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Effect of Nicotine on Cerebellar Granule Neuron Development

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Abstract

To assess the role of nicotinic cholinergic receptors (nAChR) on neuronal maturation, nAChR expression and the direct effects of nAChR activation were examined in cerebellar external granular layer (EGL) precursors isolated *in vitro*. Treatment of EGL neuroblasts with nicotine elicited a concentration-dependent increase in DNA content and synthesis, implying an increase in cell numbers. Pretreatment of cultures with the nAChR antagonist dihydro- β -erythroidine (DHBE) attenuated nicotine-induced changes in DNA abundance and synthesis. Furthermore, chronic nicotine treatment for 4–7 days promoted EGL cell survival. Epibatidine but not cytisine stimulated granule neuroblast DNA synthesis and survival. Survival effects mediated by nicotine and epibatidine were attenuated by pre-treating cultures with DHBE. Immunocytochemical analysis revealed that EGL neurons possessed $\alpha 3$, but not $\alpha 4$, nAChR immunoreactivity. Quantitative autoradiography was used to determine which nAChRs are present during the period of granule cell neurogenesis *in vivo*. On postnatal day 5, the EGL was intensely labeled by [³H]-epibatidine but virtually devoid of [³H]-A85380 binding suggesting that a high concentration of $\alpha 3$ nAChRs is present in granule neuroblasts. The pharmacology of [³H]-epibatidine displacement from EGL neurons also suggested an interaction with the $\alpha 3$ -nAChR subunits. Together these data provide novel evidence that the activation of nAChRs directly affect the development of primary cerebellar neuroblasts and further suggest that the effects are mediated through the $\alpha 3$ -nAChR subtype.

Keywords

neuroblast proliferation; cerebellar development; neurogenesis; programmed cell death; nicotinic receptors; $\alpha 3$ nicotinic receptor subunits

Introduction

Accumulating evidence suggests that the cholinergic system is important during cerebellar development. Levels of choline acetyltransferase, the enzyme that catalyzes acetylcholine

synthesis, are particularly high in cerebellum during the early postnatal period in humans (Brooksbank *et al.*, 1978; Court *et al.*, 1993) and rats (Gould & Butcher, 1987; Clos *et al.*, 1989). During this period of development, choline acetyltransferase levels are relatively higher than levels of the degradative enzyme for acetylcholine, acetylcholinesterase (Boegman *et al.*, 1988; Clos *et al.*, 1989; Court *et al.*, 1993). A developmental role for cholinergic agonists is further strengthened by several reports indicating that both muscarinic and nicotinic (nAChR) cholinergic receptor subtypes are present in perinatal periods in rats (Zoli *et al.*, 1995; Tice *et al.*, 1996; Winzer-Serhan & Leslie, 1997) and humans (Kinney *et al.*, 1993; Court *et al.*, 1995). In particular, previous studies have demonstrated nAChRs in granule and Purkinje neurons in rats (Zoli *et al.*, 1995; Dominguez *et al.*, 1997; Morley, 1997) and in the external granular layer (EGL), the location of granule neuron precursors, in humans (Court *et al.*, 1995). Multiple nAChR subtypes, including $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$, have been detected in cultured rat granule neurons (Didier *et al.*, 1995). Granule neurons are the targets for cholinergic mossy fiber innervation from the dorsal pontine brainstem nuclei occurring later in development (Jaarsma *et al.*, 1997), suggesting that acetylcholine may participate in synaptogenesis and neuromodulation (Role & Berg, 1996). Moreover, recent evidence suggests that choline itself, which is abundant in developing brain, as well as other ligands, may act as an agonist for $\alpha 7$ nAChRs and as a partial agonist for $\alpha 3$ nAChRs (Alkondon *et al.*, 1997; Albuquerque *et al.*, 1997a; 1997b). Together, these findings suggest that the cholinergic system may play an important role in the mediation of trophic events throughout cerebellar development, particularly in the maturation of granule neurons.

The cerebellum is an ideal system to explore potential developmental roles for nAChR because a considerable amount of cerebellar development, in both rats and mice, occurs during the first three postnatal weeks (Miale & Sidman, 1961; Altman, 1972a). In addition, cerebellar cytoarchitecture has been extensively characterized in both rats (Altman, 1972a) and mice (Miale & Sidman, 1961). Neuroblasts migrate from the rhombic lip and assume a position on the cerebellar surface to form the EGL (Altman & Bayer, 1978), which exclusively contains granule neuron progenitors (Zhang *et al.*, 1996). In the rodent cerebellum, the EGL consists of an outer proliferative zone, with peak rates of neuroblast division occurring between postnatal days 5–8, and an inner post-mitotic premigratory zone (Altman, 1972a; Miale & Sidman, 1961). By postnatal day 21, the EGL has disappeared with cells having migrated into the internal granule cell layer (Altman, 1972b). This developmental program corresponds to the cerebellar changes seen during the late fetal period though the first 1.5 years in humans (Bayer *et al.*, 1993). The presence of nAChRs in the EGL suggests that nicotinic agonists directly affect granule neuron precursors (Court *et al.*, 1995).

Although previous evidence implies that nicotine can alter cerebellar development (McFarland *et al.*, 1991; Slotkin *et al.*, 1993; Slotkin, 1998), the direct effects of nicotinic agonists on the genesis of isolated granule neuron precursors have not been evaluated. Furthermore, it remains unclear which nAChR subtypes reside in the mouse EGL. In the present study, the effects of nicotine treatment on granule neuroblast cell turnover were examined in an *in vitro* model of the mouse EGL. The findings of this study suggest that nAChR activation influences granule neuron maturation during the early postnatal period.

Materials and Methods

Materials

Tissue culture media, sera, and the supplements B27 and N2 were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Percoll was obtained from Pharmacia (Piscataway, NJ, USA). Cytisine, epibatidine, and dihydro- β -erythroidine (DHBE) were purchased from RBI (Natick, MA, USA). Alpha 3 and 4 nAChR subtype-specific antibodies were bought from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). The CyQuant and Live/Dead assay kits were purchased from Molecular Probes (Eugene, OR, USA). Nicotine and all other chemicals were bought from Sigma Chemical Co. (St. Louis, MO, USA).

