type cells ($p > 0.10$).

4.1.6 Analysis of APs

An analysis of the amplitude and half-width duration of the AP was examined in figure 17. Figure 17A outlined the mean AP amplitude for each firing pattern group, and showed that the pauser cells ($n = 7$) displayed a significantly smaller amplitude than both the regular cells ($n = 49$) ($p < 0.02$) ($\blacktriangle$) and the adaptation cells ($n = 13$) ($p < 0.04$) ($\circ$). The regular and adaptation cells however, showed no significant difference in their respective mean AP amplitude ($p > 0.10$). The comparison of mean half-width duration between groups is outlined in figure 17B, and only the difference between the regular and adaptation-type cells was statistically significant ($p < 0.03$).

The APs elicited in the ICx were studied further pharmacologically in order to validate the ionic basis of their conductances. For this measure, the analysis was not conducted across groups but instead was done for the entire population of ICx neurons as a whole. Figure 18 presents a representative neuron with a typical train of spikes, as seen in the top trace. The APs shown here are in response to intracellular current injection of 90 pA for 300 ms. Next, the neuron was subjected to bath application of 5 mM TEA (*blue trace*) at the same level of current injection as in the control trace, in order to block the K^+ channels. Notice that the cell was still able to fire, however the duration of the APs became longer and the overall firing frequency decreased. Next, 1 μ M TTX was added to the TEA to examine its effects upon the Na^+ channels (*red trace*). Once added to the TEA solution, the TTX completely abolished the APs and there was no obvious voltage-dependent conductance. The significant effects of both K^+ and Na^+ verified their ionic involvement in mediating the APs elicited from the neurons in the ICx. This same pharmacological analysis using TEA and TTX was conducted on twenty ICx neurons

with equivalent results. After several minutes of wash in saline to remove the TEA and TTX, the cell was eventually able to once again fire APs but a higher current injection was required (170 pA) for this neuron.

4.1.7 Ca^{2+} -mediated conductances

In some instances, after TEA and TTX had both been applied collectively an underlying response was revealed. Figure 19 shows several traces in response to positive current injection (270 pA) for 300 ms. The top trace shows a regular firing pattern, but after the application of 1 μ M TTX and 5 mM TEA the neuron could not generate classical Na^+/K^+ channel dependent APs. Removal of the Na^+ and K^+ conductances by TTX and TEA respectively, revealed an underlying response. First, there was an early “spike” indicated by the (●), followed by two “humps” designated by the (○). The early spike had a larger amplitude and shorter duration than the two latter humps, however both were smaller in amplitude and longer in duration in comparison to the APs shown in the control trace (*top trace*). In order to examine the possibility that Ca^{2+} was mediating these underlying responses, the neuron was bathed in saline that contained 1 μ M TTX and 5 mM TEA but had 0 Ca^{2+} . The results showed removal of both the spikes and the humps (figure 19; 2nd trace from bottom), thus supporting the notion that they were mediated by Ca^{2+} . The presence of these underlying Ca^{2+} mediated responses revealed after TEA/TTX application was found in 70% ($n = 14$) of the ICx neurons tested ($N = 20$). In many of the neurons where the Ca^{2+} mediated spikes/humps were evident, the cell required a higher positive current injection in comparison to the control in order to reveal Ca^{2+} mediated responses after application of TTX/TEA.

Figure 20 illustrates another Ca^{2+} mediated conductance found in ICx neurons. When hyperpolarizing current steps were injected into ICx neurons, anode-break spikes or slight depolarizing humps were typically observed upon release of the hyperpolarizing current 75% of the time (74 of 99 cells). However, in 25% of ICx neurons (24 of 99 cells) the anode-break spike was followed by a broad, “rebound” depolarization. Figure 20 shows traces from a representative neuron in response to negative current injection (*top trace: from bottom; -300, -220, -180, -120, -80 pA*). After the hyperpolarizing current steps, a spike was observed followed by a long lasting depolarizing hump (*top trace*). Once the cell was bathed in a 0 Ca^{2+} solution, the “rebound” potential was completely removed (*blue trace*). These observations suggest that this rebound potential was mediated by Ca^{2+} . This Ca^{2+} -mediated rebound potential returned once washed in normal saline (*bottom trace*). When examined by firing pattern, the results showed that only 22% of regular-type cells (15 of 67), 42% of adaptation-type cells (7 of 17) and 17% of pauser-type cells (2 of 12) in the ICx expressed this Ca^{2+} -mediated rebound potential. Another characteristic which was evident in these “rebound” cells was the latency of the initial anode-break spike. When a Ca^{2+} -mediated rebound potential was present, the anode-break spike occurred with a smaller delay after the end of the hyperpolarizing current step in comparison to the “non-rebound” cells (*data not shown*). In both types of cells (rebound/non-rebound), the anode-break spike appeared with shorter delays at increasing levels of current injection. Another important observation was that rebound cells, generally required a smaller amount of hyperpolarization to produce the anode-break spike compared to the non-rebound cells.

In response to depolarizing current injection, many ICx neurons displayed a hyperpolarizing potential which would gradually depolarize towards RMP. Figure 21 examines the comparison of the mean AHP after depolarizing current injection. Figure 21A outlines the criteria which were used in order to calculate the amplitude of the AHP after the current injection termed, *post-depolarization AHP amplitude*. In order to maintain some degree of consistency, the post-depolarization AHP amplitude measured for each of the ICx neurons in the comparison was in response to 300 pA for 200 ms. Figure 21B presents the comparison of the mean post-depolarization AHP amplitude for the firing patterns encountered in the ICx. The analysis found that there was no statistical difference ($p > 0.10$) between the any of the three groups (regular $n = 45$; adaptation $n = 12$; pauser $n = 9$). Thus, the data show that regardless of firing pattern, the neurons of the ICx display a similar post-depolarization AHP in response to positive current injection.

The AHP analysis was also examined for the single-spike undershoots seen in the ICx neurons. In response to positive current injection, the elicited AP in some ICx neurons would express a pronounced undershoot termed *single-spike AHP*. Figure 22A1,B1 outline the criteria that were used when determining the amplitude and duration of the AHP after an AP had fired. Figure 22A2 shows that there was no statistically significant difference ($p > 0.10$) in the average single-spike AHP duration between the ICx neurons in each firing group (regular $n = 22$; adaptation $n = 12$; pauser $n = 7$). The analysis of average single-spike AHP amplitude in figure 22 also shows that there was no statistically significant difference ($p > 0.10$) in the average single-spike AHP amplitude

between the ICx neurons in each firing group (regular $n = 37$; adaptation $n = 12$; pauser $n = 9$).

The single-spike AHP when closely examined, showed the undershoot was comprised of two components. First, an early sharp hyperpolarization was seen followed by a longer duration undershoot, which begins as a further hyperpolarization from the earlier component and slowly reached the threshold for the following spike. Figure 23 illustrates three traces from a representative ICx neuron displaying an obvious single-spike AHP at three different positive intracellular current injection levels (*a*) 570 pA, (*b*) 420 pA, (*c*) 280 pA. The traces show the earlier hyperpolarizing component indicated by the arrow, and the slower undershoot (●). Despite the level of positive current injection and the number of spikes in the train, the dual components of the single-spike AHP are quite evident.

In order to examine the ionic basis of the single-spike AHP, the Ca^{2+} concentration in the ACSF was manipulated. Figure 24 presents traces from a representative ICx neuron displaying a single-spike AHP in response to positive intracellular current injection at (*a*) 100 pA, (*b*) 40 pA, (*c*) 100 pA. The data show that when the neuron was bathed in 0 Ca^{2+} -saline (*trace b*) the single-spike AHP became reduced in amplitude and changed its shape. The undershoot was removed and the interspike interval increased in duration in comparison to (*trace a*). The single-spike AHP returned to that seen in the control trace once washed in normal saline (*trace c*). The ionic manipulations therefore suggest that the single-spike AHP in ICx neurons are to some extent modulated by Ca^{2+} .

4.1.8 Rate level function (RLF)

The ICx neurons encountered were subjected to varying levels of positive intracellular current injection in order to assess the cells ability to fire APs in response to increasing levels of depolarization. The protocol also revealed the threshold of current injection required in order to initiate a spike train, along with the maximum level of current injection at which the cells was able to maintain firing. Figure 25 compares the firing frequency or rate level function (RLF) from a sampled group of neurons representing each firing pattern in the ICx. The regular-type ICx neurons ($n = 10$), shown in (figure 25A) display a linear RLF and show no signs of saturation at a current injection level of 300 pA. Figure 25B illustrates the linear RLF for adaptation-type cells ($n = 10$). However, the average slope of the RLF for these cells was lower in comparison to that for the regular-type ICx neurons. The pauser-type neurons ($n = 8$) also displayed a linear RLF but initiate their spike train at a larger current injection level in comparison to the other two firing patterns. When the average RLF was derived using each of the sample neurons for each firing pattern and plotted against each other, some differences became evident (figure 25D). Within the range of 0 to 300 pA of positive current injection, the average RLF of regular-type ICx neurons was linear. These neurons required a lower level of current injection in order to initiate firing and had a higher maximum firing frequency than the other two types of firing patterns. The adaptation-type neurons displayed an average RLF which was almost parallel to the regular-type cells but had a much lower maximum firing frequency. The pauser-type cells were unique since their average RLF displayed a reprieve before the firing began, thus required a higher level of current injection for initiation. Once the spike train had been initiated, the RLF increased

at a greater rate than the adaptation-type cells at increasing levels of positive current injection. At the maximum level of current injection (300 pA), the pauser-type neurons had an average RLF which was higher than adaptation-type neurons but lower than regular-type neurons.

None of the three firing groups reached saturation of their firing frequency at current injection levels up to 300 pA, so the range of positive current injection was increased to a maximum of 1000 pA. Figure 26 displays the comparison of RLF for a sample ($n = 5$) of each firing pattern in response to the higher positive current injection. Each bar represents the RLF for an individual neuron. The regular-type ICx neurons as a group displayed a higher RLF and were able to sustain spike trains at higher frequencies at higher levels of current injection (*figure 26A*). Two of the five neurons for this subpopulation were able to sustain firing at levels over 900 pA. The adaptation-type cells (*figure 26B*) and the pauser-type cells (*figure 26C*) both displayed lower maximum firing frequencies and showed saturation of the RLF at lower levels of positive current injection in comparison to the RLF of the regular-type cells. The difference in threshold of current injection required for firing is shown graphically in figure 27. The chart compares the level of positive current injection needed in order to initiate AP trains between the three groups of ICx neurons. The data revealed that regular-type neurons ($n = 41$) displayed a significantly lower threshold of current injection required to initiate firing in comparison to both adaptation-type ($n = 13$) ($p < 0.01$)(●) and pauser-type neurons ($n = 9$) ($p < 0.01$)(▲). These results agree with those already shown for the comparison of RLF between the groups of ICx neurons (*figure 25-26*).

4.1.9 Hyperpolarization-activated current (I_h)

While in current clamp, injections of hyperpolarizing current into ICx neurons led some to exhibit an initial hyperpolarization followed by a slowly depolarizing sag toward the resting value. Figure 28A shows three traces in response to varying levels of hyperpolarizing current injection (*from bottom; -400, -260, -140 pA*). The top trace does not clearly express the depolarizing sag, however when a sufficient level of hyperpolarization was achieved the sag became evident. The rate and magnitude of the sag was dependent upon the amount of hyperpolarizing current injected. The current-voltage plots (*figure 28B*) showed an essentially linear relationship for the initial hyperpolarization (Δ), but a non-linear current-voltage relationship for the steady-state of voltage changes ($\bullet$).

In order to examine the voltage-dependence of the hyperpolarization-activated conductance, the ICx which displayed the depolarizing sag were studied in voltage clamp mode. Figure 29A presents traces from a representative ICx neuron which was clamped from a resting potential of -60 mV and subjected to hyperpolarizing voltage steps to -150 mV. The data reveal a slowly activating and deactivating inward current activated at hyperpolarizing membrane levels (I_h). The voltage steps initially caused a capacitive transient and then a sharp increase in current termed instantaneous current (I_{ins}). At the end of the 300 ms voltage step the inward current then increased slowly to a steady-state level (I_{ss}). The voltage-current relationships for both the I_{ins} ($\bullet$) and I_{ss} (Δ) are shown in figure 29B. The difference between I_{ins} and I_{ss} was representative of the hyperpolarization activated current I_h ($\circ$). A marked inward current appeared at membrane potentials more negative than -80 mV. Thus, suggesting that this slowly activating inward current

underlies the depolarizing sag observed during the hyperpolarizing current injection seen in figure 28A, in current clamp mode. In order to verify the time course of the hyperpolarization activated current, the neuron was subjected to the same protocol as outlined in figure 29A, but for a voltage clamp duration of 500 ms (*figure 30*). Figure 30 shows that the current reaches the steady-state well within the 500 ms voltage-change and the results are parallel with those shown for the 300 ms duration (*figure 29*).

ICx neurons which displayed I_h were subjected to depolarizing voltage step to -60 mV where I_h was not active, and to hyperpolarizing voltage steps reaching -150 mV from a holding potential of -130 mV in order to assess the reversal potential (E_h). The methods described by Bayliss *et al.* (1994) and Mayer and Westbrook (1983) were used to estimate the reversal potential of I_h in the ICx. Figure 31A shows traces from a representative ICx neuron in response to voltage changes from -60 to -150 mV from a holding potential of -130 mV. The voltage-current plots in figure 31B display the relationship calculated from I_{ins} ($\blacktriangle$) shown in figure 31A and from I_{ins} ($\bullet$) shown in figure 29A. The slopes of the voltage-current relationships represent the membrane conductance at each holding potential. These points were then fitted with linear regression in order to extrapolate the points to determine their point of intersection. The voltage at the intersection of the two lines was an estimate of the reversal potential of I_h for this neuron (-62 mV).

