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Developmental Nicotine Exposure disrupts dendritic arborization patterns of hypoglossal motoneurons in the neonatal rat

Gregory L. Powell^{1,3}, Joshua Gaddy^{1,3}, Fei Xu, Ralph F. Fregosi^{1,2}, and Richard B. Levine^{1,2,*}

¹Department of Physiology, University of Arizona, Tucson, Arizona

²Department of Neuroscience, University of Arizona, Tucson, Arizona

³Graduate Interdisciplinary Program in Physiological Sciences, University of Arizona, Tucson, Arizona

Abstract

Maternal smoking or use of other products containing nicotine during pregnancy can have significant adverse consequences for respiratory function in neonates. We have shown, in previous studies, that developmental nicotine exposure (DNE) in a model system compromises the normal function of respiratory circuits within the brainstem. The effects of DNE include alterations in the excitability and synaptic interactions of the hypoglossal motoneurons, which innervate muscles of the tongue. This study was undertaken to test the hypothesis that these functional consequences of DNE are accompanied by changes in the dendritic morphology of hypoglossal motoneurons. Hypoglossal motoneurons in brain stem slices were filled with neurobiotin during whole-cell patch clamp recordings and subjected to histological processing to reveal dendrites. Morphometric analysis, including the Sholl method, revealed significant effects of DNE on dendritic branching patterns. In particular, whereas within the first 5 postnatal days there was significant growth of the higher-order dendritic branches of motoneurons from control animals, the growth was compromised in motoneurons from neonates that were subjected to DNE.

Keywords

dendrite; tobacco; motor neuron; gestational; nicotine

INTRODUCTION

Nicotine exposure during embryonic and perinatal development, brought about by maternal use of tobacco or exposure to nicotine in replacement products, can have profound and persistent adverse consequences for respiratory function of human infants and children (Alm *et al.*, 1998; Mitchell & Milerad, 2006; Lavezzi *et al.*, 2010; Zuo *et al.*, 2014; England *et al.*, 2015). In animal models, developmental nicotine exposure (DNE) alters brain development

*corresponding author. Department of Physiology; University of Arizona; Tucson, AZ 85724, RBL4@email.arizona.edu; (520) 444-0970 .

(Muhammad *et al.*, 2012; Aoyama *et al.*, 2015) and compromises respiratory function by causing, among other things, more frequent apneic episodes and relative insensitivity to variations in pH, CO₂ and O₂ (Bamford *et al.*, 1996; Fewell *et al.*, 2001; Hafstrom *et al.*, 2005; Eugenin *et al.*, 2008; Campos *et al.*, 2009; Huang *et al.*, 2010). Respiratory motoneurons, the final output pathway of the respiratory system, display specific physiological consequences of DNE that likely contribute to the compromised respiratory function. Hypoglossal motoneurons, which control respiratory movements of the tongue, display reduced excitability (Pilarski *et al.*, 2011), greater spike frequency adaptation (Powell *et al.*, 2015), differences in the precision and reliability of spike timing (Powell *et al.*, 2015), reduced excitatory synaptic input (Pilarski *et al.*, 2011) and alterations in the efficacy of excitatory and inhibitory synaptic transmission (Jaiswal *et al.*, 2013; Jaiswal *et al.*, 2015) as a consequence of DNE.

DNE disrupts normal dendritic branching in multiple brain regions (Roy *et al.*, 2002; Muhammad *et al.*, 2012; Mychasiuk *et al.*, 2013) and may, therefore, affect the development of neurons in the respiratory circuits of the brainstem. In particular, the disruption of normal physiological properties prompts the hypothesis that DNE also compromises the dendritic development of hypoglossal motoneurons. The goal of the present study was to test this hypothesis as part of a broader effort to identify structural and functional effects of DNE that may contribute to compromised synaptic connectivity in essential respiratory control circuits of the brainstem. Structural and functional consequences of DNE may be related in complex ways. A failure of dendrites to arborize normally may represent both a cause and effect of the physiological changes that result from DNE. For example, the observed reduction in excitatory synaptic inputs (Pilarski *et al.*, 2011) might follow reduced growth of the postsynaptic dendrites (Sin *et al.*, 2002; Aizawa *et al.*, 2004; Hua & Smith, 2004; Niell *et al.*, 2004; Cline & Haas, 2008; Dong *et al.*, 2015). On the other hand, the abnormal activation of nicotinic acetylcholine receptors and excitation resulting from persistent nicotine exposure are likely to disrupt normal dendritic morphology, as both membrane potential and calcium influx have profound influences on dendritic development (Konur & Ghosh, 2005; Spitzer, 2006; Jan & Jan, 2010; Rosenberg & Spitzer, 2011). Clearly, changes in dendritic morphology caused by teratogens or disease, can perturb synaptic connectivity and circuit function (Kulkarni & Firestein, 2012).

