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**Development of Electrical Membrane Properties and Synaptic Transmission in the
Rat's Dorsal Nucleus of the Lateral Lemniscus Using *In Vitro* Brain Slice**

by

Tarry Ahuja

A thesis presented to the faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of

Master of Science.
Specialization in Neuroscience

Institute of Neuroscience
Carleton University
Ottawa, Ontario
August, 2000

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**“Development of Electrical Membrane Properties and Synaptic Transmission in the
Rat’s Dorsal Nucleus of the Lateral Lemniscus Using *In Vitro* Brain Slice”**

submitted by

Tarry Ahuja, B.Sc

In partial fulfillment of the requirements for the degree of

Master of Science

Specialization in Neuroscience

Chair, Department of Psychology

Thesis Supervisor

Carleton University
August, 2000

Abstract

Whole-cell patch clamp recordings were taken from dorsal nucleus of the lateral lemniscus (DNLL) neurons of rat pups at postnatal day 3 to 14. Positive current injection elicited action potentials that had attributes that were “immature” in comparison to the more mature neurons displaying longer duration, rise time, decay time, smaller amplitude and slower voltage change. Postsynaptic potentials, with excitatory predominance, were elicited by electrical stimulation of the lateral lemniscus. Young DNLL neurons displayed a longer synaptic response latency, duration and time to peak in comparison to their mature counterparts. Furthermore, these attributes begin to plateau just prior to the onset of hearing. Pharmacological treatment indicated that both types of glutamate receptors (non-NMDA and NMDA) are present throughout development as early as P3. These results suggest that both the electrical membrane and synaptic response properties undergo a developmentally influenced maturational process in DNLL neurons.

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
AP	action potential
APV	amino-5-phosphonovaleric acid
C	capacitance
CN	cochlear nucleus
CNQX	6-cyano-7-nitro-quinoxaline-2,3-dione
CP	commissure of Probst
DNLL	dorsal nucleus of the lateral lemniscus
EPSP	excitatory postsynaptic potential
GABA	γ -aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC	inferior colliculus
INLL	intermediate nucleus of the lateral lemniscus
IPSP	inhibitory postsynaptic potential
LL	lateral lemniscus
LSO	lateral superior olive
MNTB	medial nucleus of the trapezoid body
MSO	medial superior olive
NMDA	N-methyl-D-aspartate
PSP	postsynaptic potential
R_{input}	cells input resistance
SOC	superior olivary complex

τ	time constant
TEA	tetraethylammonium
TTX	tetrodotoxin
VNLL	ventral nucleus of the lateral lemniscus
V_{rest}	resting potential

Introduction

Years of investigation have furthered our knowledge of the auditory brainstem, revealing details about its anatomy, morphology and some physiological properties. Like most scientific research, the bulk of these studies have been done with mature subjects. This trend has created an incomplete representation since many of the intermediary developmental steps traveled to reach these mature structures are yet to be uncovered. Developmental studies are trying to uncover the path that these mature auditory structures take in order to reach their “functional goal”.

One such auditory structure is the dorsal nucleus of the lateral lemniscus (DNLL). The DNLL is of some interest due to recent anatomical and physiological evidence which has supported the notion that this brain region is an important structure for central processing of auditory information (Glendenning *et al.*, 1981; Hutson *et al.*, 1991; Kelly, 1997; Kudo, 1981; Oliver & Shneiderman, 1989; Shneiderman *et al.*, 1988; Wu, 1998; Wu & Kelly, 1995a,b). Therefore, any information that could shed light on how the DNLL develops would be very beneficial in our understanding of the auditory process.

Dorsal nucleus of the lateral lemniscus

The DNLL is a distinctive group of neurons within the lemniscal pathway just below the inferior colliculus (IC). In cat, rat and bat, this nucleus receives afferent projections from the bilateral superior olivary complex (SOC) and the contralateral cochlear nucleus (CN) through the lateral lemniscus (LL) (Druga and Syka 1984; Glendenning *et al.*, 1981; Hutson *et al.*, 1991; Kudo 1981; Oliver and Shneiderman 1989; Shneiderman *et al.*, 1988; Zook and Casseday 1987). The DNLL also receives descending projections from the ipsilateral central nucleus of the IC (Caicedo and Herbert, 1993) and

ascending projections from both the ventral and intermediate lemniscal nucleus (VNLL, INLL) (Glendenning *et al.*, 1981). The DNLL receives inputs from the major structures in the lower auditory brainstem, such as the CN, SOC, VNLL, and the INLL through the lemniscal pathway, and from the contralateral DNLL through the commissure of Probst (Glendenning *et al.*, 1981; Huffman & Covey, 1995; Hutson, *et al.*, 1991; Kudo, 1981; Oliver & Shneiderman, 1989; Shneiderman *et al.*, 1988; Yang *et al.*, 1996) (see Fig.1). The DNLL projects to the contralateral DNLL (Adams, 1979; Bajo *et al.*, 1993; Beyerl, 1978; Brunso-Bechtold *et al.*, 1981; Coleman & Clerici, 1987; Goldberg & Moore, 1967, Merchan *et al.*, 1994; Oliver & Shneiderman, 1989; Ross *et al.*, 1988; Shneiderman *et al.*, 1988; Shneiderman & Oliver, 1989; Wu and Kelly, 1995a,b; Zook & Casseday, 1987) and to both the ipsilateral and contralateral IC (Adams 1979; Bajo *et al.*, 1993).

Immunocytochemical studies have shown that the DNLL neurons are positively labeled with antibodies associated with γ -aminobutyric acid (GABA), indicating that these neurons are GABAergic (Adams & Mugnaini, 1984; Glendenning & Baker, 1988; Roberts & Ribak, 1987; Thompson *et al.*, 1985; Vater *et al.*, 1992; Winer *et al.*, 1995; Zhang, 1998). The axons of DNLL neurons form a bundle of fibers called the commissure of Probst (CP). The CP carries GABAergic signals to the contralateral DNLL and the central nucleus of the IC bilaterally (Bajo *et al.*, 1993; Goldberg & Moore 1967; González-Hernández *et al.*, 1996; Kudo 1981; Shneiderman *et al.*, 1988; Hutson *et al.*, 1991; Zhang *et al.*, 1998). The above mentioned findings support the notion that the DNLL is a significant source of inhibition in the auditory midbrain. The results from these studies have also shown that the DNLL receives excitatory glutamatergic projections from the contralateral lateral superior olive (LSO) and inhibitory glycinergic projections from the

ipsilateral LSO along the lemniscal pathway (Wu, 1998). Furthermore, the DNLL receives glycinergic projections from the VNLL and INLL (Wu, 1998).

Some fundamental *in vitro* brain slice studies have provided valuable information about the DNLL's membrane and synaptic response properties (Wu and Kelly, 1995a,b; Wu & Kelly, 1996), but like in most studies in which the basic properties of a brain region are trying to be established, the results were found using fully functional mature specimens. Various levels of the auditory system develop at different rates (Sanes, 1993) and may show significant physiological and synaptic differences during different periods of development (Andrew and Paterson, 1989; Puel and Uziel 1987; Rybak *et al.*, 1992; Uziel *et al.*, 1981; Wu and Oertel, 1987). There have been several attempts to describe and understand the developmental processes in the central auditory system (Andrew *et al.*, 1989; Friauf & Kandler, 1990; Sanes, 1993; Wu and Oertel, 1987; Zine and Romand 1996), but little has been uncovered about the DNLL.

Functional role of the DNLL

The complete functional role of the DNLL is still being brought to light, but enough information has been amassed to outline its function. Since the DNLL receives monaural and binaural inputs from the major structures in the auditory lower brainstem and from the contralateral DNLL, it appears that the DNLL is a crucial site of neural integration, as well as a neural substrate for binaural interaction (Gabriele *et al.*, 2000; Wu, 1998). Tonotopic studies in rat have shown a concentric tonotopic order in the DNLL with higher frequencies represented along the outer margin and lower frequencies represented centrally within the nucleus (Kelly *et al.*, 1998a,b; Merchán *et al.*, 1994).

The DNLL is also thought to play an inhibitory role in the auditory midbrain.

Immunocytochemical and *in situ* hybridization histochemical studies have shown that most DNLL neurons synthesize GABA (Adams & Mugnaini, 1984; Glendenning & Baker, 1988; Moore & Moore, 1987; Roberts & Ribak, 1987; Thompson *et al.*, 1985; Vater *et al.*, 1992; Winer *et al.*, 1995; Wynne *et al.*, 1995). Furthermore, these DNLL neurons have been shown to mediate release and high-affinity uptake of GABA in the IC (Shneiderman *et al.*, 1993), and possess a GABAergic projection from the DNLL to the IC (González-Hernández *et al.*, 1996; Zhang *et al.*, 1998). All of these observations endorse the belief that the DNLL is a notable source of inhibition in the auditory midbrain.

In comparison to the several other major structures within the central auditory pathway, the function of the DNLL has received very little attention in the past. Several recent *in vivo* physiological studies were conducted on rat, altering this structure's normal function. To illustrate how the DNLL plays an inhibitory role on binaural responses, neuronal activity in the DNLL has been blocked by local injection of the excitatory amino acid antagonist kynurenic acid or the local anesthetic lidocaine, resulting in a reduction in the strength of inhibition and a disruption of binaural responses recorded from neurons in the contralateral IC (Faingold *et al.*, 1993; Kelly *et al.*, 1998a,b; Kelly & Kidd, 1997a; Kelly & Li, 1997; Kidd & Kelly, 1996; Li & Kelly, 1992b). When unilateral lesions were employed upon the DNLL, the binaural responses in the contralateral auditory cortex were disrupted (Glenn & Kelly, 1992). Behavioral studies have shown that a lesion of DNLL or a transection of its crossed efferent projections in the CP cause deficits in the ability of animals to localize sounds in space (Kelly *et al.*, 1998a). All these results indicate that this nucleus plays an active and crucial role in shaping binaural responses and sound localization.