To examine the voltage dependence of I_h in ICx neurons, the methods described by Kamondi and Reiner (1991) were employed. Figure 32A shows traces from a representative ICx neuron when the membrane potential was held at -60 mV and then stepped towards -150 mV for 500 ms. This conditioning hyperpolarizing step was

employed to activate I_h . Next, the neuron was clamped at a hyperpolarizing test voltage step of -150 mV. The currents observed during this pulse represented the proportion of I_h that was not fully activated during the conditioning hyperpolarizing step. The voltage steps elicited the slowly deactivating tail currents (I_{tail}). The activation curve of I_h was derived by plotting the normalized current (I/I_{max}) against the membrane potential, shown in figure 32B. I_{max} represents the maximum current that was recorded at -150 mV after the conditioning hyperpolarizing step. I is the amplitude of the current recorded at a specific membrane potential after the conditioning step and $V_{1/2}$ is the membrane potential at which the current is half-maximally activated. The data derived from a representative ICx neuron (figure 32A) was fitted by a Boltzman function and showed an S-shaped curve (figure 32B). Using this curve, the $V_{1/2}$ for this ICx neuron was shown to be -100 mV. This same analysis was done for the remaining ICx neurons which displayed a prominent I_h current with similar trends.

4.2 Synaptic responses in ICx neurons

All of the synaptic responses which will be examined in this section were evoked via electrical stimulation of the inputs from the ICc. Recordings were made while in both current clamp mode and voltage clamp mode. All of the synaptic pharmacology was done while in voltage clamp mode in order to minimize shift of the RMP. Figure 33 outlines traces from an ICx neuron in response to varying levels of electrical stimulation of the ICc, in both current and voltage clamp mode. Figure 33A shows three traces exclusively in current clamp mode at increasing levels of stimulation intensity (a) 20 V, (b) 15 V and (c) 10 V. This neuron displays an EPSP at 10V, which increased in

magnitude as the stimulus intensity was increased to 15 V. A suprathreshold response was achieved when the stimulus intensity was increased to 20V with an early AP followed by a slower decaying depolarization. Figure 33B illustrates three traces in response to the same levels of stimulus intensity, however the recordings were done in voltage clamp mode. *Trace c* showed a threshold response to 10V of stimulation. The inward current was more evident at 15 V of stimulation (*trace b*). When the stimulation intensity was increased to 20V, a sharp inward current became apparent followed by a slower longer duration inward current (*trace c*).

4.2.1 Patterns of synaptic potentials

When synaptic responses were encountered in an ICx neuron, varying levels of stimulation intensity were employed to establish the threshold of response and the level of stimulation required to elicit a suprathreshold response, if possible. Furthermore, the synaptic response observed could be classified as excitatory, inhibitory or mixed based on the resultant potential evoked. Initially, no pharmacological agents were applied to the neuron and therefore recordings were made in current clamp mode to facilitate the elucidation of potentials as excitatory, inhibitory or mixed. Figure 34 outlines synaptic response in reaction to various levels of intensity of electrical stimulation in the ICc. Figure 34A shows a cell illustrating a predominantly excitatory response with an epsp visible at 15 V (*bottom trace*). As the intensity was increased to 20V, the amplitude of the epsp increased and the presence of two distinct potentials became evident, one early depolarization with a fast rising phase followed by a slower potential (*middle trace*). A suprathreshold response was achieved at 30V stimulation, with the presence of an early

AP and a slower longer duration epsp. Figure 34B presents traces from an ICx neuron which illustrated a predominantly inhibitory response with a series of ipsp's with varying levels of stimulation. With increasing levels of electrical stimulation (*from bottom, 15V, 20V, 30V*), the amplitude of the ipsp's became larger. Some neurons displayed a combination of epsp/ipsp in reaction to electrical stimulation. Figure 34C displays one such example, with the neuron showing an early epsp followed by a later ipsp at 8V and 9V. However, at 10V of stimulation the early excitatory potential became suprathreshold and displayed an AP which was followed by an ipsp. Some neurons which expressed a mixed response did not achieve a suprathreshold response even at higher levels of electrical stimulation (*figure 34D*). An ICx neuron shown in figure 34D displayed an initial epsp followed by an ipsp (*middle trace*). Even when this neuron was stimulated at 60V a suprathreshold response was not elicited (*top trace*).

For the synaptic responses that were encountered, the synaptic response latency was also measured. This latency was determined from the beginning of the stimulus artifact until the beginning of the synaptic response. For the mixed cells, the latency was measured from the initiation of the artifact until the first response (IPSP or EPSP) was encountered. The cells were categorized based on their response classification; excitatory responses displayed a mean latency of 2.0 ± 0.81 ms ($n = 21$) and inhibitory 2.8 ± 0.82 ms ($n = 4$). There was no significant difference between the mean synaptic response latency between groups. If the entire group of synaptic responses was averaged together regardless of classification, the mean synaptic response latency for ICx neurons was 2.2 ± 0.81 ms ($n = 25$).

4.2.2 Synaptic pharmacology

Once a synaptic response had been realized, and traces were recorded in voltage clamp mode at varying levels of stimulus intensity, pharmacological manipulations were employed to chemically dissect the response. Antagonists specific for certain receptors were administered on the synaptic response cumulatively to examine the effects of each agent respectively. Figure 35 outlines traces from an ICx neuron, which in normal ACSF (*trace a*) displayed a sharp inward current followed by an outward current at a stimulation intensity of 60 V. Upon application of 0.5 μ M strychnine (*trace b*), the outward current was removed but the inward current remained. This suggests that a portion of the outward current was mediated through glycinergic receptors. Addition of 20 μ M bicuculline (*trace c*) to the solution further enhanced the inward current. Removal of this inhibitory current, which was associated with GABA_A receptors, allowed for the expression of an increased inward current. In order to determine the contribution glutamatergic transmission in the synaptic response, 10 μ M CNQX was administered (*trace d*). This showed a decrease in the inward current, suggesting that AMPA receptors were in part responsible. The remaining inward current was removed with the addition of the NMDA receptor antagonist, APV (25 μ M) (*trace e*). The neuron was then bathed in normal ACSF and the inward and outward currents returned. This ICx neuron illustrated an example where several types of receptors were involved in mediating the mixed synaptic response via electrical stimulation of the ICc.

Figure 36 outlines synaptic response traces for an ICx neuron which displayed a prominent excitatory current, with a sharp inward current followed by an epsc at 20V of electrical stimulation (*trace a*). Application of 0.5 μ M strychnine (*trace b*), followed by

20 μ M bicuculline (*trace c*) to the solution had no obvious effect upon the synaptic response. Therefore, it seems likely that there was no significant inhibitory current mediated by glycinergic or GABAergic receptor respectively. When the NMDA receptor antagonist, APV (25 μ M) (*trace d*) was added the inward current was not affected. However, the entire inward current was abolished upon the addition of 10 μ M CNQX (*trace e*). Therefore, in this particular ICx neuron, the observed inward current elicited via electrical stimulation was chiefly mediated through AMPA receptors. After the neuron was washed in normal ACSF (*trace f*), the inward current returned resembling that shown in the control trace.

Figure 37 presents traces from an ICx neuron which was predominantly inhibitory, displaying a prominent outward current at 20V (*trace a*). Application of 25 μ M APV (*trace b*) and 10 μ M CNQX (*trace c*) showed no significant effect on the response. Therefore, this ICx neuron showed little or no glutamatergic receptor mediated excitation. Next, 0.5 μ M strychnine (*trace d*) was added to the existing pharmacological agents to assess the contribution of the glycinergic receptors, but no effect on the synaptic response was evident. The outward current was finally abolished upon the addition of 20 μ M bicuculline (*trace e*), demonstrating the substantial contribution of the GABA_A receptors in mediating the outward current for this neuron. After the neuron was washed in normal saline (*trace f*), the outward current returned.

In some instances an individual neuron displayed a predominantly excitatory or inhibitory response, and so a more detailed pharmacological protocol was employed to examine the response. Figure 38 displays a synaptic response recorded in current clamp mode at 65 V from an ICx neuron. The depolarizing potential was comprised of two

“humps”, with an earlier epsp which transitioned into a later depolarizing potential (*trace a*). The addition of 10 μ M CNQX (*trace b*) blocked the early portion of the and this allowed the later potential to become suprathreshold, eliciting an AP. The next trace shows that after wash in normal ACSF the earlier potential returned, but had become suprathreshold. Notice the location of the AP in *trace c*, being markedly earlier than that shown in trace b. Next, 25 μ M APV was added with no other antagonists (*trace d*), and the later depolarizing potential which was evident in *trace a* and had elicited an AP in *trace b*, had now been removed. To completely remove both excitatory potentials, 10 μ M CNQX was added to the APV solution and the results are outlined in *trace e*. The systematic analysis of the excitatory potentials in this neuron revealed that the earlier component was mediated by AMPA receptors and the latter component by NMDA receptors. The pharmacological analysis did however reveal a small amplitude ipsp (*trace e*), which was removed upon the application of 20 μ M bicuculline and 0.5 μ M strychnine to the APV/CNQX solution (*trace f*). After the neuron was washed in normal ACSF (*trace g*), the epsp returned.

Figure 39 presents traces from an ICx neuron in voltage clamp mode displaying a predominantly inhibitory outward current in response to 80V electrical stimulation (*trace a*). Application of 0.5 μ M strychnine (*trace b*) had no significant effect upon the amplitude of the outward current, thus glycinergic receptors were not mediating the response. However, when 20 μ M bicuculline (*trace c*) was added to the strychnine, the inhibitory outward current was essentially removed, revealing an underlying inward current. The outward current in this particular ICx neuron was therefore mediated mainly by GABAergic receptors. The small excitatory inward current was completely

removed with the application of 10 μ M CNQX to the existing pharmacological agents (*trace d*). Therefore, the inward response of this neuron was mediated by AMPA receptors only, since no APV was needed to remove any inward current. After the neuron was washed in normal ACSF (*trace e*), the outward current returned.

The data presented thus far outline some of the representative synaptic responses encountered in the ICx with stimulation of fibers from the ICc. Before any pharmacological analyses were conducted, the neurons were qualitatively classified as excitatory, inhibitory or mixed. Figure 40A illustrates the distribution of synaptic response found in the ICx with no pharmacological manipulation based on 25 cells. The majority of cells were predominantly excitatory (60%), followed by mixed synaptic responses (24%) and the remaining synaptic responses displayed a dominant inhibitory component (16%). However, once pharmacological manipulations were conducted using receptor specific antagonists, the distribution of each synaptic receptor that mediated the responses could be assessed for the neurons sampled in the ICx ($n = 10$). Figure 40B summarizes the occurrence of each of the receptors studied (*AMPA*, *NMDA*, *GABA*, and *glycine*) for the elicited synaptic responses in these neurons. Note some neurons expressed a certain level of contribution from more than one type of receptor, so overlap could occur. The data showed that AMPA receptors were dominant in mediating the excitatory response with 7 cells displaying their contribution (70%), and GABA_A receptors were central in mediating the inhibitory responses in 6 cells (60%). The other receptor types were present, NMDA (50%) and glycine (40%), but the chart is unable to effectively portray the magnitude of contribution each receptor-mediated conductance added to the overall response. When the contribution was examined more closely, the

AMPA and GABA receptors had a much more significant role in mediating the response when present in comparison to the two other receptor types. When the specific antagonist were applied for AMPA and GABA, the synaptic response would undergo a major reduction as opposed to the antagonists for NMDA and glycine which produced small effects. This suggests that in the ICx, the excitatory currents are mediated by glutamate primarily via AMPA receptors and the inhibitory currents are mediated chiefly by GABA through GABA_A receptors.

4.3 Effects of 5-HT

4.3.1 Modulation of synaptic responses

With the unique connectivity of the ICx in mind, the possible contribution of 5-HT on the synaptic responses was studied from a randomly sampled group of neurons ($n = 7$). Figure 41 presents traces from four individual ICx neurons each displaying a different synaptic response from electrical stimulation of the ICc. Note that the control responses presented in these traces was without any previously introduced pharmacological manipulation. Figure 41A shows a neuron with a prominent excitatory sharp inward current (*top trace*) in response to 20V electrical stimulation, however upon application of 20 μ M 5-HT a reduction in the inward current was evident (*blue trace*). After the application of 5-HT there was a small remaining epsc. When the 5-HT was removed and the neuron was washed in normal ACSF (*bottom trace*), the sharp inward current returned. Figure 41B presents a neuron with a mixed synaptic response at a stimulation intensity of 20V (*top trace*), with a small inward current followed by an outward current. When 20 μ M 5-HT was added (*blue trace*), the excitatory inward

current became suprathreshold and there was also a significant increase in the inhibitory outward current. When the 5-HT was removed and the neuron was washed in normal saline (*bottom trace*), the sharp inward current was removed and the remaining response resembled that shown in the control trace. Figure 41C presents an ICx neuron with a dominant excitatory inward current (*top trace*). Application of 20 μ M 5-HT (*blue trace*) significantly reduced the inward current and revealed an underlying inhibitory outward current. When the 5-HT was removed and the neuron was washed in normal ACSF (*bottom trace*), the inward current returned and the response resembled that shown in the control trace. The final example was illustrated in figure 41D with an ICx neuron displaying two distinct inward currents, an early and late component (*top trace*). Upon application of 20 μ M 5-HT (*blue trace*), the latter excitatory inward current was significantly reduced, but the early inward current remained. When the 5-HT was removed and the neuron was washed in normal saline (*bottom trace*), both the inward currents were present and the response resembled that shown in the control trace. From the data presented for these representative ICx neurons, it was quite evident that 5-HT had some effect upon the synaptic responses elicited via electrical stimulation of the ICc.

