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Evidence for nicotinic acetylcholine receptor activation in rat cerebellar slices

Giovanna De Filippi*, Tristan Baldwinson, Emanuele Sher

Eli Lilly and Company Limited, Lilly Research Centre, Erl Wood Manor, Sunninghill Road, Windlesham, Surrey GU20 6PH, UK

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Abstract

Neuronal nicotinic ACh receptor (nAChR) activation is known to enhance glutamate and GABA release in different brain areas. Moreover, nAChRs play an important role in neuronal differentiation. By using the patch-clamp technique, we have investigated the presence of nAChRs in cerebellar granule cells in slices from P5–P14 rats. Application of ACh (1 mM) could elicit a variety of effects. Some cells did not respond at all. In other cells, a somatic current was activated. In a proportion of cells, postsynaptic currents (PSCs), with or without somatic current, were elicited. Somatic nAChRs are likely to be of the $\alpha_4\beta_2$ subtype, but the presence of other subunit combinations (α_7- or β_4-containing receptors) cannot be ruled out. The ACh-induced PSCs were glutamatergic in nature. Thus, in a reasonable proportion of cells, nicotinic receptors are present presynaptically. They are likely to be α_7 receptors whose activation elicits Glu release via a TTX-sensitive mechanism. Our experiments are the first electrophysiological evidence showing, in a native cerebellar preparation, the presence of nicotinic receptors at the mossy fibre–granule cell synapse at early developmental stages.

Keywords: Nicotinic ACh receptors; α_7 Receptors; Cerebellum; Granule cells; Glu release; Patch clamp

1. Introduction

Neuronal nicotinic ACh receptors (nAChRs) are made of pentamers of different α (α_2–α_{10}) and β (β_2–β_4) subunits. The prevalent functional nAChRs in the mammalian brain are those composed of α_7 or $\alpha_4\beta_2$ subunits (Role and Berg, 1996). The role of these central receptors has remained obscure for many years. Only recently, significant information has been accumulated showing that nAChRs can both mediate and modulate fast synaptic transmission in the brain. Activation of presynaptic or preterminal α_7 or $\alpha_4\beta_2$ nAChRs enhances the release of many neurotransmitters in diverse brain regions (Lena et al., 1993; McMahon et al., 1994; McGehee et al., 1995; Alkondon et al., 1996, 1997, 1998; Gray et al., 1996; Lena and Changeux, 1997; Aramakis and Metherate, 1998; Frazier et al., 1998; Guo et al., 1998; Luo et al., 1998; Ji and Dani, 2000). On the other hand, the recent finding, that α_7-containing nAChRs mediate fast cholinergic synaptic transmission onto interneurons in the mammalian hippocampus, further indicates that postsynaptic and somatic nAChRs can have biologically important roles, as they have in the periphery (Alkondon et al., 1998; Frazier et al., 1998; Hefft et al., 1999). Nicotinic ACh receptors may also have a significant role during neuronal differentiation regulating early gene expression and contributing to neuronal pathfinding and target selection (Role and Berg, 1996).

In particular, a large body of evidence suggests that the cholinergic system plays an important role during cerebellar development. Levels of choline acetyltransferase (ChAT; the rate-limiting enzyme for the synthesis of ACh) are particularly high in the cerebellum during the early postnatal period, both in rats (Clos et al., 1989) and humans (Court et al., 1993). At this developmental stage, ChAT levels are relatively high compared to the levels of acetylcholinesterase (AChE; the ACh-degradative enzyme) (Clos et al., 1989; Court et al., 1993). Several reports indicate that nAChRs are present in the cerebellum at perinatal stages both in rats (Naeff et al., 1992; Zoli et al., 1995; Winzer-Serhan and Leslie, 1997) and humans (Court et al., 1995). Further studies have demonstrated the presence of nAChRs in cerebellar granule and Purkinje

* Corresponding author. Tel.: +44-1276-853-465; fax: +44-1276-853-525.

E-mail address: de_filippi_giovanna@lilly.com (G. De Filippi).

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neurones in rats (Zoli et al., 1995; Dominguez del Toro et al., 1997) and in the external granular layer (EGL) in humans (Court et al., 1995). Multiple nAChRs subunits, including α_3, α_4, α_5, α_7, β_2 and β_4, have been detected in cultured granule cells (Didier et al., 1995). Granule neurones are the targets for cholinergic mossy fibre innervation from pontine brainstem nuclei (Jaarsma et al., 1997). ACh may participate in synaptogenesis and neuromodulation of these pathways.

