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Cerebellar Nicotinic Cholinergic Receptors are Intrinsic to the Cerebellum: Implications for Diverse Functional Roles

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Abstract

Although recent studies have delineated the specific nicotinic subtypes present in the mammalian cerebellum, very little is known about their location or function within the cerebellum. This is of increased interest since nicotinic receptors (nAChRs) in the cerebellum have recently been implicated in the pathology of autism spectrum disorders. To begin to better understand the roles of these heteromeric nAChRs in the cerebellar circuitry and their therapeutic potential as targets for drug development, we used various chemical and stereotaxic lesion models in conjunction with slice electrophysiology to examine how specific heteromeric nAChR subtypes may influence the surrounding cerebellar circuitry. Using subunit-specific immunoprecipitation of radiolabeled nAChRs in the cerebella following *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride, *p*-chloroamphetamine, and pendunculotomy lesions, we show that most, if not all, cerebellar nicotinic receptors are present in cells within the cerebellum itself and not in extracerebellar afferents. Furthermore, we demonstrate that the $\beta 4$ -containing, but not the $\beta 2$ -containing, nAChRs intrinsic to the cerebellum can regulate inhibitory synaptic efficacy at two major classes of cerebellar neurons. These tandem findings suggest that nAChRs may present a potential drug target for disorders involving the cerebellum.

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Conflict of Interest None of the authors have a conflict of interest.

Keywords

Nicotinic receptor; Cerebellum; Lesion; Rat; Immunoprecipitation; Electrophysiology

Introduction

Nicotinic acetylcholine receptors (nAChRs) are ion channels that mediate fast excitatory transmission in the nervous system. In the CNS, nAChRs are also known to modulate the release of other neurotransmitters, thereby strongly influencing the surrounding neuronal circuitry. This widespread modulatory role may be one reason that nAChRs are implicated in a number of neurological disorders, including Parkinson's disease, Tourette's syndrome, Alzheimer's disease, pain, and, of course, nicotine addiction [1, 2].

Heteromeric nicotinic receptors exist as subtypes based on their composition of α - and β -subunits, which form a pentameric structure surrounding an ion pore. Upon activation by acetylcholine, the channel opens, allowing the passage of Na^+ and Ca^{++} ions into and K^+ ions out of the cell. Nine alpha subunits ($\alpha 2$ – $\alpha 10$) and three beta subunits ($\beta 2$ – $\beta 4$) are expressed in vertebrates and are well conserved across most species.

In the cerebellum, mRNA transcripts have been detected for several nAChR subunits, including $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ [3, 4], and nAChRs have been demonstrated in various neuronal populations. For example, nAChRs containing $\alpha 4$ and $\beta 2$ subunits were found in mouse cerebellar granule cells (CGCs) in culture [5], and several nicotinic receptor subunits were detected within the Purkinje cell layer, the granular layer, and in the deep cerebellar nuclei of the human cerebellum [6]. Moreover, activation of nAChRs has been shown to elicit the release of GABA and norepinephrine in the rat cerebellum [7–9]. Thus, nAChRs may have significant influence on neuronal activity within the cerebellar circuitry, and dysregulation of this activity could contribute to developmental disorders involving the cerebellum. For example, aberrations in the relative distributions of nAChRs in the cerebellum have been described in autism spectrum disorders [10–13], suggesting that these receptors may play a role in this developmental disorder.

Recently, we reported six structurally distinct heteromeric nAChR populations in the rat cerebellum, including several subtypes that have not been previously encountered in the CNS [4]. The objective of the current study was to examine the location and possible functional role of these receptors. From these studies, we conclude that cerebellar nAChRs are not on extrinsic axons to the cerebellum, but are intrinsic to the cerebellum and influence cerebellar circuitry by regulating GABA release from interneurons in a subtype-specific manner.

Materials and Methods

Materials

[^3H]Epibatidine ([^3H]EB), [^3H]nisoxetine, and [^3H]citalopram were obtained from Perkin Elmer Life Sciences (Boston, MA). Rabbit antisera directed at a bacterially expressed fusion protein containing partial sequences of the cytoplasmic domains of nAChR $\alpha 4$ and $\beta 4$

subunits were kind gifts from Drs. Scott Rogers and Lorise Gahring (University of Utah, Salt Lake City, UT). These antisera have been described previously [4, 14–16]. An antibody directed at a peptide sequence of the rat nAChR $\alpha 3$ subunit was affinity-purified from rabbit serum and was a gift from Drs. Barry Wolfe and Robert Yasuda (Georgetown University, Washington, DC). This antibody has been described previously [4, 16, 17]. A monoclonal antibody (mAb 270) to the chick $\beta 2$ subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA). This mAb was originally developed and characterized by Whiting and Lindstrom [18]. Protein G Sepharose beads were purchased from Amersham Biosciences Corporation (Piscataway, NJ). Normal rabbit serum (NRS) was purchased from Calbiochem (San Diego, CA). For simplicity, in this paper, we use the term antibody to refer to unpurified antisera as well as to affinity-purified antisera and monoclonal antibodies. Except where otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

Tissues and Animals

Lesion studies were carried out in adult male Sprague–Dawley rats (~250 g; Harlan, Indianapolis, IN, and ARC, Perth, Western Australia). Brain slices for electrophysiology studies were prepared from 29- to 31-day-old Sprague–Dawley rats (Harlan).