To test the hypothesis that DNE disrupts the normal dendritic development of hypoglossal motoneurons, we used intracellular dye introduction followed by detailed quantification of dendritic arborization patterns. Our study focused on the in utero effects of nicotine on neuron morphology in the early neonatal period, the first 5 postnatal days (P0 – P4), which is where the most profound effects of DNE on breathing in rat pups are observed in vivo (Huang *et al.*, 2004; 2010). We found that DNE did not alter basic features of hypoglossal motoneuron dendritic morphology, such as the number of primary dendrites. However there were significant effects of DNE on the pattern of dendritic branching within the neuropil. Interestingly, DNE prevented the expansion of higher-order dendrites that ordinarily occurs during the initial postnatal period.

METHODS

Animals and Treatments

Animal care and procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Pregnant Sprague-Dawley rats were implanted with an osmotic mini-pump (Alzet, Cupertino, CA) subcutaneously on gestational day 5 (Huang *et al.*, 2004; Luo *et al.*, 2004; Luo *et al.*, 2007; Pilarski & Fregosi, 2009; Huang *et al.*, 2010; Pilarski *et al.*, 2011; Jaiswal *et al.*, 2013). Rat mothers and pups were exposed to nicotine bitartrate (6 mg/kg/day) or physiologic saline for 28 days after implantation. After birth the neonates continued to receive nicotine through the mothers' milk.

Staining Protocol

Hypoglossal motoneurons (XIIMNs) were filled with Neurobiotin (Vector Labs, Burlingame, CA) during whole-cell patch clamp recordings in 300 - 400 μ m thick brain stem slices. Slices were prepared from animals at neonatal days P1 through P4 (where P0 indicates the day of birth). With the DNE protocol, levels of nicotine remain elevated over this period (Powell *et al.*, 2015). Recording was performed as described previously (Pilarski *et al.*, 2011; 2012). Cells were visualized with an Olympus BX-50WI fixed-stage microscope (40 water-immersion objective, 0.75 numerical aperture) with infrared and differential interference contrast optics and a video camera (C25400-07, Hamamatsu). Recordings were made using glass pipettes (3–6 MOhms) pulled from thick-walled borosilicate glass capillary tubes (OD: 1.5 mm; ID: 0.75 mm) and filled with the following (in mM): 135 K-gluconate, 4 KCl, 10 HEPES, 5 ATP (Mg2 salt), 0.375 GTP, and 12.5 phosphate creatine, with pH adjusted to 7.2 and osmolarity of 275–300 mosM. The pipette also contained 1% wt/vol Neurobiotin Tracer (Vector Laboratories), which diffused passively into the cell during the 10-20 minute recording. Liquid junction potentials were not corrected. Whole-cell resistance and resting membrane potential were monitored continuously to ensure continued health of the neuron. Ordinarily, one motoneuron was filled per slice, but in a few instances a second cell was filled on the opposite side of the midline.

Visualization of filled XIIMNs was obtained using a histochemical reaction protocol followed by heavy metal intensification (Adams, 1981; de Venecia *et al.*, 1998). Briefly, Neurobiotin filled cells were incubated with a primary goat anti-biotin antibody (1:10,000, Sigma), followed by a secondary biotinylated rabbit antigoat IgG (1:250; Vector Labs, Burlingame, CA). Slices were then incubated with the Elite ABC reagent (Avidin-Biotin Complex; Vector Labs, Burlingame, CA). Staining was revealed with 3,3'-diaminobenzidine (DAB; Sigma) reaction solution that included heavy metals CoCl_2 and $\text{H}_8\text{N}_2\text{NiO}_8\text{S}_2$ (nickel ammonium sulfate). This protocol produced a dark brown reaction product that clearly revealed the cell body and all dendritic processes. Stained medullary slices were mounted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO).