Current developmental research

As mentioned earlier, our knowledge of the developmental process within the auditory system is quite limited. This is most probably due to the systematic nature of research, which first discovers the auditory structures and then tries to uncover the developmental processes involved in their formation. Therefore, one can argue that until now researchers have been simply trying to realize all of the auditory structures, examining their neuroanatomy, morphology and electrophysiology, and are only now able to address the developmental aspect of this process. One of the challenges of studying structures in the central auditory brainstem is to isolate them from the peripheral components that participate in the process. Also, trying to isolate these structures from other lower brainstem structures can pose problems. Because of this, it is very difficult to isolate the contributions of one auditory structure to a developmental process, and so several studies have to be conducted to piece together the phenomenon.

Another very significant factor, which has to be addressed when dealing with auditory development, is the onset of auditory function. The onset of auditory function may be illustrated in two fundamental ways; the time when central synapses are capable of neurotransmission, and/or the time when sound first elicits a response from various auditory nuclei (Sanes & Walsh, 1998). The rat is a commonly used subject for developmental studies because it is late in its auditory development and relatively immature at birth. The auditory system of newborn rodents is unable to respond to airborne sound since the middle and inner ear structures are not yet completely developed and the outer ear canals are still closed (Kandler & Friauf, 1995a). This delay in auditory development prevents the rats from physiological hearing until postnatal day 12 (P12).

This onset of hearing can be used as a landmark, with which many electrophysiological, morphological and anatomical comparisons can be made. For example, cellular morphology can be examined prior to the onset of hearing and then following the onset. Since the onset of auditory function is such a crucial event, any significant changes seen in cellular morphology during this period could be attributed to this event. This same logic can be used for a multitude of developmental measures. Despite this, most electrophysiological studies to date have been performed during the period of development following the onset of hearing, with only a few addressing the ontogeny of auditory function in neonates (Kandler & Friauf, 1995a,b; Sanes, 1993; Wu & Oertel, 1987).

A recent study by Gleich *et al.* (1998), did examine the postnatal cellular changes in the morphology of the CN prior to the onset of hearing. The study examined volume fluctuations for regions within the CN for the gerbil, whose onset of hearing is at postnatal day 12 (P12). They found substantial growth just prior to the onset of hearing and more gradual further growth after P12. Thus, it seems that the CN increases its volume just prior to the onset of hearing and very quickly reaches its mature volume. This trend agrees with other studies which have examined changes in CN volume during development (Frisina *et al.*, 1982; Statler *et al.*, 1990).

The onset of hearing can also be used as a landmark for examining what processes are intact before the subject has begun responding to auditory stimuli. Current research has been examining which neuronal connections are present at birth, and which form postnatally in relation to the onset of hearing. Many studies have found that many of the efferent connections from many structures, such as the CN in rat (Angulo *et al.*, 1990; Kandler & Friauf, 1993) are intact prenatally. Furthermore, these projections mature postnatally even further to adult-like projection patterns prior to the onset of physiological

hearing. This was also shown for the IC in rat (Altman & Bayer, 1981; Friauf & Kandler, 1990). These studies illustrate cases where the bulk of the auditory development is done despite the presence of physiological auditory function. This raises questions as to why or even how this type of development can be established without the presence of hearing. The discovery of the schedule for what characteristics are present in relation to the onset of hearing is crucial in understanding development in the auditory system.

Neuronal properties

Another very useful tool in studying auditory development is changes in the membrane characteristics of neurons. Electrophysiological examination of neurons provides the most direct test of their membrane properties. These characteristics might be considered as “basic” properties of neurons, which are highly correlated with their maturity. Electrical membrane properties can include current-voltage relationships, membrane input resistance, membrane time constant and spike frequency. Changes in these properties during development have been noted by several studies (Kandler & Friauf, 1995a; Kandler & Friauf, 1995b; Sanes, 1993; Yancey & Dallos, 1985). Examining these properties allows researchers to study one facet of neuronal development removed from other factors that determine its function.

Another area of study which also gives researchers some insight into the developmental process, are changes in synaptic transmission. Understanding auditory development will entail uncovering when synaptic transmission can be first detected and how this changes prior to and after the onset of hearing. Earlier work has shown that synaptic transmission precedes the onset of hearing (Chuhma & Ohmori, 1998; Kandler & Friauf, 1995a,b; Sanes, 1993; Sanes & Rubel, 1988; Wu & Oertel, 1987), even though the

post synaptic responses elicited and their properties were noticeably atypical or “immature”. What role synaptic transmission plays prior to the onset of hearing and the role it plays after is undoubtedly unique since each is evoked by different means.

The synaptic properties of mature DNLL neurons has already been examined, creating a starting point for its developmental analysis (Wu & Kelly, 1995a,b; Wu & Kelly, 1996). These studies have shown that electrical stimulation of the LL evoked both excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). The EPSPs were much more predominant than the IPSPs, with some neurons displaying both types of response at varying threshold stimuli. This distribution of postsynaptic potentials supports the notion that the DNLL receives both excitatory and inhibitory projections. To better understand the synaptic transmission of DNLL neurons, Wu and Kelly (1996) used pharmacological treatments to chemically dissect the PSPs to reveal the underlying neurotransmitters controlling excitatory and inhibitory postsynaptic responses. The IPSPs elicited by stimulation of the LL were always blocked by the glycine antagonist strychnine and not by the GABA_A antagonist bicuculline. These results indicate that in the DNLL, the ipsilateral inhibition arising from the LL pathway is principally glycinergic. The majority of EPSPs evoked by lemniscal stimulation were completely blocked by application of the non-NMDA antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), and not the NMDA antagonist amino-5-phosphonovaleric acid (APV). Some of the EPSPs however, were blocked by APV and not CNQX. This variance reveals the presence of two distinct excitatory processes with lemniscal stimulation in the DNLL, one mediated by NMDA and the other by non-NMDA receptors, respectively. Developmental studies can examine whether or not this distribution of postsynaptic response is typical throughout the development of the DNLL. Does the proportion of PSPs (excitatory vs. inhibitory) remain

the same during development? Does the distribution of NMDA and non-NMDA receptors in the DNLL alter during the development of the auditory brainstem?

Our previous developmental study addressed these questions using intracellular recordings in the rat DNLL from P4 to P18 (Ahuja & Wu, 2000). The results illustrated the expected changes in electrical membrane properties, with immature attributes such as higher input resistances, longer time constants, lower spike frequency and atypical spike attributes. The PSPs distribution seem to agree with the mature data set (Wu & Kelly, 1996), however the percentage of EPSPs seemed unusually dominant in the young DNLL neurons (P4-P10). To ensure that this observation was genuine and the elevated distribution of EPSPs was not simply due to depolarized reversal potentials of inhibitory synaptic responses as seen in other studies (Kandler & Friauf, 1995a), pharmacological treatments need to be employed. Kandler & Friauf (1995a) suggest that this phenomenon may be due to developmental changes in the isoforms of the glycine receptor, as well as a developmental decrease in the intracellular Cl^- concentration. Further study is required to see if this reasoning can be applied to explain the results seen in the DNLL.

Intracellular vs. patch clamp recordings

With the introduction of *in vitro* recording from brain slices, the majority of electrophysiological data has been derived using intracellular recording techniques (Bartlett & Smith, 1999, Kandler & Friauf, 1995a,b; Li *et al.*, 1998; Oertel, 1983; Sanes, 1993; Wu & Kelly, 1995a,b; Wu & Kelly, 1996; Wu, 1999). Electrophysiological studies have the option of using two possible methods of recording, whole-cell patch clamp vs. sharp-electrode intracellular. Both, techniques are widely used and both are able to produce viable experimental results but each is best suited for different applications. The

intracellular method is a very intrusive technique of cellular recording since it impales the cell membrane and may sometimes cause damage to the neuron. Whole-cell patch clamp techniques are employed to try and combat these problems (Chen *et al.*, 1999; Chuhma & Ohmori, 1998; Tennigkeit *et al.*, 1998; Tyzio *et al.*, 1999), usually resulting in “seals” that can last for extended periods of time, producing data that is more indicative of an intact system. The patch-clamp technique uses suction to form a seal with the cell membrane tearing a portion of the membrane. Even though this technique causes some neuronal damage, it is much more agreeable than the intracellular method. This sensitivity is crucial and must be considered when doing cellular recordings in young, developing neurons. These younger neurons are far more susceptible to methodological damage, and this damage can have a much greater toll on the quality of data in comparison to mature neurons. The patch clamp technique has another advantage, in that it is able to form a “seal” with the cell for extended periods of time allowing for a more complete analysis of each neuron encountered, and the ability to conduct thorough pharmacological analyses. The sharp electrode intracellular technique might be affecting the efficacy of the results, since its protocol might be too harsh on the young, underdeveloped DNLL neurons.

Purpose of the study

The introduction of the *in vitro* brain slice technique allows researchers the unique opportunity to analyze the electrophysiological properties of a locally intact system, removed from all of the other subsystems which together make the auditory brainstem (Wu & Oertel, 1987). This isolation allows structures of interest, like the DNLL to be studied while interacting with a few other select structures like the IC, INLL and VNLL rather than every structure present. The DNLL can be studied very early postnatally, since it has

been shown to be present in the auditory brainstem prenatally at embryonic day 15 (Altman & Bayer, 1995). The level of functionality and maturity of this structure during this period however remains yet to be uncovered.