DSP-4 and PCA Lesions

Lesions of NE axons by *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP-4) and of 5-HT axons by *p*-chloroamphetamine (PCA) were performed as described previously [19, 20]. Briefly, Sprague–Dawley rats were given a single intraperitoneal injection of DSP-4 (50 mg/kg) or two separate injections of PCA on day 1 (12 mg/kg) and day 3 (15 mg/kg). Control animals received the same volume of vehicle (0.9% saline) on the same schedules. The rats were allowed to recover for 4 weeks before being lightly anesthetized with isoflurane and killed by decapitation, in accordance with the NIH and GUACUC guidelines.

Cerebellar Pedunculotomy

Axons innervating one half of the cerebellum were lesioned by transecting the inferior and middle cerebellar peduncles unilaterally [21, 22]. With this procedure, very few spinal mossy fiber afferents to the anterior lobe paravermis and catecholaminergic afferents, which pass through the superior peduncle, remain [23]. Rats were anesthetized with ketamine (50 mg/kg), xylazine (10 mg/kg), and acepromazine (0.75 mg/kg), and under aseptic conditions, the skin over the neck was incised longitudinally and the muscles retracted laterally to expose the atlantooccipital membrane. A capsulotomy knife (MSP 5-mm blade) was inserted through the membrane into the fourth ventricle parallel to the dorsal surface of the brainstem, rotated laterally to cut the left middle and inferior cerebellar peduncles, and withdrawn. The muscles were returned to their original position, the wound was sutured, and the animals were allowed to recover from anesthesia before being returned to their cage. The rats were allowed to recover for 4 days following the surgery to allow for complete degeneration of the ipsilateral axon terminals; the animals were then lightly anesthetized with isoflurane and killed by decapitation. The completeness of the lesion was assessed by visual inspection under a dissecting microscope, and only those animals that had a complete

lesion through the left middle and inferior peduncles were retained for measurements of nAChRs.

Binding Studies

Cerebellar tissues were homogenized in 50 mM Tris–HCl buffer (pH 7.4 at 24°C) and centrifuged twice at 35,000×*g* for 10 min in fresh buffer. For receptor binding studies, nAChRs were measured by adding tissue homogenates or fractions containing solubilized nAChRs to tubes containing [³H]EB. Incubations were carried out in Tris buffer, pH 7.4, for 2 h at 24°C. Bound receptors were separated from free ligand by filtration over Whatman GF/C filters wet with 0.5% polyethylenimine and mounted on a Brandell Cell Harvester (Gaithersburg, MD). The filters were then counted in a liquid scintillation counter. Nonspecific binding was measured in the presence of 300 μM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding. The norepinephrine and serotonin binding sites on their respective transporters were measured in membrane homogenates resuspended in Tris buffer (pH 7.4) containing 400 mM NaCl and 5 mM KCl. The homogenates were added to tubes containing 10 nM [³H]nisoxetine or 2 nM [³H]citalopram for measurements of the NE transporter or the 5-HT transporters, respectively. Incubations were carried out for 4 h at 4°C, and bound receptors were then separated from free ligand by vacuum filtration over GF/C glass fiber filters and counted as above. Nonspecific binding to the NE or 5-HT transporter was determined in the presence of 10 μM desipramine or 1 μM fluoxetine, respectively, and specific binding was defined as the difference between total binding and nonspecific binding.

Immunoprecipitation

Tissue membrane homogenates were prepared as above for binding assays with Tris buffer (pH 7.0). The receptors were solubilized by incubating the homogenates in 2% TritonX-100 with gentle rotation for 2 h at room temperature. After centrifuging the mixture at 35,000×*g* for 10 min, aliquots of the clear supernatant (equivalent to 9 mg original tissue weight) were added to sample tubes containing [³H]EB and either one of the subunit-specific antibodies at a concentration determined in preliminary studies to be optimal for each, or an equivalent volume of NRS. The samples were then rotated overnight at 4°C. After the addition of 50 μl of a 50% slurry of Protein G Sepharose beads, the rotation of the samples at 4°C was continued for one more hour. The samples were then centrifuged at 12,000×*g* for 1 min. The tissue pellets were washed once with 1 ml 50 mM Tris–HCl buffer (pH 7.0), dissolved in 0.1 N NaOH, and then counted in a scintillation counter. After subtracting the number of counts precipitated in tubes containing NRS, the number of [³H]EB-labeled nAChRs immunoprecipitated by each antibody was compared with the total number of labeled receptors, as measured in binding assays of the solubilized cerebellar membranes before addition of the antibodies.