4.3.2 Effects of 5-HT on isolated synaptic currents

In order to examine the effect of 5-HT on specific synaptic receptor mediated currents in the ICx, pharmacological manipulations were employed in conjunction with 5-HT application ($n = 7$). Figure 42 shows traces from an ICx neuron in voltage clamp mode in response to 20V of electrical stimulation. The neuron showed a sharp inward current (*trace a*) which was mediated by AMPA receptors since the cell had been bathed

in a solution containing 0.5 μM strychnine, 20 μM bicuculline, and 25 μM APV. Addition of 20 μM 5-HT to these pharmacological agents had no significant effect upon the excitatory inward current (*trace b*). 5-HT was then washed away, but 10 μM CNQX was added to the existing pharmacological agents remained. This completely abolished the isolated inward current (*trace c*), indicating that this inward current was mediated by glutamatergic AMPA receptors.

Figure 43 presents a similar paradigm, however this ICx neuron was manipulated with antagonists to isolate a small excitatory inward current. The ICx neuron displayed the inward current in response to 9V of electrical stimulation after the application of 0.5 μM strychnine, 20 μM bicuculline, and 10 μM CNQX (*trace a*). Application of 20 μM 5-HT to these existing pharmacological agents caused the synaptic response to express a large outward current following the preceding small inward current (*trace b*). The 5-HT was then washed away but the existing pharmacological agents remained and the outward current disappeared and the small inward current remained (*trace c*). Finally, 25 μM APV was added to the other antagonists and the small inward current was removed. Thus, from the data presented for this ICx neuron it was evident that 5-HT did interact with the excitatory inward current to some degree.

Figure 44 presents traces from another ICx neuron in response to 20V of electrical stimulation with the neuron being bathed in a solution containing 0.5 μM strychnine, 25 μM APV, and 10 μM CNQX (*trace a*). The resultant inhibitory outward current, which was mediated by GABA_A receptors, was subjected to 20 μM 5-HT in solution with the existing antagonists, but there was no significant change (*trace b*). Thus, the 5-HT had no effect upon the GABA_A receptor mediated response in this

particular ICx neuron. The 5-HT was then washed away but the existing pharmacological agents remained and 20 μ M bicuculline was added. This completely abolished the remaining outward current (*trace c*), showing that the isolated inhibitory outward current was mediated by GABA_A receptors.

I	Central nucleus		Pericentral nucleus	
II	Central nucleus		Pericentral nucleus	External nucleus
III	Central nucleus		Dorsal nucleus	External nucleus
IV	Ventrolateral division of central nucleus	Dorsomedial division of central nucleus	Pericentral nucleus	External nucleus
V	Central nucleus	Dorsomedial nucleus	Pericentral nucleus	External nucleus
VI	Central nucleus (ICc)	Dorsal nucleus (ICd)	External nucleus (ICx)	

Table 1 – Overview of the various classification schemes which have been utilized to describe the subdivisions of the inferior colliculus. The VI row highlighted in blue, indicates the classification scheme used within this study, and represents the current naming system most commonly used. (*See Huffman and Henson, 1990 for review*).

A.

B.

Figure 1 - Schematic representation of the subnuclei of the IC. (A) Coronal section through the IC showing the central (ICc), dorsal (ICd) and external (ICx) subnuclei. Lines near the dorsal and lateral surface indicate the layered cortical cytoarchitecture (layers 1-3)). (*Adapted from Huffman and Henson, 1990*). . (B) Photomicrograph of a Nissl-stained frontal section through the midbrain at bregma -8.72, indicating the various subnuclei of the IC. **ICc** – central nucleus of the IC; **ICd** – dorsal cortex of the IC; **ICx** – external cortex of the IC; **IcT** – intercollicular tegmentum (*Adapted from Winer and Larue, 2001*).

A.

B.

Figure 2 – Connectivity of the rat auditory system and the external cortex of the IC. (A) The representation of the multiple feedback loops in the ascending and descending auditory system of the rat. Each of the three parallel descending pathways provides a major projection to a nucleus in each of the three auditory centers, the IC, the medial geniculate body (MGB) and auditory cortex (AC). The three sets of connections provide a system of multiple parallel feedback loops between the auditory cortex and the IC. Note the lack of direct descending cortical input to the ICc. **ICd₁** – superficial layer 1 of the dorsal cortex of the IC; **ICd₂₊₃** – deep layers 2 and 3 of the dorsal cortex of the IC; **ICx** – external cortex of the IC; **ICc** – central nucleus of the IC. (B) A schematic representation of the connections of the ICx with acousticomotor and somatosensory systems. **CB** – cerebellum; **DCN** – dorsal column nuclei; **DLPN** – dorsolateral pontine nucleus; **SC** – superior colliculus; **STN** – spinal trigeminal nuclei; **SSCx** – somatosensory cortex (*Adapted from Huffman and Henson, 1990*).

A.

B.

Figure 3 - *In vitro* brain slice experimental chamber design. This illustration outlines the (A) design of the chamber used to house the brain slice during electrophysiological recordings. The tissue is supported between two pieces of nylon gauze and placed in a lowered section of a magnetic and Plexiglas plate. Once assembled (B) the chamber is placed under the microscope illuminated from below through a darkfield condenser. The chamber receives an inflow of heated and oxygenated perfusion medium, which bathes the tissue and is then aspirated back to the perfusion reservoir. (*Adapted from Oertel, 1985*).

Figure 4 – Overview of brain slice preparation. These photographs outline the methodology for preparing the rat brain slice for electrophysiological recordings. Panel (A) shows the open skull exposing the brain *in vivo*. The excised brain was submerged in heated oxygenated ACSF and held in place (B). Next, the tissue was blocked in the frontal plane containing the auditory midbrain (*blue lines*). The tissue was mounted on a block and placed in the vibratome where 200 μm sections were made (C,D). Once sectioned, the brain slice was placed in the recording chamber between two pieces of mesh (E). The chamber was then placed under the microscope where recordings were made.

Figure 5 – Schematic representation of a brain slice through the auditory midbrain. The figure illustrates the brain slice in the frontal plane with the subnuclei of the IC emphasized. The whole-cell patch clamp recording electrode is indicated (**R**) placed in the ICx. The stimulating electrode (**S**) is shown placed in the axon fibers from the ICc to the ICx.

Figure 6 – Whole-cell patch clamp protocol. This figure illustrates the steps involved in achieving a whole-cell patch clamp configuration using a recording electrode. The waveforms on the right of the figure depict the changes that would be seen in the current injection as the pipette interacts with the cell. (*Adapted from Kauer, 1992*).

Regular

A.

B.

Figure 7 - Membrane properties of a “Regular-type” ICx neuron. (A) Responses to intracellular current injection (*from bottom; -200, -80, 60, 110 pA*) from a representative “regular-type” ICx neuron. Note the consistent intervals between each elicited spike, providing the regular firing pattern. (B) The current-voltage relationship.

Adaptation

A.

B.

Figure 8 - Membrane properties of an “Adaptation-type” ICx neuron. (A) Responses to intracellular current injection (*from bottom*; -160, -80, 190, 260 pA) from a representative “adaptation-type” ICx neuron. Note the longer duration of the interspike intervals for the latter spikes in relation to the first interspike interval. (B) The current-voltage relationship.

Pauser

A.

B.

Figure 9 - Membrane properties of a “Pauser-type” ICx neuron. (A) Responses to intracellular current injection (*from bottom; -100, -40, 100, 130 pA*) from a representative “pauser-type” ICx neuron. Note the longer duration of the first interspike interval in relation to the latter interspike intervals. (B) The current-voltage relationship.

A.

B.

Figure 10 – Effects of fixed prehyperpolarizing and varying depolarizing current injection on a “Pauser-type” ICx neuron. (A) Responses to depolarizing current injection (*from bottom; 70, 110, 180 pA*) with a prehyperpolarizing current injection of 200 pA. Note the longer duration of the first interspike interval and its reduction as the depolarizing current injection increases. (B) The “pause” duration from the lowest depolarizing current injection was used as control to normalize the remaining data for other injection levels. The trend in the chart shows that as the depolarizing current injection level is increased, the first interspike interval in pauser-type neurons decreases.

A.

B.

Figure 11 – Effects of varying prehyperpolarizing and fixed depolarizing current injection on a “Pauser-type” ICx neuron. (A) Responses to depolarizing current injection of 100 pA with varied prehyperpolarizing current injection (*from bottom; -190, -150, -50 pA*). Note the longer duration of the first interspike interval and its reduction as the prehyperpolarizing current injection decreases. (B) The “pause” duration from the lowest hyperpolarizing current injection (200 pA) was used as control to normalize the remaining data for other injection levels. The trend in the chart shows that as the prehyperpolarizing current injection level is decreased, the interspike interval decreases.

Bursting

A.

B.

Figure 12 - Membrane properties of a “*Bursting-type*” ICx neuron. (A) Responses to intracellular current injection (*from bottom*; -100, -40, 100, 210 pA) from a representative “bursting-type” ICx neuron. Note the burst of activity followed by a prolonged undershoot. (B) The current-voltage relationship.

Figures 13 – Association of undershoot with membrane properties of “*Bursting-type*” ICx neurons. Several traces are presented here in response to positive intracellular current injection (*from bottom; 90, 110, 130, 170, 220 pA*) from a representative “bursting-type” ICx neuron. Note the long undershoot after the occurrence of the initial spike in the lowest trace (90 pA). As the level of depolarization is increased, the undershoot persists and dictates the presence of the next spike.

A.

B.

	Regular			Adaptation			Pauser		
	Mean	S.E. (±)	n	Mean	S.E. (±)	n	Mean	S.E. (±)	n
RMP (mV)	-57.7	2.9	48	-57.9	2.9	14	-58.1	2.9	9
IR (MΩ)	315.0	15.8	48	241.7	12.1	14	276.9	13.8	9
TC (ms)	18.3	0.9	48	12.0	0.6	13	12.7	0.6	7
AP amp (mV)	46.5	2.3	49	46.9	2.3	13	34.8	1.7	7
AP hw (ms)	1.4	0.07	49	1.0	0.05	13	1.1	0.06	7
PD-AHP amp (mV)	5.1	0.3	45	4.3	0.2	12	4.2	0.2	9
SS-AHP amp (mV)	10.7	0.5	37	9.6	0.5	12	11.7	0.6	9
SS-AHP dur (ms)	81.1	4.1	22	75.0	3.8	12	72.2	3.6	7

RMP - resting membrane potential; **IR** - input resistance; **TC** - time constant; **AP amp** - action potential amplitude; **AP hw** - action potential half-width; **PD-AHP amp** - post depolarization-AHP amplitude; **SS-AHP amp** - single spike-AHP amplitude; **SS-AHP dur** - single spike-AHP duration.

Figure 14 – Summary of firing pattern distribution and basic membrane properties.

(A) This chart outlines the distribution of cells based on firing pattern. The distribution is based on a total cell count ($N = 98$). Regular-type was the most prevalent firing pattern, followed by adaptation, pauser and bursting-type in the ICx neurons sampled.

(B) This summary table presents the statistics for the major basic membrane properties measured, organized by the main firing patterns (*regular, adaptation, pauser-type*).

Figure 15 – Comparison of input resistance (R_{input}) across the firing patterns of ICx neurons. This chart compares the input resistance measure across the three firing patterns observed in ICx neurons. There was a statistically significant difference between the regular and adaptation-type neurons ($p < 0.02$)(●). All other comparisons between groups showed no statistical significance ($p > 0.10$). Sample size (n) for each group is outlined in figure 13B.

Figure 16 – Comparison of time constant (τ) across the firing patterns of ICx neurons. This chart compares the time constant measure across the three firing patterns observed in ICx neurons. There was a statistically significant difference between the regular and adaptation-type neurons ($p < 0.01$)(●), and between the regular and pauser-type neurons ($p < 0.10$)(▲) . All other comparisons between groups showed no statistical significance ($p > 0.10$). Sample size (n) for each group is outlined in figure 13B.

A.

B.

Figure 17 – Comparison of action potential (AP) properties across the firing patterns of ICx neurons. (A) The AP amplitude measured across the ICx neurons are presented in this chart by firing pattern. There was a statistically significant difference in AP amplitude between regular and pauser-type neurons ($p < 0.02$)(▲) and between adaptation and pauser-type neurons ($p < 0.04$)(○). **(B)** The analysis of AP half-width duration across firing patterns is shown in the lower chart. There was a statistically significant difference in AP half-width duration between regular and adaptation-type neurons ($p < 0.03$)(●). Sample size (n) for each group is outlined in figure 13B.