Granule neuroblast culture

Granule neuronal precursors were purified from the EGL of 5 day-old ICR mouse cerebella according to procedures described by Hatten and coworkers (Hatten, 1985; Gao *et al.*, 1991). Cerebella were dissected and digested with a mixture of trypsin and DNase in Ca^{2+} - Mg^{2+} -free Tyrode's solution to produce a cell suspension. The dissociated cells were layered and centrifuged over a 35%: 60% Percoll step gradient. The granule cell fraction was preplated onto poly-D-lysine (0.1 mg/ml) substrate three times for 30 minutes each to remove contaminating glia. Nonadherent cells (granule neuroblasts) were resuspended in basal minimal essential medium containing glutamine (292 $\mu\text{g}/\text{ml}$), glucose (9 mg/ml), donor horse serum (10%) and fetal bovine (5%) serum and allowed to reaggregate into clusters in 96-well plates (3×10^5 cells/well) for 24 hours (34–35°C in 5% CO_2 /95% air). The characterization of these cultures by morphological and immunocytochemical criteria, as previously described (Gao *et al.*, 1991), indicated that >95% of the cells were granule neurons and/or precursors. In all experiments, the effects of nicotinic agents were compared in cultures containing identical cell densities (3×10^5 /well). Immediately after plating, cells were continuously exposed to nicotinic agonists and/or the antagonist DHBE for ~1 day (21 or 24 hours), or 2, 5, or 7 days.

Thymidine incorporation

Thymidine incorporation into DNA was measured by procedures similar to those previously described (Gao *et al.*, 1991; Tao *et al.*, 1997; DiCicco-Bloom *et al.*, 1993; Opanashuk & Hauser, 1998). Reaggregate cell clusters were plated in serum-free medium at 3×10^5 cells/well in 96-well plates. After 24 hours in serum-free medium, [^3H]-thymidine (2 $\mu\text{Ci}/\text{ml}$) was added continuously for 21 hours in the presence or absence of nAChR agonists and/or DHBE, a competitive nAChR-antagonist. DHBE was added for 15 minutes prior to the addition of nicotine. Cell clusters were harvested onto filter paper with trichloroacetic acid washes and repeated water elution using a Skatron cell harvester as previously described (Opanashuk & Hauser, 1998). After the filters were dried, thymidine labeling was measured by liquid scintillation counting. At least four independent determinations from separate cell preparations were evaluated for each treatment.

Measurement of DNA content

Cells were grown as described for the [³H]-thymidine incorporation studies and treated with drugs for 48 hours. Total DNA content of EGL neurons was assessed using the CyQuant DNA assay according to the procedure provided by the manufacturer as previously described (Opanashuk & Hauser, 1998). Plates were frozen at -80°C until assayed. For determination of total DNA content, cells were thawed and resuspended in a detergent-based lysis buffer containing 1 mM EDTA and 1 µg/ml DNase-free RNase. After 1 hour, the CyQuant reagent, a green fluorescent dye that binds to nucleic acids, was added for 5 minutes and fluorescence measured using a Cytofluor 2300 microplate reader (Millipore, Bedford, MA, USA) with 480 nm excitation and 520 nm emission filters. The amount of DNA in each culture was extrapolated by using linear regression with bacteriophage DNA as a standard. At least 4 independent determinations from separate cell preparations were made for each treatment.

Cell Survival Assay

Reaggregate cell clusters were plated onto poly-L-lysine-coated glass coverslips (3×10^5 cells/coverslip/culture) for 2 hours to permit cell adhesion to the substrate. Cells were subsequently grown in serum free medium for 24 hours before continuous exposure to nAChR agonists and/or DHBE for an additional 1–7 days. Cultures were rinsed and incubated in Dulbecco's phosphate buffered saline containing 4.0 µM ethidium homodimer and 3.5 µM calcein-AM at 34°C in 5% CO₂/95% air. After 30 minutes, coverslips with attached cells and cell clusters were removed from culture plates, mounted on microscope slides using Prolong Antifade (Molecular Probes, Eugene, OR, USA), and counted within 24 hours. Cells were counted using a Nikon Diaphot fluorescence microscope with a long working-distance 100x-oil immersion objective and a 485 nm excitation filter. The emission filters were 530 nm for calcein and 590 nm for the ethidium ion. Live cells were distinguished by their intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent calcein-AM to calcein, which is fluorescent green in live cells. Ethidium is excluded by an intact plasma membrane, and enters cells with damaged membranes, binds to nucleic acids and fluoresces bright red. To arbitrarily sample cells, the microscope stage controller was moved along a line through the center of the coverslip and all cell clusters within the field were sampled. For each value, a random sample of 1000 cells was counted. At least 4 independent determinations from separate cell preparations were evaluated for each treatment.

Immunocytochemistry

After a 24-hour reaggregation period, EGL neuroblasts were plated onto poly-L-lysine-coated glass coverslips (3×10^5 cells/coverslip/culture) and given 2 hours to adhere. The media was subsequently removed and cells were rinsed 3 times with Dulbecco's minimal essential medium then maintained in serum free medium, comprised of Dulbecco's minimal essential medium with glutamine (292 µg/ml), glucose (9 mg/ml), B27, and N2. After 24 hours in serum free medium, EGL neuronal clusters were fixed in 100% ethanol containing 5% acetic acid for 15 minutes at 4°C then rinsed 3 times with phosphate buffered saline, pH 7.2. Fixed cells were incubated in phosphate buffered saline (pH 7.2) containing 1.0 %

bovine serum albumin, 0.1% Triton X-100, and 10% rabbit serum for 30 minutes at room temperature. Cell clusters were then incubated with the appropriate dilution of primary antibodies (2 µg/ml) in phosphate buffered saline (pH 7.2) containing BSA and Triton-X-100 for 24 hours at 4°C for 24 hours on an orbital shaker. After 3 rinses with phosphate buffered saline (pH 7.2) cells were incubated with the rhodamine-conjugated rabbit anti-goat antibodies for one hour. After several rinses, coverslips were mounted on to glass slides in Prolong Antifade mountant (Molecular Probes). For peptide neutralization experiments, the primary antibodies were incubated with a five-fold excess of peptide antigen for 2 hours at room temperature prior to accomplishing the remainder of the immunocytochemical procedure. Controls lacking primary antiserum were used to determine the specificity of the reaction.

Receptor autoradiography

Receptor autoradiography was used to evaluate the expression of cholinergic receptor subtypes in the developing mouse cerebellum. Nicotinic binding was compared on adjacent tissue sections from P5 mice. Brain sections (16 µm thick) were sliced using a Leica CM1850 cryostat and thaw-mounted on sections coated with gelatin, chromium potassium sulfate and poly-L-lysine to increase tissue adherence. All binding assays were performed in parallel. [³H]-epibatidine autoradiography was performed as described by Perry and Kellar (1995), with minor modifications. Sections were incubated for 40 minutes in buffer containing 50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 2.5 mM CaCl₂ (pH 7.0) and 200 pM [³H]-epibatidine (DuPont/New England Nuclear, Boston, USA; specific activity = 59.7 Ci/mmol). The sections were then washed twice in ice-cold buffer (5 minutes each), once in diluted ice-cold buffer (10 sec) and once in ice-cold deionized water (10 sec). Identical binding conditions were used for ³H-A85380 autoradiography (specific activity = 69.5 Ci/mmol). The sections were then washed 3 times in 50 mM Tris-HCl (4 min each; 4°C), once in diluted Tris-HCl (10 sec; 4°C) and once in deionized water (10 sec; 4°C).