Cerebellar Slices and Electrophysiology

Cerebella from P29–32 rats were placed in an ice-cold slicing solution containing (in mM) NaCl (85), KCl (2.5), CaCl₂ (1), MgCl₂ (4), NaH₂PO₄ (1), NaHCO₃ (25), glucose (25), sucrose (75), pH 7.4, and then continuously bubbled with 95% O₂ and 5% CO₂. Sagittal slices of the cerebellum (200 μm) were prepared using a Vibratome 3000 Plus Sectioning

System (Vibratome, St. Louis, MO) and incubated in the slicing solution at 34°C for 30 min before being transferred to the recording solution. Slices were viewed under an upright microscope (E600FN, Nikon) equipped with Nomarski optics and an electrically insulated 60× water immersion objective with a long working distance (2 mm) and high numerical aperture (1.0). Recordings were done in visually identified CGCs and Purkinje cells at 24–26°C. The continuously perfused extracellular recording solution contained (in mM): NaCl (120), KCl (3.1), CaCl₂ (2), MgCl₂ (1), KH₂PO₄ (1), NaHCO₃ (25), glucose (2.5), sucrose (30), pH 7.4. Recording electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA). For cerebellar granule cell recordings, the electrodes were filled with the intracellular solution containing (in mM): KCl (145), HEPES (10), ATP.Mg (5), GTP.Na (0.2), and BAPTA (10), adjusted to pH 7.2 with KOH. For Purkinje cell recordings, the intracellular solution contained (in mM): CsCl (135), HEPES (10), EGTA (10) MgCl₂ (2), adjusted to pH 7.2 with CsOH. Pipette resistance was 5–10 MΩ. Whole cell voltage clamp recordings were made at –60 mV (granule cells) or –70 mV (Purkinje cells) with an Axopatch-1D or Multiclamp 700B amplifiers (Molecular Devices Inc., Sunnyvale, CA), and access resistance was monitored throughout the recordings. Currents were filtered at 2 kHz with a low-pass Bessel filter and digitized at 5 kHz using a personal computer equipped with Digidata 1322A or Digidata 1440A digitizer (Molecular Devices). Stock solutions of gabazine, ACh, cytosine, and I-A85380 were prepared in water and diluted to the desired concentration in extracellular medium. All drugs were locally applied through a “Y tube” drug application system [24] modified for optimal solution exchange in brain slices [25]. Recordings were analyzed using Clampfit 9 software (Molecular Devices). The average spontaneous inhibitory postsynaptic current (sIPSC) amplitude was determined from an average trace of >30 individual sIPSCs. Frequency and amplitude changes in the presence of a drug were calculated as a percent increase over frequency and amplitude in the absence of the drug within the same cell. Tonic GABA_A receptor-mediated currents were measured as the difference in the mean baseline current during and before application of nAChR agonists. Mean baseline currents were computed from continuous 1-s current traces devoid of synaptic events.

Data Analysis

In the electrophysiological studies, statistical comparisons were done using two-tailed Student's *t* tests. All data are expressed as the mean±standard error of the mean, unless otherwise indicated. In all other studies, statistical analyses of the differences between groups were assessed using two-way analysis of variance followed by Bonferroni's multiple comparison test using the GraphPad Prism 5.0 software package (GraphPad Software, San Diego, CA).

Results

Nicotinic Receptors Are Not Located on Norepinephrine Axon Terminals

Previous studies found that nicotine stimulates NE release in rat cerebellar slices [9]. To determine whether this nicotine-stimulated release reflects the presence of nAChRs directly on NE terminals in the cerebellum or an indirect effect through other neurons, we lesioned NE axons in young adult rats with the neurotoxin DSP-4, which selectively targets NE

axons. Four weeks after the DSP-4 injection, [^3H]nisoxetine binding to the NE transporter (NET) in the cerebellum was decreased by >75% (Fig. 1a), indicating a successful lesion of NE axons and their consequent degeneration. Despite the extensive loss of NE axons in the cerebellum, we found no change in the number of cerebellar nAChRs containing $\alpha 3$, $\alpha 4$, $\beta 2$, or $\beta 4$ subunits, as measured in immunoprecipitation assays (Fig. 1b). These data indicate that nAChRs in the adult cerebellum are not likely to be located on NE axons originating in the locus coeruleus.

