Pre and Postnatal Differences in Membrane, Action Potential and Ion Channel Properties of Rostral Nucleus of the Solitary Tract Neurons

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Running head: Properties of rNST neurons during development

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Abstract

There is little known about the prenatal development of rNST neurons in rodent or the factors that influence circuit formation. With morphological and electrophysiological techniques in vitro, we investigated differences in the biophysical properties of rNST neurons in pre and postnatal rat from embryonic day (E) E14 through postnatal day 20. Developmental changes in passive membrane and action potential properties, and the emergence and maturation of ion channels important in neuron function were characterized. Morphological maturation of rNST neurons parallels changes in passive membrane properties. Mean soma size, dendritic branch points, neurite endings and neurite length all increase prenatally whereas neuron resting membrane potential, input resistance and time constant decrease. Dendritic spines, on the other hand, develop after birth. Action potential discharge patterns alter in pre and postnatal stages. At E14 neurons generated a single TTX-sensitive, voltage-gated Na\(^+\) action potential when depolarized; a higher discharge rate appeared at older stages. Action potential amplitude, halfwidth, rise and fall times all change during development. Responses to current injection revealed a number of voltage gated conductances in embryonic rNST, including a hyperpolarization-activated inward current I_h and a low-threshold Ca\(^{2+}\) current that initiated Ca\(^{2+}\) spikes. A hyperpolarization-activated, transient outward potassium current, I_{KA}, was also present in the developing neurons. Although the properties of these channels change during development, they are present before synapses form and therefore can contribute to initial establishment of neural circuits as well as to the changing electrophysiological properties in developing rNST neurons.
The rostral nucleus of the solitary tract (rNST) is the initial integrative relay for multiple sensory inputs from the oral cavity. In rats the taste system is functional at birth as demonstrated by stereotypic behaviors to orally applied taste stimuli (Johanson and Shapiro 1986; Moe 1986). Therefore, chemical responses and central connections via rNST neurons to muscles of facial expression, with functional synapses, are present in late fetal and newborn rats.

Despite the fact that the rat has been used for decades to study taste function, there is little information on the prenatal development of rNST in rat or the factors that potentially influence circuit formation. There are data about temporal formation of the solitary tract (ST), the projection for taste nerves into the brainstem. Classic studies by Altman and Bayer (1982) identified afferent fibers projecting into the ST shortly after generation of ganglion neurons in rat. Reportedly, trigeminal nerve (V) fibers appear in the ST first at E12, followed closely by facial nerve (VII) at E13, and then finally glossopharyngeal fibers (IX) at about E15. By E14, V and VII axons are reported to project medially and enter the anlage of the rNST, and to steadily increase in density from E15-17 (Zhang and Ashwell 2001b). Synapses onto NST neurons have been identified as early as E17, and by E19, synaptic vesicles were described, suggesting the establishment of synaptic connections (Zhang and Ashwell 2001b).

Compared to initial formation of the ST, much less attention has been given to development of rNST neurons. Presumptive target neurons in the rNST for ST sensory afferents are reportably produced between E11 and E14 in rat, with peak production at E12 (Altman and Bayer 1982). The rNST is clearly visible by E15, individual subnuclei
are apparent by E19, and mature expression patterns of acetylcholinesterase activity and a
number of other biochemical markers (e.g., tyrosine hydroxylase, substance P, calbindin,
and calretinin) have been demonstrated at E19 (Zhang and Ashwell 2001a). However,
nothing is known of the cellular origin of these neurons, or the development of their
intrinsic membrane characteristics.

Early events in gestation can influence establishment of afferent terminations and
the nature of neurophysiological responses of second order rNST neurons (Vogt and Hill
1993). Restriction of maternal dietary sodium (0.03% sodium chloride) maintained into
adulthood results in a 2- and 3-fold increase in volume of afferent taste nerve terminal
fields (May and Hill 2006) and 4-fold increase in the number of synapses made by chorda
tympani nerve terminals (May et al., 2007), when compared to controls. Importantly, a
brief critical period of sodium restriction from E3-E12 is sufficient to achieve this
terminal field expansion (Krimm and Hill 1997; Mangold and Hill 2007).

Understanding how and when rNST neurons begin to function during embryonic
development is a necessary first step for any future studies about regulatory factors in
taste plasticity. Because a remarkable plasticity is documented in the postnatal taste
system, based on prenatal interventions, study of temporal events in development of
rNST function is necessary to understand the biological underpinnings and limitations of
taste plasticity. In the current study we investigated the biophysical properties of rNST
neurons in prenatal rodents during a broad gestational period. Developmental differences
in passive membrane and action potential properties are demonstrated, with ion current
emergence and changes that support these functional characteristics.

METHODS
Preparation of brainstem slices

Sprague-Dawley rats between E14 days and postnatal age (P) 20 days (39 embryos and 61 postnatal rats) were used. All procedures were conducted under National Institutes of Health and University of Michigan Animal Care and Use Committee approved protocols. Embryos were obtained from timed pregnant dams (Charles River) by hysterectomy under halothane anesthesia on the day of experiment. The day on which a vaginal plug was found was designated E0. Postnatal rats were obtained from timed pregnant dams. The day on which pups were born was P0.

Embryos and postnatal rats were decapitated. For postnatal rats, the decapitation was carried out under halothane anesthesia. The brain was rapidly removed and cooled for 5–8 min in an oxygenated physiological saline solution in which NaCl was replaced with isosmotic sucrose at 4°C (Aghajanian and Rasmussen 1989). The brainstem was transected at the level of the pons and just caudal to the obex and secured onto a Vibratome (Technical Products International) stage with agarose. The brainstem was sectioned horizontally into 200- or 250-μm-thick slices. Slices were incubated for at least 1 hour in an oxygenated artificial cerebrospinal fluid (ACSF) at room temperature before transfer to a recording chamber. ACSF contained (in mM) 124 NaCl, 5 KCl, 2.5 CaCl2, 1.3 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, and 25 dextrose, and was gassed with a 95% O2-5% CO2 mixture to achieve a solution pH of 7.4.