Only a few electrophysiological studies, mainly in vivo, have been reported so far on the action of ACh in the cerebellum. The results have been often contradictory. Iontophoretic application of ACh to granular layer cells in the anaesthesised cat cerebellum has been shown to cause excitation by some authors (McCance and Phillis, 1964, 1968) and to have no effect by others (Crawford et al., 1966). Similarly, McCance and Phillis propose that part of the mossy fibre population may be cholinergic, while Crawford et al. show that cholinergic antagonists do not affect mossy fibre-evoked field potentials. Lack of ACh effects on lobules IX and X neurones in the internal granular layer (IGL) has also been reported in rat cerebellar slices (Crepel and Dhanjal, 1982). In these experiments, bath application of nAChR antagonists did not show any detectable effect on excitatory and inhibitory synaptic potentials evoked in Purkinje cells via mossy and climbing fibre stimulation, thus making unlikely the presence of a significant contingent of these fibres using ACh as the neurotransmitter. Nicotinic receptor activation in cerebellum has been demonstrated by De la Garza et al. (1987a,b). In anaesthesised rats, pressure ejection of nicotine had an inhibitory effect on Purkinje cells and a strong excitatory effect on ''interneurons'' in IGL of lobules VI and VII. These effects were shown to be due to postsynaptic mechanisms. Also, the excitatory effect on ''interneurons'' was shown to be irreversibly blocked by α-bungarotoxin (αBTX) application, this being the first electrophysiological evidence suggesting that mammalian brain contains putative functional nicotinic receptors sensitive to αBTX. More recently, bath application of ACh has been reported to evoke a robust inward current in visually identified unipolar brush cells (UBCs) in the nodulus and uvula of thin cerebellar slices (Rossi et al., 1995).

Here, we present some new data on the presence of nAChRs in the cerebellum, influencing the activity of granule cells. Whole-cell patch-clamp experiments have been carried out in slices from 5- to 14-day-old rats. We report here new evidence that nAChRs can be present either pre- and/or postsynaptically in granule cells at early developmental stages. Somatic nAChRs are likely to be of the $\alpha_4\beta_2$ subtype, even if the presence of other subunit combinations cannot be completely ruled out. Presynaptic nAChRs are likely to be of the α_7 receptor subtype. The latter are ''preterminal'' receptors, the activation of which elicits Glu release via a TTX-sensitive mechanism.

2. Method

Whole-cell patch-clamp recordings in the voltage-clamp configuration ($n = 80$) were made from granule cells in the IGL of different lobules in rat cerebellar slices, by using the blind patch approach (Edwards et al., 1989).

2.1. Cerebellar slices

Slices were obtained from 5- to 14-day-old Wistar rats (day of birth = 1) by a procedure in compliance with the UK Animal Scientific Procedures Act 1986. Briefly, rats were anaesthesised with an overdose of fluothane and killed by decapitation. The cerebellar vermis was isolated, glued to the stage of a vibroslicer (Campden Instruments, Loughborough, UK) and rapidly immersed in oxygenated Krebs solution at 4 °C. Slices, 250–300 μm thick, were cut in the parasagittal plane and maintained at room temperature (19–21 °C) in oxygenated Krebs solution for at least 30 min before being transferred to the recording chamber. Krebs solution for cutting and recovery contained (mM): 120 NaCl, 2 KCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$, 11 D-glucose (equilibrated with 95% O_2 and 5% CO_2, pH 7.4).

2.2. Recordings and analysis

Whole-cell currents were recorded from the soma of granule cells according to the standard patch-clamp technique (Hamill et al., 1981), using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA; output cut-off frequency = 5 kHz) at a holding potential (V_h) of −60 mV. The signals were digitised every 50 μs and analysed off-line with Pclamp8 software (Axon Instruments).

During recording, the preparation was continuously superfused (3 ml/min) with Krebs solution to which 10 μM bicuculline ($GABA_A$ receptor antagonist) and 0.1–1 μM atropine (muscarinic ACh receptor antagonist) were added. Recordings were performed at 29 ± 2 °C.

Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter; Clark Electromedical Instruments, UK), and when filled with the intracellular solution, they had resistance between 5 and 7 MΩ. The intracellular solution contained (mM): 81 Cs_2SO_4, 4 NaCl, 2 $MgSO_4$, 0.02 $CaCl_2$, 0.1 BAPTA, 15 D-glucose, 10 HEPES, 3 ATP and 0.5 GTP, 2 QX-314, 5 Na-phosphocreatine and 25 Uml^{-1} creatine phosphokinase (pH adjusted to 7.2 with CsOH).

The series resistance (R_s) was monitored throughout the recordings, before and after each drug application ($R_s = 25.3 \pm 7.3$ MΩ, $n = 80$). Only experiments in which R_s did not change by more than 5% during the recording were considered in our analysis.

The peak amplitude, rise time and decay time constants of nicotinic whole-cell currents were determined by using Clampfit8 (from Pclamp8 software; Axon Instruments).

Glu-mediated postsynaptic currents (PSCs) were analysed using Clampfit8 and Minis Analysis Software (Synaptosoft, Leonia, NJ).

The delay in onset was defined as the time between the switching on of the valve and the time at which 10% of the peak amplitude was reached, or the peak of the first PSC in a series of PSCs induced by the agonist was reached.

Results are presented as mean ± S.D.

2.3. Agonists and antagonists application

Since bath application of nAChR agonists can dramatically desensitise the receptors, drugs were locally applied by gravity via a multibarelled pipette. The pipette tip had a diameter between 70 and 100 μm and was positioned at the surface of the slice, about 100–150 μm from the recording pipette. Solution changes were manually switched. With this system, the delay in onset of the ACh-induced whole cell current or PSCs was between 0.5 and 1.5 s.

A number of control experiments were carried out to test that ACh-induced inward currents were not an artifact due to solution exchange (not shown).

All nicotinic ACh receptor agonists were applied for 10 s every 3 min.

2.4. Drugs and toxins used

Drugs were obtained from the following sources: ACh chloride, atropine sulphate, αBTX, (−)bicuculline methobromide, choline chloride, cytisine, dihydro-β-erythroidine hydrobromide (DHβE) and tetrodotoxin (TTX) from Sigma-Aldrich (Poole, UK); D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), 7-chlorokynurenic acid (7-Cl-kyn), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and methyllycaconitine citrate (MLA) were from Tocris Cookson (Bristol, UK). Stock solutions of all drugs were made up in distilled water, stored at −20 °C and diluted in the Krebs solution at the final concentration before each use.

3. Results

3.1. Nicotinic ACh receptors activation in cerebellar granule cells

The presence of nicotinic ACh receptors in the granule cell-mossy fibre synapse at early developmental stages has been investigated in rat cerebellar slices with the patch-clamp technique. Granule cells from lobules III to VIII were