Following binding, the sections were gently dried with ambient airflow from a desktop fan, desiccated overnight under a vacuum at room temperature and then exposed to Amersham Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 5 weeks. Films were processed in Kodak (Kodak, Rochester, NY, USA) D-19 developer (5 min), stop bath (30 sec) and Kodak rapid fixer (5 min). After the films were developed, the sections were stained with thionin so that the autoradiographic images could be directly compared to the corresponding histological section.

Results

Nicotine increases granule neuroblast DNA synthesis and content

Nicotine caused significant increases in DNA content in EGL cultures at 48 hours (Fig. 1A, B) that were preceded by increased rates of [³H]-thymidine incorporation at 21 hours (Fig. 1C, D). As noted, [³H]-thymidine incorporation was used to estimate the proportion of dividing neuroblasts, while DNA content was used to estimate EGL cell numbers (all non-dividing diploid cells contain the same amount of DNA). DNA content was assessed at 48 h

following nicotine treatment to allow sufficient time for changes in DNA synthesis (determined at 24 h) to be manifest as actual changes in cell numbers, as previously determined using other drugs (Hauser *et al.*, 2000). The effect of nicotine on DNA synthesis was concentration-dependent (Fig. 1C) and markedly suppressed by pretreating cultures with DHBE at (Fig. 1D). Similarly, the effect of nicotine on DNA content was concentration-dependent (Fig. 1A) and significantly attenuated by DHBE (Fig. 1B) at the later (48-hour) time point. Maximal increases in thymidine incorporation and/or DNA content were observed following 1 μ M nicotine and were approximately 1.5-fold higher than untreated controls. Considering that the number of cells was identical in each culture at the start of each experiment and that nicotinic agonists did not significantly affect the rate of cell death at 24–48 hours, the drug-induced increases in granule neuroblast DNA synthesis and content could result from increases in cell proliferation.

Epibatidine but not cytosine stimulates DNA synthesis in cultured EGL neuroblasts

To elucidate the selectivity of the effect of nicotine on DNA synthesis, thymidine incorporation was evaluated in EGL cells treated with the nicotinic agonists epibatidine or cytosine. Treatment of EGL cells with epibatidine stimulated a concentration-dependent increase in DNA synthesis (Fig. 2A). Significant increases in thymidine incorporation were observed following 1 pM epibatidine. Furthermore, the nAChR antagonist DHBE (Fig. 2B) attenuated the increases in DNA synthesis following epibatidine treatment. In contrast, treatment with cytosine failed to elicit significant changes in DNA synthesis (Fig 2A) even at 100 nM concentrations (data not shown). Baseline [3 H]-thymidine counts were $2.9 \times 10^3 \pm 878$ cpm and $1.6 \times 10^3 \pm 364$ (for epibatidine A and B, respectively) and $2.4 \times 10^3 \pm 1103$ (for cytosine) in untreated controls.

Nicotine promotes granule neuroblast cell survival

To evaluate whether the alterations in DNA synthesis and content could be attributed to altered rates of cell survival, EGL cell viability was assessed following nicotine treatment. As a control for the viability assay, EGL cells were lysed with 0.1% saponin, resulting in 100% cell death (data not shown). The live/dead markers did not overlap in the same cell and all cells were labeled as either living or dead. In general, there was a low percentage of EGL cell death 24 hours after nicotine was added, between 5–8%, in control cultures (Fig. 3). After 4 days, approximately 20 % of untreated EGL cells were dead; cell survival was slightly enhanced in response to continuous nicotine treatment (Fig. 3). Larger and more significant effects on EGL neuroblast survival were observed following 7 days of nicotine exposure. Because the greatest nicotine response on cell survival was observed 7 days following treatment, all subsequent experiments were assessed on day 7. Nicotine effects on granule neuroblast survival at 7 days were completely reversed by DHBE (Fig 3B). Furthermore, DHBE alone had no effect on cell survival.

Epibatidine but not cytosine is a survival factor for EGL neuroblasts in vitro

Continuous treatment with epibatidine simulated a concentration-dependent increase in EGL neuroblast survival, compared to untreated controls, at 7 days in vitro (Fig. 4 A). The epibatidine-mediated increases in granule neuroblast survival were attenuated by DHBE (Fig

4B). In contrast, treatment with cytosine had no effect on EGL cell survival (Fig. 4A) even at concentrations up to 100 nM (data not shown).

EGL neuronal precursors display $\alpha 3$ nAChR subunit immunoreactivity and binding

EGL neuronal precursors displayed $\alpha 3$ nAChR subunit immunoreactivity (Figs. 5A, C), which was not evident in controls lacking primary antiserum (Fig. 5B) or in preabsorbed controls (5D). In contrast, $\alpha 4$ nAChR immunoreactivity was not seen in the reaggregate cultures. Alpha 3 immunoreactivity was typically diffuse and associated with neuronal cell bodies and neurites, but not the nucleus.

Pseudocolor enhanced photomicrographs depicting nicotinic acetylcholine receptors in the mouse brainstem labeled by [3 H]-epibatidine (200 pM) and [3 H]-A85380 binding (200 pM) are shown in Figures 5F and 5G, respectively. In most regions of the mouse brainstem, the binding of these two radioligands was nearly identical. For example, prominent binding of both radioligands was identified in the superior colliculus (SC), inferior colliculus (IC), medial vestibular nucleus (MVN) and the deep nuclei of the cerebellum. Epibatidine binding was also present in the EGL of the cerebellum (Figure 5F, arrows); in contrast, there was no detectable binding of [3 H]-A85380 in the EGL (Figure 5G). The discrepancy between [3 H]-epibatidine and [3 H]-A85380 binding in the EGL may indicate that epibatidine binds to multiple nAChR subtypes in developing mouse brain, similar to what has been reported in adult rodent brain. The pharmacological profile of [3 H]-epibatidine binding in the mouse cerebellum was also evaluated in the present study. Sets of adjacent sections were incubated in either 200 pM [3 H]-epibatidine alone (Figure 5H) or with 200 pM [3 H]-epibatidine plus 50 nM unlabeled cytosine (Figure 5I). The addition of cytosine to the incubation buffer reduced [3 H]-epibatidine binding to the level of film background in all brain regions except the inferior colliculus, the medial vestibular nucleus and the EGL of the cerebellum. This result supports the notion the [3 H]-epibatidine binds to multiple nicotinic receptor subtypes in the developing mouse brain and that the EGL expresses a receptor with a unique pharmacological profile.