Neuron Tracing

A light microscope (Nikon) and NeuroLucida® software/equipment (MBF Bioscience, Williston, VT) were used to measure stained motoneurons. Images were first obtained with a

MicroFire™ camera (Optronics, Goleta, CA) attached to the microscope. Next, the Neurolucida program was used to trace the outer edges of the medullary slice and edges of the fourth ventricle at 5× magnification. Stained cells were located and marked with a “reference point” so that the cell was easily located as the magnification was increased with a 63× oil emersion lens (Zeiss). After the soma outline was complete the cursor was relabeled as “dendrite”. The cursor was placed on the edge of the soma and the first primary dendrite was traced, with the cursor adjusted according to the thickness of the dendrite. If a primary dendrite branched it was labeled as “bifurcation” or “trifurcation” depending on how many branches were observed. The branches beyond this point were called “secondary” until the next branch point occurred. Once one branch was traced to completion, the cursor would return to the original branch node so that other branches could be traced. This process was repeated for all branch orders. When a branch was traced completely it was labeled as “complete” or “incomplete”. Complete endings had a clear termination point, whereas incomplete endings faded into the tissue or were cut at the surface of the slice. A “Branched Structure Analysis” was performed with Neurolucida Explorer (MBF Bioscience, Williston, VT) to obtain measurements of the number of branches, branch order, branch length and branch surface area. Examples of filled and reconstructed hypoglossal motoneurons for each treatment condition can be seen in Figure 1.

Another method of assessing morphometric parameters, the Sholl Analysis (Sholl, 1953), was also performed in Neurolucida Explorer. This analysis was useful for comparing the distribution of the dendrites in the neuropil as a function of distance from the soma. Concentric spheres, centered at the cell body, were placed around the neuron. The spheres were equidistant (10 µm) from one another, starting with a 5 µm origin sphere at the soma. Within each concentric sphere the dendritic length, surface area, and number of intersections were measured. For dendritic length, each branch was categorized by order.

Statistical Analysis

Parameters quantified in the Branched Structure Analysis included the number, total length and surface area of complete primary dendrites, as well as the lengths of higher order branches. As branches were sometimes incomplete due to fading of the stain in distal processes or truncation during slice preparation, only complete processes were included in the analysis. Measured cells were separated into two age groups, postnatal day 1 and 2 (P1+P2), and P3+P4, to assess postnatal dendritic growth. A 2-way ANOVA was used, with age (P1+P2 and P3+P4) and treatment (Control and DNE) as the two main factors, and the relevant morphometric properties as the dependent variables.

Dendritic length, surface area, and the number of intersections at particular radial distances from the soma, as measured in the Sholl Analysis, were analyzed using 3 or 4 factor, first-order autoregressive ANOVAs. The first-order autoregressive analysis assumes that radii that are next to one another are closely related and radii further away are unrelated, i.e. 35µm is closely related to 25 and 45µm, whereas 200µm is unrelated. Branches beyond 345µm were excluded due to the large number of incomplete branches at this distance. We first used a 3-factor mixed-model ANOVA, with treatment, age and distance from the soma (radius) as the main factors. This model yields four interaction tests: treatment × age, treatment × distance,

age $\times$ distance and treatment $\times$ age $\times$ distance (Table 2). Secondly, a first-order autoregressive, mixed model 4-factor ANOVA was used to analyze how each of the factors mentioned above (age, treatment, distance) interacted with branch order to influence dendritic length. This model yields 6 two-way interaction tests: treatment $\times$ age, treatment $\times$ distance, treatment $\times$ branch order, age $\times$ distance, age $\times$ branch order and distance $\times$ branch order (Table 3).