Control

TEA

TTX &
TEA

Wash

30 mV
50 ms

Figure 18 - Pharmacological analysis of ionic basis for APs in ICx neurons. Responses from a typical ICx neuron with a prominent Na^+/K^+ mediated conductance. The firing pattern was in response to intracellular current injection of 90 pA (*top trace*). The elicited APs showed a longer duration in response to bath application of 5 mM TEA (*blue trace*). The remaining ionic conductance was completely blocked by the addition of $1\mu\text{M}$ TTX to the TEA (*red trace*). Once the pharmacological agents were washed away, the control response returned at a current injection of 170 pA (*bottom trace*).

Control

TTX/TEA

0 Ca^{2+}
in TTX/TEA

Wash

30 mV
50 ms

Figure 19 - Pharmacological analysis of underlying Ca^{2+} mediated conductance. Responses from a representative neuron with a prominent Na^+/K^+ mediated conductance blocked by $1\mu\text{M}$ TTX and 5 mM TEA respectively. This revealed an underlying Ca^{2+} mediated “spike”(●) and “hump” (○) in response to positive current injection (270 pA) (*2nd trace*). Both of these Ca^{2+} mediated responses were removed once the neuron was bathed with TTX and TEA in a 0 Ca^{2+} ACSF (*3rd trace*). The control response returned after the slice was washed with ACSF (*bottom trace*).

Control

0 Ca^{2+}

Wash

30 mV
50 ms

Figure 20 – Calcium mediated rebound potential in an ICx neuron. Responses from a representative neuron from a subpopulation of ICx neurons with a Ca^{2+} mediated rebound potential in response to negative current injection (*top trace: from bottom; -300, -220, -180, -120, -80 pA*). The rebound potential was removed when subjected to 0 Ca^{2+} ACSF (*blue trace*). The rebound potential reappeared once the ICx neuron was returned to Ca^{2+} containing ACSF (*bottom trace*).

Figure 21 – Comparison of the post depolarization-AHP amplitude across firing patterns in ICx neurons. (A) This representative trace depicts the criteria used when measuring the post depolarization-AHP amplitude for ICx neurons, indicated by the arrowheads. This AHP was present in a subpopulation of ICx neurons and was expressed in response to positive intracellular current injection of 300 pA. (B) The analysis of post depolarization-AHP amplitude showed that there was no statistical significant difference between the groups of ICx neurons based on firing pattern ($p > 0.10$). Sample size (n) for each group is outlined in figure 13B.

A1.

A2.

B1.

B2.

Figure 22 – Comparison of single-spike AHP properties across firing patterns in ICx neurons. (A) *A1*. This representative trace depicts the criteria used when measuring the single-spike AHP duration for ICx neurons, indicated by the arrowheads. This AHP was expressed in response to positive intracellular current injection at the threshold level. *A2*. The analysis of single-spike AHP duration showed that there was no statistical significant difference between the groups of ICx neurons based on firing pattern. (B) *B1*. This representative trace depicts the criteria used when measuring the single-spike AHP amplitude for ICx neurons, indicated by the arrowheads. *B2*. The analysis of single-spike AHP amplitude showed that there was no statistical significant difference between the groups of ICx neurons based on firing pattern ($p > 0.10$). Sample size (n) for each group is outlined in figure 13B.

30 mV
30 ms

Figure 23 – Dual components associated with single-spike AHP in ICx neurons. These representative traces illustrate the two components underlying the single-spike AHP in a subpopulation of ICx neurons. These traces were in response to positive intracellular current injection (*a*) 570 pA, (*b*) 420 pA, (*c*) 280 pA. After the “classic” falling phase of the spike there seemed to be an earlier undershoot (arrow) followed by a relatively longer AHP (●). The two components of the single-spike AHP persisted throughout the range of positive current injection.

Figure 24 – Effects of calcium on single-spike AHP in an ICx neuron. These traces were in response to positive intracellular current injection (*a,c*) 100 pA, (*b*) 40 pA. Once the neuron was bathed in a 0 Ca²⁺ environment the single-spike AHP was eliminated (*b*; *blue trace*). The AHP returned after the ICx neuron was washed in normal saline (*c*).

A.

Regular

C.

Pauser

B.

Adaptation

D.

Comparison

Figure 25 - Comparison of rate-level function to depolarizing current injection across ICx neurons. Both the (A) regular ($n = 10$) and (B) adaptation-type ($n = 10$) ICx neurons display a linear firing rate increase in response to increasing depolarizing current. The (C) pauser-type ($n = 8$) also displays a general linear trend but express a higher threshold to initiate firing. Each trace shown in (A,B,C) represent the rate-level function of an individual neuron for that specific firing pattern. (D) Comparison of increasing rate level firing frequency between three types of ICx neurons. The trace shown for each firing pattern represents the average rate-level function derived from the traces shown in (A,B,C).

A.

B.

C.

Figure 26 - Comparison of rate-level function at higher levels of depolarizing current injection across ICx neurons. Each coloured bar in the chart represents the maximum firing frequency at a particular level of current injection for an individual neuron for that firing pattern. A sample population ($n = 5$) was used for each firing pattern. The neurons of the (A) regular-type firing pattern displayed the ability to respond to higher levels of depolarizing current injection without saturation and also had a higher maximum firing frequency. Both the (B) adaptation-type and (C) pauser-type ICx neurons saturated at lower levels of positive current injection and had a lower maximum firing frequency.

Figure 27 - Comparison of rate level threshold across ICx neurons. The regular-type neurons ($n = 41$) had a statistically significant lower threshold of current required in order to initiate firing in comparison to both adaptation ($n = 13$) ($p < 0.01$)(●) and pauser-type neurons ($n = 9$) ($p < 0.01$)(▲).

A.

B.

Figure 28 - Membrane properties of an ICx neuron in response to hyperpolarizing current injection. (A) Responses to intracellular current injection (*from bottom*; -400, -260, -140 pA). At increasing levels of hyperpolarizing current injection a depolarizing sag becomes evident. (B) The current-voltage relationship was examined from both the peak (Δ) and from the steady-state of voltage changes ($\bullet$).

A.

B.

Figure 29 – Effects of hyperpolarizing voltage steps on a ICx neuron. (A) Responses to voltage steps (300 ms) from a holding potential of -60 mV to -150 mV, revealing a slowly activating and slowly deactivating inward current, I_h . The voltage steps initially caused a capacitive transient and then a jump in current termed instantaneous current (I_{ins}). The inward current then increased slowly to a steady-state level (I_{ss}). **(B)** The voltage-current (VI) relationship for the instantaneous ($\bullet$) and steady-state (Δ). The difference between the instantaneous and steady-state currents represents I_h ($\circ$).

Figure 30 – Effects of long duration hyperpolarizing voltage steps on an ICx neuron. (A) Responses to voltage steps from a holding potential of -60 mV to -150 mV, revealing a slowly activating and slowly deactivating inward current, I_h . The voltage steps in this protocol were conducted for a duration of 500 ms. (B) The voltage-current (VI) relationship for the instantaneous ($\bullet$) and steady-state (Δ). The difference between the instantaneous and steady-state currents represent I_h ($\circ$).

Figure 31 – Measurements of the reversal potential for I_h on a ICx neuron. (A) Responses to both depolarizing and hyperpolarizing steps from a holding potential of -130 mV. The symbol below the current traces ($\blacktriangle$) indicates the time at which the current was measured and was used to calculate the voltage-current plot in (B). The voltage-current plot displays the relationship calculated from I_{ins} for this neuron from the voltage-steps shown in (A) and from the control voltage-steps (as seen in figure 28, $\bullet$). The slopes of the voltage-current relationships represent the membrane conductance at each holding potential. The voltage at the intersection of the two lines was an estimate of the reversal potential of I_h for this neuron (-62 mV).

Figure 32 – Voltage dependence of I_h on a ICx neuron. (A) Responses to steps from a holding potential of -60 mV to -150 mV. The voltage steps elicited the slowly deactivating tail current (I_{tail}). (B) Plot of relative I_h current against the membrane potential from the cell shown in (A). The data were fitted using a Boltzman function, with a calculated $V_{1/2} = -100$ mV.

Current Clamp

A.

B.

Voltage Clamp

Figure 33 – Comparison of current clamp and voltage clamp recordings of synaptic responses from an ICx neuron. (A) Three traces obtained from recordings in current clamp mode from an ICx neuron. The graded postsynaptic potentials are in response to various levels of electrical stimulation of the ICc. (B) Three traces from the same ICx neurons however, the synaptic currents are recorded in voltage clamp mode at a holding potential of -60 mV. Note that the responses are at the exact same stimulus intensity.

A.

B.

C.

D.

Figure 34 – Typical graded postsynaptic potentials from four ICx neurons. The traces outlined in this figure are synaptic potentials in response to electrical stimulation of the ICc. **(A)** Responses which display a predominantly excitatory response (epsp) and become suprathreshold at 30V. **(B)** Responses which display a predominantly inhibitory response (ipsp) and no suprathreshold response was visible. **(C)** Traces which demonstrate a mixed epsp/ipsp response at 8 V and 9 V and suprathreshold at 10V. **(D)** A neuron which produced a mixed epsp/ipsp response, but did not produce a suprathreshold response. The arrow indicates the electrical stimulus artifact.

Cell 1

Figure 35 - Pharmacological analysis of synaptic currents elicited via electrical stimulation of the ICc on an ICx neuron (*cell 1*). The control response displayed both an inward and outward current (*a*). The outward current was partially blocked by 0.5 μ M strychnine (*b*) and 20 μ M bicuculline (*c*). The inward current was partially blocked by 10 μ M CNQX (*d*) and by 25 μ M APV (*e*). After saline wash, the control response returned (*f*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

Cell 2

Figure 36 - Pharmacological analysis of synaptic currents elicited via electrical stimulation of the ICc on an ICx neuron (*cell 2*). The control response displayed a predominantly inward current (*a*). Application of 0.5 μ M strychnine (*b*) and 20 μ M bicuculline (*c*) had no significant effect on the synaptic response. The inward current was not visibly affected by 25 μ M APV application (*d*) but was completely removed once 10 μ M CNQX was applied (*e*). After saline wash, the control response returned (*f*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

Cell 3

Figure 37 - Pharmacological analysis of synaptic currents elicited via electrical stimulation of the ICc on an ICx neuron (*cell 3*). The control response displayed a predominantly outward current (*a*). Application of 25 μ M APV (*b*) had no significant effect upon the synaptic response. Application of 10 μ M CNQX (*c*) slightly reduced an underlying inward current as can be seen by the slight increase in the outward current. The outward current was not visibly affected by 0.5 μ M strychnine (*d*) application but was completely removed once 20 μ M bicuculline was applied (*e*). After saline wash, the control response returned (*f*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

25 mV
100 ms

Figure 38 - Pharmacological analysis of excitatory synaptic potentials elicited via electrical stimulation of the ICc on an ICx neuron. The traces outlined here were obtained from recordings of an ICx neuron in current clamp mode. The control response displayed a predominantly excitatory potential with two visible components, an earlier response followed by a slower longer potential (*a*). Application of 10 μ M CNQX (*b*) inhibited the earlier component and elicited a suprathreshold response from the latter component. Once washed in normal saline, the earlier component of the synaptic current returned (*c*). Application of 25 μ M APV (*d*) removed the longer lasting potential and isolated the earlier response. When both APV and CNQX were added (*e*), the excitatory potential had been completely removed and a very small underlying inhibitory potential was revealed. This slight inhibitory response was completely removed with the application of 20 μ M bicuculline and 0.5 μ M strychnine added to the APV and CNQX (*f*). After saline wash, the control response returned (*g*). Note pharmacological application was cumulative unless a wash was applied. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

300 pA
30 ms

Figure 39 - Pharmacological analysis of inhibitory synaptic currents elicited via electrical stimulation of the ICc on an ICx neuron. The traces outlined here were obtained from recordings of an ICx neuron in voltage clamp mode. The control response displayed a predominantly inhibitory outward current (*a*). Application of 0.5 μ M strychnine (*b*) had no effect upon the outward current. The remaining outward response was completely blocked by 20 μ M bicuculline (*c*), revealing a small underlying inward current. This inward current was removed with the application of 10 μ M CNQX (*d*). Once washed in normal saline, the dominant outward current returned (*e*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

A.

B.

Figure 40 – Summary of synaptic response distribution across ICx neurons. The synaptic response patterns were examined in response to electrical stimulation of the ICc. **(A)** The distribution of responses that were observed with no pharmacological manipulation. The synaptic responses were qualitatively judged as predominantly “excitatory”, “inhibitory” or “mixed”. The majority of the cells (60%) were primarily excitatory, followed by mixed (24%) and finally inhibitory (16%) (N = 25). **(B)** The contribution of the various synaptic receptors in the synaptic responses of the ICx neurons tested (n = 10).

A.

B.

C.

D.

Figure 41 – Effects of 5-HT on synaptic currents in ICx neurons. The traces outlined in this figure are synaptic currents in response to electrical stimulation of the ICc from four distinct ICx neurons recorded in voltage clamp mode. **(A)** Responses which displayed a predominantly excitatory response (epsc) with a suprathreshold component at 20 V. Application of 20 μ M 5-HT caused a reduction in the inward current (*blue trace*). **(B)** Traces which displayed a mixed inward/outward response at a stimulation intensity of 20 V. Application of 20 μ M 5-HT caused a sharp increase in the inward current (*blue trace*). **(C)** Responses which demonstrated a predominantly inward current at 40 V, which was reduced upon the application of 20 μ M 5-HT (*blue trace*). **(D)** Responses which displayed a strong inward current, with two distinct components at 20 V. Application of 20 μ M 5-HT significantly reduced the later inward current (*blue trace*). The original response returned in all four neurons when bathed in saline. The arrow indicates the electrical stimulus artifact.