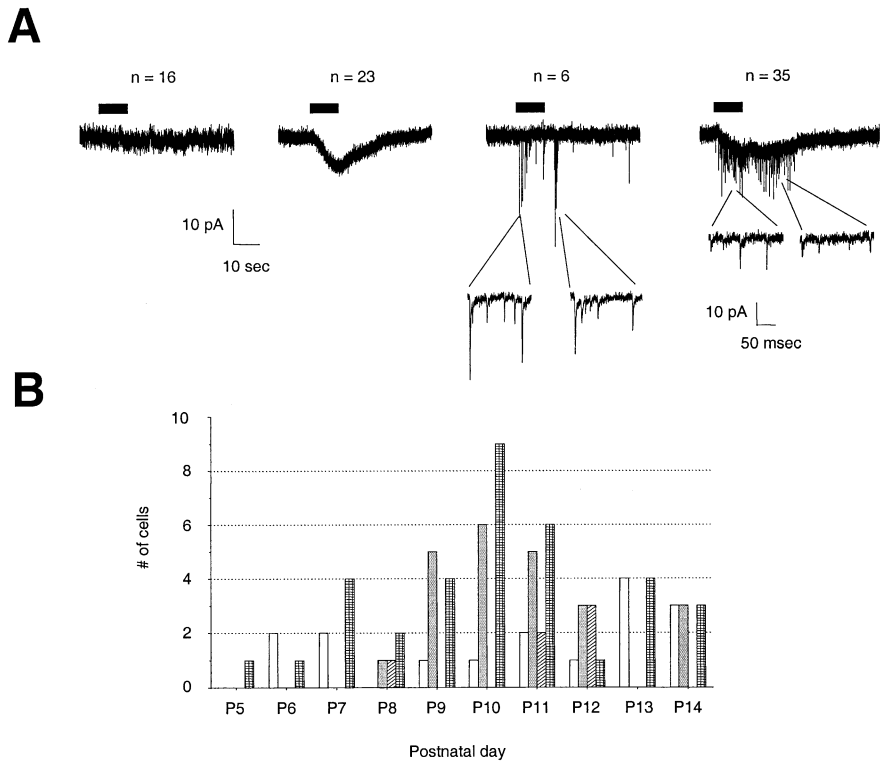

Fig. 1. ACh application induced distinct responses in cerebellar granule cells in situ. (A) A concentration of 1 mM ACh application (horizontal bar on top of the traces) could elicit a variety of effects. In 16 cells, there was no effect (example trace from cell at P11); in 23 cells, a somatic current was induced (example trace from cell at P11); in 6 cells, only PSCs were elicited (example trace from cell at P12); in 35 cells, both somatic current and PSCs were elicited (example trace from cell at P11). The insets show a magnification of the PSCs. (B) Histogram showing the different effects of ACh application at the different ages covered by this study. The white bar represents cells with no effect; the grey bar represents cells with somatic current only; the dashed bar represents cells with PSCs only; the grid bar represents cells with somatic current and PSCs.

voltage-clamped at −60 mV; 1 mM ACh (in the presence of 0.1–1 μM atropine) was locally applied for 10 s every 3 min.

In 80 cells tested, 1 mM ACh application elicited a variety of effects (Fig. 1A). No response was found in 20% of the cells, while in a further 29%, only an inward current was induced. In 7% of the cells, only PSCs were elicited, and in 35% of the cells, we observed both an inward current and PSCs.

ACh-induced inward currents were likely to be generated by activation of somatic nAChRs. The delay in onset was 1.1±0.6 s (n=46). Currents were generally small in amplitude, ranging from 3 to 14 pA (mean amplitude 5.8±3.5 pA, n=46). However, in four cells, the somatic current was about 20 pA. Both the rising and decaying phases of the ACh-induced current were fitted with a single exponential function. The activation and decaying time constant were 5.1±3.7 and 23.7±14.8 s, respectively (n=46). The total current could last up to 30–70 s, thus outlasting the time of ACh perfusion.

In a proportion of cells, ACh induced PSCs, with or without the somatic current. The delay in onset for the PSCs was 1.3±0.3 s (n=6). PSCs were usually elicited during ACh application and they could last up to 10–30 s after ACh washout. Cells then became silent again. PSCs kinetic parameters were measured in different cells. The amplitude could vary from 5 to 50–60 pA and occasionally bigger PSCs were measured. The 10–90% rise time ranged between 0.3 and 1.7 ms, while the decay time, fitted with a single exponential function, was between 6 and 10 ms. The majority of granule cells did not show spontaneous activity before ACh application. However, when some spontaneous activity was present, kinetic parameters were similar to those of PSCs induced by ACh application (not shown).

In the histogram shown in Fig. 1B, we summarise the effects of ACh application in granule cells at different ages. The probability of finding granule neurones responsive to ACh is quite high in the age window between 5 and 14 days after birth. The probability of evoking somatic current and/or PSCs appears to be the same at all the ages investigated.

In another set of experiments, 100–300 μM nicotine application elicited inward currents, PSCs or both, similar to that described for ACh (not shown). However, the proportion of cells not responding was much higher. Also, in a number of cells, we observed that ACh was able to induce a response where nicotine seemed to be ineffective. We have therefore been using ACh as a pharmacological tool to activate nicotinic receptors in our system.

3.2. Pharmacological characterisation of the nicotinic receptors in granule cells

We characterised the nicotinic receptors in our preparation using commercially available agonists and antagonists.

In 16 cells, we compared the action of 1 mM ACh to that of 10 mM choline (selective α_7 agonist). In 12 cells, choline elicited only PSCs, while ACh induced both somatic currents and release (Fig. 2A). In two cells, choline had no effect, while ACh induced a somatic current. However, in another two cells, both ACh and choline induced a somatic current (not shown). These results suggest that in the majority of granule cells, the somatic current is not α_7-mediated. More striking was the evidence that PSCs are likely to be mediated by α_7 receptor activation.

In seven experiments, we tested the effects of 100 μM cytisine (α_7 and β_4 agonist, β_2 partial agonist), and we compared it with 1 mM ACh- and 10 mM choline-induced effects. In four cells, cytisine induced only PSCs (Fig. 2B). In these same cells, choline mimicked the cytisine effect (not shown), while ACh induced both a somatic current and PSCs. In one cell, cytisine (and choline) had no effects, while ACh induced only a somatic current. Finally, in two cells, both cytisine and ACh induced a somatic current, while choline had no effects (not shown).