Recording

Brainstem slices were transferred to a recording chamber attached to the stage of a microscope (ECLIPSE E600-FN, Nikon) and anchored with nylon mesh. During recording, the slice was superfused at 2–2.5 ml/min with oxygenated ACSF. The
recording chamber was kept at 32°C by a heating unit. The ST was used as an anatomical landmark to search for rNST (asterisks in Figs. 1A - 1C). Recording sites were approximately 1.2, 1.0 and 0.9 mm rostral to the narrowest point of the fourth ventricle in the brainstem slices at E14, E16 and E18 respectively. These recording sites are in an area in which collateral branches were observed in a study of the developing ST (Tsukamoto et al., 2005, inset in Figs. 1B and C). At E20 and older, recording sites were in the NST area that was rostral to the level where the NST abuts the fourth ventricle.

Neurons in rNST were observed using infrared-differential interface contrast optics (IR-DIC) via a CCD camera (IR-1000, DAGE-MTI). The neurons were recorded in whole-cell mode using a patch-clamp amplifier (AxoClamp 2B, Axon Instruments).

Signals were recorded through 2-kHz low-pass filter, digitized at 20–50 kHz (DigiData 1200, Axon Instruments), and stored on a computer hard disk. Data were acquired using a data acquisition module Clampex in pCLAMP 8 software (Axon Instruments). Patch pipettes were made of borosilicate glass capillaries (TW150F-4, World Precision Instruments) using a two-stage puller (PP-83, Narishige) and filled with a solution that contained (in mM) 130 K-gluconate, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 MgCl₂, 1 CaCl₂, and 2 ATP, buffered to pH 7.2 with KOH. Lucifer yellow (Sigma) was dissolved in the pipette solution at a concentration of 0.1% to label recorded neurons. Tip resistance of filled pipettes was 6–8 MΩ.

Neuron reconstruction

After patch-clamp recording, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h. Fixed slices were rinsed in the phosphate buffer for 30 min and
embedded in 4% agar, then cut into 100-μm-thick sections on a Vibratome. The sections were mounted on glass slides, dried overnight, and then coverslipped with an antifade medium (ProLong Gold, Molecular Probes).

Neurons filled with Lucifer Yellow were scanned and serial images were captured in 1 μm image stacks using a laser confocal microscope (Nikon C-1 confocal microscope). Projection images were made from the stacked serial images and neurons were traced on the projection images using a neuroanatomical analysis program (Neurolucida, MBF Bioscience). Analyses were: soma size, number of primary dendrites, branch points, neurite length, number of neurite endings, and number and density of neurite spines.

Data analysis

Electrophysiological data were analyzed using a data analysis module Clampfit in pCLAMP 8 (Axon Instrument). The junction potential due to potassium gluconate (10 mV) was subtracted from the membrane potential (V_m) values. Whole-cell configuration was first established in current-clamp recording mode and resting membrane potential (V_rest), input resistance (R_input) and membrane time constant measured. R_input was calculated from change in V_m evoked by a hyperpolarizing current (10 pA, 2 s long). Membrane time constant was measured by fitting a single exponential function to the data points in the hyperpolarizing phase of the same recording. Membrane capacitance (C_m) was calculated by dividing the time constant by R_input. Action potentials and afterhyperpolarization (AHP) were evoked by depolarizing currents. During the recording of action potential and AHP, baseline V_m was maintained at -55 mV by constant DC current. Action potential threshold was defined as V_m at the inflection point of the rising
phase. Action potential amplitude was defined as the difference in $V_m$ between the peak and threshold. AHP amplitude was defined as the difference in $V_m$ between the peak AHP and action potential threshold. The time to peak of the AHP was defined as elapsed time of the change in $V_m$ from action potential threshold to the peak AHP.

Recording mode was switched to voltage-clamp to obtain the hyperpolarization-activated, transient outward potassium ($I_{KA}$) and inward currents ($I_h$). Holding potential was set at -60 mV.

We evoked the transient outward current containing $I_{KA}$ using standard protocols (Tell and Bradley, 1994: membrane hyperpolarization to -120 mV for 1 s followed by depolarization to various $V_m$ between -70 and -10 mV in 10 mV steps for 1.2 s total time). Sustained outward current was obtained by depolarization steps between -70 and -10 mV (10 mV increment) for 1.2 s without a hyperpolarizing pre-pulse. $I_{KA}$ was isolated by digital subtraction of the sustained current from the transient current. $I_{KA}$ amplitude and decay time constant were measured at membrane depolarization to -30 mV. $I_{KA}$ amplitude was defined as the difference between peak and steady-state values. $I_{KA}$ decay time constant was calculated by fitting single exponential curve to the data points of the decay phase included in 10 - 90% of the $I_{KA}$ amplitude. The voltage-dependent inactivation of $I_{KA}$ was observed by depolarizations to -30 mV for 1.2 s following hyperpolarizations to various $V_m$ between -110 and -30 mV (10 mV step) for 1 s. $I_h$ was evoked by hyperpolarizing steps between -60 mV and -150 mV for 2 s. The amplitude of $I_h$ was defined as the difference between the peak and steady-state values of currents in response to the hyperpolarizing steps. Activation curve for $I_{KA}$ and
I_h as well as inactivation curve for I_{KA} were fit with the Boltzmann function using a data analysis Origin software (OriginLab).

Statistical analysis was conducted using PASW Statistics 18 (SPSS) software. Normality of data was assessed using the Shapiro-Wilk test. When data were normally distributed, statistical differences between groups were tested using ANOVA with a post-hoc tests. Scheffe and Games-Howell post-hoc tests were used in cases of equal and unequal variances of data, respectively. When normal distributions were rejected, statistical differences between groups were tested using Kruskal–Wallis test followed by multiple Mann-Whitney tests with the Bonferroni correction. Significance level was set at 0.05. Mean values and the number of observations (n) for all stages and variables are shown in Table 1.