Nicotinic Receptors Are Not Located on Serotonin Axon Terminals

The cerebellum receives 5-HT axons from several reticular and Raphe nuclei of the brainstem [26]. To determine whether nAChRs are located on 5-HT axons in the cerebellum, we lesioned these axons in young adult Sprague–Dawley rats with the specific serotonergic neurotoxin, PCA. In the cerebella from PCA-treated rats, [^3H]citalopram binding to the 5-HT transporter (SERT) was decreased by ~70% (Fig. 2a), reflecting the loss of 5-HT axons. Again, despite this extensive loss of 5-HT axons, no significant change in the number of any of the nAChR subunits was found in the cerebellum (Fig. 2b). These data indicate that nAChRs are not likely to be located on 5-HT axons that innervate the cerebellum.

Nicotinic Receptors Are Not Located on Glutamate or Acetylcholine Axon Terminals

With the exception of NE and most 5-HT projections, which enter the cerebellum through the superior peduncle, the middle and inferior peduncles carry all other afferent fibers to the cerebellum. Since these fibers do not cross the midline within the cerebellum [22], the contralateral hemi-cerebellum can serve as a control for unilateral pedunculotomy. Figure 3 demonstrates that after transection of virtually all fibers leading into the cerebellum through the middle and inferior peduncles, no significant change in density of any of the four nAChR subunits measured was detected, indicating that axons entering the cerebellum through these anatomical pathways do not contain nAChRs on their terminals.

Taken together, these measurements of nAChRs after chemical lesions of axons with DSP-4 and PCA and after unilateral pedunculotomy indicate that virtually all nAChRs within the rat cerebellum are on cells intrinsic to the cerebellum.

$\beta 2$ - and $\beta 4$ -Containing Nicotinic Receptors Differentially Control GABAergic Neurotransmission on Granule and Purkinje Cells

Our binding results indicate that the several heteromeric nAChR subtypes found in the cerebellum [4] are intrinsic to the cerebellum rather than being expressed on axons from extrinsic cells. Since GABAergic signals are also generated from exclusively within the cerebellum [27], we investigated the influence of these intrinsic nAChR subtypes on neuronal function by examining GABA_A receptor-mediated neurotransmission in CGCs and Purkinje neurons. In our experiments, the application of ACh (100 μM) led to a fourfold increase in the frequency of sIPSCs in CGCs (Fig. 4). The application of cytosine (100 μM), a full agonist at nicotinic receptors containing $\beta 4$ subunits, but a weak partial agonist at those containing $\beta 2$ subunits [27, 28], resulted in a similar increase of sIPSC frequency. In contrast, I-A-85380 (30 nM), a potent agonist at $\beta 2$ -containing nAChRs, did not affect the

frequency of sIPSCs (Fig. 4). Neither ACh nor the $\beta 2$ or $\beta 4$ subunit-selective nicotinic agonists affected the amplitude of the sIPSCs (Fig. 4). Modulation of sIPSC frequency, but not amplitude, implies pre-synaptic mechanisms; thus, our results are consistent with several studies that demonstrated regulation of synaptic function in the cerebellum by pre-synaptically expressed nAChRs [8, 29]. Importantly, our results indicate that control over synaptic inhibition of CGCs is exerted primarily by the $\beta 4$ -containing nAChR subtypes rather than the $\beta 2$ -containing subtypes.

While Golgi interneurons are the primary source of GABAergic input to the CGCs, a different population of interneurons, made up of basket and stellate cells, forms GABAergic synapses onto Purkinje neurons, which generate the sole output from the cerebellar cortex [30]. Thus, we investigated the role of the nAChRs expressed by the basket and stellate cells in recordings from the Purkinje neurons. Similarly to our observations in the CGCs, both ACh and cytisine, but not I-A-85380, increased the frequency of sIPSCs recorded in the Purkinje neurons (Fig. 5). The magnitude of this increase, however, was much smaller than that seen in CGCs and ranged from 7% to 83%. As with CGCs, the sIPSC amplitude in the Purkinje neurons was not affected by any of the three drugs (Fig. 5). Thus, synaptic inhibition of Purkinje cells is also regulated by the $\beta 4$ -containing, but not the $\beta 2$ -containing, nAChRs, although the strength of this regulation appears to be weaker than in the CGCs.