The present study has tried to describe the ontogeny of the electrical membrane properties and synaptic transmission in DNLL neurons of rat during different stages of development postnatally. More specifically, focus was placed on how these electrical and synaptic properties change quantitatively during postnatal development. The study employed whole-cell patch clamp recordings from DNLL neurons in *in vitro* brain slices, to assess the changes seen during development prior to the onset of hearing. Subjects included neonates with very little auditory development and those closer to the onset of hearing, to create a sample encompassing the entire period before physiological hearing.

To ensure that the data derived from this study agrees with those already achieved, the electrical membrane and synaptic properties were obtained for comparison. These included the current-voltage relationships, neurons input resistance, time constant, spike frequencies and spike attributes. When examining synaptic transmission, the latency and duration of the PSPs, their distribution and the schedule of their appearance was investigated. Pharmacological tests were included to better understand the developmental changes in both electrical membrane properties and synaptic transmission. The ionic compositions of the action potentials (APs) were monitored during development, examining Na^+ currents, K^+ currents and Ca^{2+} currents. When PSPs were realized, chemical dissection via pharmacology would verify the transmitters and receptors associated with them. These pharmacological techniques were mostly qualitative, used to help determine the initial presence of particular types of responses ontogenetically in the DNLL. The receptor specific antagonists were further used to reveal the individual

components, which together comprised the synaptic response, possibly revealing any changes in their proportion during development.

With these measures in hand, the current study made systematic comparisons with the DNLL developmental study by Ahuja & Wu (2000). This previous study also examined postnatal developmental shifts in electrical membrane properties and synaptic properties in the DNLL, but did so using intracellular techniques. The data derived from the present whole-cell patch clamp study therefore verified these findings and substantiated these results using pharmacological techniques.

Materials and Methods

Brain slice preparation

Brain slices were obtained from young albino rats (Wistar, Charles River Co., Quebec Canada) between 3 and 14 days of age. The animals were first anesthetized with halothane gas in an isolated delivery chamber for approximately 60-90 seconds. They were then euthanized by decapitation, and their brains were removed. Once removed, the brain was placed in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature (20°C-22 °C). The auditory midbrain was blocked and mounted on the stage of a tissue slicer using cyanoacrylate adhesive. Brain slices were cut at 400 μm in the frontal plane through the auditory midbrain with a Vibratome tissue slicer. An intact slice containing the DNLL was then selected on the basis of structural clarity, and placed in a small recording chamber. The remaining tissue slices were then transferred to a beaker containing oxygenated saline at room temperature for subsequent recordings.

An oxygenated saline solution circulates 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 was completely submerged in the saline (Fig. 2). The volume of the chamber was ~1.5 ml and the flow rate of the saline was between 10-12 ml/min. The temperature of the saline was monitored and maintained at 29°C - 30 °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 distilled water, saturated with 95% O₂-5% CO₂ before it was circulated through the slice chamber. The pH was 7.4 after complete saturation with O₂-CO₂ mixture. The slice was kept in the recording chamber for approximately one hour before any recordings were made.

Whole-cell patch clamp recording

The brain slice chamber was illuminated from below by light passed through a Zeiss microscope, so that the structures of the auditory brainstem were visible. The outlines of the DNLL, and lateral lemniscus (LL) were identified. The recordings were made using a blind whole-cell patch procedure. A single recording electrode was inserted into the DNLL while a stimulating electrode was placed along the LL (Fig. 3).

The patch pipettes were produced using a Flaming/Brown micropipette puller (model P-80/PC), from 1.1 mm OD and 0.8 mm ID thin-walled glass tubing (Kimax-51, Kimble). The glass microelectrodes was then filled with a 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 impedance of the electrodes was between 4-7 MΩ. The blind whole-cell patch was achieved utilizing the “blow and suck” procedure. First, the recording electrode

was positioned over the DNLL and then lowered with a gross three-dimensional manipulator towards the surface of the DNLL. While being lowered, positive pressure was applied by mouth through the electrode holder to prevent the electrode from being clogged by tissue or debris. After, the electrode had touched the tissue there was an increase in the impedance of the electrode. The electrode was then finely advanced into the tissue using a Narishige hydraulic three-dimensional micromanipulator. Once the tip of the electrode had hit the neuron and suction was applied, a gigaohm ($G\Omega$) seal was formed. A whole-cell patch was achieved by further application of sudden negative pressure by mouth through the electrode holder, tearing the cell membrane. An Axopatch 200A amplifier was used for the whole-cell patch-clamp recordings. The data were collected and analyzed using pClamp 6 software on an IBM-compatible PC.

Once a whole-cell patch had been successfully made, membrane properties and synaptic responses were examined under current clamp mode. Current injections were made between -200 to 200 pA, applied in 10 pA steps for 200 ms. The current-voltage traces generated were analyzed using the same pClamp6 software. Synaptic responses were elicited by electrical stimulation of the LL. The bipolar stimulating electrode was constructed from paired tungsten wires with a tip separation of $50\ \mu\text{m}$. Electrical stimuli were either positive or negative square waves, $100\ \mu\text{s}$ in duration, obtained from a Grass S-8800 stimulator and stimulus isolator unit. When neural responses were encountered, the current strength was adjusted to produce reliable postsynaptic responses. When an EPSP or IPSP was apparent without the other, a graded postsynaptic response was examined by the systematic increase of stimulus intensity. This sometimes led to a stepwise increase in PSP amplitude, and in the case of EPSPs led to a suprathreshold response. The reversal potential of the PSPs was realized by examining the evoked potential produced by

stimulation of the lemniscal pathway, while the cells membrane potential was shifted by the injection of current from -160 to 140 pA in 30 pA steps. The reversal potential for these responses were investigated to better recognize the postsynaptic responses elicited in the DNLL.

When a neuron was encountered, some criterion were used to determine whether an acceptable recording was achieved and further analysis should be done. The resting membrane potential had to be at least -45 mV. The resting potential had to be relatively stable staying at the same position with little fluctuation during the period of recording.

Pharmacology

Once a postsynaptic response had been elicited, pharmacological tests were conducted depending on the type of response; *excitatory* or *inhibitory*. Concentrated stock solutions of the following drugs were prepared in distilled water, aliquoted, and stored at -15°C : Tetrodotoxin (TTX), Tetraethylammonium (TEA), CNQX, APV. When required, small amounts of the stock solutions were diluted into oxygenated ACSF and delivered through the chamber bathing the slice with final concentrations as follows: TTX ($1\mu\text{M}$), TEA (5 mM), CNQX ($10\mu\text{M}$), APV ($10\mu\text{M}$). The Na^+ channel blocker, TTX and the K^+ channel blocker TEA, were employed to verify the ionic components of the action potentials during development. The EPSPs were studied using the non-NMDA glutamate receptor antagonist CNQX and the NMDA glutamate antagonist APV. This pharmacological analysis was used to qualitatively assess the developmental changes in NMDA receptor mediated and non-NMDA receptor mediated responses, after blocking one or the other. The above mentioned chemicals were purchased from Sigma.

Experimental group assignment

The subjects were classified by postnatal day, and were not divided into groups. Subjects ranged from postnatal day 3 (P3) to postnatal day 14 (P14), with emphasis placed on subjects from P3 to P12. Data collection included at least five neurons for each postnatal day in each experimental group (except P13 [n=2], P14 [n=1]). All experimental comparisons were made between these postnatal age groups.

Data analysis

The change in membrane potential elicited by intracellular injection of negative current, was obtained by measuring between the baseline membrane potential and the peak hyperpolarization. These values were used to plot the current voltage relationships. From these curves, the slope was calculated from a range of values that were approximately linear. This slope represents input resistance (R_{input}) of the cell membrane, measured in megaohms ($M\Omega$). The time constant (τ) of the cell membrane from these neurons was obtained by measuring the time it took the membrane potential to move 63% of the way towards its final value in response to a hyperpolarizing pulse current. The action potential duration measurements were taken at 50% amplitude of the spike. The action potential amplitude was taken from the threshold required to elicit the response to its peak. The rise time was simply the time required for the spike to go from threshold to peak amplitude, and the decay time is the return to threshold from peak amplitude. The spike threshold was the absolute resting membrane potential required to evoke a response.

Synaptic latency was obtained by measuring the duration between the beginning of the stimulation and the initiation of a postsynaptic potential. Synaptic duration was obtained by measuring the duration between the beginning of the postsynaptic potential

and the point at which the synaptic potential returns to baseline. Pharmacological analysis of the blocking effects were qualitative and not quantitative, simply observing the changes to the PSPs upon drug application. These changes were also analyzed in correlation with developmental changes in the DNLL, to investigate their contribution in the process. The pharmacological technique may also be used to assess changes in PSP latency.

Statistical Analysis

Means and standard errors ($\pm$ SEM) were calculated for all measured variables. For each parameter, the statistical difference between age groups was tested using a linear regression analysis (Excel). In the figures, levels of statistical significance have been indicated as follows; * $P < 0.05$, ** $P < 0.01$. The correlation (r) and sample size (n) are shown for each figure, where applicable.

The Animal Care Committee of Carleton University 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.

Results

Membrane properties

Whole-cell patch clamp recordings were obtained from a total of 69 healthy DNLL neurons. The mean resting potential (V_{rest}) for P3 neurons was -61.7 ± 2.6 mV and for

P12 was -57.6 ± 2.4 mV. Figure 4 shows that there was no ontogenetic difference in the distribution of resting potentials across the sampled postnatal ages ($n=64$) ($P>0.1$).

The current-voltage curves for each of the 69 neurons tested across each of the postnatal days were all linear in shape. Injection of positive current produced a graded depolarization that in some cases led to a continuous train of action potentials at suprathreshold values. The number of action potentials was related to the strength of the current delivered, with an increase in frequency seen with increased current injection. Injection of negative current produced a graded hyperpolarization of the cell membrane, displaying a more negative membrane potential away from resting values with continued application.