RESULTS

Branched Structure Analysis of XIIMNs

Figure 1 shows micrographs, at one focal plane, of neurobiotin filled hypoglossal motoneurons from a control (A) and DNE animal (C). The corresponding Neurolucida tracings are shown in Panels B and D. The number of primary dendrites (i.e. dendrites emerging directly from the soma before the first branch point), primary dendrite length and primary dendrite surface area were quantified in the Branched Structure Analysis program in Neurolucida. There were no significant treatment or age effects on these morphological parameters, but note that in control animals the total length and surface area of primary dendrites increased with age (P1+P2 vs. P3+P4), but these values decreased with age in DNE motoneurons (Table 1). We also assessed the effects of DNE on the length of all complete branches of orders 1 – 6 by considering all dendritic branches independently from their neuron of origin. Figure 2 shows the length distribution of complete dendrites of each branch order in control and DNE groups for the two age groups. When considered in this manner, independently of their neuron of origin or location within the neuropil, there were no significant differences between groups.

Sholl Analysis

An alternative approach to morphometric analysis of dendritic branching, the Sholl Analysis, provides an interesting perspective on dendrite distribution within the neuropil and the mode of dendritic development. Figure 3 shows a XIIMN with concentric rings radiating from its soma. Each ring represents a sphere separated by 10 μ m to capture dendritic morphology in 3-dimensional space. Due to the large number of incomplete dendrites further from the soma, distance was restricted to 345 μ m in our analysis. A 3-factor ANOVA was used to compare dendritic morphological properties against 3 factors: age, treatment, and distance from the soma (Table 2). Note that there was a significant overall effect of distance from the soma for all three parameters examined (number of dendritic intersections, dendrite length and dendrite surface area).

For dendritic intersections, there was a significant interaction between treatment and age. Note, in particular, that the number of dendritic intersections at a given distance from the soma increased with age in control animals (P1+P2 vs. P3+P4), whereas this age-dependent increase did not occur in motoneurons from DNE animals (Table 2; Figure 4). Similarly, there was a significant interaction between treatment and age for dendritic length (Table 2; Fig. 5). Again, the dendritic length at multiple Sholl radii increased with age in motoneurons from control but not DNE pups. Thus, unlike stage P1+P2, at age P3+P4 dendritic length at a range of distances from the soma was greater in control cells than DNE cells. As might be

expected given these results, a significant treatment $\times$ age interaction was also revealed for dendritic surface area (Table 2; Fig. 6). There was an increase in surface area over a range of distances as the animal reached postnatal days 3 and 4 in control motoneurons, but not in DNE motoneurons. Interestingly, there was actually a clear decrease in surface area over a range of distances from the soma in DNE animals.

To gain additional insight into the pattern of dendritic growth and branching, we analyzed dendritic length at distinct branch orders (1- 6), as a function of distance from the soma, using a 4-factor ANOVA. There were overall effects of treatment, distance and branch order (Table 3), and multiple 2-way interactions were observed. Specifically, distance, treatment and age interacted significantly with branch order (Table 3). Thus, dendritic length depends on both distance from the soma and branch order, but distance and branch order are interdependent. Note that dendrite length falls with distance in branch orders 1-3, but not in branches 4-6 (Figs 7 & 8).

The interdependence of branch order and postnatal age can also be observed in Figures 7 and 8. Note, for example, that in younger neonates (P1+P2) the average length of primary dendrites (branch order 1) was similar to that in older neonates (P3+P4), whereas there were clear age differences in the average length of third order dendrites (Figs 7 & 8). Similarly, the significant treatment $\times$ branch order interaction (Table 3), is revealed by the similarity in branch lengths between DNE and control dendrites in branch order 1, but the clear difference between treatment groups in branch order 3 (Figs 7 & 8). Treatment did not have a significant influence on the change in dendrite length as a function of distance from the soma, and there were no significant 3 or 4-way interactions.

Electrophysiologic Parameters

Despite the significant effects of age and treatment on higher order dendritic branching parameters, measurements of electrophysiologic characteristics did not reveal significant differences in resting membrane potential, input resistance or spike threshold (Table 4). Similar results have been reported previously (Powell et. al., 2015).