RESULTS

Neuron morphology

rNST neuron morphology was analyzed at prenatal (E14/16; n = 14; E18/20; n = 25) and postnatal ages 0-2 days (P0-2; n = 32) and 6-8 days (P6-8; n = 15). Soma size and neurite parameters increased substantially during the prenatal period (Fig. 2). The mean soma size, number of branch points, neurite endings, and neurite length at E14/16 were all significantly different from values at P0-2 (Fig. 2). Spines were not detected on the prenatal rNST neurons, but were obvious after birth. Spine numbers and density increased significantly after birth (Fig. 2).

The different rNST neuron morphologies characteristic of the adult rNST (ovoid, fusiform and multipolar) are not apparent in embryonic neurons. Embryonic neurons generally resemble the ovoid neurons described in the adult rNST. The different adult
morphologies become apparent in the postnatal animals. Tracings of an E14 and P2 neuron illustrate changes in neuron complexity (Fig. 2).

Passive membrane properties

Passive membrane properties of rNST neurons changed significantly during the prenatal period (Table 1). The mean $V_{\text{rest}}$ significantly shifted in a hyperpolarizing direction between E14/16 and E18/20 and then was maintained at the lower level (Fig. 3A). $R_{\text{input}}$ decreased across embryonic and postnatal stages with a notable decrease in variability between stages (Fig. 3B). Membrane time constant also decreased and the wide variability observed at embryonic stages was much reduced at P14-20 (Fig. 3C). $C_m$ significantly increased between E14/16 and P0-2 and then stabilized (Fig. 3D). Overall, major differences were observed in membrane characteristics between E14/16 or E18/20 and the earliest postnatal stage.

Action potential

Action potential discharge patterns and underlying ion currents were observed in pre and postnatal rNST neurons. Baseline $V_m$ was maintained at $-55$ mV with constant DC current during recording of action potentials and AHP.

Neurons in rNST generated a single action potential in response to depolarizing current at E14. The transient spikes were suppressed by the voltage-gated Na$^+$ channel blocker tetrodotoxin (TTX, 1 μM) in 3 of 5 neurons at E14 and all neurons tested at E16 and older ($n = 5$ each at E16, P0-2 and P6-8) indicating that TTX-sensitive, voltage-gated Na$^+$ channels are functional in rNST neurons as early as E14. In a subset of rNST neurons at E16 and older ($n = 10$), the Ca$^{2+}$ channel blocker CdCl$_2$ (200 μM) was applied to determine any contribution of voltage-gated Ca$^{2+}$ current ($I_{\text{Ca}}$) to action potential.
TTX suppressed transient spikes evoked by depolarizing currents but some spikes were still evoked in the presence of TTX (Fig. 4A). CdCl₂ eliminated the remaining spikes (Fig. 4B). These results indicate that Ca²⁺ channels are also functional and contribute to the action potential in rNST neurons as early as E16.

Action potential properties altered significantly before birth. The threshold for spike initiation shifted in a hyperpolarizing direction between E14/16 and P0-2 (Fig. 5Aa, Table 1). Action potential amplitude steadily increased between E14/16 through P14-20 (Fig. 5Ab). Only one of 14 neurons tested had overshooting action potentials at E14/16. Mean value of the action potential peak first exceeded 0 mV at P0-2, and all neurons (n = 39) were able to generate overshooting action potentials at P14-20 (Fig. 5Ac).

Action potential rising and falling slope velocities (Figs. 5Ad and 5Ae) became faster and duration measured at half-maximal amplitude decreased between E14/16 through P14-20 (Fig. 5Af), resulting in sharper action potentials. Properties of the AHP also changed. Amplitude increased between E14/16 and P0-2 (Fig. 5Ag) and the time to peak became faster postnatally (between P6-8 and P14-20; Fig 5Ah).

Examples of action potentials in each age group are illustrated in Fig. 5B (a-e), demonstrating how changes in action potential characteristics influence action potential shape. Developmental changes in AHP are illustrated in Fig. 5C. In the younger age groups (E14/16 through P6-8, Fig. 5a - d) AHPs have slow kinetics. At P14-20 neurons had AHPs with fast (filled arrowhead in Fig. 5D) and slow (open arrowhead in Fig. 5D) components.

At E14/16, almost all neurons (34 of 35 neurons) generated only a single action potential (Fig. 6Aa) even though depolarizing currents above rheobase were injected.
Neurons with higher discharge rate appeared at older stages (Fig. 6Ab). Thus, action potential pattern changed and maximal discharge frequency steadily increased during development (Fig. 6B).

Low-voltage-activated Ca\(^{2+}\) current

Membrane hyperpolarization induced neuronal excitation via triangular depolarization, referred to as post-inhibitory rebound (PIR). PIR results in an action potential (PIR spike) when the amplitude of the transient depolarization exceeds action potential threshold (Guido et al., 1998). We obtained PIR spikes immediately after the end of hyperpolarizing current injection pulses that resulted in a \(V_m\) between -100 to -110 mV (Figs. 7A and 7B). About 30% of the neurons displayed PIR spikes in prenatal animals (28% or 16 of 57 neurons at E14/16; 28% or 14 of 51 neurons at E18/20). Incidence of PIR spikes was significantly higher at P0-2 (52% or 16 of 31 neurons) and at P6-8 (67% or 10 of 15 neurons). The incidence was 35% at P14-20 (13 of 37 neurons).