Figure 5 illustrates the traces seen in representative DNLL neurons tested at P3, P7 and P12, with current injections of 200 ms between -200 and 200 pA. These ages were chosen to give samples over the entire subject set. P3 represented the earliest age at which recordings were made, P12 is of significance because it marks the onset of physiological hearing in rats, and P7 is simply a midpoint over this scale. These neurons displayed the typical hyperpolarization with the injection of negative current (-120 , -60 pA), with the maximum deflection in potential most pronounced under the -120 pA condition. When injected with positive current (60 , 120 pA), the neurons displayed a gradual decrease in the membrane potential away from resting values with continued application. These depolarizations then led to suprathreshold responses, which varied in response to stimulus strength. Note the differences in the characteristics of action potentials evoked by positive current injection across age. The young P3 neuron did not elicit a suprathreshold response at 60 pA, while the P7 and P12 neurons did show spikes. Once the P3 neuron was able to elicit action potentials, they seemed “stunted” or “truncated” and broad in duration. Also,

the number of spikes evoked increases as the animal develops. By P7, the spikes seemed more mature and uniform in shape, and the P12 neurons continued this maturational trend.

The relationship between current and voltage as measured by the peak or maximum shift in membrane potential is plotted in Figure 6. The representative neurons were once again chosen at P3 (A), P7 (B) and P12 (C) to give samples over the observed postnatal ontogenetic period. The relationship was essentially linear over the range of negative current strengths tested for each of the three plots and this also held true for all the neurons encountered in this study. Despite this similarity, the neurons did however differ in their range of membrane potential or slope (Fig. 7). Notice the more negative membrane potential range in the young neuron (P3; 0 to -134 mV) in comparison to the middle neuron (P7; 0 to -90 mV) and late neuron (P12; 0 to -55 mV). Thus, it seems that the slope of the current-voltage curves decreases during development. This trend was seen for the other neurons with increasing postnatal age.

With these data in hand, two other membrane properties were evaluated to compare the developing DNLL neurons, their input resistance (R_{input}) and time constant (τ). The cells R_{input} is simply the slope of the current-voltage curves presented earlier. Figure 8 illustrates the significant declining trend in R_{input} ($P < 0.01$) of the neurons as they develop, with the younger neurons showing a higher resistance in comparison to the more mature DNLL neurons ($n=64$). The mean R_{input} for P3 was 484.0 ± 63.2 M Ω , while the P12 neurons had an average of 258.4 ± 19.5 M Ω . The P3 DNLL neurons had a mean τ of 35.8 ± 6.4 ms while the P12 neurons had a mean of 46.4 ± 10.1 ms. The analysis of τ ($n=63$) did not show any significant ontogenetic changes ($P > 0.1$), with the relevance of this discussed later (Fig. 9).

Action potential characteristics

The development of spike threshold in DNLL neurons is illustrated in Figure 10. When sufficiently depolarized, 64 neurons were able to generate action potentials. The average spike threshold for P3 neurons was -28.4 ± 1.2 mV and -41.3 ± 0.7 mV for the P12 neurons tested. There was a significant declining trend in the absolute spike threshold across age ($P < 0.01$) for the neurons tested ($n=64$). As the DNLL neurons developed the spike threshold became increasingly negative.

It is evident that the action potentials elicited by the injection of positive current during development are different, and this is illustrated in Figure 11. This figure presents two traces evoked in response to 100 pA and 200 pA of current injection for three neurons at postnatal ages; P3, P7 and P12. When the characteristics of the spikes are examined closely, it is clear that there are some developmental differences. The most prominent difference seems to be in spike frequency, or the number of spikes elicited within the stimulus duration of 200 ms. There is an increasing trend in spike frequency, with the younger neuron displaying a lower frequency at both injection levels (100, 200 pA) in comparison to the developing P7 and P12 neurons. Examination of these traces also reveals that the spike amplitude and duration show an developmental correlation, with the younger neuron displaying “immature” spikes with a smaller amplitude and longer duration. The developing neurons on the other hand display spikes with a larger amplitude and shorter duration. Note however, that the changes in these attributes are not dramatic, but instead show a consistent improvement.

Figure 12 illustrates this increasing trend in spike amplitude for all of the neurons tested ($n=64$). The statistically significant increase ($P < 0.01$) in spike amplitude shows that this characteristic is developmentally influenced. The mean spike amplitude for P3 was

24.6 $\pm$ 3.1 mV, while the P12 neurons had an average of 42.7 $\pm$ 1.9 mV. The duration of these spikes clearly display developmental differences among the neurons tested. The data shown in Figure 13 display the significant decrease ($P < 0.01$) in spike duration measured at 50% of peak amplitude ($n=64$). As the neurons develop, the duration of the action potentials decreases significantly, with the mean duration for P3 at 4.2 $\pm$ 0.3 ms, while the P12 neurons had an average of 1.7 $\pm$ 0.1 ms.

The action potentials from these 64 neurons, can be further examined to reveal any changes they might experience during development. The spike can be divided into two phases; a *rising phase* and a *decay phase*. Figure 14 displays the decreasing trend seen in rise time with development in the DNLL neurons ($P < 0.01$). The mean spike rise time for P3 neurons was 3.7 $\pm$ 0.4 ms and for P12 neurons was 1.2 $\pm$ 0.1 ms. The decay time for the developing DNLL neurons also displayed the same decreasing ontogenetic trend ($P < 0.01$) (Fig. 15). The mean spike decay time for P3 neurons was 6.5 $\pm$ 1.1 ms and for P12 neurons was 2.1 $\pm$ 0.1 ms.

The slope of these parts of the spike gives rise to the voltage change (mV/ms) of both the rising phase and the falling phase. Figure 16 illustrates the significant developmental increase in voltage change in the spikes rising phase in DNLL neurons ($P < 0.01$). The mean voltage change in spike rising phase for P3 neurons was 7.0 $\pm$ 1.1 mV/ms and was 37.6 $\pm$ 3.4 mV/ms for P12 neurons. The corresponding voltage change in the spikes decay phase also displays the ontogenetic increase ($P < 0.01$), as seen in Figure 17. The mean voltage change in spike decay time for P3 neurons was 4.3 $\pm$ 0.9 mV/ms and was 20.9 $\pm$ 2.1 mV/ms for P12 neurons.

Figure 18 illustrates the voltage change rate (dV/dt) for both the rising phase (red) and the decay phase (blue) simultaneously. Both rise and decay show a significant increase ($P < 0.01$) in voltage change during development, explaining the observed decrease in spike duration. When each is examined individually, it seems that the slope of the rise time increases developmentally quicker than the decay time slope. Thus, it seems reasonable to propose from this data that the early rising phase of the spike matures before the falling or decay phase of the spike.

Action potential pharmacology

To verify the ionic components of the action potentials elicited via current injection, several neurons of varying postnatal ages were perfused with 1 μ M TTX, and 5 mM TEA. Of all of the neurons tested ($n=8$), 100% were blocked by the application of TTX and TEA. TEA was only added in half of the neurons tested ($n=4$), since TTX almost completely removed the suprathreshold responses. In the cases where TEA was added, the additive blocking effects with TTX were minimal.

Figure 19 illustrates the effects of applying TTX and TEA to a neuron while being injected with 60 pA of current for 200 ms. The first trace displays a P8 neuron under normal ACSF, eliciting 4 action potentials in response to the current injection. After TTX application the spikes were completely removed (2nd trace). TEA application had no additional blocking effects (3rd trace). The bottom trace shows that the TTX effects were reversible, since perfusion with normal ACSF for 15 minutes recovered the suprathreshold response.

Synaptic response properties

Electrical stimulation of the lateral lemniscus (LL) could elicit both short-latency excitatory postsynaptic potential (EPSPs) and inhibitory postsynaptic potentials (IPSPs) from DNLL neurons in brain slice preparations. These neurons had the ability to display EPSPs, IPSPs or a combination of both EPSPs and IPSPs. Figure 20 shows synaptic responses of a representative neuron from P3, P7 and P12 to stimulation of the LL at varying stimulus strengths. This P3 neuron required 30V to evoke a synaptic response, while both P7 and P12 responded to stimulation at 10V. Notice, that the PSPs illustrated here show developmental differences in their characteristics, including latency, rise time and duration. Examination of these traces shows that the postsynaptic response elicited in the P3 neuron seems “stunted” or immature in comparison to both other responses. The P12 PSP seems more mature than that evoked in the other two younger neurons.

Figure 21 addresses the synaptic response latency changes during development in the encountered DNLL neurons. The traces illustrated here focus on the very short-lived synaptic latency in response to electrical stimulation of the LL at 10V, displaying a definite ontogenetic decrease; P3 (15.5 ms), P7 (5.3 ms) and P12 (4.0 ms). Figure 22 accounts for all of the PSPs in the DNLL neurons encountered (n=36), with the data exhibiting a clear declining trend ($P < 0.01$) in latency. The mean synaptic response latency for P3 was 11.6 ± 0.6 ms, while the P12 neurons had an average of 3.3 ± 0.1 ms. However, the latency for the more mature neurons were very close, suggesting that the measure begins to plateau just prior to the onset of hearing (P12); P9 (3.5 ± 0.1 ms), P10 (3.7 ± 0.1 ms) and P11 (4.0 ± 0.9 ms). The changes in synaptic response duration are shown in Figure 23 (n=37). The significant declining trend ($P < 0.01$) indicates that the younger DNLL neurons have a longer synaptic response duration in comparison to the older neurons. The mean PSP

duration for P3 was 680.3 ± 24.8 ms, while the P12 neurons had an average of 310.8 ± 11.8 ms. Note that like synaptic response latency, duration decreases as the neuronal age increases, with LL stimulation. Also, the developmental synaptic response duration changes seem to begin to plateau just prior to the onset of hearing; P9 (356.5 ± 24.7), P10 (349.9 ± 9.6) and P11 (359.0 ± 35.8).