Discussion

Neurotransmitters can act as signals to influence neuronal maturation and can differentially regulate cellular proliferation, migration, survival and differentiation (Lipton & Kater, 1989; Schwartz, 1992; Hauser & Mangoura, 1998; Hauser *et al.*, 2000). The abundance of cholinergic synthetic enzymes and receptors in the postnatal cerebellum (Brooksbank *et al.*, 1978; Court *et al.*, 1993; 1995) is consistent with the notion that acetylcholine is important during postnatal cerebellar development. Granule neurons contain both muscarinic (Aloso *et al.*, 1990) and nicotinic receptors (Didier *et al.*, 1995), and receive cholinergic innervation from mossy fiber subpopulations (Jaarsma *et al.*, 1997). Periods of peak developmental change in granule neurons (Miale & Sidman, 1961; Altman, 1972a) coincide with the maturation of cholinergic systems during the first 3 postnatal weeks in rodents (Brooksbank *et al.*, 1978; Court *et al.*, 1993; 1995; Jaarsma *et al.*, 1997).

The results of cell growth, immunohistochemical, receptor autoradiography and pharmacological displacement experiments in the present study provide some information

concerning the nAChR phenotype expressed in EGL neurons. We speculate that $\alpha 3$ nAChR subunits are involved with nicotine-induced proliferation and survival of EGL neuronal precursors. This is based immunohistochemical evidence that $\alpha 3$, but not $\alpha 4$ nAChR's are expressed in cultured EGL cells (Fig. 5A–E) and radioligand binding showing robust and specific binding of [^3H]-epibatidine in the EGL, but negligible binding of [^3H]-A85380 in the EGL in vivo. Previous autoradiographic binding studies have concluded that [^3H]-epibatidine binds with high affinity to both $\alpha 3$ and $\alpha 4$ receptor subunits, whereas other radioligands such as [^3H]-nicotine and [^3H]-cytisine only identify receptors that express $\alpha 4$ subunits (Marks *et al.*, 1998; Perry & Kellar, 1995; Flores *et al.*, 1996). Alternatively, A-85380 reportedly has high affinity for the $\alpha 4$ nAChR, but not $\alpha 3$ receptors (Pauly *et al.*, 1996). Findings that epibatidine, but not cytisine, affect neuronal growth and survival further support this notion.

$\alpha 3$ nicotinic receptor binding in the developing cerebellum has not been reported in the mouse. In the developing rat cerebellum, $\alpha 3$ messenger RNA is expressed in the early postnatal EGL using in situ hybridization techniques (Zoli *et al.*, 1995; Morley, 1997). In the adult rat cerebellum, $\alpha 3$ mRNA expression is largely restricted to the Purkinje cell layer (Wada *et al.*, 1989). Our findings indicate that $\alpha 3$ nAChR subtypes are expressed by immature EGL neurons in the mouse and by isolated EGL neurons in vitro. Interestingly, in addition to the $\alpha 3$ variant, granule neurons appear to express other type nAChR subtypes. In rats, $\alpha 7$ nAChR mRNA is expressed by Purkinje cells and the deep cerebellar nuclei (Dominguez *et al.*, 1997). In adult granule neurons, $\alpha 7$ -subunit immunoreactivity has a limited pattern of expression and is associated with dendrites but not the cell body (Caruncho *et al.*, 1997). We saw little $\alpha 7$ binding ([^{125}I]-BTX) binding associated with the EGL on postnatal day 5 (data not shown). Thus, although the present study strongly implicates the $\alpha 3$ nAChR in EGL neuroblast proliferation, other nAChR subtypes may also be present and influence development.

Heterologous expression studies demonstrate that $\alpha 3$ nAChRs exhibit some unique pharmacological properties that may influence development. Chronic exposure to nicotinic agonists causes a dose dependent increase in the density of brain $\alpha 4$ nAChRs, possibly as a result of desensitization or permanent inactivation (Pauly *et al.*, 1991; Schwartz & Kellar, 1983; Collins & Marks, 1996; Changeux *et al.*, 1998). However, $\alpha 3$ receptors respond differently to chronic agonist treatment in terms of receptor number compared to other nAChR isoforms (Olale, *et al.*, 1997). Alpha 3 nAChRs are also more resistant than $\alpha 4$ subunits in terms of upregulation following agonist exposure (Flores *et al.*, 1997; Peng *et al.*, 1997). Why $\alpha 3$ receptors fail to desensitize like other subtypes is not known but repeated exposure to mainstream (or side-stream) cigarette smoke may have unique properties in neuronal populations expressing the $\alpha 3$ phenotype.

The effects of nicotine on development are complex, as nAChR activation appears to either stimulate or inhibit cell proliferation depending on the target cell type and experimental paradigm used. For instance, in many in vitro studies, nAChR activation can increase cell proliferation, e.g., in a thymic cell line, lung cell carcinoma, subsets of normal and neoplastic cervical cells, neuroendocrine and vascular smooth muscle cells (Tominaga *et al.*, 1989; Maneckjee & Minna, 1990; Quik *et al.*, 1994; Strohschneider *et al.*, 1994; Waggoner &

Wang, 1994; Carty *et al.*, 1997). In contrast, several studies report that nicotine administration decreases rat brain DNA synthesis in several regions including the cerebellum (Navarro *et al.*, 1989; McFarland *et al.*, 1991; Slotkin *et al.*, 1994), although one recent report suggests that increased numbers of mitotic neural cells are present in rat embryos exposed to nicotine *in vitro* (Roy *et al.*, 1998). Studies *in vivo* have shown that lesions of forebrain cholinergic neurons reduce the thickness of cortical lamina, and decrease neuronal cell body size and dendritic field size in the developing neocortex (Hohmann *et al.*, 1988; Robertson *et al.*, 1998), suggesting that cholinergic input is trophic. Furthermore, nAChRs reportedly regulate the migration and differentiation of developing retinal ganglion neurons (Wong, 1995).