A previous report identified the activation of a tonic GABA_A receptor current by pre-synaptic nAChRs at inhibitory synapses onto CGCs [8]. Tonic GABA_A receptor-mediated inhibition is characterized by a persistently active current, the strength of which may have profound implications for cerebellar function [31, 32]. In our experiments, tonic GABA_A receptor-mediated currents in the CGCs could be elicited by all three nAChR agonists (Fig. 6a, c), including I-A-85380, which activates nAChR subtypes containing $\beta 2$ subunits and which did not affect synaptic events. Furthermore, the magnitude of the tonic current elicited by the I-A-85380 was only slightly smaller than that evoked by ACh and cytisine (ACh 7.9 ± 2 pA, $n=9$; cytisine 5.5 ± 2.8 pA, $n=6$; I-A-85380 4.8 ± 3.2 pA, $n=4$). In the Purkinje neurons, the effects of ACh and the two nAChR subunit-selective compounds on tonic GABA_A receptor-mediated currents were highly variable. All three compounds could elicit a downward shift in baseline current (ACh and cytisine in 3 cells, I-A-85380 in 2 cells), an upward shift in baseline current (ACh in 7 cells, cytisine in 3 cells, I-A-85380 in 4 cells), or remained without effect (ACh in 1 cell, cytisine and I-A-85380 in 2 cells). This variability may be due to the simultaneous stimulation of GABA release at the basket/stellate to Purkinje cell synapse and at the Golgi to CGC synapse. Golgi cell inhibition of CGCs (Figs. 4 and 5) will decrease CGC output to basket/stellate cells [33] and may reduce tonic GABA current in the Purkinje cells, while stimulation at the basket/stellate terminals may act to increase it. Altogether, our electrophysiology experiments highlight two important observations: first, that nAChRs intrinsic to the cerebellum can regulate inhibitory synaptic efficacy at two major classes of cerebellar neurons and, second, that the character of nAChR-mediated regulation of inhibition in the cerebellum depends upon the nAChR subtype activated.

Discussion

The rat cerebellum expresses several mixed heteromeric subtypes of nAChRs [4]. Our studies indicate that these receptors are on cells intrinsic to the cerebellum, as opposed to being on afferent fibers originating from cells outside the cerebellum. This conclusion is based on radioligand measurements after selective lesions of the NE and 5-HT afferents and unilateral middle and inferior cerebellar pedunculotomy, which eliminates cholinergic and glutamatergic cerebellar afferent axons. Furthermore, though previous studies have shown that nicotinic stimulation is responsible for modulating GABAergic inhibition on granule cells of the cerebellum [8], this is the first study to evaluate subtype specificity of such modulation in both CGCs and Purkinje cells.

Although our findings indicate that heteromeric nAChRs in the cerebellum are located exclusively on cells intrinsic to the cerebellum, O'Leary and Leslie [9] observed nicotine-stimulated NE release in cerebellar slices from very young rats, which markedly decreased during the first 30 days after birth. Furthermore, this release, observed between P0 and P30, was only partially (45%) prevented by tetrodotoxin, which suggests that approximately half of these nAChRs are in fact present on NE terminals during postnatal development. Moreover, in the adult cerebellum, a small amount of nicotine-stimulated NE release was observed even in the presence of tetrodotoxin, suggesting that a fraction of these nAChRs remain on NE terminals into adulthood. This discrepancy could suggest that a very small number of nAChRs on NE terminals are present in the adult rat cerebellum, but are below our level of detection; however, our method of immunoprecipitation of [³H]EB-labeled nAChRs is very sensitive and is able to detect significant changes in specific nAChR subunits of approximately 0.1 fmol/mg tissue [4]. Alternatively, the neurotoxin DSP4 is highly specific for noradrenergic afferents arising from the locus coeruleus, but spares extra-coeruleus noradrenergic innervation [34]. Although our neurotoxic lesion was successful to the degree previously observed in this strain of rat [34], we cannot rule out a contribution or extra-coeruleus noradrenergic afferents containing a small number of nAChRs. However, in our functional experiments, we show that blockade of GABA_A receptors with gabazine completely blocks the electrophysiological effects of the nicotinic agonists. These data, too, suggest that nAChRs in the cerebellum are predominantly present in the interneuronal cell population.

Based on our previous sequential immunoprecipitation studies, the cerebellum expresses several complex heteromeric nAChR subtypes, including two subtypes that contain both $\beta 2$ and $\beta 4$ subunits [4]. The cerebellar cortex possesses three major cell types: CGCs, Purkinje cells, and interneurons. Therefore, heteromeric nAChRs in the cerebellum, including those containing $\beta 2$, $\beta 4$, or both subunits, could be present in some, if not all, of these cell types. Consistent with this possibility, autoradiographic studies with nicotinic ligands found the granular/Purkinje cell layer to possess nAChR subtypes containing both $\beta 2$ (36%) and $\beta 4$ (64%) subunits [35].

In electrophysiology experiments, current pharmacological tools can differentiate between $\beta 2$ - and $\beta 4$ -containing receptors. For example, while acetylcholine activates all nicotinic receptors, I-A-85380 and cytosine preferentially activate $\beta 2$ - and $\beta 4$ -containing receptors,

respectively. We found no evidence for direct activation of nAChRs by nicotinic agonists on either the granule cells or the Purkinje cells since all the observed effects of these agonists were completely blocked by the GABA_A receptor antagonist, gabazine (Figs. 4a and 5a). A previous study found that ACh increases the frequency of GABA_A receptor-mediated IPSCs in CGCs via a pre-synaptic mechanism [8]. Likewise, we observe an ACh-induced increase in sIPSC frequency, but not sIPSC amplitude, in both CGCs and Purkinje neurons. These results are consistent with synaptic GABA release activated by the stimulation of nAChRs located on GABA-containing terminals pre-synaptic to these cells. Thus, GABA release from both the Golgi cells that provide inhibitory input to the CGCs and the basket/stellate interneurons that provide inhibitory input to the Purkinje cells can be modulated by nAChRs. Furthermore, we find that cytosine similarly increases sIPSC frequency in both CGCs and Purkinje cells, while the application of I-A-85380 does not. These results suggest that the β 4-containing, but not the β 2-containing, nAChRs regulate the strength of inhibition at GABAergic synapses onto the CGCs and Purkinje cells.