Synaptic responses can be dissected further to reveal if there are any developmental changes in other attributes. The time required for the synaptic response to go from baseline resting potential to its peak amplitude is indicative of how quickly a neuron is able to respond to stimulation. Figure 24 displays the developmental changes in the time required for these neurons to respond to stimulation and reach peak amplitude, or the *time to peak* (n=33). The mean time to peak for P3 was 17.0 ± 1.5 ms, while the P12 neurons had an average of 5.4 ± 0.9 ms. There is a clear decrease in the time required for the synaptic response to reach its peak amplitude as the DNLL neurons mature ($P < 0.01$). Note, that like synaptic response latency and duration, the developmental changes in time to peak seemed to begin to plateau just prior to the onset of hearing; P10 (6.1 ± 0.8 ms) and P11 (5.5 ± 0.5 ms).

The distribution of types of synaptic responses (EPSPs only, IPSPs only, or both) for the PSPs encountered in this study are illustrated in Figure 25. The presence of “EPSPs only” were dominant throughout ontogenetic development (Fig. 25A), accounting for 62% of all PSPs encountered across all neurons (Fig. 25B). The remaining 38% of response also showed EPSPs but these were seen in coincidence with IPSPs. It seems from this data that the EPSPs were dominant in the early DNLL neurons and were first seen at P3. The EPSPs were seen in all of the neurons where synaptic responses could be elicited. The

IPSPs on the other hand were never seen alone, but could be evoked in conjunction with EPSPs as early as P4.

Synaptic response pharmacology

Once an excitatory postsynaptic potential (EPSP) was encountered in the DNLL from electrical stimulation of the LL, 10 μ M CNQX, 10 μ M APV or both were applied to chemically dissect the response (n=24). These pharmacological data illustrated which glutamate receptors (NMDA vs non-NMDA) mediated that particular synaptic response. Figure 26 depicts some PSPs that were elicited in DNLL neurons at P4, P8 and P12. These postnatal ages were chosen to present samples from a very young neuron (P4), a neuron at the onset of hearing (P12), and a neuron in between (P8). The control traces were simply the EPSPs evoked in response to electrical stimulation prior to any pharmacological treatment. The same intensity of electrical stimulation was used throughout the pharmacological treatment for each individual neuron. The representative P4 neuron was stimulated at 10V, evoking an EPSP. Upon CNQX application, the presence of the EPSP was drastically reduced. Introduction of APV into this treatment further reduced the EPSP. The effects of both drugs were reversible after the neuron was bathed with normal ACSF, shown in the wash trace. Both the P8 and P12 neurons, which were stimulated at 8V, also responded in this fashion to the pharmacological treatment. The representative P12 neuron however revealed an underlying IPSP after the pharmacological treatment had removed the excitation. No wash trace was available for this neuron because the patch-clamp seal was lost before an appropriate recording could be made. The effects of these drugs were similar in all of the neurons tested in this study. This pharmacological treatment also showed that in all of the neurons tested, there was both a non-NMDA component and a

NMDA component in the EPSPs as early as P3, since both CNQX and APV were effective. Table 1 lists all of the neurons tested, and whether these drugs were effective in blocking a portion of the elicited EPSP. This summary also illustrates that the effects of these drugs could be implemented as early as P3.

Once an EPSP was encountered, we examined the reversal potential of the response. Positive and negative current pulses were injected into the cell at its resting membrane potential. This process was repeated after pharmacological treatment to verify if the response had been effectively blocked and to confirm that the depolarizing response was not a reversed IPSP. Examination of the reversal potentials showed that the EPSPs tested had a reversal potential greater than approximately -24 mV (P3) and even greater in the more mature neurons (> 7 mV in P12).

A

B

Figure 1. DNLL and its surrounding structures. *(A)* Schematic diagram of the major excitatory and inhibitory projections to the DNLL. Excitatory synapses are shown in (blue) and the inhibitory synapses in (red). *(B)* Photomicrograph of a cresyl violet-stained frontal section through the midbrain and lateral lemniscus indicating the nuclei surrounding the DNLL.

Figure 2. Experimental chamber design. (*A*) The experimental chamber as it would sit on the stage of the microscope, illuminated from below through a darkfield condenser. (*B*) The layered composition of the brain slice chamber, illustrating how the tissue is “sandwiched” between mesh while being perfused. (Adapted from Oertel, 1983).

D
L

Figure 3. Schematic representation of a brain slice through the auditory midbrain.

The figure illustrates the slice in the frontal plane with the nuclei of interest indicated. The whole-cell patch recording electrode is indicated (R) submerged in the DNLL. The stimulating electrode (S) is shown in the LL.

Figure 4. Development of DNLL neuron membrane resting potential (V_{rest}). A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The resting membrane potential of each neuron was recorded once a stable “seal” had been achieved. Note the non-significant change in V_{rest} across postnatal age ($P>0.10$).

३५

P7

P12

200 ms

10 mV
50 ms

Figure 5. Development of action potentials in DNLL neurons. Traces from representative DNLL neurons are shown for three postnatal age groups; P3, P7, and P12 (from left to right). Top traces for all three age groups are the responses elicited by injecting 120 pA of positive current for 200 ms. The next depolarizing traces are the responses to 60 pA of positive current. The bottom two hyperpolarizing traces are the responses to -60 pA and -120 pA of negative current, respectively. Notice the characteristic differences in the action potentials elicited across age groups. The resting membrane potential for each of the representative neurons was as follows; P3 (-62 mV), P7 (-58 mV) and P12 (-60 mV).

A.

P3

B.

P7

C.

P12

Figure 6. Development of current-voltage relationships across age in DNLL neurons.

Representative current-voltage curves were chosen at three postnatal ages; (A) P3, (B) P7 and (C) P12. The curve was plotted as measured by the peak shift in membrane potential as a function of current strength from -200 to 0 pA. Shifts in membrane potential could not be measured if a suprathreshold response was elicited by a depolarizing response (A, B). Note the general linear relationship over the range of negative currents for all three neurons. Also, note the decreasing range of membrane potentials with increasing postnatal age (i.e. decreasing slope).

Figure 7. Comparison of current-voltage relationships across age in DNLL neurons.

The representative current-voltage curves from Fig. 6 chosen at three postnatal ages; P3 (dark blue), P7 (red) and P12 (light blue) are plotted together. Each curve was plotted as measured by the peak shift in membrane potential as a function of current strength from –200 to 0 pA. Note the decreasing range of membrane potentials with increasing postnatal age (i.e. decreasing slope). The younger P3 neuron shows a significantly steeper current-voltage curve in comparison to the other two more mature neurons, while the P7 neuron has a steeper slope when compared to the P12 neuron.

Figure 8. Development of DNLL neurons membrane input resistance (R_{input}). A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The R_{input} was measured as the slope of the current-voltage relationships for each neuron. Note the significant decrease in R_{input} across postnatal age ($P < 0.01$).

Figure 9. Development of DNLL neurons membrane time constant (τ). A

comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. τ is measured as the time it takes V_{rest} to move 63% of the way towards its final value. Note the non-significant change in τ across postnatal age ($P > 0.10$).

Figure 10. Development of action potential threshold. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The spike threshold was the absolute membrane resting potential required to elicit a suprathreshold response. Note the significant decrease in action potential threshold across postnatal age ($P < 0.01$).

P3

P7

P12

100 pA

200 pA

25 mV
50 ms

Figure 11. Development of DNLL neurons spike frequency. Traces from representative DNLL neurons are shown from three age groups; P3, P7 and P12. The top trace for each neuron is the response elicited by injecting 100 pA of positive current for 200 ms. The next depolarizing traces are the responses to 200 pA of positive current. Notice the characteristic differences in the action potentials spike frequency, amplitude, and interspike interval across age groups. The resting membrane potential for each of the representative neurons was as follows; P3 (-62 mV), P7 (-58 mV) and P12 (-60 mV).

Figure 12. Development of action potential amplitude. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. Spike amplitude was measured from the threshold of the suprathreshold response to its peak. Note the significant increase in spike amplitude with increasing postnatal age ($P < 0.01$).

Figure 13. Development of action potential duration. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The spike duration was obtained by measuring the duration of the spike at 50% of its peak amplitude. Note the significant decrease in spike amplitude with increasing postnatal age ($P < 0.01$).

Figure 14. Development of action potential rise time. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The spike rise time was obtained by measuring the duration required by the spike to reach peak amplitude from threshold. Note the significant decrease in spike rise time with increasing postnatal age ($P < 0.01$).

Figure 15. Development of action potential decay time. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The spike decay time was obtained by measuring the duration required by the spike to return to threshold from peak amplitude. Note the significant decrease in spike decay time with increasing postnatal age ($P < 0.01$).

Figure 16. Development of action potential voltage change rate in rise time. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The voltage change rate in spike rise time (dV/dt) indicates the slope of the rising phase of the elicited AP. Note the significant increase in spike rise time with increasing postnatal age ($P < 0.01$).

Figure 17. Development of action potential voltage change rate in decay time. A

comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The voltage change rate in spike decay time (dV/dt) indicates the slope of the falling phase of the elicited AP. Note the significant increase in spike decay time with increasing postnatal age ($P < 0.01$).

Figure 18. Comparison of action potential voltage change rate. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The voltage change rate (dV/dt) was determined for both the rising phase (red) and the falling or decay phase (blue). Note the significant increase in voltage change for both rise time and decay time with increasing age. Also, notice that the magnitude of increase in voltage change for rise time is larger than that of decay time.