The major component of PIR is membrane depolarization via low-voltage-activated (LVA) \(I_{Ca}\) in adult rNST neurons (Tell and Bradley 1994; Guido et al., 1998). To characterize the LVA Ca\(^{2+}\)-spike, we used NiCl\(_2\) to block \(I_{Ca}\) with a voltage protocol in which \(V_m\) was hyperpolarized between -100 and -110 mV. Baseline \(V_m\) was maintained at -55 mV with constant DC current during recording of LVA Ca\(^{2+}\)-spikes. Membrane hyperpolarization between -100 and -110 mV completely releases LVA \(I_{Ca}\) from its inactivation state in both embryonic (see plots of the ratio of the amplitude of the maximum PIR and the PIR amplitude at different membrane hyperpolarization; Fig. 7H) and adult rNST neurons (Tell and Bradley 1994). This experiment was performed in the presence of 1 \(\mu\)M TTX to block action potentials and 5 mM 4-AP to block \(I_{KA}\).
Detectable LVA Ca\(^{2+}\)-spikes were obtained at E16 and older (Figs. 7C, 7E and 7F), and these were suppressed by 200 μM NiCl\(_2\) (Fig. 7D). Because a similar concentration of NiCl\(_2\) preferably blocks the T-type Ca\(^{2+}\) channel in neurons of various brain areas (reviewed in Perez-Reyes 2003), sensitivity of the Ca\(^{2+}\)-spikes to 200 μM NiCl\(_2\) suggests that T-type Ca\(^{2+}\) channels are functional in rNST neurons as early as E16.

LVA Ca\(^{2+}\)-spikes were observed in rNST neurons at all developmental ages. There was no significant difference in amplitude of the Ca\(^{2+}\)-spike between E18/20 and P14-20 (Fig. 7G).

**Hyperpolarization-activated, transient outward potassium current**

The transient outward potassium current, I\(_{KA}\), influences the repetitive firing pattern of adult rNST neurons (Tell and Bradley 1994; Uteshev and Smith 2006; Suwabe and Bradley 2009). We determined prenatal development of I\(_{KA}\) in rNST neurons using voltage protocols and 4-AP blocking. Depolarization with a hyperpolarizing pre-pulse (see Methods) evoked a transient outward current (TOC in Figs. 8Aa and 8Ab) whereas depolarization without a hyperpolarizing pre-pulse failed to evoke a transient outward current (sustained, SUS in Figs. 8Aa and 8Ab). The transient outward current was suppressed by 5 mM 4-AP (\(n = 2\), E14; 6, E16; 2, E18; 7, P6-8; 5, P14-20; Figs. 8Ac and 8Ad). These electrophysiological and pharmacological properties indicate presence of functional I\(_{KA}\) channels in rNST neurons as early as E14. Many neurons exhibited I\(_{KA}\) at E14/16 (Fig. 8B). The incidence of I\(_{KA}\) was significantly smaller at P14-20 in comparison with the other ages (\(p = 0.03\) by Fisher’s exact test, Fig. 8B).

Although I\(_{KA}\) was detected as early as E14, electrophysiological properties of this current changed during development. When inactivation curves were compared between
E14/16 \( (n = 5; \text{ filled circles in Fig. 8C}) \) and P14-20 neurons \( (n = 6; \text{ filled triangles in Fig. 8C}) \), the curve was shifted in the depolarizing direction postnatally. In contrast, the activation curve did not significantly change between these two ages (open circles and triangles in Fig. 8C). The size (combination of amplitude with decay time constant) of \( I_{KA} \) was too small to suppress action potential discharge in some prenatal neurons. The amplitude and decay time constant were larger (Fig. 8Da) and longer (Fig. 8Db) during development possibly relating to maturation of spike pattern generation.

Hyperpolarization-activated, inward current

\( I_h \) has also been reported in adult rNST neurons (Tell and Bradley 1994; Uteshev and Smith 2006; Suwabe and Bradley 2009) as a subthreshold current. \( I_h \), indicated as a voltage sag, was already detectable in current-clamp recording at E14 when 65% of rNST neurons exhibited voltage sag (Fig. 9D). The percentage of neurons with voltage sag was significantly higher E18/20 neurons \( (p = 0.007 \text{ by Fisher’s exact test}) \). Neurons with voltage sag showed a nonohmic current response in voltage-clamp recording. \( I_h \) was confirmed using a specific blocker, ZD 7288 (100 \( \mu \text{M} \)). Voltage sag (arrowhead in Fig. 9A) and inward current (difference between filled and open circles in Fig. 9B) were eliminated by ZD 7288 (three neurons each at E16, E18, E20, P2 and P20; Figs. 9A and 9C). These results demonstrated that \( I_h \) is functional in rNST neurons as early as E14.

Between E14/16 \( (n = 6) \) and P14-20 neurons \( (n = 7) \), the \( I_h \) activation curve was shifted postnatally in the depolarizing direction (Fig. 9E). The amplitude of \( I_h \) increased between E14/16 and P0-2 (Fig. 9F).

DISCUSSION
With studies of developing morphological, intrinsic membrane, action potential and ion channel properties, we have shown that rNST neurons are functional as early as E14. However there are major developmental differences in neuron morphology and neurophysiology before birth, and changes continue postnatally. Notably, whereas soma size progressively increases as does neuron complexity, spines are not observed on embryonic rNST neurons but are seen only after birth; threshold to spike decreases, the action potential becomes shorter, and there is a steady shift in discharge pattern from one action potential in the embryonic neuron to a sustained train in postnatal stages.

Underlying the numerous, detailed changes in passive membrane and action potential characteristics not only are basic neuron morphology, but importantly, ion channel properties shift remarkably during development. As early as E14-16 there is evidence of Ca$^{2+}$ and K$^+$ channel function in rNST neurons; but channel properties change in the embryo and continue to alter after birth. As neurons differentiate, voltage gated Ca$^{2+}$ channels might regulate neuron death, migration and proliferation and neurite outgrowth (Spitzer et al., 1994). These channels are important in driving ‘activity-dependent’ development also (Rakic et al., 1994).

In short, we demonstrate very early function in rNST neurons but in turn show that there are substantial embryonic changes and neurophysiological changes that continue after birth. Thus, there is an embryonic emergence of function in rNST before taste buds have appeared; yet changes continue after birth with opportunities to “shape” taste responses by incoming taste input from developing taste buds. The embryonic and early postnatal differences in rNST that we have documented provide a clear base for the extreme plasticity of the taste system, widely reported in the field (Hill and May 2006).
The early rNST

Presumptive rNST neurons are generated in the ventricular zone between E11 and E14 in rat (Altman and Bayer 1980). An identifiable NST is detectable by Nissl staining at E15 and all subnuclei of NST are discernable at E19 (Zhang and Ashwell 2001a).