DISCUSSION

This study was initiated to test the hypothesis that DNE would perturb the normal pattern of dendritic development in hypoglossal motoneurons. Although previous studies have shown clearly that nicotine exposure during development has significant consequences for dendritic branching in multiple brain regions (Muhammad *et al.*, 2012; Mychasiuk *et al.*, 2013), the importance of the hypoglossal motoneurons in respiration warranted a detailed examination of these neurons. DNE did not alter the basic features of hypoglossal motoneuron dendritic branching, including the number, length and surface area of primary dendrites. Nor did it significantly affect the lengths of higher order branches, when considered independently of their neuron of origin. This basic level of analysis, however, does not provide substantial information about how branches are oriented and distributed within the neuropil of the hypoglossal motor nucleus. Nor does it consider branch location relative to other dendrites or the soma of the same neuron. Such information is critical for eventual understanding of how the dendritic branching of hypoglossal motoneurons is related to the locations, within

the XII nucleus, of synaptic inputs from specific sources. Similarly, from a computational standpoint, it is essential to know where these synaptic inputs are located relative to the spike initiation site and other dendrites of the motoneuron. For example, although the average length of third-order dendritic branches was not affected by DNE (Figure 2), changes in the location of these branches within the neuropil or relative to the soma (Figs 7 & 8) will influence synaptic integration.

The Sholl analysis, which has been widely used for many years to describe dendritic arborization (Sholl, 1953; O'Neill *et al.*, 2015), provides useful information about how dendritic branches are distributed within the neuropil relative to some reference point, in our case the cell body of the motoneuron. Thus, to again use third-order branches as an example, the Sholl analysis can reveal whether DNE influences the length of these dendritic components at specific points relative to the cell body. This method of analysis is particularly illuminating since dendritic branches may bend or curve in three dimensions in certain regions of the neuropil rather than grow straight through them. This type of information may, therefore, be particularly illuminating in understanding how teratogens, such as nicotine, can affect how and where dendrites elongate and branch during development.

Indeed the Sholl analysis revealed that DNE had a significant effect on the pattern of dendritic branching within the neuropil, and how the patterns changed over the first 5 postnatal days (P0-P4). Whereas the number of intersections, length and surface area of dendrites over a range of distances from the motoneuron cell body increased over the first 5 postnatal days in control animals, these parameters remained constant or actually decreased in motoneurons from DNE animals. Moreover, the changes were particularly evident in higher order branches. Although the average length of third-order branches was similar in the two treatment groups in the younger neonates, in the older neonates the average length of these branches was greater, over a range of distances from the cell body, in control than in DNE motoneurons. Thus, nicotine exposure may reduce the growth of certain dendrites, and perhaps cause abnormal regression or pruning.

With the experimental model that we have employed, the developing fetus is exposed during embryonic development to levels of nicotine approximating those observed in a moderate to heavy smoker (Powell *et al.*, 2015). These elevated levels of nicotine are maintained through ingestion of the maternal milk at least until age P5 (Powell *et al.*, 2015). As a potent nicotinic acetylcholine receptor (nAChR) agonist, nicotine depolarizes hypoglossal motoneurons and can evoke high levels of action potential activity (Chamberlin *et al.*, 2002; Lamanauskas & Nistri, 2006; Shao & Feldman, 2009; Pilarski *et al.*, 2012). In addition, DNE leads to persistent desensitization of nAChRs (Gentry & Lukas, 2002; Pilarski *et al.*, 2012). Both clearly have deleterious consequences for normal respiratory function and are likely to perturb dendritic development by changing neuronal activity levels and synaptic transmission. Maintenance of the normal balance of excitatory and inhibitory synaptic inputs is critical for normal development and function of the respiratory system (Berger, 2011; Gao *et al.*, 2011).