Control

TTX

TTX
+
TEA

Wash

Figure 19. Pharmacological treatment of action potentials in developing DNLL neurons. Traces from a representative DNLL neuron (P8) are shown in response to 200 ms of current injection at 60 pA. The first trace illustrates the APs elicited prior to any drug application (control). The next trace shows the effects of applying 1 μ M TTX, to the same level of current injection. The following trace exhibits the effects of adding 5 mM TEA to the existing TTX. The final trace shows the return of the suprathreshold response after washing the neuron with ACSF for 15 min. The resting membrane potential of the neuron was -62 mV.

P3

P7

P12

Figure 20. Development of postsynaptic responses in DNLL neurons. Traces from representative DNLL neurons are shown from three age groups; P3, P7 and P12. The trace for each sample neuron shows a postsynaptic response elicited by electrical stimulation of the LL at varying levels of intensity. The levels of stimulus intensity for each neuron were as follows; P3 (30V), P7 (10V) and P12 (10V). Notice the characteristic differences in the PSP duration and amplitude across age groups. The resting membrane potential for each of the representative neurons was as follows; P3 (-56 mV), P7 (-72 mV) and P12 (-60 mV).

P3

P7

P12

2.5 mV
5 ms

Figure 21. Development of synaptic response latency in DNLL neurons. Traces from representative DNLL neurons are shown from three age groups; P3, P7 and P12. The trace for each sample neuron, shows a postsynaptic response elicited by electrical stimulation of the LL at 10V. The traces focus on the short-lived period between stimulus introduction and the initiation of the PSP or *synaptic response latency*. The stimulus artifact is indicated by the (*filled circle*), and the (*arrow*) indicates the initiation of the PSP. Notice the decrease in synaptic response latency as the neurons mature. The resting membrane potential for each of the representative neurons was as follows; P3 (-59 mV), P7 (-70 mV) and P12 (-60 mV).

Figure 22. Development of synaptic response latency. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The latency was measured for each responsive DNLL neuron to electrical stimulation of the LL pathway. Synaptic response latency was obtained by measuring the duration passed from the stimulus artifact until the PSP appeared. Note the significant decrease in synaptic response latency with increasing postnatal age ($P < 0.01$).

Figure 23. Development of synaptic response duration. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The duration was measured for each responsive DNLL neuron to electrical stimulation of the LL pathway. Synaptic response duration was obtained by measuring the duration passed from the initiation of the PSP at baseline until the response returned back to baseline. Note the significant decrease in synaptic response duration with increasing postnatal age ($P < 0.01$).

Figure 24. Development of synaptic response time to peak. A comparative scatterplot where each data point represents an individual DNLL neuron at its specified postnatal age, between P3 and P14. The time to peak was measured for each responsive DNLL neuron to electrical stimulation of the LL pathway. Time to peak was obtained by measuring the duration passed from the initiation of the PSP at baseline until the response reached peak amplitude. Note the significant decrease in synaptic response time to peak with increasing postnatal age ($P < 0.01$).

A

B

**EPSPs and
IPSPs
38%**

**EPSP only
62%**

Figure 25. Development of synaptic response distribution. *(A)* A histogram illustrating the distribution of PSPs during development. PSPs can be either excitatory (EPSPs only), inhibitory (IPSPs only) or a combination of both. Note the elevated percentage of EPSPs throughout the developmental sample. *(B)* The distribution of PSPs across all of the DNLL neurons sampled.

P4

P8

P12

5 mV
100 ms

Figure 26. Pharmacological treatment of postsynaptic responses in developing DNLL neurons. Traces from representative DNLL neurons are shown from three age groups; P4, P8 and P12. The traces for each neuron are postsynaptic responses elicited by electrical stimulation of the LL at varying levels of intensity. The first trace for each neuron is the PSP elicited prior to any drug application (control). The next trace shows the effects of applying 10 μ M CNQX, to the same intensity of electrical stimulation. The next trace shows the effects of adding 10 μ M APV to the existing CNQX. The final trace presents the PSP after the drugs are removed (wash) [except in P12 due to incomplete recording period]. The levels of stimulus intensity for each neuron were as follows; P4 (10V), P8 (8V) and P12 (8V). Note that the pharmacological treatments systematically reduced the excitatory components of the PSPs. The resting membrane potential for each of the representative neurons was as follows; P3 (-56 mV), P7 (-65 mV) and P12 (-60 mV).

Summary Chart of Drug Application on Excitatory Synaptic Responses in the DNLL.

Postnatal Age (days)	CNQX (AMPA receptor antagonist)	APV (NMDA receptor antagonist)
P3		
Cell 41C	+	N/A
Cell 41E	+	+
P4		
Cell 22B	+	+
Cell 31D	+	+
Cell 39A	+	+
P5		
Cell 23A	+	+
Cell 26A	+	+
P6		
Cell 13B	+	N/A
Cell 18B	+	N/A
Cell 24B	+	N/A
Cell 27A	+	+
Cell 28A	+	+
P7		
Cell 17D	+	N/A
Cell 19B	+	+
Cell 38C	+	+
P8		
Cell 20A	+	+
Cell 33C	+	+
Cell 33E	+	+
P9		
Cell 34F	+	+
P10		
Cell 35B	+	+
Cell 35C	+	+
P11		
Cell 36A	+	+
Cell 36B	+	+
P12		
Cell 40A	+	+

+ = drug blocked synaptic response

N/A = drug not applied

**** Note – drugs were applied additively, with CNQX followed by APV
or vice versa.**

Table 1. Summary of pharmacological treatments on excitatory synaptic responses in DNLL neurons. The table lists the cells by postnatal age, showing which drug was applied and whether it was effective in blocking the elicited PSP. A (+) denoted that the drug blocked the synaptic response. The drugs were introduced additively to the DNLL neurons in either sequence.

Discussion

It seems reasonable to assume that there is a postnatal developmental process, which the auditory system undergoes prior to the onset of hearing. Previous studies have already shown that this system does undergo developmental changes after the onset of hearing, eventually reaching functional maturity (Backus et al., 1998; Friauf and Kandler, 1990; Kandler and Friauf, 1995a,b; Kotak et al., 1998; Kotak and Sanes, 1995,1996; Sanes, 1993; Wu and Oertel, 1987; Zine and Romand, 1996). The present study addresses the developmental processes that the DNLL undergoes, prior to the onset of physiological hearing using subjects very early in development (P3) through to the onset of hearing (P12) and even some later neurons (P13-P14). The results from the current study support the view that there is a maturation process, within which differences in the electrical membrane and synaptic properties of the DNLL neurons could be distinguished.

Intrinsic membrane properties

The intrinsic membrane properties that were examined in developing DNLL neurons in this study undergo dramatic changes during the first 2 postnatal weeks. The current-voltage (IV) curves for these neurons illustrate a linear function over the range of negative current injection. The linear IV curves from these DNLL neurons were similar to those found in adult DNLL neurons (Wu & Kelly, 1995a,b), stellate cells in the ventral CN (Oertel *et al.*, 1990; Wu & Oertel, 1984), various cell types in the dorsal CN (Oertel & Wu, 1989; Zhang & Oertel, 1993) and neurons in the lateral SOC (Sanes, 1990,1993; Wu & Kelly, 1991). However, the DNLL neurons from the younger subjects showed some differences in their current-voltage (IV) curves in comparison to the maturing subjects. The IV curves obtained from the younger neurons were much steeper than both the middle

and later neurons, resulting in a higher slope. This slope was representative of the R_{input} , which showed a significant decreasing trend as the neurons matured ($P < 0.01$). This type of relationship would be expected if the younger cells had a smaller surface area in comparison to their more mature counterparts. The hippocampal pyramidal cells in young rabbits also display higher R_{input} when compared to the mature cells (Schwartzkroin, 1982). The same relationship is seen in bushy and stellate cells of mouse (Wu and Oertel, 1987) and in cells from LSO in gerbil (Sanes, 1993) in early development.

Time constant (τ) is another membrane property which in some neurons expresses ontogenetic differences (Ahuja & Wu, 2000; Kandler & Friauf, 1995a; Sanes, 1990, 1993; Wu & Oertel, 1987), but the DNLL neurons in this study did not. τ is the product of the resistance and capacitance of the membrane [$\tau = (R_{input})(C)$]. The above mentioned data has shown a developmental decrease in R_{input} and other studies have shown significant increases in neuronal somal size during development (Gleich *et al.*, 1998; Kandler & Friauf, 1995b; Webster, 1988). Since the capacitance of the membrane is directly related to the surface area of the cell membrane, increases in the membrane surface area will lead to an ontogenetic increase in the cell membrane capacitance. If the R_{input} in the DNLL neurons are developmentally increasing at the same rate as the decrease in the cell capacitance, it is reasonable to believe that their effects upon τ will neutralize one another. Thus, it will appear as though the τ displays a non-significant developmental change in these DNLL neurons.