Synaptic inhibition in the CGCs is complemented by tonic inhibition that may exert powerful control over the efficacy of inhibitory neurotransmission in the cerebellum [32]. Our results indicate that both β 2- and β 4-containing nAChRs play a role in controlling the level of tonic GABA_A receptor-mediated currents in the CGCs. The application of either cytosine or I-A-85380 elicited an inward current that was similar in amplitude between the two agonists. We have not explored the reasons for the β 2-containing nAChR involvement in the regulation of tonic, but not synaptic, inhibition of the CGCs. It is possible that the pre-synaptic location of the two subunits differs such that the β 2-containing nAChRs are located distal to the synapse, preventing them from influencing fast synaptic events but still allowing them to modulate the GABAergic tone. Alternatively, the β 2-containing nAChRs may be less effective at stimulating the release of vesicular GABA, which is necessary for synaptic inhibition, but still able to maintain coupling to non-vesicular mechanisms that could underlie the generation of tonic inhibition in the CGCs [32, 36].

Since different nAChR subtypes appear to be associated with select populations of cells within the cerebellum rather than with extracerebellar afferents, nicotinic cholinergic transmission could provide a basis for fine tuning the cerebellar signal processing of extrinsic information, thus selectively influencing cerebellar function. For example, a recent study demonstrated that coordinated network-level rhythm generation patterns occur in the cerebellum during nicotinic receptor subtype stimulation [37]. Moreover, these receptors could play a role in disorders involving the cerebellum. For example, recent autopsy evidence points to the possible involvement of cerebellar nAChRs in developmental disorders such as autism spectrum disorders [11–13, 29]. Therefore, drug therapies targeting select nAChR subtypes could result in the coordinated activation and subsequent modulation of these cerebellar networks and result in novel treatments for disorders involving the cerebellum.

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Abbreviations

ACh	Acetylcholinep
nAChR	Nicotinic cholinergic receptor
NE	Norepinephrine
EB	Epibatidine
CGC	Cerebellar granule cell

References

1. Lindstrom J. Nicotinic acetylcholine receptors in health and disease. *Mol Neurobiol.* 1997; 15(2): 193–222. [PubMed: 9396010]
2. Gotti C, Zoli M, Clementi F. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol Sci.* 2006; 27(9):482–91. [PubMed: 16876883]
3. Tribollet E, et al. Comparative distribution of nicotinic receptor subtypes during development, adulthood and aging: an autoradiographic study in the rat brain. *Neuroscience.* 2004; 124(2):405–20. [PubMed: 14980390]
4. Turner JR, Kellar KJ. Nicotinic cholinergic receptors in the rat cerebellum: multiple heteromeric subtypes. *J Neurosci.* 2005; 25(40):9258–65. [PubMed: 16207885]
5. Didier M, et al. Characterization of nicotinic acetylcholine receptors expressed in primary cultures of cerebellar granule cells. *Brain Res Mol Brain Res.* 1995; 30(1):17–28. [PubMed: 7609640]
6. Graham A, et al. Immunohistochemical localisation of nicotinic acetylcholine receptor subunits in human cerebellum. *Neuroscience.* 2002; 113(3):493–507. [PubMed: 12150770]
7. De Filippi G, Baldwinson T, Sher E. Evidence for nicotinic acetylcholine receptor activation in rat cerebellar slices. *Pharmacol Biochem Behav.* 2001; 70(4):447–55. [PubMed: 11796144]
8. Rossi DJ, Hamann M, Attwell D. Multiple modes of GABAergic inhibition of rat cerebellar granule cells. *J Physiol.* 2003; 548(Pt 1):97–110. [PubMed: 12588900]
9. O'Leary KT, Leslie FM. Developmental regulation of nicotinic acetylcholine receptor-mediated [³H]norepinephrine release from rat cerebellum. *J Neurochem.* 2003; 84(5):952–9. [PubMed: 12603820]
10. Court JA, et al. Nicotinic receptors in human brain: topography and pathology. *J Chem Neuroanat.* 2000; 20(3–4):281–98. [PubMed: 11207426]
11. Lee M, et al. Nicotinic receptor abnormalities in the cerebellar cortex in autism. *Brain.* 2002; 125(Pt 7):1483–95. [PubMed: 12076999]
12. Martin-Ruiz CM, et al. Molecular analysis of nicotinic receptor expression in autism. *Brain Res Mol Brain Res.* 2004; 123(1–2):81–90. [PubMed: 15046869]
13. Perry EK, et al. Cholinergic activity in autism: abnormalities in the cerebral cortex and basal forebrain. *Am J Psychiatry.* 2001; 158(7):1058–66. [PubMed: 11431227]
14. Flores CM, et al. A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol.* 1992; 41(1):31–7. [PubMed: 1732720]
15. Rogers SW, et al. The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. *J Neurosci.* 1992; 12(12):4611–23. [PubMed: 1464760]
16. Marritt AM, et al. Nicotinic cholinergic receptors in the rat retina: simple and mixed heteromeric subtypes. *Mol Pharmacol.* 2005; 68(6):1656–68. [PubMed: 16129735]