There are numerous potential mechanisms whereby DNE may be affecting the dendritic development of hypoglossal motoneurons. It is clear, for example, that depolarization and altered levels of neuronal activity can directly influence normal dendritic arborization, including the formation of new dendritic branches, the growth of existing dendrites and the pruning or regression of dendrites (Dong *et al.*, 2015). In many cases, this can be linked to alterations in calcium influx or buffering by internal stores (Konur & Ghosh, 2005; Rosenberg & Spitzer, 2011), which can influence cytoskeletal elements and the function of transcription factors (Jan & Jan, 2010; Dong *et al.*, 2015). In addition, nicotine activates glutamatergic interneurons that provide excitatory synaptic input to hypoglossal motoneurons, both through its agonistic effect on nAChRs in the pre-Botzinger complex and by modulating presynaptic terminals within the XII motor nucleus (Shao & Feldman, 2009). Thus, DNE may change the level of synaptic drive experienced by the hypoglossal motoneurons during development. In addition, DNE reduces the number of excitatory synaptic events recorded in neonatal hypoglossal motoneurons (Pilarski *et al.*, 2011). Both would alter the activity of the motoneuron and could influence dendritic growth through mechanisms mediated by contact or the release of diffusible morphogens and growth factors (Kuczewski *et al.*, 2009; Wong *et al.*, 2015). Furthermore, it is clear that early cholinergic transmission is essential for the normal development of the cortex, among other brain regions (Slotkin, 2008; Dwyer *et al.*, 2009; Muhammad *et al.*, 2012). By desensitizing nAChRs, DNE may disrupt these influences. Of course, since in our model the whole animal is exposed to nicotine, the mechanisms responsible for altered dendritic arborization could be rather indirect. For example, nicotine may alter the release of growth factors or hormones from distant sites. Although DNE has only minimal effects on the overall size of neonates (Huang *et al.*, 2004; Huang *et al.*, 2010), there may be tissue or neuron-specific effects.

The alteration, by DNE, of the normal pattern of dendritic growth during the first 5 postnatal days, can be viewed in the context of previous information about the postnatal development of hypoglossal motoneuron dendrites. In our study, DNE prevented normal elongation and perhaps caused regression of branches, particularly at higher orders. Indeed, there is normally an overall increase in the total dendritic length of hypoglossal motoneuron dendrites over the initial postnatal weeks, but there is also a distinct phase of pruning during which some higher-order branches are lost while remaining branches increase in length (Nunez-Abades *et al.*, 1994; Nunez-Abades & Cameron, 1995; Carrascal *et al.*, 2005; Greer & Funk, 2005). These changes take place over a longer time course than the 4 days that we have examined in this study, but it is possible that DNE alters the timing and/or magnitude of growth and regressive events.

Regardless of the underlying mechanisms, the alterations of dendritic morphology are likely to have functional consequences at cell, circuit and systems levels. Changes in dendritic surface area could alter the number and location of synaptic contacts, as well as synaptic integration. In addition, changes in the nature of dendritic branching (i.e. the lengths of branches of particular orders at different distances relative to the cell body) will alter the efficacy of synaptic inputs, as those more distant from the cell body or with more intervening branch points may have less influence on the timing and number of action potentials produced by the motoneuron. The effects of DNE and age on the more distal dendritic branches, such as those reported here, are unlikely to affect the basic

electrophysiologic characteristics that are measured from the cell body. Consistent with previous reports (Powell et al., 2015) we did not detect effects on input resistance, resting potential or spike threshold. It is important to recognize, however, that such findings do not preclude localized effects on the passive membrane properties of distal dendrites, such as membrane resistance and length constant, or voltage-sensitive conductances in these distal dendrites that would be difficult to measure with an electrode placed on the cell body but have significant consequences for synaptic transmission.

These findings raise a number of interesting questions for further analysis. In particular, it will be important to determine directly whether the number, locations or types of synaptic inputs to hypoglossal motoneurons are affected by DNE. We know, for example, that DNE increases the density of GABA_A receptors (Jaiswal et al., 2015) and reduces the density of glutamate receptor subunits 2 and 3 (Jaiswal et al., 2013) in the hypoglossal motor nucleus. Both effects are consistent with changes in the efficacy of these major inhibitory and excitatory neurotransmitters due to DNE (Jaiswal et al., 2013; 2015; Luo et al., 2004, 2007; Pilarski and Fregosi, 2009). Given that the efficacy of nicotinic cholinergic agonists is reduced by DNE (Pilarski et al., 2012), it will be important to determine whether the density or subtype of AchRs is affected in the hypoglossal motor nucleus. Importantly, there may be a relationship between such changes in the expression of ligand-gated ion channels and alterations in dendritic branching. On a related point, it will be important in further analysis to determine the effects of DNE on the number and location of postsynaptic dendritic spines and the relationship to the sites of synaptic inputs from different sources.