100 pA
15 ms

Figure 42 – Effects of 5-HT on AMPA receptor mediated responses in an ICx neuron. Responses were recorded in voltage clamp mode at a stimulus intensity of 20V. An inward current was elicited with the neuron bathed in 0.5 μ M strychnine, 20 μ M bicuculline, and 25 μ M APV (*a*). Upon application of 20 μ M 5-HT the inward synaptic current remained unchanged (*b*; *blue trace*). Application of 10 μ M CNQX removed the inward current (*c*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

Strychnine / Bicuculline / CNQX

150 pA
15 ms

Figure 43 – Effects of 5-HT on NMDA receptor mediated responses in an ICx neuron. Responses were recorded in voltage clamp mode at a stimulus intensity of 9V. A small inward current was elicited with the neuron bathed in 0.5 μ M strychnine, 20 μ M bicuculline, and 10 μ M CNQX (*a*). Upon application of 20 μ M 5-HT the inward synaptic current reversed in polarity and revealed a large outward current (*b*; *blue trace*). When 5-HT was removed, and the other pharmacological agents remained, the small inward current returned (*c*). Application of 25 μ M APV to the existing strychnine, bicuculline and CNQX removed the inward current (*d*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

Figure 44 – Effects of 5-HT on GABA_A receptor mediated responses in an ICx neuron. Responses were recorded in voltage clamp mode at a stimulus intensity of 20V. An outward current was elicited with the neuron bathed in 0.5 μ M strychnine, 25 μ M APV, and 10 μ M CNQX (*a*). Upon application of 20 μ M 5-HT the outward synaptic current showed no significant change (*b*; *blue trace*). Application of 20 μ M bicuculline removed the outward current (*c*). Note pharmacological application was cumulative. Conditions are indicated by the first letter of the antagonist. The arrow indicates the electrical stimulus artifact.

5. Discussion

5.1 Cell types

Neurons in the ICx expressed different firing patterns classified as *regular-type*, *adaptation-type*, and *pauser-type* in response to positive current injection. A small group of cells was shown to display a firing pattern termed *bursting-type*, but due to its low occurrence (3%) it was not included in the overall analysis and classification of ICx neurons. The three major types of ICx neurons showed some differences between their membrane properties, but generally seemed to be a homogenous population in regard to these measures (figure 14B). The regular-type neurons were by far the most commonly encountered firing pattern constituting 68% of the population, followed by adaptation-type (17%) and pauser-type (12%). The current classification scheme differs from those that are based on responses to sounds (Le Beau *et al.*, 1996; Rees *et al.*, 1997), in which firing patterns of the neuron were likely in response to the interaction of the intrinsic membrane properties and with its synaptic inputs. Studies in rat (Sivaramkrishnan and Oliver, 2001) have shown a diverse group of physiologically defined firing patterns in ICc neurons similar to those found in the ICx, but with some differences. Their results suggested that the firing patterns of the ICc neurons were intrinsically present, however do not exclude the possibility that the firing patterns could also be evoked via synaptic stimulation.

In this study, the ICx firing patterns were examined exclusively in relation to intrinsic membrane properties. The regular-type cells were shown to display a firing pattern in which the interspike intervals were quite regular, unlike the adaptation- and pauser-type cells which showed different interspike intervals. In adaptation-type cells,

the earlier interspike intervals were shorter in comparison to the later intervals. The pauser-type cells displayed a pronounced pause after the first spike. This relatively long interspike interval was followed by a more regular firing pattern. These three firing patterns from the ICx were compared with those found in the ICc (Sivaramkrishnan and Oliver, 2001). The analysis of the ICc revealed the presence of six distinct firing patterns in response to depolarizing current injection; two variety of regular-type cells, adaptation-type, pauser-type, onset-type, and transient-type cells. Thus, the ICc seems to possess a wider array of neurons based on firing pattern in comparison to the ICx. This does however agree with the notion that the ICc plays a more integral role in auditory processing and would therefore require a greater selection of neurons for task allocation.

Some studies have also tried to correlate firing pattern with neuronal morphology in the IC (Smith, 1992; Wagner, 1994) with limited success. Neurons in the ICc have been cited as having two morphologically distinct populations; *disc* and *multipolar* shaped cells (Faye-Lund and Osen, 1985). The neurons of the ICd and ICx on the other hand have been described overall as *multipolar*. Faye-Lund and Osen (1985) reported that the ICd and ICx neuronal morphology in the rat was relatively homogeneous. More recently, Malmierca *et al.*, (1995) used extensive three-dimensional reconstructions to show that the cell types in ICc are more homogeneous than the terms *disc* and *multipolar* suggest. The group instead proposed a new classification of *flat* and *less flat* neurons. Taken together, the morphological data suggest that the cellular population in each subnucleus may be relatively homogeneous. With these observations in hand it seems possible that the firing patterns seen in this study for the ICx and those encountered in

other studies for the IC, may be independent of neuronal morphology and are instead influenced by other intrinsic factors.

5.1.1 Pauser-type neurons in ICx & A-type K^+ channels

The classification of pauser-type cells was more detailed than the other cell types, since each cell underwent two protocols that included a prehyperpolarizing current injection, before the neuron received depolarizing steps. The prehyperpolarizing steps were employed to remove any inactivation of the A-type K^+ channel. This pauser-type firing pattern and the presence of the A-type K^+ channel has been shown in the ICc (Sivaramkrishnan and Oliver, 2001), and has been described in other vertebrate and invertebrate neurons (Connor and Stevens, 1971; Kanold and Manis, 1999; Neher, 1971). This fast transient K^+ conductance can be observed from activated A-type channels when the cell is depolarized following a prehyperpolarization. If the neuron is not prehyperpolarized, a depolarizing step will elicit K^+ currents from delayed-rectifier K^+ channels and Ca^{2+} -activated K^+ channels, but not from A-type K^+ channels (Hille, 1992; Sivaramkrishnan and Oliver, 2001). Some neurons encountered in the ICx, initially appeared to be regular-type cells, however when the prehyperpolarization protocols were employed, they revealed a pauser-type firing pattern. Therefore, at rest the delayed-rectifier K^+ channels and Ca^{2+} -activated K^+ channels were responsible for the outward current and were instrumental in shaping the observed firing pattern. Once the prehyperpolarizing current injection was engaged, this first removed the inactivation of the A-type channels, and then after depolarization the faster transient K^+ current were activated. In vivo, under non-experimental conditions this prehyperpolarizing current

step may be similar to the observed AHP seen in response to sustained levels of depolarization or a synaptically evoked inhibitory response, inactivating the A-type channels.

The “pause” seen in the pauser-type ICx neurons in this study was attributed to the presence of the A-type K^+ current. The first prehyperpolarization protocol (figure 10) examined the effect of increasing levels of depolarization after administering a fixed level of prehyperpolarization, on the pause duration. The results showed, a negative linear correlation between pause duration and levels of positive current injection. Therefore, as the level of depolarization increased following the prehyperpolarization, the pause duration shortened. This suggests, that at low levels of depolarization the effects of the A-type current are more pronounced. When the level of the prehyperpolarizing current injection was varied and the depolarizing pulse was maintained at a level which produced a reliable spike train, pause duration was also influenced (figure 11). The data illustrated a positive correlation between pause duration and level of prehyperpolarization, with the pause duration at its maximum at the most negative levels of current injection. This suggests that the degree of removal of the inactivation of the A-type channels prior to a fixed level of depolarization determines the pause duration. At low levels of prehyperpolarization, only the non-A-type K^+ -conductances are activated and therefore the firing pattern is more comparable to regular-type.

What purpose does this pause serve in terms of auditory processing? Sivaramkrishnan and Oliver (2001) suggest that the pauser-type cells may have the ability to mediate two time-dependent tasks; the time interval between successive

excitatory inputs to the cell, and the pairing of excitation with inhibition. In this cell type, sustained firing in response to an excitatory stimulus is followed by an intrinsic AHP of the cell membrane that would be sufficient to remove the inactivation of the A-current. If a secondary excitatory stimulus was to arrive during this AHP, the A-current would be activated resulting in a pause in the firing. Since the magnitude of the A-current is directly proportional to the magnitude of the AHP, the pause would be directly proportional to the time interval between the two excitatory stimuli. If the secondary excitatory input arrived during the initial falling phase of the AHP, the amount of inactivation would be lower, and would result in a smaller A-current. This same relationship would be illustrated if the input were to arrive during the decaying rising phase of the AHP. If the input was to interact with the AHP at its peak amplitude, this would result in maximum inactivation of the A-type channels and thereafter produce a larger A-current by depolarization. Therefore, the length of the pause duration could indirectly be used to decipher the duration between stimuli. The ability of detecting short stimulus intervals in pauser-type neurons is, however limited to <100 ms since the A-current would have decayed to zero (Sivaramkrishnan and Oliver, 2001).

The second time-dependent task the pauser-type cells may be suited to perform involves registering the temporal order of inhibitory and excitatory inputs to the cell. IPSPs can serve to remove the inactivation of the A-type channels, but the level of removal from inactivation would depend on the magnitude of the IPSP. Then the arriving EPSP could activate the A-current and the pause could be an indicator of the interval between the two synaptic events, with the same mechanisms outlined above. Although these theories are in agreement with the findings from the present study and

those outlined in previous studies (Rhode *et al.*, 1983; Sivaramkrishnan and Oliver, 2001), further experimentation would be required to substantiate them.

5.2 *Passive membrane properties*

The current-voltage relationships for each of the ICx neurons tested showed a linear relationship, across all cell types. Using the linear portion of the relationship, the slope was used to calculate R_{input} and the τ was derived from the responses to negative current injection. The analyses showed that the regular-type cells displayed a significantly higher mean R_{input} and a longer mean τ in comparison to the other cell types. This indicated that in the ICx, the regular-type neurons were relatively smaller in size, since R_{input} is inversely related to cell size. The longer τ means that in these cells the membrane potential changes slower in response to current injection. Furthermore, the longer τ in regular-type cells represented a larger capacitance for the cell, since $\tau = RC$; where R is input resistance and C is capacitance (Koester and Siegelbaum, 2000). The R_{input} value is known and was shown to be small therefore, a high τ value is correlated with an increased C . This increased C in regular-type cells may be a direct measure of neuronal surface area (Nitzan *et al.*, 1990). Since the various cell types of the ICx have been suggested to have similar cell soma size, the differences in surface area may be equated to varying levels of dendritic arborization. The data suggest that regular-type cells have a relatively larger surface area and so may possess greater levels of dendritic arborization. The trends of these two membrane properties for this study in the ICx show no significant correlation with those illustrated in the ICc (Sivaramkrishnan and Oliver, 2001). The ICc study did however have twice the amount of cell types which makes

direct comparisons between cell types difficult. The mean R_{input} across all of the cell types in the ICc, was however similar to that obtained in the present study. The study also found that the average τ of ICx neurons was longer in comparison to the ICc neurons. Li *et al.*, (1998) examined the membrane properties across the three subnuclei of the IC and found that the ICx displayed the highest mean R_{input} . They propose that the higher R_{input} in ICx neurons will increase their tendency to fire APs in response to depolarizing current injection. The current ICx study provided a more detailed analysis of R_{input} , examining this measure across the cell types instead of across subnuclei. In relation to the present study, regular-type cells displayed a higher tendency to fire APs in response to depolarizing current injection in comparison to the other two cell-types. The threshold for firing was examined (figure 27) across cell types and showed that the regular-type cells of the ICx did have a significantly lower threshold for eliciting APs in response to current injection relative to adaptation- and pauser-type cells.

5.3 *Properties of action potentials (APs)*

When the properties of the APs were examined more closely, some differences were found across groups. The average AP amplitude of the pauser-type neurons was significantly lower than both other cell types. This could be partly due to the smaller contribution of this cell type to the overall population. The average half-width duration of the APs was found to be longer in the regular-type neurons in comparison to the other two cell types. Despite these differences, the overall range of each measure was quite close for both AP properties across groups. The APs in the ICx also displayed an undershoot, termed *single-spike AHP*. The average duration and amplitude of this

measure was calculated and compared across the three cell types. The analysis showed that there was no significant difference in either the average amplitude or duration, thus these properties were homogeneous for the neurons in the ICx.

At the end of the depolarizing current injection, many ICx neurons displayed another type of AHP, termed *post-depolarization AHP*. The amplitude of this AHP was analyzed across the cell types at an equivalent level of current injection for each neuron sampled in the ICx, and no significant difference was found between groups. The previous studies on ICc and ICx neurons have shown the presence of both of these AHPs, but its properties were not analyzed (Li *et al.*, 1998; Sivaramkrishnan and Oliver, 2001).

5.4 Contribution of Ca^{2+}

5.4.1 Ca^{2+} spikes/humps

The ionic basis of the APs was verified using pharmacological manipulations. The “classical” Na^+ and K^+ conductances were systematically abolished using the ion channel blockers, TTX and TEA respectively. This substantiated the notion that in the APs encountered, the inward current was mediated by voltage-dependent Na^+ channels and the outward current was mediated by delayed-rectifying slow inactivating K^+ channels. In 70% of the ICx neurons sampled, after the removal of Na^+ and K^+ conductances, a group of underlying excitatory potential was revealed. The first response was seen as a “*spike*”, but expressed a significantly smaller amplitude and longer duration than the Na^+/K^+ – mediated APs. These were followed by an even smaller amplitude, longer duration set of “*humps*”. These depolarizing potentials of

varying time course both seem to be mediated by Ca^{2+} since they were eliminated by perfusion with 0 Ca^{2+} ACSF. Other physiological studies have shown these same Ca^{2+} -mediated potentials in both ICc and other ICx neurons (Sivaramkrishnan and Oliver, 2001; Smith, 1992). Li *et al.*, (1998) also noted the presence of these Ca^{2+} -mediated potentials in their analyses of the subnuclei of the IC, but only realized them in the ICx and ICd and failed to observe them in the ICc. Similar Ca^{2+} -mediated potentials have been shown in other neuronal structures such as the hippocampus (Disterhoft *et al.*, 1993).