Afferent fibers of ST that originate in the geniculate ganglion enter the developing brainstem as early as E13 and run throughout the brainstem by E14 (Tsukamoto et al., 2005). The ST begins to send collateral fibers medially toward the presumptive rNST at least as early as E16 (Tsukamoto et al., 2005). Synaptic thickenings are apparent in pre- and postsynaptic membranes at E15 although synaptic vesicles were not reported until E19 (Zhang and Ashwell 2001b). The terminal fields of gustatory afferent fibers have been reported to become larger in postnatal rats (Lasiter 1992) but more recent investigations report that the terminal field volume is greater at P15 and P25 than in rats aged P35 and older (Sollars et al., 2006). Detailed data on embryonic terminal fields are not available. However, available neuroanatomical reports, in combination with our electrophysiological data indicate that newly forming rNST neurons from as early as E14 are able to generate action potentials and the afferent ST fibers are within the brainstem and positioned to form connections for early circuit formation.

Development of passive membrane properties and neuron morphology

Combined intracellular injection of Lucifer Yellow and electrophysiological recordings demonstrate morphological maturation of rNST neurons in parallel with changes in passive membrane properties. $V_{rest}$ alters substantially in the hyperpolarizing direction before birth. As reported in our previous study (Bao et al., 1995), $V_{rest}$ is
relatively stable postnatally. These findings indicate that cellular mechanisms responsible for $V_{\text{rest}}$ develop well before birth in rodent.

$R_{\text{input}}$ decreases prenatally whereas $C_m$ increases only after birth paralleling the marked changes in total membrane volume and dendritic complexity. This discrepancy in time course also suggests developmental changes in membrane kinetics as well as a postnatal developmental increase in density of resting channels.

Associated with development of biophysical properties are morphological modifications in rNST neurons. At P0 -2, neurons have much increased dendritic trees compared with E14. By P6-8, the neurons are more similar to adult rNST, with a large soma from which 2–4 dendrites emerge with very few ramifications. Others have reported that dendritic length increases approximately three fold between P8 and P25 (Lasiter et al., 1989) while dendritic branching decreases with age (Bao et al., 1995).

**Development of action potential characteristics**

Action potential threshold shifts in the hyperpolarizing direction between E14/16 and P0-2 and then stabilizes. This demonstrates that rNST neurons acquire adult-like excitability in terms of threshold to generate a spike by a few days after birth. The amplitude, and maximal rising and falling velocities of the action potential, change with a similar time-course. Increased action potential amplitude would suggest increased density of voltage-gated Na\(^+\) channels. Increases in both maximal rise and fall rates of action potential reflect developmental changes in kinetics of voltage-gated Na\(^+\) and K\(^+\) channels (Hille, 2001). The slow action potential kinetics at younger stages may be advantageous for neurons to increase influx of cations (reviewed in Spitzer 1991). Entry of cations such as Na\(^+\) and Ca\(^{2+}\) is important in establishing neural circuits (see discussion below).
Both the amplitude and rise time to peak amplitude increase during development of the AHP. Between E14-16 and P6-8 AHPs consist of a single slow component. At P14-20 neurons have AHPs with slow and fast components suggesting that at least two types of K$^+$ channels underlie developmental changes in electrophysiological properties of the AHP. In other neurons the slow AHP is generated by a small-conductance calcium-activated potassium channel (SK) and the fast AHP is due to a second voltage-gated K$^+$ channel (Hille, 2001). Thus, the ion channels responsible for the fast and slow components of the AHP develop at different pre and postnatal ages in the rodent rNST.

**Development of ionic currents**

We identified TTX-sensitive action potential components, and the membrane currents that influence action potential firing patterns, -I$_{KA}$ and I$_h$, - as early as E14 in rNST neurons. At this stage, the facial nerve already enters the brainstem but few collateral fibers extend toward the presumptive rNST. Our data demonstrate that prenatal rNST neurons are already excitable and channels for I$_{KA}$ and I$_h$ are functional before formation of synaptic connections. Ca$^{2+}$-channel-mediated components (HVA and LVA components) were also detectable in current-clamp recordings in the prenatal period, indicating that HVA and LVA I$_{Ca}$ together with Na$^+$-current are involved in neural excitation in the developing rNST. However, because voltage-gated I$_{Ca}$, in particular HVA I$_{Ca}$, show rapid rundown (Hille, 2001), the age when Ca$^{2+}$-channel-mediated components first appear in patch-clamp recording could be biased. Thus, these components could potentially become functional at an earlier age.

As discussed, intrinsic membrane properties tested in the present study change significantly before birth. Because early collections of cells that are apparently the
presumptive taste buds appear at about the time of birth, and taste buds develop postnatally in rat (Mistretta, 1972), our data suggest that the prenatal intrinsic membrane properties develop independently of prenatal taste bud input. Thus, it is unlikely that afferent sensory activity derived from stimulation of taste buds \textit{per se} contributes to embryonic rNST circuit development. Chemosensory stimulation, by amniotic fluid components, of nerve endings in the lingual epithelium of developing papillae is theoretically possible before birth.

Neurons with slowly decaying $I_{KA}$ emerge at a later stage of development, shown in a statistically significant increase in the decay time constant. There may be several reasons for the change, for example, increase in complexity of neural morphology could amplify electrical errors in voltage-clamp recording. If new $I_{KA}$ channels were inserted in distal sites from the tip of recording pipette (e.g. dendritic trees), decay time constant for $I_{KA}$ could be overestimated. Emergence of a new channel subunit for $I_{KA}$ may also account for the increase in the decay time constant. Slowly decaying $I_{KA}$ that is sensitive to 4-AP and fast decaying $I_{KA}$ that is relatively insensitive to 4-AP have been reported in rNST (Tell and Bradley 1994) and spinal dorsal horn neurons (Ruscheweyh et al., 2004).