The complexity of nAChR subtypes and rich diversity of developmental actions among different species, ages, and brain regions indicates that nicotinic agonists convey highly selective and individualized information depending on the particular cell type and brain region that is targeted. In addition, the pharmacodynamics of nicotine administration and particular experimental paradigms used (e.g., *in vivo* versus *in vitro*) may affect outcome. Moreover, nAChR activation has potent systemic effects on the cardiovascular, respiratory, and endocrine systems that are likely to indirectly influence neurogenesis. For these reasons, it was important to define the intrinsic effects of nicotine on granule neuron maturation. Alternatively, because granule neurons do not normally develop in isolation, other factors (e.g., interactions with other cell types or non-nicotinic trophic factors) are likely to modify the intrinsic response of granule neurons to nAChR activation. For example, isolated EGL neurons stop proliferating when astroglia are present (Gao *et al.*, 1991) and no longer respond to morphine when epidermal growth factor-like ligands are present (Opanashuk & Hauser, 1998). Thus, we feel that caution should be used when comparing our results to other studies because of significant differences in the developmental models utilized. It is premature to speculate whether granule neurons might respond similarly to nAChR activation *in vivo*, or whether the untimely exposure to nicotine during maturation might influence cerebellar development. Lastly, our data indicate that EGL cells predominantly express an $\alpha 3$ nAChR phenotype. The effects of nicotine on cell proliferation and survival may be exclusive to cells that express this receptor subtype. The mixed populations of cells *in vivo* may express alternative nAChR subunit types ($\alpha 4$ and $\alpha 7$) that may mask/overshadow the $\alpha 3$ mediated-decreases in DNA synthesis seen in an isolated population of neurons.

Granule neuron death occurs during normal cerebellar development *in vivo* (Wood *et al.*, 1993) and in EGL cell cultures (Gao *et al.*, 1991). It has previously been reported that nicotine prevents apoptosis of immune cells (Aoshiba *et al.*, 1996) and lung cancer cells (Maneckjee & Minna, 1994; Heusch & Maneckjee, 1998), and is a survival factor for spinal motor neurons (Messi *et al.*, 1997). Alternatively, in other systems nicotine can promote cell death, e.g., in embryonic rat brains (Roy *et al.*, 1998) and at high concentrations in vascular cells (Villablanca, 1998). Like its effects on cell replication, it is likely that nicotine's apoptotic actions are highly individualized based on cell type and developmental stage. We found that nicotine did not alter EGL neuroblast survival at early time-points *in vitro*, when the range of percentages of dying cells in untreated control cultures varied between 5–8%. However, as the rate of cell death increased in more mature cultures, significant effects of

nicotine on survival became evident. Together the cell proliferation and survival data suggest that nicotine is initially mitogenic and later serves as a survival factor when EGL cells are dying at a higher rate. Interestingly, Yan and coworkers (1995) reported that acetylcholine prevented apoptosis in cultured granule neurons via an interaction with muscarinic AChRs. It is conceivable that activation of both nAChR and muscarinic AChRs regulate the maturation of cerebellar granule neurons, as has been suggested in retina (Wong, 1995). Last, there are numerous examples in which nicotine is neurotoxic. For example, at high concentrations (1, 10 or 100 μ M), nicotine is neurotoxic to the brains of whole rat embryos (Roy *et al.*, 1998) and causes apoptosis in cultured hippocampal neurons (Berger *et al.*, 1998). Nicotine neurotoxicity may differ depending on the particular nAChR subtype affected. Hippocampal neurotoxicity appears to be preferentially associated with the presence of $\alpha 7$ nAChR subtypes, which permit large inward Ca^{2+} currents (Berger *et al.*, 1998).

The mechanisms by which nAChR activation modulates granule neuron maturation are not understood. Recent reports suggest that nicotine can regulate the synthesis and/or degradation of trophic factors, including PDGF, TNF α , and TGF β , each of which can affect cellular growth (Rakowicz-Szulczynska, *et al.*, 1994; 1996). Nicotine stimulates the production and secretion of basic fibroblast growth factor in smooth muscle cells (Carty *et al.*, 1996; Cucina *et al.*, 1999). Although similar scenarios involving trophic factors could be proposed for cholinergic systems during cerebellar development see Tao *et al.*, 1997), our findings suggest that nicotinic agonists intrinsically affect the development of isolated neuroblasts in the absence of many of the extracellular signals normally present. For example, the proliferative effects of nicotine could be related to the high Ca^{2+} permeability of nAChRs (Wong, 1995; Role & Berg, 1996; Berger *et al.*, 1998). Intracellular Ca^{2+} signals regulate cell entry into and exit from mitosis (Reddy, 1994). Cells deprived of Ca^{2+} cannot progress through the G₁/S phase of the cell cycle (Means & Rasmussen, 1988; Reddy, 1994). Although the specific mechanisms underlying trophic events following nAChR activation require further evaluation, this study provides evidence for a direct cholinergic role in neurogenesis.

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Abbreviations

AChR	acetylcholinergic receptor
BrdU	5'-bromodeoxyuridine
DAB	diaminobenzidine
DHBE	dihydro- β -erythroidine
EGL	external granular layer
G₁	pre-DNA synthesis phase of the cell cycle

IC	inferior colliculus
MVN	medial vestibular nucleus
nAChR	nicotinic cholinergic receptor
PDGF	platelet-derived growth factor
PBS	phosphate buffered saline
S-phase	DNA synthesis phase of the cell cycle
SC	superior colliculus
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α

References

- Albuquerque EX, Alkondon M, Pereira EF, Castro NG, Schrattenholz A, Barbosa CT, Bonfante-Cabarcas R, Aracava Y, Eisenberg HM, Maelicke A. Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. *J Pharmacol Exp Ther.* 1997a; 280:1117–1136. [PubMed: 9067295]
- Albuquerque EX, Pereira EF, Alkondon M, Schrattenholz A, Maelicke A. Nicotinic acetylcholine receptors on hippocampal neurons: distribution on the neuronal surface and modulation of receptor activity. *J Recept Signal Transduct Res.* 1997b; 17:243–266. [PubMed: 9029494]
- Alkondon M, Pereira EF, Cortes WS, Maelicke A, Albuquerque EX. Choline is a selective agonist of $\alpha 7$ nicotinic acetylcholine receptors in the rat brain neurons. *Eur J Neurosci.* 1997; 9:2734–2742. [PubMed: 9517478]
- Aloso R, Didier M, Soubrie P. [^3H]-methylscopolamine binding studies reveal M2 and M3 muscarinic subtypes on cerebellar granule cells in primary culture. *J Neurochem.* 1990; 55:334–337. [PubMed: 2355226]
- Altman J. Postnatal development of the cerebellar cortex in rat. I The external germinal layer and the transitional molecular layer. *J Comp Neurol.* 1972a; 145:353–398. [PubMed: 4113154]
- Altman J. Postnatal development of the cerebellar cortex in rat. III Maturation of the components of the granule layer. *J Comp Neurol.* 1972b; 145:465–514. [PubMed: 4114591]
- Altman J, Bayer SA. Prenatal development of the cerebellar cortex in rat. I Cytogenesis and histogenesis of the deep nuclei and the cortex of cerebellum. *J Comp Neurol.* 1978; 179:23–48. [PubMed: 8980716]
- Aoshiba K, Nagai A, Ysui S, Konno K. Nicotine prolongs neutrophil survival by suppression of apoptosis. *J Lab Clin Med.* 1996; 127:186–194. [PubMed: 8636647]
- Bayer SA, Altman J, Russo RJ, Zhang X. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicol.* 1993; 14:83–144.
- Berger F, Gage FH, Vijayaraghavan S. Nicotinic receptor-induced apoptotic cell death of hippocampal progenitor cells. *J Neurosci.* 1998; 18:6871–6881. [PubMed: 9712657]
- Boegman RJ, Parent A, Hawkes R. Zonation in rat cerebellar cortex: patches of high acetylcholinesterase activity in the granular layer are congruent with Purkinje cell compartments. *Brain Res.* 1988; 448:237–251. [PubMed: 3259899]
- Brooksbank BWL, Martinez M, Atkinson DJ, Balazs R. Biochemical development of the human brain. I Some parameters of the cholinergic system. *Dev Neurosci.* 1978; 1:267–284.
- Carty CS, Soloway PD, Kayastha S, Bauer J, Marsan B, Ricotta JJ, Dryjski M. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. *J Vascular Surg.* 1996; 24:927–935.