5.4.2 Ca^{2+} mediated rebound potential

When hyperpolarizing current steps were injected into the ICx neurons, anode-break spikes or depolarizing potentials were typically (75%) observed upon release of the hyperpolarizing current. However, in a small percentage (25%) of ICx neurons, the anode-break spike was followed by a broad, *rebound* depolarization. When the cells that illustrated this rebound potential were bathed in a 0 Ca^{2+} ACSF, the rebound hump disappeared indicating that it was mediated by Ca^{2+} . Once the neuron was returned to normal saline the anode-break spike and rebound depolarization returned. This post hyperpolarization rebound potential has been shown to be mediated by Ca^{2+} in studies examining ICc and ICx neurons (Sivaramkrishnan and Oliver, 2001; Smith, 1992). Sivaramkrishnan and Oliver (2001) showed that the anode-break spike was distinct from the rebound potential. With TTX application the former was removed and the latter potential persisted, suggesting the rebound potential is not mediated by Na^+ ions. A characteristic difference between neurons that exhibited the Ca^{2+} -mediated rebound and

those that did not was the latency of the Na^+ driven anode-break spike from the end of the hyperpolarizing current step. When the rebound potential was evident, the anode-break spike occurred with a shorter latency in comparison to the non-rebound ICx neurons in response to the similar levels of negative current injection.

Another important observation was that rebound cells, generally required a smaller amount of hyperpolarization to produce the anode-break spike compared to the non-rebound cells. These observations are in agreement with those shown by Sivaramkrishnan and Oliver (2001) for ICc neurons. It seems plausible that the Ca^{2+} entry mediated rebound depolarization may function to increase the probability that anode-break spikes are produced at less negative levels of hyperpolarizing current injection, and also decreasing the latency of the anode-break spike. This Ca^{2+} entry may therefore be making the cell more excitable after hyperpolarization. In non-rebound cells, an increased level of hyperpolarization may be required to remove the Na^+ inactivation, allowing the anode-break spike to fire.

Casseday *et al.*, (2000) have suggested that this Ca^{2+} - mediated rebound potential may be involved in duration tuning. For many auditory midbrain neurons, their response as a function of duration can be classified as band-pass in that the neuron responds to only a narrow range of sound durations and is unresponsive to sounds outside of this range. Other midbrain neurons can be classified as short-pass, since they respond to brief sounds but not longer duration sounds. The authors describe these neurons as *duration-tuned* neurons. Previous research has shown that duration tuning is produced at neurons in the IC through specific temporal sequence of excitatory and inhibitory inputs (Casseday *et al.*, 1994; Covey *et al.*, 1996). Covey *et al.*, (1996) showed

that in the IC, duration-tuned cells respond to sound with an outward current, followed by a net inward current. The initial outward current is temporally locked to the onset of sound, and the inward current is temporally locked to the offset of sound. As the sound duration is lengthened, the magnitude of the offset inward current increased until spiking occurs. This latter inward current the model refers to in IC neurons could be representative of the Ca^{2+} – mediated rebound potential seen in the ICc and from the ICx neurons in the present study. Therefore, if the incoming input coincides temporally with the rebound potential expressed in the target neuron, the neuron will be activated. However, if the duration between the incoming input is either too early or too late, it will not interact with the rebound potential. Thus, the neuron is tuned to a certain inter-synaptic response duration (i.e. duration-tuned). Recall that the studies examining the ICc (Sivaramkrishnan and Oliver, 2001) observed a higher proportion of rebound cells (57%) in comparison to the present ICx study with only 25% expressing the Ca^{2+} – mediated rebound potential. The lower occurrence of rebound cells in the ICx has also been expressed in a previous study (Li *et al.*, 1998). These findings correlate well with the assumption that ICc neurons are involved in the temporal processing of auditory information, unlike ICx neurons that are not usually associated with these tasks.

5.4.3 Ca^{2+} and AHP

The contribution of Ca^{2+} to the single-spike AHP seen in the ICx neurons in response to depolarizing current injection was also investigated. The properties of this AHP have already been discussed, but when the undershoot was examined more closely there appeared to be two distinct components. The first earlier component was of shorter

duration, followed by a slower longer lasting portion (figure 23). In several cases, the dual components of the single-spike AHP were not so evident and instead were seen as a continuous phenomenon (figure 24). When a representative neuron was bathed in ACSF with 0 Ca^{2+} , the AHP was greatly reduced. Therefore, the undershoot which was mediated mostly by the slower component in this neuron was abolished by the removal of Ca^{2+} . The effects of 0 Ca^{2+} on the single-spike AHP were reversed once the neuron was bathed in normal saline. These observations for the presence of the dual components suggest that the single-spike undershoot in some ICx neurons were mediated by two different K^+ channels. The first early component of the AHP may be mediated by the classical voltage-dependent K^+ channel activated by depolarization. The second slower activating conductance could be mediated by voltage-dependent Ca^{2+} -activated - K^+ channels. In the present study, two types of single-spike AHP were evident. One group of ICx neurons displayed an AHP with two obvious components temporally separated, and each mediated by a different type of K^+ channel. The second group displayed an AHP which seemed to be one continuous phenomenon, and thus mediated predominantly by one type of K^+ channel (i.e. Ca^{2+} -activated - K^+ channels). The results from the present study for the contribution of Ca^{2+} to the single-spike AHP agrees with the findings of other studies in the various subnuclei of the IC (Li *et al.*, 1998; Sivaramkrishnan and Oliver, 2001; Smith, 1992).

This presence of Ca^{2+} may have significant effects upon the membrane properties of the ICx neurons. The results from the present study have shown that the single-spike AHP was mediated by Ca^{2+} -activated K^+ -channels. Therefore, this K^+ current is modulating the interspike interval, which influences both firing frequency and firing

pattern. For example, the firing pattern of the adaptation-type cells may be in part mediated by the AHP. When a sustained depolarizing current injection was applied a train of APs was elicited but the interspike interval became progressively longer. The increase in interspike interval may be associated with the accumulating slow AHP mediated by Ca^{2+} -activated K-currents. Some studies on ICd and ICc neurons have blocked Ca^{2+} entry or Ca^{2+} -activated K^{+} -channels using Cd^{2+} , or charybdotoxin (a specific blocker for the Ca^{2+} -activated K^{+} -channels), which significantly reduces the presence of the slow AHP. The resultant train of APs to positive current injection display more regular interspike intervals and resemble regular-type cells (Li *et al.*, 1998; Sivaramkrishnan and Oliver, 2001).

5.5 RLF

The firing frequency or RLF of the ICx neurons was examined across cell types and differences between the groups were evident. In response to depolarizing current injection, the regular-type cells had a significantly lower threshold for firing and displayed a linear increase with a steeper slope in comparison to the other two cell types. The adaptation-type cells displayed a linear relation, but for a lower RLF. The pauser-type cells had the highest threshold for firing and also displayed a linear RLF. Initially, the adaptation-type had a higher RLF in comparison to the pauser-type, but this was due to the “pause” seen in the first interspike interval. However, it is interesting to note that as the level of current injection increased, the RLF of pauser-type neurons increased at a greater rate than adaptation-type cells. The RLFs were then tested at even higher levels of depolarizing injection (> 300 pA), to see when the firing frequencies of the three cell

types would saturate or max-out. The maximum current injection level with which both adaptation-type and pauser-type ICx neurons could fire APs, was significantly lower than the regular-type cells. At the maximum current injection, the cells no longer produced an increase in their firing frequency and were considered *saturated*. The regular-type cells could continue to fire at injection levels above 900 pA. Also, the average firing frequency for the regular-type cells was also higher than the other two cell types at these higher levels of positive current injection. The ability of the regular-type cells to display a higher average RLF and higher maximum current injection, could be linked to a difference in distribution of Ca^{2+} -activated K-channels between groups but further evidence would be required to substantiate this hypothesis.

When the RLF and max firing frequency were compared across the subnuclei of the IC, the ICx and ICc neurons had a significantly greater rate of firing for the same strength of current injection compared to the ICd neurons (Li *et al.*, 1998). The average RLF shown in this study for the regular-type ICx neurons are similar to the values produced by Li *et al.*, (1998), however analysis of RLF in their study was not differentiated by cell type. Their study based the RLF analysis for each subnucleus on the sustained firing pattern group of neurons, which are equivalent to the regular-type cells of the present study. It is also interesting to note that the maximum firing frequency, maximum current injection level at which APs could be elicited, and threshold for current injection from ICx neurons shown in the present study were all lower than the ICc neurons shown by Peruzzi, *et al.*, (2000). When these measures are compared against results found in other auditory neurons such as the VNLL (Zhao and

Wu, 2001), and CN (Oertel, 1997), the ICx still appear to display a relatively lower threshold for firing APs and a lower maximum firing frequency.

5.6 Hyperpolarization- activated cation current (I_h)

Two distinct types of hyperpolarization activated conductances have been described in previous studies, described together as an inward rectification or an increased conductance on hyperpolarization (*see Pape, 1996 for review*). The first type is labeled as the classical inward rectifier discovered in muscle cells by Katz, termed I_{KIR} . This rectification arises from voltage-dependent K^+ channels and is sensitive to extracellular cations, including Cs^+ and Ba^{2+} and displays fast kinetics.

Another type of mixed-cation current, called I_h . These channels open at negative potentials and close at positive potentials, and have been shown to be blocked by Cs^+ and Rb^+ ions, and mediate a mixed cation-current, almost as permeable to Na^+ as K^+ . The gating of I_h channels is directly associated by the binding of cAMP (DiFrancesco and Tortora, 1991), making their depolarizing effect dependent upon the status of second-messenger systems. The effect of I_h channels is to initiate slow depolarization if the membrane potential becomes very negative. Further, an elevation of cAMP makes I_h channels easier to open and promotes depolarization.

The present study on ICx neurons, found the presence of I_h underlying the observed inward rectification. The data illustrate a close temporal correlation between the depolarizing sag of the membrane potential and the development of I_h , indicating that I_h underlies the inward rectification.

5.6.1 Voltage sensitivity of I_h

The data show that in response to increasing levels of hyperpolarizing current injection, the inward conductance proposed to be I_h , showed an increase in magnitude. Furthermore, when the neuron was clamped at increasingly hyperpolarized voltage steps, the conductance for I_h increased. This voltage dependence of I_h in ICx neurons was illustrated by a Boltzman function, derived from tail current measurements (figure 32). The data shows threshold activation for I_h at membrane potentials more negative than -70 mV. Various studies have examined the properties of I_h and have found similar threshold values for activation. Mercuri *et al.*, (1995) showed a threshold activation of -69 mV in rat midbrain dopaminergic neurons, which is in agreement with the findings in this study. Similar values have been shown in guinea pig bulbospinal neurons (-70 mV)(Dekin, 1993), rat hypothalamus histaminergic neurons (-80 mV) (Kamondi and Reiner, 1991), and rat DNLL neurons (-70 mV) (Fu *et al.*, 1997). The data for the current study showed an activation range for I_h at voltage levels of between -70 to -140 mV. The activation curve with the Boltzman function also shows a $V_{1/2}$ of -100 mV. The voltage dependence illustrated here is similar to range of values found in various other neurons (Bayliss *et al.*, 1994, Fu *et al.*, 1997; Mayer and Westbrook, 1983).

5.6.2 Reversal potential of I_h

Examination of the reversal potential from the representative ICx neuron showed a reversal potential of -62 mV (figure 31). When other neurons from the present study are included the reversal potential of I_h ranged from -28 to -65 mV. Measurements of reversal potential of I_h in other cells derived using the same methodology show similar

results, ranging from -17 to -50 mV (Fu *et al.*, 1997; Maccaferri *et al.*, 1993; Spain *et al.*, 1987). The reversal potential encountered for the sampled ICx neurons was more positive than the RMP, suggesting that it was mediated by both Na^+ and K^+ . Other studies have also manipulated the extracellular concentration of these ions to provide further evidence that I_h is a mixed-cation current mediated by these ions (Bal and Oertel, 2000; Fu *et al.*, 1997).

5.6.3 Functional role of I_h

The presence of I_h has been shown throughout the CNS in both auditory and non-auditory neurons (Bal and Oertel, 2000; Bayliss *et al.*, 1994; Fu *et al.*, 1997; Maccaferri *et al.*, 1993; Mayer and Westbrook, 1983; Spain *et al.*, 1987). In the auditory system, the most prominent currents were illustrated in octopus cells of the cochlear nucleus (Bal and Oertel, 2000; Golding *et al.*, 1995). The findings in these studies showed that octopus cells express I_h and have a lower R_{input} , which is due to the low threshold K^+ currents ($I_{\text{K(L)}}$). The interplay of I_h and $I_{\text{K(L)}}$ accelerates voltage changes in response to synaptic currents, thus improving the temporal precision of the synaptic response and increasing the ability to follow stimuli at high frequencies (Bal and Oertel, 2000; Golding *et al.*, 1995; Koch and Grothe, 2003). These studies support the notion that I_h in the octopus cells regulate the “gain” of synaptic responses to activation of the auditory nerve when processing and encoding the temporal aspects of sound (Bal and Oertel, 2000).