17. Yeh JJ, et al. Neuronal nicotinic acetylcholine receptor alpha3 subunit protein in rat brain and sympathetic ganglion measured using a subunit-specific antibody: regional and ontogenic expression. *J Neurochem.* 2001; 77(1):336–46. [PubMed: 11279289]
18. Whiting P, Lindstrom J. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc Natl Acad Sci USA.* 1987; 84(2):595–9. [PubMed: 3467376]
19. Gonzalez MM, Debilly G, Valatx JL. Noradrenaline neurotoxin DSP-4 effects on sleep and brain temperature in the rat. *Neurosci Lett.* 1998; 248(2):93–6. [PubMed: 9654350]
20. Stockmeier CA, Kellar KJ. Serotonin depletion unmasks serotonergic component of [³H]dihydroalprenolol binding in rat brain. *Mol Pharmacol.* 1989; 36(6):903–11. [PubMed: 2557537]
21. Desclin J. Early terminal degeneration of cerebellar climbing fibres after destruction of the inferior olive in the rat. Synaptic relationships in the molecular layer. *Anat Embryol.* 1976; 149:87–112. [PubMed: 1267191]
22. Cicirata F, et al. Different pontine projections to the two sides of the cerebellum. *Brain Res Rev.* 2005; 49:280–94. [PubMed: 16111556]
23. Ito M. The modifiable neuronal network of the cerebellum. *Jpn J Physiol.* 1984; 34(5):781–92. [PubMed: 6099855]
24. Murase K, Ryu PD, Randic M. Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. *Neurosci Lett.* 1989; 103(1):56–63. [PubMed: 2476693]
25. Hevers W, Luddens H. Pharmacological heterogeneity of gamma-aminobutyric acid receptors during development suggests distinct classes of rat cerebellar granule cells in situ. *Neuropharmacology.* 2002; 42(1):34–47. [PubMed: 11750914]
26. Bishop GA, Ho RH. The distribution and origin of serotonin immunoreactivity in the rat cerebellum. *Brain Res.* 1985; 331(2):195–207. [PubMed: 3986565]
27. Luetje CW, Patrick J. Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J Neurosci.* 1991; 11(3):837–45. [PubMed: 1705971]
28. Papke RL, Heinemann SF. Partial agonist properties of cytosine on neuronal nicotinic receptors containing the beta 2 subunit. *Mol Pharmacol.* 1994; 45(1):142–9. [PubMed: 8302273]
29. Kawa K. Acute synaptic modulation by nicotinic agonists in developing cerebellar Purkinje cells of the rat. *J Physiol.* 2002; 538(Pt 1):87–102. [PubMed: 11773319]
30. Kondo S, Marty A. Synaptic currents at individual connections among stellate cells in rat cerebellar slices. *J Physiol.* 1998; 509(Pt 1):221–32. [PubMed: 9547395]
31. Hansel C, Linden DJ, D'Angelo E. Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat Neurosci.* 2001; 4(5):467–75. [PubMed: 11319554]
32. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci.* 2005; 6(3):215–29. [PubMed: 15738957]
33. Ito M. Cerebellar circuitry as a neuronal machine. *Prog Neurobiol.* 2006; 78(3–5):272–303. [PubMed: 16759785]
34. Fornai F, et al. Region- and neurotransmitter-dependent species and strain differences in DSP-4-induced monoamine depletion in rodents. *Neurodegeneration.* 1996; 5(3):241–9. [PubMed: 8910902]
35. Nguyen HN, Rasmussen BA, Perry DC. Subtype-selective up-regulation by chronic nicotine of high-affinity nicotinic receptors in rat brain demonstrated by receptor autoradiography. *J Pharmacol Exp Ther.* 2003; 307(3):1090–7. [PubMed: 14560040]
36. Cavalier P, et al. Tonic excitation and inhibition of neurons: ambient transmitter sources and computational consequences. *Prog Biophys Mol Biol.* 2005; 87(1):3–16. [PubMed: 15471587]
37. Middleton S, et al. High-frequency network oscillations in cerebellar cortex. *Neuron.* 2008; 58:763–74. [PubMed: 18549787]

Fig. 1.