- Caruncho HJ, Guidotti A, Lindstrom J, Costa E, Pesold C. Subcellular localization of the $\alpha 7$ nicotinic receptor in rat cerebellar granule cell layer. *Neuroreport*. 1997; 8:1431–1433. [PubMed: 9172148]
- Changeux J-P, Bertrand D, Corringer JP, Dehaene S, Edelstein S, Lena C, Le Novère N, Marubio L, Picciotto M, Zoli M. Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. *Brain Res Rev*. 1998; 26:198–216. [PubMed: 9651527]
- Clos J, Ghandour S, Eberhart R, Vincendon G, Gombos G. The cholinergic system in developing cerebellum: comparative study of normal, hypothyroid, and underfed rats. *Dev Neurosci*. 1989; 11:188–204. [PubMed: 2766963]
- Collins AC, Marks MJ. Are nicotinic receptors activated or inhibited following chronic nicotine treatment? *Drug Dev Res*. 1996; 38:231–242.
- Court JA, Perry EK, Johnson M, Piggott MA, Kerwin JA, Perry RH, Ince PG. Regional patterns of cholinergic and glutamate activity in the developing and aging human brain. *Dev Brain Res*. 1993; 74:73–82. [PubMed: 8104741]
- Court JA, Perry EK, Spurdin D, Griffiths M, Kerwin JM, Morris CM, Johnson M, Oakley AE, Birdsall NJM, Clementi F, Perry RH. The role of the cholinergic system in the development of the human cerebellum. *Dev Brain Res*. 1995; 90:159–167. [PubMed: 8719339]
- Cucina A, Corvino V, Sapienza P, Borrelli V, Lucarelli M, Scarpa S, Strom R, Santoro-D'Angelo L, Cavallaro A. Nicotine regulates basic fibroblastic growth factor and transforming growth factor beta1 production in endothelial cells. *Biochem Biophys Res Commun*. 1999; 257:306–312. [PubMed: 10198208]
- Didier M, Berman SA, Lindstrom J, Bursztajn S. Characterization of nicotinic receptors expressed in primary cultures of cerebellar granule cells. *Mol Brain Res*. 1995; 30:17–28. [PubMed: 7609640]
- DiCicco-Bloom E, Friedman WJ, Black IB. NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. *Neuron*. 1993; 11:1101–1111. [PubMed: 7903858]
- Dominguez del Toro E, Juiz JM, Smillie FI, Lindstrom J, Criado M. Expression of $\alpha 7$ neuronal nicotinic receptors during postnatal development of the rat cerebellum. *Dev Brain Res*. 1997; 98:125–133. [PubMed: 9027411]
- Flores CM, Davila-Garcia MI, Ulrich YM, Kellar KJ. Differential regulation of neuronal nicotinic receptor binding sites following chronic nicotine exposure. *J Neurochem*. 1997; 69:2216–2219. [PubMed: 9349569]
- Flores CM, DeCamp RD, Kilo S, Rogers SW, Hargreaves KM. Neuronal nicotinic receptor expression in sensory neurons of the rat trigeminal ganglion: demonstration of $\alpha 3/\alpha 4$, a novel subtypes in the mammalian nervous system. *J Neurosci*. 1996; 16:7894–7901.
- Gao WQ, Heintz N, Hatten ME. Cerebellar granule cell neurogenesis is regulated by cell-cell interactions in vitro. *Neuron*. 1991; 6:705–715. [PubMed: 2025426]
- Gould E, Butcher LL. Transient expression of choline acetyl transferase-like immunoreactivity in Purkinje cells of the developing rat cerebellum. *Dev Brain Res*. 1987; 34:303–306.
- Hatten ME. Neuronal regulation of astroglial morphology and proliferation in vitro. *J Cell Biol*. 1985; 100:384–396. [PubMed: 3881455]
- Hauser KF, Houdi AA, Turbek CS, Elde RP, Maxson W III. Opioids intrinsically inhibit the genesis of mouse cerebellar granule cell precursors *in vitro*: Differential impact of μ and δ receptor activation on proliferation and neurite elongation. *Eur J Neurosci*. 2000; 12:1281–1293. [PubMed: 10762357]
- Hauser KF, Mangoura D. Diversity of the endogenous opioid system in development: novel signal transduction translates multiple extracellular signals into neural cell growth and differentiation. *Perspect Dev Neurobiol*. 1998; 5:337–449. [PubMed: 10533524]
- Heusch WL, Maneckjee R. Signalling pathways involved in nicotine regulation of apoptosis of human lung cancer cells. *Carcinogenesis*. 1998; 19:551–556. [PubMed: 9600337]
- Hohmann CF, Brooks AR, Coyle JT. Neonatal lesions of the basal forebrain cholinergic neurons result in abnormal cortical development. *Brain Res*. 1988; 470:253–264. [PubMed: 3219585]
- Jaarsma D, Ruigrok TJH, Caffè R, Cozzari C, Levey AI, Mugnani E, Voogd J. Cholinergic innervation and receptors in the cerebellum. *Prog Brain Res*. 1997; 114:67–96. [PubMed: 9193139]
- Kinney HC, O'Donnell TJ, Kriger P, White WF. Early developmental changes in [3 H]nicotine binding in the human brainstem. *Neuroscience*. 1993; 55:1127–1138. [PubMed: 8232902]