The neurons of the ICx on the other hand are not usually associated with temporal processing, and failed to produce any onset-type neurons and therefore the role of I_h must be different. It is interesting to note that I_h is modulated by various substances, such as

cAMP, Ca^{2+} , and even neuromodulators like noradrenalin and 5-HT (McCormick and Pape, 1990). Therefore, it is quite plausible that in the ICx, I_h could be influenced by the behavioural state of the subject, since changes could lead to fluctuations in the normal levels of these substances (i.e. 5-HT) (*discussed later*).

Another possible theory for the functional role of I_h in the ICx, or any other structure for that matter, comes from the observation that this conductance is activated when the neuron hyperpolarized. Even more specifically, I_h is generally activated at levels of hyperpolarization more negative than the cells resting potential and its magnitude increases as the level of hyperpolarization increases. Therefore, I_h may act as a *safety mechanism*, which is activated when the cell becomes increasingly hyperpolarized and helps depolarize the cell to “normal” working conditions. This modulation of the membrane would prevent the cell from becoming “too hyperpolarized”, which would make normal firing and synaptic transmission difficult. Some studies have shown that I_h may also assist in modulating the RMP of the cell. However, this observation is associated with neuronal structures which displayed activation ranges close to or more positive than the normal RMP of those neurons (Bayliss *et al.*, 1994; Maccaferri *et al.*, 1993; McCormick and Pape, 1990). The ICx neurons sampled in the present study displayed an activation threshold for I_h which was more negative than the average RMP, thus it is quite unlikely that I_h was contributing to the setting of the normal RMP of the cells in this structure. Similar conclusions were drawn from I_h studies in other auditory and non-auditory neurons where the activation threshold of I_h was more negative than the neuron’s RMP (Fu *et al.*, 1997; Kamondi and Reiner, 1991, Mercuri *et al.*, 1995).

5.7 *Synaptic response patterns*

Many of the previous studies, which have studied the synaptic response properties in ICc and ICx neurons, examined responses elicited via electrical stimulation of the CoIC or lower auditory structures in the case of the ICc. The ICx receives some minor input from the contralateral ICc through the CoIC and also receives its most significant lower auditory brainstem projection through a local connection from the ipsilateral ICc (Adams, 1980; Aitkin, *et al.*, 1975, 1978; Moore and Goldberg, 1963; Willard and Ryugo, 1979). To my knowledge, the present study is the first to examine the synaptic responses derived from stimulation of the direct inputs from the ICc to the ICx. This stimulation site was chosen because stimulation of the CoIC undoubtedly activates inputs from various nuclei such as the contralateral subnuclei of the IC, lower auditory brainstem projections via the contralateral ICc and even descending cortical input. This wide array of inputs would therefore complicate the analysis of the synaptic response from ICx neurons. Also, the inputs the ICx receives via the CoIC would most likely be polysynaptic, making direct conclusions about the elicited responses quite difficult. The synaptic responses presented in this study show that this direct input between these two subnuclei of the IC, does exist.

Stimulation of the ICc fibers produced EPSPs and/or IPSPs in all three types of ICx neurons. The synaptic responses that were produced were categorized as predominantly excitatory, inhibitory, or a mixed response composed of both excitatory and inhibitory components. The data show that the majority of neurons in the ICx (60%) produced synaptic responses that were predominantly excitatory. Meanwhile, inhibitory only synaptic responses were only encountered in 16% of the neurons, but mixed

EPSP/IPSP responses were shown in 24% of the neurons. If the proportion of excitatory and mixed responses are combined, then the ICx neurons display excitation in its synaptic response in 84% of the neurons sampled. Using this same logic, the proportion of inhibitory responses would be 40%. Therefore, from the synaptic responses elicited in these ICx neurons, it seems that this subnucleus is mostly an excitatory structure accompanied by inhibition to a lesser extent.

The synaptic responses that were evoked by stimulation of the ICc, were graded and increased in magnitude with increasing levels of stimulation intensity. Also, generally low levels of stimulation ($< 10V$) were required to reach the threshold for eliciting a response, supporting the notion of the direct inputs from the ICc. Furthermore in some neurons, slightly increased levels of electrical stimulation ($\sim 20V$) produced a suprathreshold response. The response latency was examined for the ICx neurons, and no significant differences were observed between the excitatory, inhibitory, and mixed-type synaptic responses. The average synaptic response latency was 2.3 ms collapsed across all three cell types. This value was low enough to substantiate that the synaptic responses elicited were via a monosynaptic pathway, however some may argue that lower values ($< 1-1.5$ ms) would be expected for a direct pathway (i.e. ICc to ICx). Even though, the latency in the present study was well within expected limits, the slightly longer value may be due to the age of the animals used. Most other studies, with which comparisons are being made, used young adult rats between 3-6 weeks of age (Wu and Kelly, 1995; Wu and Oertel, 1986), while the current study utilized rats between P8-11. Onset of hearing has not even been achieved at this age range, and the process of myelination is still on going. Myelination commences around the P6 and continues so

that 85% of the fibers are myelinated by P28 (Foster *et al.*, 1982). Furthermore, it has been shown throughout the auditory brainstem, that neurons undergo developmental changes both before and after the onset of hearing and these changes can significantly affect the synaptic response properties (Ahuja and Wu, 2000; Kandler and Friauf, 1995; Wu and Oertel, 1987). Therefore, the slightly inflated latency time seen here may be due to lags in conduction velocity and transmission in general between these two subnuclei due to age.

5.7.1 Pharmacological analysis

Once the nature of the synaptic responses was classified, pharmacological agents were employed to elucidate the neurotransmitters responsible. Specific antagonists verified the presence of the “classical” neurotransmitters; GABA, glycine and glutamate (mediated by NMDA and AMPA receptors). It is interesting to note that these same transmitters are also found in the ICc (Koch and Grothe 2003; Wu *et al.*, 2004), further evidence supporting the direct input from ICc to ICx.

However, the presence of each of these transmitters was not equivalent, and differences in proportion became evident. The pharmacological analysis utilized a sequential application of the antagonists that was cumulative, therefore the contribution that each receptor-type made to the overall synaptic response could be qualitatively determined. The present study found that in ICx neurons, the inhibition is primarily mediated through GABAergic transmission via GABA_A receptors. On the other hand, the involvement of glycinergic transmission and its receptors was limited at best. Whenever an IPSC or an inhibitory component of a response was evident, application of

the GABA_A receptor antagonist, bicuculline would greatly suppress or even completely abolish the inhibition. If any inhibitory response was remaining, the glycine receptor antagonist, strychnine would remove it. This same type of proportional analysis using pharmacological manipulations was utilized to examine the contributions of AMPA and NMDA receptors on glutamatergic transmission. The results show that the contribution of AMPA receptors in glutamatergic transmission was much more significant than that observed for the NMDA receptors in ICx neurons. When an EPSC or an excitatory component was evident, blockade of the AMPA receptors using the antagonist, CNQX caused a much larger decrease upon the overall excitatory response than the NMDA receptor antagonist, APV. In several cases, blockade of the AMPA receptor completely removed the excitatory component of the response.

Taken together, the pharmacological data in the present study suggests that in the synaptic responses elicited via stimulation of the ICc, the excitatory transmission is chiefly mediated by glutamate through AMPA receptors and the inhibitory transmission is primarily mediated by GABA through GABA_A receptors. Some neurons in the ICc have also been shown to be immunoreactive for GABA and the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), while the terminals of these neurons sending inputs to ICx neurons possess the characteristic features of inhibitory synapses as seen in other areas of the CNS (i.e. flattened or pleomorphic vesicles) (Chakravarty and Faingold, 1996; Moore and Moore, 1987; Wynne, *et al.*, 1995).

Other studies, which have pharmacologically examined synaptic responses in the IC, have reported that ICx neurons display epileptiform activity with the application of blocking agents (Li *et al.*, 1998; Smith, 1992). This activity was shown to be mediated

by NMDA receptors. This phenomenon was not observed in any of the ICx neurons encountered in the present study. The data shows that after pharmacological blockade of the inhibitory conductance, the excitatory components of the response appear to be “normal” (i.e. no epileptiform-type activity). The absence of the epileptiform-type in the ICx neurons from the current study could be due to several factors. Firstly, in the two studies mentioned, (Li *et al.*, 1998; Smith, 1992) this activity was expressed by application of NMDA and/or used 0 Mg^{2+} ACSF. Secondly, both studies expressed the epileptiform-type activity in the ICx in response to electrical stimulation of the commissure of the IC (CoIC). The authors themselves realize that this intercollicular projection contains many excitatory and inhibitory inputs from both auditory and non-auditory structures. Taken together, if the CoIC is electrically stimulated this would activate a larger number of inputs directed towards the ICx, in comparison to the direct inputs from the ipsilateral ICc (as seen in the current study). Therefore, if the inhibition is blocked using the specific antagonists (i.e. bicuculline, strychnine), the proportional increase in excitatory transmission and its postsynaptic effects will be more pronounced in the stimulated CoIC inputs than those of the ipsilateral ICc. Once the inhibition is removed, both exogenously applied NMDA and/or used 0 Mg^{2+} ACSF would further exaggerate the responses at the NMDA receptors. Thus, these two factors combined may be inflating the responses at the ICx in response to electrical stimulation, manifested as epileptiform-type activity. Another difference between the current study and those which showed this activity, was with differences in subject age and pharmacological application. Both of the previous studies used rats that were more mature than those used in the present study, and this may have had some influence in the various receptor

distributions which are known to be developmentally linked. Furthermore, both previous studies applied the inhibitory receptor antagonists for a longer duration (>10 min) and in several cases used a larger stimulation intensity than the control condition to elicit the epileptiform activity. The present study maintained stimulation intensity throughout the pharmacological manipulations, and only applied the drugs until their antagonistic effects were seen. These factors together may explain the presence of epileptiform-type activity in the ICx in those previous studies (Li *et al.*, 1998; Smith, 1992).

An earlier study from our lab on ICc neurons (Ma, *et al.*, 2002) utilizing the identical recording procedures and methods as the present study, found that blockade of the inhibition via bicuculline and strychnine, resulted in bursting activity much like that reported in the ICx (Li *et al.*, 1998; Smith, 1992). This epileptiform-type activity lasted for several hundred milliseconds, and was effectively blocked by the NMDA receptor antagonist, APV. This supports the notion that the ICc may be a initiation site for AGS (discussed later) and this activity is mediated by NMDA receptors. Therefore, the absence of the epileptiform activity in ICx neurons for the present study may have not been due to methodological or recording concerns, but perhaps is not present under normal conditions, supporting ICx's role as a relay center for AGS.

5.8 Contribution of 5-HT

Despite the global presence of 5-HT in the brain, its relationship with auditory neurons is unclear at best. Further complicating matters is the limited data that is known about the types of 5-HT receptors and more specifically the types present in the ICx. There is a vast array of 5-HT receptors in the CNS that can act both pre- and

postsynaptically. Current research has revealed seven serotonin receptor families, designated 5-HT₁ through 5-HT₇, and each is convoluted further with varying subunit compositions (Li *et al.*, 2004; Peroutka, 1994). With regards to the auditory system, the serotonergic system has been shown to innervate most nuclei of the ascending auditory system (Klepper and Herbert, 1991; Thompson *et al.*, 1994; Willard *et al.*, 1984), however the influence of this innervation is poorly understood. Interestingly, a significant amount of information about the effects of 5-HT in non-auditory cells has already been realized (Byrne and Kandel, 1996; Wang *et al.*, 1996). Only a limited number of electrophysiological studies have been done which examine the influence of 5-HT on auditory brainstem nuclei (Ebert and Oswald, 1992; Wang and Robertson, 1997), and even fewer have done so *in vitro*. The results from these previous studies are inconclusive and limited at best, and only begin to introduce the possible effects of 5-HT on auditory neurons. To my knowledge, no other study has examined the contribution of 5-HT on pharmacologically isolated receptors in the IC using *in vitro* brain slice techniques.

In the present study, when a synaptic response was observed in an ICx neuron, in response to electrical stimulation of the ICc, 5-HT was applied with no other pharmacological manipulations. The results from four representative neurons are outlined in figure 41, chosen on the diversity of their control responses. The overall finding was that 5-HT did indeed have some effect upon the control response, and these were reversible once the 5-HT was removed. In some neurons, application of 5-HT caused an apparent increase in the inward/outward current in comparison to the non-drug response. Other cells showed a decrease or even reversal of the synaptic current from

control conditions. Therefore, 5-HT showed that it had some modulatory effect upon the electrically evoked synaptic responses.