Our data indicate that fast decaying $I_{KA}$ emerges earlier than slowly decaying $I_{KA}$ in developing rNST.

$I_h$ is activated by membrane hyperpolarization. Combination of this characteristic with high $R_{input}$ at early stages raises a possibility that the effect of $I_h$ on $V_m$ is enhanced in rNST prenatal neurons. In fact, when the amplitude of voltage \textit{sag} (difference between peak and steady-state $V_m$) induced by 40-pA hyperpolarizing current was directly compared between neurons at E14/16 and those at P14-20, the amplitude was
significantly larger in neurons at E14/16 (18 ± 3 mV vs. 6 ± 2 mV; Mann-Whitney test, \( p < 0.01 \)) in association with a more hyperpolarized \( V_m \) (-126 ± 7 mV vs. -74 ± 2 mV; Mann-Whitney test, \( p < 0.01 \)). Our data demonstrate that the amplitude of \( I_h \) increases during development but the effect of \( I_h \) on the membrane potential is large in rNST neurons at young stages.

Channels for LVA \( I_{Ca} \), \( I_{KA} \) and \( I_h \) need membrane hyperpolarization to deactivate LVA \( I_{Ca} \) and \( I_{KA} \) and activate \( I_h \). One possible source of hyperpolarization (inhibitory input) is inhibitory amino acids such as \( \gamma \)-aminobutyric acid (GABA) and glycine from interneurons. Several studies have reported that activation of GABA or glycine receptors causes membrane depolarization in developing neural systems (Yamada et al., 2004; Nakamura et al., 2008). The reversal potential of Cl\(^{-}\) in rNST neurons is more negative than \( V_{rest} \) as early as P0-7 (Grabauskas and Bradley 2001), suggesting that activation of GABA or glycine receptor may cause membrane hyperpolarization and induce LVA \( I_{Ca} \), \( I_{KA} \) and \( I_h \) in prenatal period.

**Significance to development of rNST gustatory circuits**

Neuronal activity is tightly connected with development of neuronal morphology and neural circuits in the visual system (Shatz and Stryker 1988; Sretavan et al., 1988). Influx of Na\(^{+}\) and/or Na\(^{+}\)-dependent action potentials, confirmed by TTX experiments, plays important roles in segregation of afferent projections in the lateral geniculate nucleus (Shatz and Stryker 1988), axonal arborization of projection neurons in the retinogeniculate (Sretavan et al., 1988) and thalamocortical connections (Herrmann and Shatz 1995; Catalano and Shatz 1998). Further, the influx of Ca\(^{2+}\) through LVA Ca\(^{2+}\) channels confirmed by NiCl\(_2\) plays an important role in activity-dependent cell
growth/differentiation (Holliday and Spitzer 1993) and neurotransmitter synthesis
(Spitzer et al., 1993). In the present experiments we found that $I_{Na}$ and LVA $I_{Ca}$ are
functional in presumptive rNST neurons suggesting that these ionic currents could be
important in the development of rNST and synaptic connections. These currents act as a
form of intrinsic (spontaneous) activity in the visual system. We could not detect
spontaneous activity in prenatal and early postnatal rNST. Spontaneous neural activity
similar to that demonstrated in the developing visual system may occur at an earlier stage
and/or a less invasive recording method such as Ca$^{2+}$-imaging may be required to
preserve native cellular function.

Gustatory information is encoded by spiking pattern (rate, inter-spike interval and
synchronicity of spikes) in rNST neurons (reviewed in Hallock and Di Lorenzo 2006). In
adult rNST neurons, $I_{KA}$ modulates the patterns of stimulus-driven repetitive firing (Tell
and Bradley 1994; Suwabe and Bradley 2009). Thus $I_{KA}$ would play an important role in
gustatory information processing in adult rNST. In the present study, most excitable
neurons in rNST fired only one action potential in response to depolarizing current
injections at E14/16. The basic peripheral gustatory system (i.e., taste buds or synaptic
connections between nerve fibers and presumptive taste bud epithelium) is not
established at this age (Mbiene and Farbman 1993; Mbiene and Mistretta 1997). $I_{KA}$,
therefore, is not likely to contribute to gustatory information processing in the early rNST.

It has been reported that neural progenitor cells in rat subventricular zone express
$I_{KA}$ prior to the differentiation to neurons, suggesting that $I_{KA}$ supports proliferation of the
cells (Smith et al., 2008). $I_{KA}$ may play the same role in the germinal zone for rNST
during neurogenesis and later contribute to modulation of gustatory information in rNST.
The present work revealed that the amplitude of $I_{KA}$ increases and the decay becomes slower during development.

The existence of $I_h$ has been reported in adult rNST neurons (Tell and Bradley 1994; Uteshev and Smith 2006; Suwabe and Bradley 2009). We found that almost all neurons (96%) exhibited $I_h$ at E18/20 and there was a trend for the incidence of this current to decrease postnatally. In adult rat rNST, $I_h$ has been reported in only 26% of neurons (Uteshev and Smith 2006). These data suggest that $I_h$ plays an important role in the developing rNST. Numerous studies have reported that $I_h$ sets $V_{rest}$, influences the rhythmicity of action potential firing and regulates neuronal excitability (reviewed in Bender and Baram 2008). While the present study could not determine the role of $I_h$ in developing rNST neurons it is possible that it may regulate the LVA-$I_{Ca}$-induced membrane excitation by reduction of $R_{input}$ that decreases $Ca^{2+}$ influx.

In conclusion, voltage-gated channels emerge and become functional in rNST neurons before synaptic connections with ST fibers. Thus, currents through the channels would contribute to initial establishment of neural circuits. Development of the channels would change the electrophysiological properties of the currents and influence modulation of afferent inputs in rNST neurons.
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FIGURE LEGENDS

FIG. 1. Horizontal brainstem slices from rat embryos. A - C: Brightfield images of brainstem slices at E14, E16 and E18. The trajectory of ST is indicated by asterisks. Approximate recording site is circled by dashed line. B and C inset: DiI anterograde transport from the geniculate ganglion to illustrate collateral, medially directed, branching of the solitary tract.