- Lipton SA, Kater SB. Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends in Neurosci.* 1989; 12:265–270.
- Maneckjee R, Minna JD. Opioid and nicotine receptors affect growth regulation of human lung cancer cell lines. *Proc Natl Acad Sci USA.* 1990; 87:3294–3298. [PubMed: 2159143]
- Maneckjee R, Minna JD. Opioids induce while nicotine suppresses apoptosis in human lung cancer cells. *Cell Growth Differ.* 1994; 5:1033–1040. [PubMed: 7848904]
- McFarland BJ, Seidler FJ, Slotkin TA. Inhibition of DNA synthesis in neonatal rat brain regions caused by acute nicotine administration. *Dev Brain Res.* 1991; 58:223–229. [PubMed: 2029766]
- Marks MJ, Smith KW, Collins AC. Differential agonist inhibition identifies multiple epibatidine binding sites in mouse brain. *J Pharmacol Exp Ther.* 1998; 285:377–386. [PubMed: 9536034]
- Miale IL, Sidman RL. An autoradiographic analysis of histiogenesis in the mouse cerebellum. *Exp Neurol.* 1961; 4:277–296. [PubMed: 14473282]
- Means AR, Rasmussen CD. Calcium, calmodulin and cell proliferation. *Cell Calcium.* 1988; 9:313–319. [PubMed: 3224371]
- Messi ML, Renanathan M, Grigorenko E, Delbono O. Activation of $\alpha 7$ nicotinic acetylcholine receptors promotes survival of spinal cord motoneurons. *FEBS Lett.* 1997; 411:32–38. [PubMed: 9247137]
- Morley BJ. The embryonic and postnatal expression of the nicotinic alpha 3-subunit in lower brainstem. *Mol Brain Res.* 1997; 48:407–412. [PubMed: 9332738]
- Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, Slotkin TA. Effects of prenatal nicotine exposure in development of central and peripheral cholinergic neurotransmitter systems: Evidence for cholinergic trophic influences in developing brain. *J Pharmacol Exp Ther.* 1989; 251:894–900. [PubMed: 2600820]
- Navarro HA, Seidler FJ, Schwartz RD, Baker FE, Dobbins SS, Slotkin TA. Prenatal exposure to nicotine impairs nervous system development at a dose which does not affect viability or growth. *Brain Res Bull.* 1989; 23:187–192. [PubMed: 2819477]
- Olale F, Gerzanich V, Kuryatov A, Wang F, Lindstrom J. Chronic nicotine exposure differentially affects the function of human $\alpha 3$, $\alpha 4$, and $\alpha 7$ neuronal nicotinic receptor subtypes. *J Pharm Exp Ther.* 1997; 283:675–683.
- Opanashuk LA, Hauser KF. Opposing actions of the EGF family and opioids: heparin binding-epidermal growth factor (HB-EGF) protects mouse cerebellar neuroblasts against the antiproliferative effect of morphine. *Brain Res.* 1998; 804:87–94. [PubMed: 9729296]
- Pauly JR, Anderson DJ, Sullivan JP. Autoradiographic comparison of [3 H]-A85380, [3 H]-epibatidine and [3 H]-cytisine binding in rat and mouse brain. *Soc Neurosci Abst.* 1996; 22:1264.
- Pauly JR, Marks MJ, Gross SD, Collins AC. An autoradiographic analysis of cholinergic receptors in mouse brain after chronic nicotine treatment. *J Pharmacol Exp Ther.* 1991; 258:1127–36. [PubMed: 1890617]
- Peng X, Gerzanich V, Anand R, Wang F, Lindstrom J. Chronic nicotine treatment up-regulates $\alpha 3$ and $\alpha 7$ acetylcholine receptor subtypes expressed by the human neuroblastoma cell line SH-SY5Y. *Mol Pharmacol.* 1997; 51:776–784.
- Perry DC, Kellar KJ. [3 H]-Epibatidine labels nicotinic receptors in rat brain: an autoradiographic study. *J Pharmacol Exp Ther.* 1995; 275:1030–1034. [PubMed: 7473129]
- Quik M, Chan M, Patrick J. α -Bungarotoxin blocks the nicotinic receptor mediated increase in cell number in a neuroendocrine cell line. *Brain Res.* 1994; 655:161–167. [PubMed: 7812768]
- Rakowicz-Szulczynska EM, McIntosh DG, Smith ML. Growth factor-mediated mechanisms of nicotine-dependent carcinogenesis. *Carcinogenesis.* 1994; 15:1839–1846. [PubMed: 7923576]
- Rakowicz-Szulczynska EM, McIntosh DG, Perry M, Smith ML. PDGF AA as a mediator in nicotine-dependent carcinogenesis. *Carcinogenesis.* 1996; 17:1813–1818. [PubMed: 8824500]
- Reddy GP. Cell cycle: regulatory events in G1→S transition of mammalian cells. *J Cell Biochem.* 1994; 54:379–386. [PubMed: 8014186]
- Robertson RT, Gallardo KA, Claytor KJ, Ha DH, Ku KH, Yu BP, Lauterborn JC, Wiley RG, Yu J, Gall CM, Leslie FM. Neonatal treatment with 192 IgG-saporin produces long-term forebrain cholinergic deficits and reduces dendritic branching and spine density of neocortical pyramidal neurons. *Cereb Cortex.* 1998; 8:142–155. [PubMed: 9542893]