Effect of DSP-4 on nAChR subunits in the rat cerebellum. **a** Specific binding of 10 nM [³H]Nisoxetine at a single saturating concentration to cerebellar membrane homogenates from both DSP-4 lesioned rats and control rats to demonstrate the reduced NET density following DSP-4 treatment. Values are the mean±SEM from six control and six DSP-4-treated samples; $p < 0.001$. **b** Rat cerebellar nAChRs from both control and DSP-4-treated rats were solubilized, labeled with 2 nM [³H]EB, and immunoprecipitated with each of the antibodies shown. Nonspecific immunoprecipitation was measured with normal rabbit serum and has been subtracted. There was no significant difference between the control and treated groups. Data shown are the mean±SEM from six control and six DSP-4-treated samples

Fig. 2.

Effect of PCA on nAChR subunits in the rat cerebellum. **a** Specific binding of 2 nM [³H]Citalopram at a single saturating concentration to cerebellar membrane homogenates from both PCA lesioned rats and control rats to demonstrate the reduced SERT density following PCA treatment. Values are the mean±SEM from four control and four PCA-treated samples; $p<0.001$. **b** Rat cerebellar nAChRs from both control and PCA-treated rats were solubilized, labeled with [³H]EB, and immunoprecipitated with each of the antibodies shown. Nonspecific immunoprecipitation was measured with normal rabbit serum and has been subtracted. There was no significant difference between the control and treated groups. Data shown are the mean±SEM from four control and four PCA-treated samples

Fig. 3.

Effect of unilateral pedunculotomy on nAChRs in the rat cerebellum. **a** Nissl-stained section of cerebellum from pedunculotomized rat. *Left side* Lesion; *right side*, control. *Scale bar*, 1 mm. **b** Rat cerebellar nAChRs from either the control side or lesioned side were solubilized, labeled with [^3H]EB, and immunoprecipitated with each of the antibodies shown. Nonspecific immunoprecipitation was measured with normal rabbit serum and has been subtracted. There was no significant difference between the control and treated groups. Data shown are the mean $\pm$ SEM from four control and four pedunculotomized rats

Fig. 4. nAChR agonists modulate inhibitory synaptic transmission in cerebellar granule neurons. **a** Representative traces illustrate the effects of ACh (100 μM), cytisine (100 μM), and I-A-85380 (30 nM) on GABA_A receptor-mediated sIPSCs. Notice that baseline noise is increased during the application of all three nAChR agonists. This is indicative of the activation of a tonic inhibitory current (see Fig. 6). Both synaptic and tonic inhibitory current components are blocked by the application of GABAzine (10 μM) in the presence of ACh (100 μM, *bottom trace*). **b** Bar histograms demonstrate a prominent increase in sIPSC frequency (*top*) during the application of ACh and cytisine, but not during the application of I-A-85380. Neither of the three nAChR agonists affected the amplitude of GABA_A receptor-mediated sIPSCs (*bottom*). ** $p < 0.01$; * $p < 0.05$; $n=9$, 6, and 4 cells for ACh, cytisine, and I-A-85380, respectively

Fig. 5. nAChR agonists modulate inhibitory synaptic transmission in cerebellar Purkinje neurons. **a** Representative traces illustrate the effects of ACh (100 μ M), cytosine (100 μ M), and I-A-85380 (30 nM) on GABA_A receptor-mediated sIPSCs. As in cerebellar granule cells, the application of GABA_A receptor antagonist (10 μ M) in the presence of ACh blocks spontaneous synaptic events (*bottom trace*). **b** Bar histograms demonstrate an increase in sIPSC frequency (*top*) during the application of ACh and cytosine, but not during the application of I-A-85380. Neither of the three nAChR agonists affected the amplitude of GABA_A receptor-mediated sIPSCs (*bottom*). ** $p < 0.01$; $n = 9, 6,$ and 8 cells for ACh, cytosine, and I-A-85380, respectively

Fig. 6. nAChR agonists elicit tonic GABA_A receptor-mediated current in CGCs. **a, b** Current traces illustrate the effects of ACh (100 μ M), cytisine (100 μ M), and I-A-85380 (30 nM) on GABA_A receptor-mediated tonic currents in a representative CGC (**a**) and a Purkinje neuron (**b**). *White dotted lines* are centered at the mean baseline current before nAChR agonist application. Notice that all three nAChR agonists reliably elicit an inward tonic current (i.e., downward shift in the mean baseline current) in the CGC with inconsistent effects in the Purkinje neuron. **c, d** Tonic current measurements obtained from all recorded CGCs (**c**) and Purkinje neurons (**d**) following nAChR agonist applications. *Horizontal black bars* show the means of measurements with each of the agonists. Negative values represent inward tonic currents; positive values represent outward tonic currents. Again, notice widely variable responses in the Purkinje neurons