Further analysis was conducted in a group of ICx neurons which included pharmacological blockade of all of the receptors except one, followed by the application of 5-HT on the isolated receptor. All but one of the inward/outward currents had been pharmacologically removed via the specific antagonists (i.e. bicuculline, strychnine, CNQX, APV) and the effects of 5-HT on the remaining current, assumingly mediated by a specific type of receptor, were observed. Then to verify that the isolated receptor was the one in question, its specific antagonist was added to completely remove the synaptic response. The results found that 5-HT had its most pronounced effects on the glutamatergic NMDA receptor and very limited effects on the associated AMPA receptor. Figure 42 shows traces from an ICx neuron with the middle trace showing an inward current theoretically mediated solely by AMPA receptors. When 5-HT was applied, there was no recognizable change in the magnitude of the inward current, thus 5-HT had no significant effect on the isolated AMPA receptor mediated response. The AMPA receptor antagonist, CNQX was then added to abolish the inward current after the 5-HT is removed to verify the identity of the receptor. The present study also found that GABA and glycine receptor mediated responses were not significantly affected by the application of 5-HT. Using the same methodology as above, figure 44 shows that 5-HT application had no obvious effect on the isolated GABA_A receptor mediated outward current. Figure 43 presents an ICx neuron with the isolated glutamatergic NMDA receptor mediated response and 5-HT application revealed an outward current. Using the theory that 5-HT significantly influences NMDA receptors, this phenomenon can be

explained as follows. The 5-HT may have caused a decrease in the activation or even block the NMDA receptors and this revealed an outward current that may be a result of incomplete blockade of an inhibitory current. When the 5-HT was removed, the response returned to control conditions. The small inward current was then blocked with APV.

This "*NMDA theory*" can also be used to as a possible mechanism to explain the results seen in the neurons where the individual receptors were not isolated (figure 41). In figure 41A, modulation of NMDA by 5-HT caused a decrease in the inward synaptic current, with more prominent effects seen on the later component. Meanwhile, the neuron in figure 41B expresses a large inward current and a larger outward current after 5-HT application. The inward current may be mediated by AMPA receptors, which were allowed to go suprathreshold once NMDA receptor mediated current had been removed. This removal may have also revealed an underlying outward current which was opposing the inward current under normal conditions. This same logic can be used to explain the revelation of an outward current after the application of 5-HT in figure 41C. Figure 41D illustrates an ICx neuron with an early and late inward current possibly mediated by AMPA and NMDA receptors respectively based on time course. Once 5-HT was applied, the later component was significantly reduced and returns after wash. Therefore, from the data presented in this study it is quite plausible that 5-HT expresses a significant modulatory interaction with glutamatergic NMDA receptors in ICx neurons. The NMDA theory presented here is speculation on how 5-HT might be modulating the ICx synaptic responses. This model is one of many possibilities for this observed phenomenon. The present data show that the modulation of the responses by 5-HT is likely through action

on the glutamate receptors. Again, the complexity and mode of action of the mechanisms is still unclear and further experimentation is required to elucidate this question.

This data is by no means conclusive, but it does shed light on the possible contribution that 5-HT may have on normal auditory function in the ICx. Also, the data presented in this study has not considered the potential diversity of 5-HT receptors in the ICx, since each would potentially have different effects upon activation. Several recent studies have shown the presence of 5-HT_{1A} and 5-HT_{1B} receptors in the ICx and also in the other subnuclei of the IC (Peruzzi and Dut, 2004; Thompson *et al.*, 1994). The distribution of the various subtypes of 5-HT remains unclear, with different studies producing conflicting results. For example some studies have illustrated the presence of 5-HT_{2A} receptors (Li *et al.*, 2004) in IC neurons, while other have described its absence (Peruzzi and Dut, 2004). Understanding of the distribution of 5-HT receptors in the auditory brainstem is in its infancy, and this hinders the interpretation and comprehension of the modulatory effects for this neurotransmitter.

One question which arises when trying to understand the complicated observations of 5-HT modulation involves the possible mechanism of action. It is interesting to note, that in the present study 5-HT was shown to display significant effects upon NMDA receptors while only limited interaction with AMPA receptors. Both are glutamate receptors, yet one is more greatly affected by 5-HT than the other. Some have suggested that 5-HT effects are presynaptic while others have proposed postsynaptic actions (Ebert and Oswald, 1992; Faingold *et al.*, 1991). Presynaptic actions alone would not explain the results from the present study, because if this were the case 5-HT would modulate the presynaptic release of glutamate. However, this

should theoretically influence both NMDA and AMPA receptor equally or very closely. The present study shows significant effects of 5-HT on NMDA receptors and only limited modulation of AMPA receptors. It is most likely a combination of both pre- and postsynaptic effects. In order to explain the discrepancy in efficacy of glutamatergic receptor modulation via 5-HT, one may have to look postsynaptically.

One possible explanation may be that NMDA receptors found postsynaptically may have specific binding sites for 5-HT or even an associated binding protein which interacts with the NMDA receptor. Therefore, certain receptors found presynaptically, most probably mediated by a specific subtype of 5-HT receptor, could modulate glutamate release. Then different subtypes of 5-HT receptors found postsynaptically could influence the NMDA receptors directly/indirectly. Evidence from other studies have shown 5-HT to influence presynaptic release of GABA in ICc neurons (Peruzzi and Dut, 2004), with the actions suggested to be both presynaptically and postsynaptically modulated, further supporting the above mentioned theory. Unpublished results in our lab from ICc neurons using methods similar to those outlined in this study, show modulation of glutamate release by GABA_B receptors with results on glutamate receptors similar to those shown here. Activation of the GABA_B receptors via specific agonists is believed to be causing a decrease in the presynaptic glutamate release. However, the data show that the glutamatergic NMDA receptor mediated currents are more significantly affected than the AMPA receptor mediated currents. These findings can be explained using the same logic for the proposed theory for 5-HT modulation of presynaptic glutamate release and postsynaptic action. It is quite evident that the effects of 5-HT and their methods of action are quite intricate and convoluted. Further

immunohistochemical and more specific pharmacological analyses will be crucial in understanding how 5-HT may modulated transmission in ICx and other auditory neurons.

The role of 5-HT in the ICx still remains unclear, however the current study shows that there is some interaction. Previous research has shown that 5-HT depresses or facilitates the responsiveness of IC neurons (Hurley and Pollak, 1999) and other auditory neurons (Ebert and Ostwald, 1992; Faingold *et al.*, 1992). The findings also showed that in the IC, the type of modulatory effects of 5-HT in the ICc were dependent upon the type of auditory signal presented. The *in vivo* data showed that 5-HT predominantly caused depression on responses evoked by tone bursts, and depression or facilitation of responses evoked by FM sweeps. Even though, these results are from the ICc and are from *in vivo* recordings from Mexican free-tailed bats, they illustrate the modulatory role 5-HT can have on auditory processing. These same general mechanisms of 5-HT modulation might be present in the ICx, with depression and/or facilitation of responses to varying types of acoustical stimuli, in association with the behavioural states of the subject.

5.9 Functional role of the ICx

Our understanding of the functional role of the ICx remains elusive. The focus of the majority of IC studies has and still remains to be the ICc. The data presented from the present study and the limited earlier findings show that the ICx is indeed a distinct subnucleus unique from its other counterparts. The ICx is generally believed to have two main responsibilities; first the ICx may be involved in processing auditory information for the reflexive orienting responses evoked by auditory stimulation (Aitkin,

et al., 1978, 1981; Faye-Lund, 1985). Secondly, the ICx may have involved in genetically-inherited and ethanol withdrawal-induced AGS (Chakravarty and Faingold, 1996; McCown, *et al.*, 1984; Ribak, *et al.*, 1994; Wada, *et al.*, 1970).

Studies on the ICc have shown the presence of at least six physiologically distinct cell types, each attributed with a different role in terms of auditory processing (Bal and Oertel, 2000; Peruzzi *et al.*, 2000; Sivaramkrishnan and Oliver, 2001). It is widely accepted that the ICc is a major integrative center, receiving inputs from essentially every lower auditory brainstem structure. Various studies have illustrated the ICc involvement in temporal processing of auditory information, spatial mapping, frequency tuning, binaural processing, duration tuning, and processing of complex sounds to name a few. Thus, it makes sense that this subnucleus would possess the varying cell types with different properties. The ICx on the other hand displays a lower diversity of physiologically distinct cell types. Only four cell types were observed in the ICx for the present study; regular-, adaptation-, pauser-, and bursting-type which was not analyzed because of its low occurrence (<3%). Of the three cell types that were evident, the regular-type constituted almost 70% of the population. This regular-type or sustained-type as some other studies denote, displays not characteristic properties which associate them with precise temporal processing or frequency mapping. Instead, this cell type is usually coupled with simple signal transformation.

Furthermore, the ICx neurons have been shown to display a lower maximum firing frequency, lower maximum current injection level at which APs could be elicited, and lower threshold for firing than the ICc neurons (Peruzzi, *et al.*, 2000). These same trends for these measures are also seen when compared against results found in other

auditory neurons such as the VNLL (Zhao and Wu, 2001), and CN (Oertel, 1997). The ICx neurons also display a higher average R_{input} especially for the more prevalent regular-type cells, than most other auditory neurons, including the ICc. When taken together, all of these findings suggest that the ICx is a more easily excitable cell. The lower threshold of current injection required to elicit APs, indicates that *in vivo* a smaller excitatory input would still allow for firing/activation of the ICx neurons. However, once activated the neuron has a limited range of firing frequency. These properties support the notion that the ICx may act as a relay station receiving significant direct input from the ipsilateral ICc. The ICx might simply be relaying the auditory information already processed by the ICc to the higher structures (i.e. thalamus, primary auditory cortex) in the hierarchical auditory processing order.

Also, since the ICx receives significant projections from somatosensory structures (i.e. contralateral cuneate and gracile nuclei, and the contralateral spinal trigeminal nucleus), and other non-auditory structures (i.e. deep superior colliculus, substantia nigra par lateralis, raphe nucleus and motor areas), it may be involved in integrating sensor-motor information with auditory information. Furthermore, because of the reciprocal connections with the ICc, and these non-auditory inputs, the ICx may be modulating processing at the ICc and indirectly other lower auditory structures. Research has shown that the activity of the raphe nucleus varies markedly with an animal's arousal state and with the direction of attention to a stimulus (Jacobs and Fornal, 1999; Trulson and Jacobs, 1979). The raphe nucleus has serotonergic projections to the ICx, and the ICx has been shown to be modulated by arousal states. The auditory

processing characteristics in the ICc also get affected, possibly by the modulation by the ICx.

The contribution of 5-HT in ICx neurons remains unclear. Extracellular electrical stimulation of the IC has been shown to cause fearful and aversive behaviour in rats (Melo and Branão, 1995). The IC is thought to be involved in linking external stimuli and aversive behaviour, primarily through the ICx. This aversive behaviour has been shown to be in part modulated by 5-HT. 5-HT levels in the IC were increased using the reuptake inhibitor, zimelidine, or α -methyl-5-hydroxytryptamine, a 5-HT agonist, and reductions in fear and anxiety influenced behaviour were observed. The ICx could then itself modulate the auditory processing occurring at the ICc. These changes in processing and responsiveness could be viewed as functional reconfiguration of the neural circuitry, making the process highly dynamic.

The ICx has also been associated with the AGS activity, because of its higher level of excitability and the presence of spontaneous or epileptiform-like activity shown in some studies. The present study failed to show epileptiform-like activity and found only a small proportion of bursting-type cells. However, studies in the ICc have shown spontaneous activity, and epileptiform-like activity along with comparatively higher levels of excitability. Sakamoto and Niki (2001) utilized acoustic priming to examine what effect it had on the threshold for inducing AGS in the areas of the IC. The results clearly demonstrate the contribution of the ICx and the ICc in eliciting AGS. Thus, it might be possible that the ICc is the initiation point for seizure-like activity and the ICx acts as a relay station, receiving input from the ICc and acts as the output site for the sensory-motor integration. Furthermore, the ICx then propagates this information to the

deep layers of the superior colliculus, which has been noted as the primary generator for wild running (Faingold and Casebeer, 1999; Faingold and Randall, 1999; Faingold, *et al.*, 1998; Sakamoto and Niki, 2001), which is a characteristic of AGS.

6. Conclusions

The current electrophysiological study of ICx neurons was able to demonstrate its distinctiveness from the other subnuclei within the IC. Whole-cell patch clamp recordings showed the presence of three different cell types based on firing pattern; *regular-*, *adaptation-*, and *pauser-type*. Furthermore, it seems that ICx is composed mainly of regular-type neurons and generally displays homogeneity of its intrinsic membrane properties across all cell types. The present study was also able to illustrate the presence of a Ca^{2+} -mediated component in action potentials and rebound potential after hyperpolarization. The post-depolarization AHP and single-spike AHP may be mediated by certain channels including Ca^{2+} -activated K^+ channels. Some ICx neurons were also shown to express the hyperpolarization-activated cation current I_h , with characteristic properties that were similar to those found in other auditory neurons. The I_h was shown to have an activation threshold and range more negative than the neuron's RMP.

The present study was the first of its kind to examine synaptic responses via electrical stimulation of the inputs from the ipsilateral ICc, allowing for direct analysis of this projection. The ICx neurons displayed predominantly excitatory responses in response to the stimulation. This excitatory transmission was shown to be primarily mediated by glutamate AMPA receptors and to a lesser extent, NMDA receptors. The inhibitory component of the synaptic responses was mediated chiefly by GABA_A receptors and some limited glycinergic transmission. These electrically elicited synaptic response showed changes in magnitude upon application of 5-HT. When specific receptor mediated responses were isolated, 5-HT was shown to have modulatory effects

upon the NMDA and AMPA receptor mediated responses, with greater effects seen in the former. Insignificant effects were seen upon the GABA and glycine receptor mediated responses.

The data presented in this study support the proposed functional role of the ICx as a relay center which is modulated by behavioural states, and does not have a significant role in the complex characteristics of auditory processing. The ICx may modulate the auditory processing occurring in the ICc and may also serve as an output site for the sensory-motor integration.

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