All together, these results strongly suggest that PSCs are induced by α_7 receptor activation, while somatic currents are mediated by non-α_7 receptor activation. The cytisine results suggest that β_2-containing receptors are more likely to be responsible for the somatic currents, but do not completely rule out the possible contribution of β_4 and α_7 receptors in a subpopulation of cells.

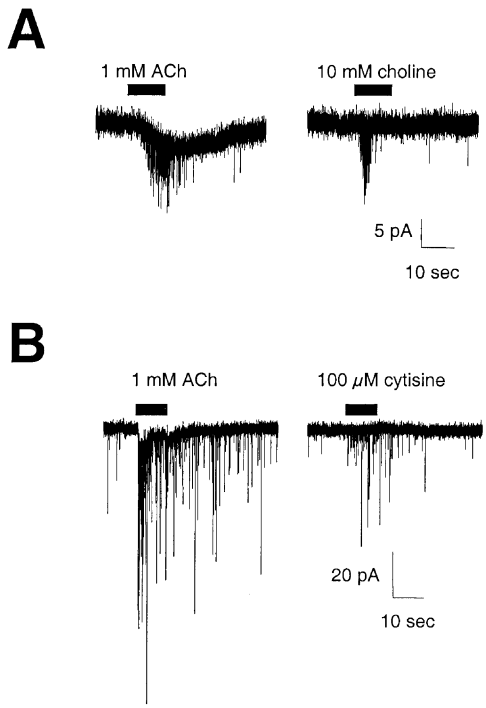

Fig. 2. Pharmacological characterisation of ACh-induced effects on granule cells by nicotinic receptor agonists application. The horizontal bar on top of all traces represents the duration of agonist application. (A) 1 mM ACh application is compared to 10 mM choline application in the same cell (cell at P11). (B) 1 mM ACh application is compared to 100 μM cytisine application in the same cell (cell at P5).

We also tried commercially available nicotinic receptor antagonists. A concentration of 100 nM MLA application blocked both the ACh-induced somatic currents and the PSCs ($n=2/2$) (Fig. 3A), thus confirming that both effects are due to nicotinic receptor activation. At this concentration, MLA blocks all nicotinic receptors nonselectively, while lower MLA concentrations should selectively block α_7 nAChRs. In three out of three cells, 1–10 nM MLA application did not affect the somatic current, thus ruling out the presence of somatic α_7 receptors, at least in these cells (Fig. 3B). As showed before, subsequent addition of 100 nM MLA completely blocked these currents. In another two cells, the inward current was reversibly blocked by 10 μM DHβE ($\alpha_4\beta_2$-preferring antagonist; Fig. 3C). These experiments with selective antagonists confirm our hypothesis that the somatic current is carried, in the majority of the cells, by $\alpha_4\beta_2$-containing receptors, with PSCs are mainly induced by α_7 receptor activation.

3.3. Nicotinic receptor modulation of Glu release

To characterise further the pharmacology of ACh-induced PSCs, we performed the following experiments. The application of a cocktail of Glu receptor antagonists (20 μM CNQX + 100 μM D-AP5 + 50 μM 7-Cl-kyn, $n=5$) completely blocked the PSCs induced by ACh (without affecting the somatic current). The effect was reversed 5–10 min after antagonist washout (Fig. 4A). This result suggests that activation of presynaptic nAChRs at the synapse between mossy fibre and granule cells can modulate Glu release. Ten to fifteen minutes of application of low concentrations of MLA (1–10 nM) completely prevented ACh-induced Glu release ($n=4$). The blocking effect was partially reversed after 15–20 min washout ($n=4$; Fig. 4B). A concentration of 100–500 nM αBTX irreversibly blocked ACh-induced Glu release ($n=2$, not shown). These results are in line with the effects of nAChRs agonists on neurotransmitter release, and strongly suggest that presynaptic α_7 receptors can modulate Glu release at this synapse. In four experiments, ACh-induced release was largely inhibited after voltage-dependent Na^+ channel block by 1 μM tetrodotoxin (TTX) (Fig. 4C). TTX sensitivity suggests that nicotinic receptors have a "preterminal" localisation at this synapse (Lena et al., 1993; Wonnacott, 1997).