It will also be important to determine whether the changes we have described are persistent. Although the neonates continued to experience nicotine exposure through the mother's milk over the 4 postnatal days that we examined, we do not know whether arborization patterns (or motoneuron function) will recover over the course of days and weeks after weaning, and how the time course of dendritic growth in DNE animals will compare with that described in normal postnatal development (Carrascal et al., 2005). In this context it is also interesting to speculate on the potential impact of nicotine withdrawal.

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Figure 1. Filled and traced control and DNE hypoglossal motoneurons
(A) An example of a control motoneuron that was filled with Neurobiotin (magnification 40×), underwent DAB staining, and (B) traced with NeuroLucida system. (C) A filled and (D) reconstructed DNE motoneuron. Scale bars represent 25 μm.

Figure 2. Complete dendrite length as a function of branch order

This graph shows the distribution of total length for each complete (un-truncated) dendrite in branch order 1 – 6, independent of the motoneuron that the dendrite came from. The two age groups (P1+P2) and (P3+P4) are graphed separately. Branch order is represented on the x axis; “1” = order 1 dendrites, “2” = order 2 dendrites; etc. For each order there is a column for control dendrites followed by a column for DNE dendrites; filled circles represent control dendrites and open squares represent branches from DNE animals. The black bar represents the median of each treatment.

Figure 3. Diagram of the Sholl Analysis

(A) Fourth ventricle; (B) Neurolucida tracing of one of the motoneurons in this study. The tracing is shown with a black cell body, colored dendrites (colors indicate distinct dendrite trees; six dendrites are shown), and black axon (exiting diagonally at the bottom of the rings); (C) Rings represent the spheres that radiate from the cell body, separated from one another by 10 μm .

Figure 4. The number of dendritic intersections does not increase with age in motoneurons from DNE preparations, unlike control

The number of dendritic intersections as a function of distance from the soma. The data were separated into two age groups: P1+P2 and P3+P4. Note that there was a significant treatment $\times$ age interaction. The number of intersections increased across a range of distances as the animal aged in motoneurons from control animals (filled black circles) but not in DNE (open squares). There was also a significant overall effect of distance from the soma. Note that the number of intersections peaked around 50 µm and fell as the distance from the soma increased. See Table 2.

Figure 5. Unlike the dendrites of control motoneurons, those from cells in DNE preparations do not increase in length with age

Dendritic length as a function of distance from the soma. There were no significant overall effects of treatment or age alone on dendritic length (see Table 2). However, there was a significant 2-way interaction between treatment and age, where control dendritic length increased with age across a range of distances, but not dendrites from DNE animals. Again, there was a significant overall effect of distance. See Table 2.

Figure 6. The dendritic surface area of motoneurons from DNE preparations does not increase with age, unlike control

Dendritic surface area as a function of distance from the soma. There were no overall treatment or age effects on the dendritic surface area, although there was a significant interaction between treatment and age. As with intersections and length, control dendrites across a range of distances from the soma gained more dendritic surface area as they aged. By contrast, dendrites from motoneurons that were subjected to DNE did not increase in surface area but actually showed a reduction at distances ranging approximately from 50 to

150 microns from the soma. As with intersections and length, there was a significant overall effect of distance from the soma. See Table 2.

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Figure 7. Whereas dendritic length increased with age in certain branch orders in motoneurons from control preparations, this did not occur for DNE motoneurons

Mean dendritic length as a function of distance from the soma for branch orders 1-3. Each branch order is plotted separately at two age groups. For statistical evaluation see Table 3. Age, treatment and distance interacted significantly with branch order. Note, in particular, that the lengths at some branch orders (e.g. orders 3-5; see also figure 8) increased between ages P1+P2 and P3+P4 for dendrites from control subjects unlike those from DNE motoneurons.

Figure 8. Mean dendritic length as a function of distance from the soma for branch orders 4-6
 Same coordinates as Figure 7 but for branch orders 4-6. See Figure 7 and Table 3 for further explanation and information.

Table 1
The effects of DNE on XIIMN primary dendrites

Developmental nicotine exposure had no treatment or age effects. Values represent the mean $\pm$ the standard error of the mean (SEM). P-values were obtained using a 2-way ANOVA, with $P = 0.05$ considered statistically significant.