FIG. 2. Morphometric analysis of pre and postnatal neurons in rNST. Developmental increases in all measures are observed except number of neurites. Spines were not observed in embryonic stages. Neuronal tracings of E14 and P2 rNST neurons are shown in upper right panels. Values are medians, with the 25th – 75th percentile range. Horizontal bars above each graph indicate pairs with significant differences. Single and dual asterisks indicate $p < 0.01$ and $p < 0.05$ (Mann-Whitney test), respectively.

FIG. 3. Developmental changes in passive membrane properties. A: Resting membrane potential ($V_{\text{rest}}$). B: Input resistance ($R_{\text{input}}$). C: Membrane time constant. D: Membrane capacitance ($C_m$). Values of $V_{\text{rest}}$ and membrane time constant are means ± SE, and values of $R_{\text{input}}$ and $C_m$ are medians, with the 25th – 75th percentile range. Statistical differences in $V_{\text{rest}}$ and membrane time constant between groups were tested using Scheffe and Games-Howell tests, respectively. Statistical differences in $R_{\text{input}}$ and $C_m$ between groups were tested using Mann-Whitney tests. Horizontal bars above each graph indicate pairs with significant differences. Single and dual asterisks indicate $p < 0.01$ and $p < 0.05$, respectively.
FIG. 4. Cd^{2+}-sensitive component of action potential in a neuron at E20. A: Membrane depolarization and spikes (filled arrowhead) evoked by depolarizing current steps (0, 20, 40, 50, 55 and 60 pA) in the presence of TTX. B: Membrane depolarizations evoked by the same current protocol in the presence of CdCl$_2$ and TTX. The spikes seen in the control recording are eliminated (open arrowhead). C: Washout to demonstrate return of spikes. $V_m$ was maintained at -55 mV with constant DC current during recording.

FIG. 5. Developmental changes in electrophysiological properties of action potential (AP) and AHP. A: Changes in action potential ($a-f$) and AHP ($g$ and $h$) properties. Values of AP threshold and AP peak potential are means ± SE, and other values are medians, with the 25th – 75th percentile range. Statistical differences in AP threshold and AP peak potential between groups were tested using Scheffe and Games-Howell tests, respectively and the differences in other values between groups were tested using Mann-Whitney tests. Horizontal bars above each graph indicate pairs with significant differences. Single and dual asterisks indicate $p < 0.01$ and $p < 0.05$, respectively. B: Representative traces to illustrate action potential shape at different ages. C: Representative traces showing AHPs at different ages. The peak of AHP is indicated by an arrowhead below each trace. D: Peaks of fast (filled arrowhead) and slow (open arrowhead) AHPs in a neuron at P14-20.

FIG. 6. Action potential discharge in response to tonic membrane depolarization. A: action potential spikes evoked by depolarizing current steps (1s duration) in neurons at E16 ($a$) and P20 ($b$). The current intensities are indicated on the upper-left of each trace.
10 and 60 pA depolarizing currents resulted in maximal spike counts in the E16 and P20
neurons, respectively. B: Developmental change in maximal spike count. Values are
medians, with the 25th – 75th percentile range. Horizontal bars above the graph indicate
pairs with significant differences. Single and dual asterisks indicate $p < 0.01$ and $p < 0.05$
(Mann-Whitney test), respectively.

FIG. 7. Post-inhibitory rebound (PIR) and LVA Ca$^{2+}$-spike in a neuron at E18. A:
Membrane depolarizations and action potential spikes (asterisks) induced by PIRs after
membrane hyperpolarizations. Hyperpolarizations were evoked by negative current
injection steps (0 - 30 pA in 5 pA step, 2s duration). B: Termination of hyperpolarizations
of Fig. 7A with expanded time scale. C: Membrane depolarizations induced by PIRs in
the presence of TTX (filled arrowhead). D: Membrane depolarizations in the presence of
NiCl$_2$ and TTX. Transient membrane depolarizations were eliminated by NiCl$_2$ (open
arrowhead). E: Ni$^{2+}$-sensitive spikes (filled arrowhead) computed by subtraction of traces
in Fig. 7D from those in Fig. 7C, indicating LVA Ca$^{2+}$-spikes. F: Expanded voltage and
time scale of traces in Fig. 7E. Intensities of hyperpolarizing currents are indicated on the
right of each trace of the Ca$^{2+}$-spikes. G: Relationship between Ca$^{2+}$-spike amplitudes and
different age groups. H: Relationships of hyperpolarized $V_m$ with Ca$^{2+}$-spike amplitude in
pre- (circles) and postnatal (triangles) neurons. The spike amplitudes were normalized to
the maximum amplitude.

FIG. 8. $I_{K_A}$ in neurons at E16 and P14. A: Transient (TOC) and sustained (SUS) outward
currents at E16 ($a$) and P14 ($b$). The transient outward currents were evoked by
hyperpolarization to -120 mV followed by depolarization to -30 mV. The sustained
outward currents were evoked by depolarization to -30 mV without the hyperpolarizing
pre-pulse. The transient currents were eliminated by 4-AP in the same neurons at E16 (c)
and P14 (d). Dashed lines indicate 0 pA level. B: Incidence of neurons with $I_{KA}$ at
different ages. C: Activation and inactivation curves for $I_{KA}$ at E14/16 (filled and open
circles) and P14-20 (filled and open triangles). Values are means ± SE. D:
Developmental changes in amplitude ($a$) and decay time constant ($b$) of $I_{KA}$. Values are
medians, with the 25th – 75th percentile range. Horizontal bars above each graph indicate
pairs with significant differences. Single and dual asterisks indicate $p < 0.01$ and $p < 0.05$
(Mann-Whitney test), respectively.