Injection of positive current in DNLL neurons produced a proportional depolarization of the cell membrane and in some cases, generated a sustained train of action potentials (APs). The number of resulting APs was dependent upon the magnitude

of the depolarization. Very little spontaneous spike activity was observed in the sampled developing DNLL neurons. Many other *in vitro* studies in both young and mature auditory brainstem neurons also described the low incidence of spontaneous spike activity (Kandler & Friauf, 1995a; Oertel *et al.*, 1990; Sanes, 1990; Smith, 1992; Wu & Kelly, 1991). This lack of spontaneous activity agrees with the finding of high spike thresholds (<30 mV above V_{rest} in P3), indicating that a large amount of voltage was required to sufficiently depolarize the cell to generate an AP. Once elicited, examination of the APs showed that they varied with age and their attributes showed ontogenetic differences. The cells from the earlier neurons displayed APs with smaller amplitudes in comparison to the more mature neurons ($P < 0.01$), seeming “stunted” or immature. As the DNLL neurons sampled became increasingly mature, the amplitude of these spikes seemed more mature as well. The spikes also differed in their duration, with the younger neurons displaying a much longer duration in comparison to the older DNLL neurons ($P < 0.01$). The APs of the younger neurons seemed much broader when compared to the tighter more mature spikes of the older neurons. The improvement in action potential duration allows the cells to recover from the suprathreshold response by reaching its resting membrane potential quicker. The cell is then better prepared to respond to the next stimulus provoking yet another AP. The final attribute of the spikes that was addressed here, was their ability to respond to current injection producing a train of APs. The spike frequency that the neurons were able to elicit improved as they matured. The younger neurons had trouble eliciting a train of spikes, but as they matured they were able to maintain a train of spikes that had the attributes of mature spikes (i.e. larger amplitude, shorter duration). Examination of the interspike intervals from the traces reveals that in the younger neurons, more time is required for the cell to recover from one AP to the next. As the neurons

mature however, this interspike interval decreases. Similar results have been shown for developmental improvements in AP properties in cells of SOC (Kandler and Friauf, 1995a; Sanes, 1993), and in bushy and stellate cells from the CN (Wu and Oertel, 1987).

To better understand what aspect of the APs were developmentally influenced, the change in the spike rise time and decay time were measured. Using this measure and incorporating the voltage change in the membrane potential the voltage change in rise time or decay time could be determined (dV/dt). This attribute can reveal whether the rising phase or the falling phase of the action potential is improving with age. Furthermore, it can also explain which is responsible for the developmental improvement in AP duration. The data show that both rise time and decay time show a significant developmental increase in voltage change. This reinforces the idea that there is some developmental regulation of both Na^+ and K^+ channels, which are usually responsible for the rise and fall of an action potential, respectively (Kandler & Friauf, 1995a). Therefore, the younger APs have the broader duration because of both a slow rising phase and decay phase. The more mature DNLL neurons on the other hand show significant improvements ($P < 0.01$) in voltage change for both the rising phase and decay phase. However, the voltage change for the rising phase shows a marginal developmental advantage in comparison to that for the decay phase. This improved voltage change for the rising phase coincides with previous research which has shown that the density of the fast Na^+ channels increases with development for neocortical neurons (Huguenard *et al.*, 1988) and for mammalian sympathetic neurons (Nerbonne & Gurney, 1989). This might therefore explain why the rising phase of the APs becomes shorter in duration quicker than the falling phase. The longer decay time is the rate-limiting step in the “recovery” process of an individual spike, constraining when the next AP can be elicited. The spike threshold showed a

developmental decrease across age, indicating that a less positive depolarization was required to activate the Na^+ channels, initiating an AP. Therefore, it seems that the increase in the voltage change for the rising phase is a direct measure of the overall Na^+ channels conductance characterizing the density of these channels, whereas the decrease in absolute spike threshold is an indirect measure of the activation threshold or kinetics of these Na^+ channels (Kandler & Friauf, 1995a).

To verify the ionic components of the spike generated by positive current injection, TTX and TEA were administered. All of the neurons tested, including even the youngest neurons sampled (P3) were able to generate TTX-sensitive action potentials. In most cases, TEA application was unnecessary since the spikes were all but essentially removed with TTX alone. TEA did help remove residual depolarizations, initially seen as “humps” before application in some cases. These pharmacological results verify that Na^+ -dependent APs can be evoked in the DNLL as early as P3. Na^+ -dependent APs have been described in other developing mammalian neurons (Huguenard *et al.*, 1988; Kandler & Friauf, 1995a; Nerbonne & Gurney, 1989). Action potential maturation can take two possible routes; the first pattern is typified by the early presence of Ca^{2+} spikes with the eventual development of Na^+ spikes (Spitzer *et al.*, 1994). The other pattern is characterized by the presence of Na^+ -dependent spikes from the beginning, with underlying Ca^{2+} spikes as well seen only when the K^+ currents are blocked (Spitzer *et al.*, 1994). Therefore, of these two possible patterns it seems clear that the DNLL neurons in this study follow the first AP maturation scenario, since TTX was so effective in all of the tested neurons. This same pattern has been described in other neurons as well (Friauf & Kandler, 1995a; Krieger & Sears, 1988; Zisking-Conhaim, 1988). The addition of TEA in the DNLL neurons tested here at a maximum current injection of 200 pA, did not however reveal any underlying

Ca²⁺ spikes. This absence does not suggest that these Ca²⁺ spikes cannot be elicited, since increased current injection might expose their presence.

Synaptic response properties

The DNLL neurons responded to electrical stimulation of the LL with either EPSPs and/or IPSPs. The attributes of these PSPs could be analyzed to reveal any developmental differences. Simple examination of the PSP traces for the developing neurons shows that the younger neurons seem to produce PSPs that are smaller in amplitude, much broader in duration and less pronounced. When a quantitative analysis was done, these differences become much more evident. The synaptic responses elicited in the younger neurons had a significantly longer latency ($P < 0.01$) than seen in the more mature neurons. Extended latencies are considered signs of immaturity or diminished synaptic capabilities. This trend of decreasing synaptic response latency with age has been shown in gerbil LSO neurons (Sanes, 1993), in the CN of chicken embryo (Hackett et al., 1982) and mouse CN cells (Wu and Oertel, 1987), and in rat SOC neurons (Kandler and Friauf, 1995a). This longer synaptic response latency in younger neurons can be explained by several factors. Deficiencies in conduction may be attributing to the latency trends. Conduction in the presynaptic fiber along the LL, from where the fiber is activated to the synaptic terminals must take time since the fibers are not fully myelinated. Myelination commences around the sixth postnatal day and proceeds rapidly so that approximately 85% of the fibers are myelinated at 28 days of age (Foster, *et al.*, 1982). Furthermore, these fibers undergo a significant increase in diameter during early development, experiencing a moderate increase during the later stages (Foster, *et al.*, 1982; Franson & Hildebrand; 1975; Song, *et*

al., 1995). Both myelination and neuronal diameter have a direct effect upon synaptic transmission.

The duration of the PSPs also followed the same trend, with the younger neurons displaying a significantly longer synaptic response duration ($P < 0.01$) in comparison to the more mature DNLL neurons. In several cases, for the very young neurons the falling phase of the PSP did not return to baseline within the allotted recording time (750 ms). Deficiencies in this measure are also indicative of synaptic immaturity, and so synaptic response duration is clearly developmentally linked. This trend of decreasing duration with age has been shown in gerbil LSO neurons (Sanes, 1993), in mouse CN cells (Puel and Uziel, 1987; Rybak et al., 1992; Wu and Oertel, 1987), and in rat SOC neurons (Kandler and Friauf, 1995). The longer PSP duration in younger neurons may be explained by receptor properties and distribution. The kinetics of the postsynaptic receptors have been shown to be slower and the density of these receptors lower, in developing neurons in comparison to their mature counterparts (Gutierrez, *et al.*, 1994; Kandler & Friauf, 1995b; Sanes & Wooten, 1987). Slower receptor kinetics include reuptake mechanisms, which, if compromised, would increase transmitter presence, resulting in a prolonged response duration. Previous research has shown the proportion of NMDA receptors is higher in younger neurons in comparison to more mature samples (Isaacson and Walmsley, 1995; Zhang and Trussel, 1994). NMDA receptors have a much slower time course in comparison to non-NMDA receptors, and thus may account for the longer EPSP duration. Since the proportion of NMDA receptors are high, the late portion of the EPSP may be exaggerated in the younger neurons.

Another indicator of synaptic competence is the time required for the PSP to reach its peak amplitude. This illustrates just how quickly a neuron is able to respond to a

stimuli and reach its peak synaptic amplitude. As expected, the developing DNLL neurons showed a decreasing trend for the time required to reach peak response amplitude ($P < 0.01$). The younger neurons required much more time to reach their peak amplitude in comparison to their mature counterparts. One must also consider the PSP amplitude when examining the measure of time to peak, since the time to peak only reveals how quickly the response reaches its peak but doesn't consider the amplitude of the peak. Therefore, when a developmental decrease in synaptic response time to peak is seen with increasing synaptic amplitude, it makes the results that much more significant. Since, it has been shown that presence of NMDA receptors is dominant in younger neurons (Isaacson and Walmsley, 1995; Zhang and Trussel, 1994) and that the NMDA receptors have slower kinetics than non-NMDA receptors (Fu, *et al.*, 1997), the longer synaptic response time to peak can be attributed to the lower proportion of non-NMDA receptors. Also, the higher proportion of NMDA receptors in younger neurons may also be used to explain the longer synaptic response duration, because of their slower time course. The current study only verified the presence of both types of glutamate receptors, non-NMDA and NMDA, but did not quantitatively address their distribution during development. Therefore, using ontogenetic changes in their distribution to explain the changes in synaptic responses during development cannot be substantiated. The presence of both types of receptors throughout development does however, support the notion that they influence the attributes of the synaptic responses.

Ontogenetic development

Even though hearing onset occurs relatively late during the ontogeny of rats, anatomical connections are formed much earlier and are present before birth. The

structure of the DNLL is present by embryonic day 15 (Altman, 1995) and many of its afferent and efferent projections are intact, however the competence of these connections is not yet known. On a nuclear level, adult-like projection patterns are achieved one week prior to the onset of hearing, with the same being seen in the CN (Angulo, *et al.*, 1990; Kandler & Friauf, 1993) and the SOC (Kandler & Friauf, 1995). These findings indicate that a substantial portion of maturation occurs without acoustic input. The data from the present study agree with these observations, since the very young neurons were still able to respond to synaptic stimulation and displayed basic membrane properties comparable to the more mature neurons. As the neurons approached the onset of hearing, these properties became increasingly congruent with those derived from mature samples.