	P1+P2		P3+P4		P-Value	N (Control/DNE)
	Control	DNE	Control	DNE		
Number of Primary dendrites per neuron	5.2 $\pm$ 0.4	5.8 $\pm$ 0.4	4.8 $\pm$ 0.3	5.4 $\pm$ 0.4	$P > 0.05$	35/37
Complete primary total dendritic length (μm)	187.4 $\pm$ 31.1	254.6 $\pm$ 43.9	201.4 $\pm$ 45.0	163.1 $\pm$ 31.4	$P > 0.05$	35/35
Complete primary total dendritic surface area (μm^2)	707.5 $\pm$ 118.9	1214.6 $\pm$ 228.9	830.0 $\pm$ 158.0	778.8 $\pm$ 150.7	$P > 0.05$	35/35

Table 2
Results of 3-factor ANOVA of the Sholl analysis

The main factors are age, treatment and distance from the soma. Two-way interactions between treatment and age, treatment and distance from the soma and age and distance from the soma are also given. We also examine the more complex three-way interaction between age, distance from the soma and treatment. In all cases, each cell of the table displays the degrees of freedom (df), F value (F), and P value (P), displayed as (df, F, P). Significant comparisons are *bold and italicized*.

	Treatment	Age	Distance From Soma	Treatment × Age	Treatment × Distance	Age × Distance	Treatment × Age × Distance
Dendrite intersections	(1, 3.14, 0.079)	(1, 0.35, 0.56)	(33, 24.5, <0.0001)	(1, 6.8, 0.01)	(33, 0.95, 0.56)	(33, 0.98, 0.5)	(33, 0.87, 0.67)
Dendrite length	(1, 3, 0.086)	(1, 0.7, 0.4)	(33, 24.4, <0.0001)	(1, 6.1, 0.015)	(33, 1.1, 0.27)	(33, 0.87, 0.68)	(33, 1.07, 0.37)
Dendrite surface area	(1, 0.03, 0.96)	(1, 1, 0.3)	(33, 26.3, <0.0001)	(1, 8.4, 0.004)	(33, 1, 0.46)	(33, 0.48, 0.96)	(33, 0.87, 0.67)

Table 3
The effects of DNE at different branch orders

Results of ANOVA for Sholl analysis of dendrite length, as a function of treatment, age, distance from the soma and branch order. For each main factor and interaction, degrees of freedom (df), F value (F), and P value are provided. Significant comparisons are ***bold and italicized***.

	df	F	p
• <i>Treatment</i>	<i>1</i>	<i>8.4</i>	<i>0.004</i>
• Age	1	1.32	0.250
• <i>Distance from Soma</i>	<i>33</i>	<i>3.76</i>	<i><0.0001</i>
• <i>Branch Order</i>	<i>5</i>	<i>3.12</i>	<i>0.008</i>
Interactions			
• Treatment × Age	1	1.0	0.31
• Treatment × Distance	33	0.74	0.87
• <i>Treatment × Branch Order</i>	<i>5</i>	<i>4.95</i>	<i><0.0001</i>
• Age × Distance	33	0.36	1.0
• <i>Age × Branch Order</i>	<i>5</i>	<i>3.90</i>	<i>0.002</i>
• <i>Distance × Branch Order</i>	<i>151</i>	<i>2.2</i>	<i><0.0001</i>

Table 4
Select electrophysiologic data by age and treatment group

Values are mean $\pm$ SEM (N). There were no significant effects of treatment, age or interactions.

Age	P1 + P2		P3 + P4	
Treatment Group	Control	DNE	Control	DNE
Resting membrane potential (mV)	-59.8 ± 1.6 (30)	-58.9 ± 2.7 (19)	-57.1 ± 4.3 (9)	-57.9 ± 2.8 (16)
Input resistance (MOhms)	209.3 ± 23.8 (30)	249.7 ± 35.4 (19)	191.4 ± 43.7 (9)	209.7 ± 42.3 (15)
Spike threshold (mV)	-44.9 ± 1.4 (30)	-44.5 ± 2.2 (19)	-46.2 ± 4.5 (9)	-41.8 ± 2.6 (16)