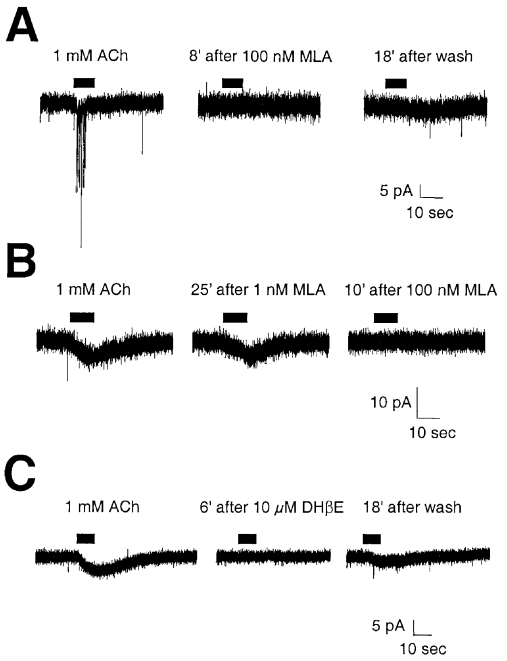

Fig. 3. Pharmacological characterisation of ACh-induced effects on granule cells by application of nicotinic receptor antagonists. The horizontal bar on top of all traces represents the duration of 1 mM ACh application. (A) ACh-induced effects (left trace) are completely and reversibly blocked (middle and right trace, respectively) by 100 nM MLA application (cell at P8). (B) ACh-induced somatic current (left trace) is not affected by 1 nM MLA application (middle trace), and it is completely blocked by 100 nM MLA application (right trace) (cell at P8). (C) ACh-induced somatic current is completely and reversibly blocked (middle and right trace, respectively) by 10 μM DHβE application (cell at P12).

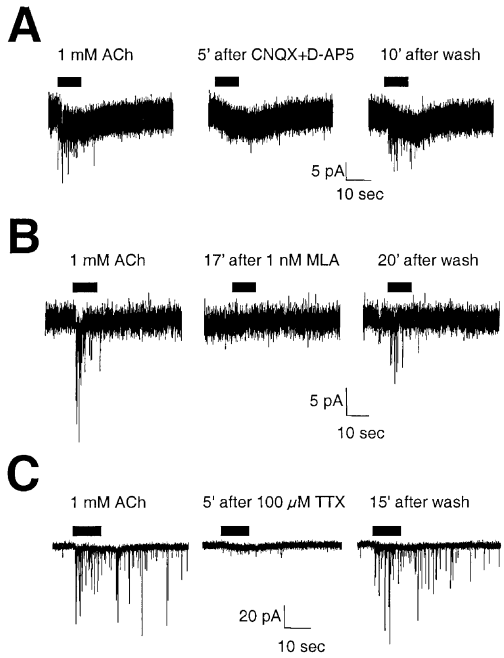

Fig. 4. ACh-induced enhancement of Glu release at the mossy fibre–granule cell synapse. The horizontal bar on top of all traces represents the duration of 1 mM ACh application. (A) ACh-induced PSCs (left trace) are completely and reversibly blocked (middle and right trace, respectively) by a cocktail of Glu receptor antagonists (20 μM CNQX + 100 μM D-AP5 + 50 μM 7-Cl-kyn) (cell at P13). (B) ACh-induced PSCs (left trace) are completely blocked by 1 nM MLA (middle trace). The block is partially reversed after long washout (right trace) (cell at P12). (C) ACh-induced PSCs (left trace) are almost completely and reversibly blocked by 100 μM TTX application (middle and right trace, respectively) (cell at P6).

4. Discussion

The present study represents the first electrophysiological evidence in cerebellar slices for activation of nicotinic

receptors in granule cells and modulation of Glu release by activation of presynaptic α_7 nicotinic receptors. The presence of nicotinic receptors in the IGL of the cerebellar cortex has been investigated by patching granule cells scattered in lobules III to VIII. Our results suggest that the probability of finding functional nicotinic receptors in granule cells within the cerebellar cortex at early developmental stages is quite high.

4.1. Somatic nicotinic currents in granule cells

A significant proportion of granule cells expresses nicotinic receptors on their soma or dendrites. These nAChRs give rise to a rather small and slow current that persists longer than ACh perfusion. The pharmacological profile indicates that in most granule cells, this current is mediated by $\alpha_4\beta_2$-containing receptors. However, we do not rule out the possibility that other receptor subunit combinations may be present. In fact, experiments with selective agonists such as choline and cytisine indicate that α_7, and possibly β_4, subunits may also be present in a number of granule cells.