All of the synaptic response attributes examined here agree with the notion that the DNLL has some capabilities before physiological hearing. The PSPs elicited in the younger DNLL neurons were immature in comparison to the neurons sampled closer to the onset of hearing, displaying longer measures in synaptic response latency, duration and time to peak. Thus, it seems on the whole that synaptic transmission is definitely developmentally linked and this process undergoes a postnatal maturational process. Another point of interest, is the observation that the measured synaptic response properties seem to approach a plateau just prior to the onset of hearing (P9 to P11). Thus, it seems that the synaptic transmission in DNLL neurons undergo a dramatic maturational process during early postnatal life and has reached near functional maturity by the onset of hearing. The system undoubtedly undergoes continued development but the bulk of the process will have been completed. What needs to be clarified is what specific factors are influencing the ontogenetic improvements in the synaptic responses. These factors include changes in receptor density and kinetics, proportion of subtypes and subunits of the receptors and

morphological changes of the neurons. Pharmacology and immunohistochemistry would be useful in examining these measures further.

Synaptic pharmacology

This study introduces a pharmacological treatment to reveal the various components, which produce the recorded PSPs. Revealing these components also allows us to examine if the proportion or presence of these receptors changes during development, in a qualitative manner. The present study examined two receptors in this manner (non-NMDA and NMDA), using CNQX and APV respectively. The drugs were applied in an additive manner so that one drug was added and its effects were recorded, and then the second was introduced with the first drug still present. In every neuron where the non-NMDA receptor antagonist CNQX was added, the EPSP was diminished to some capacity. This same observation held true when the NMDA receptor antagonist APV was added. This indicates that the EPSPs in the DNLL, share both a non-NMDA and a NMDA component as early as P3. Other studies have shown the NMDA receptors to be far more abundant in early developing neurons (Isaacson and Walmsley, 1995; Zhang and Trussel, 1994), but this clearly shows the presence of both receptor types throughout development. However, one must keep in mind that this observation is purely qualitative since it does not give any measure of the proportion of each receptor type throughout development, it simply indicates its presence. A more quantitative analysis of this measure would be required to firmly understand the underlying receptor changes in developing DNLL neurons.

Examination of the reversal potential for the encountered synaptic responses verified several notions. Reversal potentials were executed both before and after

pharmacological treatment of the synaptic responses. The measure showed that the reversal potential for all of the responses encountered across all postnatal ages, were depolarized enough to rule out the possibility that they were reversed IPSPs (Kandler & Friauf, 1995b). The Kandler & Friauf study (1995b) explained the high incidence of depolarizing responses in LSO neurons, by illustrating a more depolarized reversal potential of IPSPs (~ -40 mV) than normally found (~ -70 mV). Thus, the IPSPs appeared as depolarizing responses at resting membrane potential. In the current study, when pharmacology techniques were employed (CNQX or APV), the synaptic responses were attenuated but the reversal potential remained constant for the neuron tested. Further application of the complementary drug completely removed the EPSP. This showed that the EPSP was glutamatergic in nature and was not a reversed IPSPs. Thus, the EPSPs elicited in the DNLL neurons in response to electrical stimulation, were “genuine” EPSPs. The combined presence of IPSPs and EPSPs was visible by as early as P4, but was not obvious until later development. In some cases when the EPSP was completely blocked by drug application, an underlying IPSP was revealed. The observations from the pharmacological treatment complemented by the reversal potentials, suggest that the glutamatergic pathway develop earlier than the glycinergic pathway since the majority of synaptic responses were excitatory and were completely blocked by the glutamatergic antagonists, CNQX and APV.

Study comparison

Many of these same analyses have already been conducted on mature DNLL neurons (Wu and Kelly, 1995a,b; Wu and Kelly, 1996) and developing DNLL neurons in the rat (Ahuja & Wu, 2000), however methodological differences set the latter and present

developmental studies apart from one another. The current study has tried to verify many of the findings from this previous study, implementing the whole-cell patch clamp technique rather than the sharp-electrode intracellular recording technique. Both studies found the current-voltage relationships to be linear for DNLL neurons. More importantly, the slope of these curves (R_{input}) decreased significantly as the neurons matured. Furthermore, the attributes of the elicited APs also shared developmental trends in frequency, amplitude, and duration. The present study, however, introduced the measure of voltage change for the rising phase and decay phase revealing that both are developmentally linked and may be contributing to the slower AP duration in younger DNLL neurons. Also, that the rising phase of the spikes seems to be maturing quicker than the falling phase.

The synaptic response analyses of the two studies were also in agreement, with latency, duration and time to peak all improving (i.e. decreasing) with age. The current study did take the synaptic response analysis one step further by introducing a pharmacological component to it. The chemical dissection revealed that all of the postsynaptic responses elicited shared both a non-NMDA and a NMDA receptor mediated component to it, even as early as P3.

Despite the above mentioned similarities, these two studies differed in two very important ways. First, was the method of recording, whole-cell patch clamp vs. sharp-electrode intracellular. The present study chose to employ the whole-cell patch clamp method for reasons already discussed. The second important difference that these two studies shared was in group assignment and analysis. The previous study sampled DNLL neurons from P4 to P18, and these were grouped together by age. The neurons from P4 to P8 were grouped as “Early”, P10 to P12 as “Intermediate” and P16 to P18 as “Late”. This

data grouping had some drawbacks because the averaging of postnatal data may have hidden some of the underlying trends or even introduced some of its own. The current study has improved upon this by examining each postnatal age individually, from P3 to P14. All of the analyses were done in this manner, so each individual neuron regardless of age is represented in the measure. This method also allows for differences to be seen on a postnatal day to day basis rather than a postnatal group to group basis. These two experimental differences have “sharpened” the quality of the data from the current study and allowed for a more precise and true analyses, while still agreeing with previous DNLL data.

Further study

The current study was able to establish that even in the early EPSPs (P3), there was both a non-NMDA component and a NMDA component to the evoked responses. However, investigation was unable to quantitatively examine changes in the proportion of glutamate receptors or their attributes. Future studies should definitely address these changes, but will have to employ different experimental techniques. The current study utilized current clamp to make whole-cell patch clamp recordings, but this has its inherent drawbacks. Once a “seal” has been made with a neuron, the cell membrane resting potential experiences some fluctuation ($\sim \pm 5-7$ mV). This fluctuation makes it very difficult to make accurate measurements of PSP changes during pharmacological treatment. Also, since the NMDA receptors are voltage-sensitive, any oscillation in the membrane resting potential would compromise the validity of the results. This problem can be resolved by using voltage clamp whole-cell patch clamp recordings. This method maintains the resting potential at a chosen level, and so the acquisition of all measurements

are equivalent. This allows for a clear analysis of the ontogenetic receptor changes and their effects upon the elicited PSPs.

Another very important area of DNLL development that is lacking is the investigation of developmental morphological changes. Many studies have found ontogenetic changes in neuronal morphology in the auditory system (Gleich *et al.*, 1998; Kandler & Friauf, 1993, 1995a; Rogowski & Feng, 1981; Sanes *et al.*, 1993). Future studies should include this developmental morphological analysis for the DNLL neurons. Ontogenetic changes in morphology could be correlated with developmental changes seen in the electrical membrane and synaptic response properties in the DNLL. This analysis will be of some significance because previous studies on DNLL neurons have already established the various subtypes of neurons and their morphology (Wu & Kelly, 1995b). This study revealed several types of DNLL neurons based on their somal and dendritic fields. Future studies could examine if these subtypes of neurons undergo processes of maturation during early development.

Conclusion

The major findings of the current study, with its methodological differences, still agree with the previous developmental DNLL findings (Ahuja and Wu, 2000). It seems evident from these data that the DNLL neurons undergo developmental changes expressed as differences in membrane properties and synaptic responses. The neurons encountered across all of the postnatal ages sampled here can be described as “immature” to some degree in comparison to mature adult DNLL neurons. During early ontogeny, many anatomical connections are established even though the DNLL is devoid of any acoustically driven activity. Nevertheless, even the very young neurons (P3-P5) are able to respond to electrical stimulation, however, they seem to be especially immature. Analysis of the electrical membrane properties, showed a decreasing trend for R_{input} , spike threshold, spike duration, spike time to peak and an increasing trend for spike amplitude and voltage change for both the rising phase and decay phase. Thus, the younger DNLL neurons display “immature” electrical membrane properties. The synaptic response properties of the developing DNLL neurons also display ontogenetic improvements. The data showed a decreasing trend in synaptic response latency, duration and time to peak. Also, the synaptic responses displayed an excitatory dominance, which could be elicited as early as P3. Each of these measures improved or reaches adult-like levels before the onset of physiological hearing. From the data presented here it seems that the synaptic response properties of DNLL neurons seem to plateau just prior to the onset of hearing at P12. Thus, a large portion of the maturation process occurs very early postnatally and begins to subside just prior to the onset of hearing. After physiological hearing has been initiated these measures must undoubtedly continue to mature, but the bulk of the process has been completed. Pharmacological analysis revealed that the glutamate receptors, non-NMDA

and NMDA mediate the excitatory synaptic responses. These two types of receptors are present throughout the neurons tested, as early as P3. These drugs were able to completely block the synaptic responses even in the early neurons, while IPSP presence became evident later in development. This indicates that the glutamatergic pathway develops earlier. Changes in the receptors throughout the maturational process undoubtedly influence synaptic transmission in the DNLL, although further experimentation is required.

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