FIG. 9. Voltage sag and $I_h$ in an E16 neuron. A: Voltage sag (arrowhead) evoked by
hyperpolarizing current (30 pA) was eliminated by $I_h$ blocker, ZD 7288. B: Current trace
in response to hyperpolarization to -130 mV in control ACSF. C: Current trace in
response to hyperpolarization to -130 mV in the presence of ZD 7288. $I_h$ (difference
between filled and open circles) was eliminated by ZD 7288. D: Incidence of neurons
with $I_h$ at different ages. E: Activation curves for $I_h$ at E14/16 (circles) and P14-20
(triangles). F: Developmental change in amplitude of $I_h$. Values are medians, with the
25th – 75th percentile range. Horizontal bars above the graph indicate pairs with
significant differences. An asterisk indicates $p < 0.05$ (Mann-Whitney test).
A  
TTX (Control)

B
TTX + CdCl₂

C
TTX (Washout)

-55 mV

45, 50, 55, 60 pA

20 pA

0 pA

300 ms

20 mV
Table 1. *Electrophysiological properties of prenatal and early postnatal neurons in rNST.*

<table>
<thead>
<tr>
<th></th>
<th>E14/16</th>
<th>n</th>
<th>E18/20</th>
<th>n</th>
<th>P0-2</th>
<th>n</th>
<th>P6-8</th>
<th>n</th>
<th>P14-20</th>
<th>n</th>
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<tbody>
<tr>
<td>V&lt;sub&gt;rest&lt;/sub&gt; (mV)</td>
<td>-36 ± 2</td>
<td>19</td>
<td>-49 ± 2</td>
<td>18</td>
<td>-52 ± 2</td>
<td>23</td>
<td>-49 ± 2</td>
<td>15</td>
<td>-56 ± 1</td>
<td>36</td>
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<tr>
<td>R&lt;sub&gt;input&lt;/sub&gt; (MΩ)</td>
<td>1986 ± 424</td>
<td>19</td>
<td>1886 ± 163</td>
<td>18</td>
<td>1315 ± 154</td>
<td>23</td>
<td>1036 ± 108</td>
<td>15</td>
<td>742 ± 59</td>
<td>36</td>
</tr>
<tr>
<td>Membrane time constant (ms)</td>
<td>92 ± 14</td>
<td>19</td>
<td>75 ± 6</td>
<td>22</td>
<td>70 ± 9</td>
<td>23</td>
<td>55 ± 5</td>
<td>19</td>
<td>41 ± 3</td>
<td>36</td>
</tr>
<tr>
<td>C&lt;sub&gt;m&lt;/sub&gt; (pF)</td>
<td>31 ± 1</td>
<td>14</td>
<td>-34 ± 1</td>
<td>20</td>
<td>-36 ± 1</td>
<td>25</td>
<td>-38 ± 2</td>
<td>18</td>
<td>-39 ± 1</td>
<td>39</td>
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<tr>
<td>AP height (mV)</td>
<td>18 ± 2</td>
<td>14</td>
<td>34 ± 3</td>
<td>20</td>
<td>43 ± 3</td>
<td>25</td>
<td>50 ± 3</td>
<td>18</td>
<td>61 ± 2</td>
<td>39</td>
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<tr>
<td>AP peak potential (mV)</td>
<td>-12 ± 2</td>
<td>14</td>
<td>-0.4 ± 3</td>
<td>20</td>
<td>7 ± 2</td>
<td>25</td>
<td>12 ± 2</td>
<td>18</td>
<td>22 ± 1</td>
<td>39</td>
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<tr>
<td>AP rising velocity (mV/ms)</td>
<td>8 ± 1</td>
<td>24</td>
<td>19 ± 2</td>
<td>26</td>
<td>34 ± 4</td>
<td>29</td>
<td>44 ± 5</td>
<td>19</td>
<td>82 ± 4</td>
<td>40</td>
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<tr>
<td>AP half-width (ms)</td>
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<td>14</td>
<td>6 ± 1</td>
<td>20</td>
<td>4 ± 0.3</td>
<td>25</td>
<td>3 ± 0.3</td>
<td>18</td>
<td>1.7 ± 0.1</td>
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<td>AHP amplitude (mV)</td>
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<td>7 ± 1</td>
<td>20</td>
<td>12 ± 1</td>
<td>25</td>
<td>15 ± 1</td>
<td>18</td>
<td>15 ± 1</td>
<td>39</td>
</tr>
<tr>
<td>AHP peak time (ms)</td>
<td>20 ± 3</td>
<td>14</td>
<td>14 ± 2</td>
<td>20</td>
<td>16 ± 1</td>
<td>25</td>
<td>16 ± 2</td>
<td>18</td>
<td>6 ± 1</td>
<td>39</td>
</tr>
<tr>
<td>AP discharge rate (/sec)</td>
<td>1 ± 3</td>
<td>35</td>
<td>3 ± 1</td>
<td>27</td>
<td>8 ± 1</td>
<td>30</td>
<td>11 ± 2</td>
<td>20</td>
<td>17 ± 1</td>
<td>39</td>
</tr>
<tr>
<td>I&lt;sub&gt;ka&lt;/sub&gt; amplitude (pA)</td>
<td>77 ± 12</td>
<td>29</td>
<td>143 ± 18</td>
<td>23</td>
<td>182 ± 30</td>
<td>28</td>
<td>242 ± 32</td>
<td>14</td>
<td>247 ± 33</td>
<td>25</td>
</tr>
<tr>
<td>I&lt;sub&gt;ka&lt;/sub&gt; decay time constant (ms)</td>
<td>23 ± 2</td>
<td>14</td>
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<td>17</td>
<td>44 ± 8</td>
<td>20</td>
<td>43 ± 12</td>
<td>11</td>
<td>99 ± 12</td>
<td>19</td>
</tr>
<tr>
<td>I&lt;sub&gt;h&lt;/sub&gt; amplitude (pA)</td>
<td>12 ± 3</td>
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<td>17 ± 2</td>
<td>18</td>
<td>32 ± 5</td>
<td>22</td>
<td>36 ± 7</td>
<td>13</td>
<td>29 ± 4</td>
<td>27</td>
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Values are means ± SE.