Autoradiographic studies show that in the rat cerebellar cortex, [^{3}H]nicotine, [^{3}H]ACh and [^{3}H]cytisine binding sites are concentrated in the IGL and the deep cerebellar nuclei, while the molecular layer and the white matter display no significant binding (Clarke et al., 1985; Naeff et al., 1992; Happe et al., 1994; Jaarsma et al., 1997). The density of labelling in the cerebellar cortex is moderate compared to other cortical areas; labelling appears at the cerebellar anlage at embryonic day 15 and is still present at adulthood (Naeff et al., 1992). A number of in situ hybridisation and immunocytochemical studies have shown that several nicotinic receptor subunits are expressed in rat cerebellum during development and into the adulthood.

Granule cells have been found to express mRNA for α_4 and β_2 receptor subunits, and most granule cells are stained by monoclonal antibodies against those two subunits. The pattern of α_4 and β_2 immunoreactivity is very similar to that of in situ hybridisation (Wada et al., 1989; Hill et al., 1993; Nakayama et al., 1997, 1998). Within the cerebellar cortex, α_4 and β_2 mRNA signals are also detected quite strongly in the Purkinje cell soma and dendrites, and in several neurones in deep nuclei (Hill et al., 1993; Nakayama et al., 1998). Immunocytochemical localisation of the α_4 subunit at the EM level shows that the α_4 subunit is present in the plasma membrane on the soma of granule cells (Nakayama et al., 1997). α_4 mRNA levels in the whole cerebellar cortex have been shown to undergo postnatal changes. The transcript level is quite high during the first two postnatal weeks, and decreases thereafter to adult levels. However, β_2 transcript levels remain fairly stable during development (Zhang et al., 1998). Recently, the presence of functional $\alpha_4\beta_2$ -containing receptors has been shown in rat cultured granule cells by using microphysiometry (Zago and Markus, 2000).

A series of in situ hybridisation studies has shown that α_3 and β_4 mRNA can be detected in the developing cerebellum (Winzer-Serhan and Leslie, 1997). Both gene transcripts are expressed during embryonic development and expression of both subunit declines after birth (Zoli et al., 1995; Morley, 1997; Winzer-Serhan and Leslie, 1997). Low overall β_4 hybridisation was detectable in the IGL at P5, increasing to moderate intensity during the second postnatal week. Scattered cells had strong hybridisation signal in the IGL and Purkinje cell layer, starting at P11 and into adulthood. α_3 mRNA expression was first detected at P7, but limited to scattered cells in the IGL and to some Purkinje cells, with no detectable signal in the rest of cerebellum. The same low expression, restricted to few cells, was maintained up to adult age. α_3 mRNA is expressed in the early postnatal EGL (Zoli et al., 1995; Morley, 1997; Opanashuk et al., 2001). Weak α_3 mRNA levels within the IGL have been detected also by Zhang et al. (1998) at first stages of development and by Wada et al. (1989) in adult rats.

Although very weak density of [^{125}I]αBTX labelling and very weak hybridisation signals for α_7 mRNA have been reported by some authors (Clarke et al., 1985; Seguela et al., 1993), detectable levels of [^{3}H]αBTX binding and α_7 mRNA have been detected in the cerebellar cortex during development (Zhang et al., 1998). Maximal levels of αBTX binding sites were achieved during the first postnatal week, with adult levels reached at P14. Similarly, α_7 mRNA expression was 10-fold higher at P1 than at adulthood and rapidly decreased to adult levels at P14. Immunohistochemistry experiments suggest that α_7 immunoreactivity is widely distributed in the cerebellar cortex (Swanson et al., 1987; Dominguez Del Toro et al., 1994, 1997). High levels of expression were found in Purkinje cells, while granule cells in the IGL were mostly unlabelled. The IGL contained very few, scattered positive neurones, interpreted by the authors as Golgi cells. A similar pattern of α_7 labelling was obtained at the immunogold EM level (Caruncho et al., 1997). In this study, the gold immunolabelling for α_7 was greater in Purkinje neurones and neighbouring processes than in molecular or granular layer. In the IGL, immunolabelling was not found in the granule cell soma, but in granule cell dendrites, mostly at perisynaptic sites.