- Role LW, Berg DK. Nicotinic receptors in the development and modulation of CNS synapses. *Neuron*. 1996; 16:1077–1085. [PubMed: 8663984]
- Roy TS, Andrews JE, Seidler FJ, Slotkin TA. Nicotine evokes cell death in embryonic rat brain during neurulation. *J Pharmacol Exp Ther*. 1998; 287:1136–1144. [PubMed: 9864303]
- Schwartz JP. Neurotransmitters as neurotrophic factors: A new set of functions. *International Rev Neurobiol*. 1992; 34:1–23.
- Schwartz RD, Kellar KJ. Nicotinic cholinergic receptor binding sites in the brain: regulation in vivo. *Science*. 1983; 220:214–216. [PubMed: 6828889]
- Slotkin TA. Fetal nicotine or cocaine exposure: which is worse? *J Pharmacol Exp Ther*. 1998; 285:931–945. [PubMed: 9618392]
- Slotkin TA, Lappi SE, Seidler FJ. Impact of fetal nicotine exposure on development of rat brain regions: critical sensitive periods or effects of withdrawal? *Brain Res Bull*. 1993; 31:319–328. [PubMed: 8490731]
- Strohschneider T, Oberhoff M, Hanke H, Hannekum A, Karsch KR. Effect of chronic nicotine delivery on the proliferation rate of endothelial and smooth muscle cells in experimentally induced vascular wall plaques. *Clin Investig*. 1994; 72:908–912.
- Tao Y, Black IB, DiCicco-Bloom E. In vivo neurogenesis is inhibited by neutralizing antibodies to basic fibroblast growth factor. *J Neurobiol*. 1997; 33:289–296. [PubMed: 9298766]
- Tice MAB, Hashemi T, Taylor LA, McQuade RD. Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. *Dev Brain Res*. 1996; 92:70–76. [PubMed: 8861724]
- Tominaga K, Kinoshita Y, Hato F, Masuda A, Matsuyama M. Effects of cholinergic agonists on the proliferation and protein synthesis in a cultured thymic epithelial cell line. *Cell Mol Biol*. 1989; 35:679–686. [PubMed: 2627706]
- Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Appl Physiol*. 1998; 84:2089–2098. [PubMed: 9609804]
- Waggoner SE, Wang X. Effect of nicotine on proliferation of normal, malignant, and human papillomavirus-transformed human cervical cells. *Gynecol Oncol*. 1994; 55:91–95. [PubMed: 7959275]
- Wada EK, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol*. 1989; 284:314–335. [PubMed: 2754038]
- Winzer-Serhan UH, Leslie FM. Codistribution of nicotinic acetylcholine receptor subunit $\alpha 3$ and $\beta 4$ mRNAs during rat brain development. *J Comp Neurol*. 1997; 386:540–554. [PubMed: 9378850]
- Wong RO. Cholinergic regulation of $[Ca^{2+}]_i$ during cell division and differentiation in the mammalian retina. *J Neurosci*. 1995; 15:2696–2706. [PubMed: 7722623]
- Wood KA, DiPasquale B, Youle RJ. In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron*. 1993; 11:621–632. [PubMed: 8398151]
- Yan G-M, Lin S-Z, Irwin RP, Paul SP. Activation of muscarinic cholinergic receptors blocks apoptosis of cultured cerebellar granule neurons. *J Pharm Exp Ther*. 1995; 47:248–257.
- Zhang L, Goldman JE. Generation of cerebellar interneurons from dividing precursors in white matter. *Neuron*. 1996; 16:47–54. [PubMed: 8562089]
- Zoli M, Le Novère Hill JA, Changeux JP. Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous system. *J Neurosci*. 1995; 15:1912–1939. [PubMed: 7891142]

Figure 1.

Effect of nicotine on DNA content (A and B) and [³H]-thymidine incorporation into DNA (C and D) in external granule layer (EGL) neuroblasts. (A) Nicotine treatment stimulated a significant increase in DNA levels after 48 hours. The effect of nicotine on DNA levels was (A) concentration-dependent and (B) antagonized by the nicotinic acetylcholine receptor (nAChR) antagonist DHBE (10 μM). Results are expressed as percent of control where DNA content in control cultures was 945 ± 242 ng/well. (C) Treatment of EGL cells with nicotine (nic) for 21 hours resulted in a modest but significant elevation in [³H]-thymidine incorporation into DNA. Increases in [³H]-thymidine incorporation were (C) concentration-dependent and (D) attenuated by pretreatment with the nAChR antagonist DHBE (10 μM). Results are expressed as percent of control defined as thymidine incorporation in serum-free media alone. In controls, there were $2.3 \times 10^3 \pm 162$ c.p.m. Data represent the mean $\pm$ SEM from at least 4 separate experiments and were analyzed by ANOVA with Newman-Keuls post hoc analysis, ^{*} $P < 0.05$ vs. untreated cultures, ^b $P < 0.025$ vs. untreated cultures, [#] $P < 0.05$ vs. nicotine -treated and untreated cultures, ^a $P < 0.05$ vs. nicotine -treated cultures.

Figure 2.

Effect of nicotinic acetylcholine receptor (nAChR) agonists on EGL neuroblast DNA synthesis. (A) Treatment of EGL cells with epibatidine (epi) but not cytosine for 21 hours resulted in elevated rates of [^3H]-thymidine incorporation into DNA. Increases in [^3H]-thymidine incorporation were (A) concentration-dependent and (B) attenuated by pretreatment with DHBE (10 μM). Results are expressed as percent of control defined as thymidine incorporation in serum-free media alone. Data represent the mean $\pm$ SEM from at least 4 separate experiments and were analyzed by ANOVA with Newman-Keuls post hoc analysis, * $P < 0.02$ vs. untreated cultures, # $P < 0.005$ vs. nicotine -treated cultures.

Figure 3.

Effect of nicotine on EGL cell survival. (A) Chronic nicotine (nic) (1 μ M) treatment for 4–7 days results in enhanced granule neuroblast survival. (B) Nicotine -mediated survival effects at 7 days were reversed by pretreatment with DHBE. Data represent the mean $\pm$ SEM from at least 4 independent experiments and were analyzed by ANOVA with Duncan's post hoc analysis, * $P < 0.05$ vs. untreated cultures, # $P < 0.05$ vs. nicotine.

Figure 4. Effect of nicotinic acetylcholine receptor (nAChR) agonists on EGL cell survival. (A) Epibatidine, but not cytisine, treatment for 7 days displays a concentration-dependent increase in granule neuroblast survival. (B) Epibatidine (10 pM) -mediated survival effects at 7 days were reversed by pretreatment with DHBE. Data represent the mean $\pm$ SEM from at least 4 independent experiments and were analyzed by ANOVA with Duncan's post hoc analysis, * $P < 0.05$ vs. untreated cultures, # $P < 0.05$ vs. epibatidine treatment.

Figure 5.

EGL neuronal precursors display α3 nicotinic acetylcholine receptor (nAChR) subunit immunoreactivity and radioligand binding. (A–D) EGL neuronal precursors form neurosphere clusters in vitro. EGL neuronal precursors isolated from 5-day-old mice displayed α3, but not α4, nAChR subunit immunoreactivity. α3 immunoprotein was associated with the cell body and neurites as visualized using (A) nickel -intensified DAB or (C) using immunofluorescence. Specific immunoreactivity was not evident in (B) controls lacking primary antiserum or in (D) preabsorbed controls. In contrast, α4-nAChR immunoreactivity was not seen in the neuronal cultures. (F–I) Autoradiographs showing nicotinic acetylcholine receptor (nAChR) binding using radioligands selective for nAChR subtypes. Radioligands were bound to coronal sections from 5-day-old mice. Note the external granule layer (arrows) at the cerebellar surface is prominently labeled with [3H]-

epibatidine (F and H), and not labeled with [^3H]-A85380 (G), suggesting the presence of $\alpha 3$ nAChR subunits in cerebellar neuroblasts. Specific [^3H]-epibatidine binding was evident in the presence of unlabeled cytosine, suggesting the presence of $\alpha 3$ sites. Note sections F and G are more rostral than H and I; superior colliculus (SC); inferior colliculus (IC); medial vestibular nucleus (MVN). Scale bars, 20 μm (A and B); 25 μm (C–E).