Furthermore, early expression of nAChRs during in vitro development has been shown in mouse cerebellar granule cells primary cultures (Didier et al., 1995). Autoradiography and immunocytochemical experiments showed the presence of α_4, α_7 and β_2 subunits in these mouse cultured granule neurones. Most, but not all, cells showing granule cell morphology appear to express α_4 and β_2 subunits; some of them appear to express α_7 subunit on the soma or neurites. PCR experiments in the same preparation revealed the additional presence of α_3, α_5 and β_4 mRNA after 10 DIV. No signals were detected for α_2 or β_3. No signals were detected for any nAChR subunit in cerebellar glial cells in primary culture.

Although all the reported studies show that cerebellar granule cells can express nicotinic receptors, there is lack of evidence on the co-expression and the co-assembly of

different nAChRs subunits in the same cellular type within the cerebellar cortex. Unfortunately, the lack of subunit-selective compounds did not allow us to make a detailed pharmacological characterisation of somatic nicotinic receptors on the granule cells. Our electrophysiological data suggest the presence of somatic $\alpha_4\beta_2$ receptors, although some cells express α_7 or β_4-containing receptors. Moreover, an attempt to classify different receptor subunit combinations by measuring the current kinetics (as has been done in hippocampal slices; Sudweeks and Yakel, 2000) is very difficult. Distortion in the kinetics could be caused by slow drug diffusion into the depth of the slice, and cannot be excluded that receptors with a given subunit combination in native preparations might have different kinetics from the same subunit combination studied in recombinant systems.

Somatic nicotinic receptor activation could cause sufficient depolarization to trigger action potentials. This has, in fact, been demonstrated in CA1 interneurons in hippocampal slices (Alkondon et al., 1999; Ji and Dani 2000). The functional role of somatic nicotinic receptors in granule cells is currently under investigation.

4.2. Preterminal nicotinic receptors

Even more interesting was our finding that in a significant proportion of granule cells, we could detect Glu release from the presynaptic terminal induced by nicotinic receptor activation. The majority of cells, in which we detected ACh-induced Glu release, did not show any spontaneous activity at all. In few cells, some spontaneous PSCs were detected before ACh application. However, the frequency of these events was much lower than during ACh application (not shown). ACh-induced Glu release could be mimicked by drugs known to act as α_7 receptor agonists (i.e., choline and cytisine) and could be blocked by αBTX or low concentrations of MLA. Therefore, we believe that Glu release enhancement is mediated by activation of presynaptic α_7 nicotinic receptors. The ability of TTX to attenuate ACh-induced Glu release suggests that the α_7 receptors responsible for this effect are present in somatodendritic regions and/or in preterminal sites of axons (Lena et al., 1993; Wonnacott, 1997).

In line with our findings, some data have been recently presented on [^{3}H]glutamate release from adult rat cerebellar slices induced by stimulation of nAChR activation (Renó and Markus, 2000). Nicotine-induced [^{3}H]glutamate release could be prevented by 100 nM αBTX, by 1 μM TTX and by Glu receptor antagonists.

There is evidence for the presence of cholinergic fibre terminals in the rat cerebellar cortex. Immunohistochemistry studies show that a number of mossy fibres immunoreactive to ChAT are present in all lobules (Ojima et al., 1989; Jaarsma et al., 1996). Other fibres, which are distinct from mossy fibres and Golgi cell axons and express ChAT reactivity in axon-like structures, are present in all cortical layers throughout all lobules (Ojima et al., 1989; Barmack et al., 1992a,b).

Various histochemistry studies have shown that AChE is present in mossy fibre rosettes in the vestibulocerebellum (Silver, 1967). AChE-positive mossy fibres appear to be situated in close proximity of the UBCs (Harris et al., 1993). [^{125}I]αBTX binding studies have shown small patches (with the size of glomeruli) of intense labelling in the IGL of rat cerebellum (Frostholm and Rotter, 1986). The distribution of [^{125}I]αBTX binding sites largely corresponds to that of ChAT-immunoreactive mossy fibres. These observations suggest that αBTX binding sites may be pre- or postsynaptic to ChAT-immunoreactive mossy fibres, but they could also be associated with noncholinergic mossy fibre terminals.

The role of cholinergic mechanisms in synaptic transmission within the cerebellar cortex has not yet been elucidated with electrophysiological methods. Most data so far are inconsistent with the idea that ACh mediates fast synaptic transmission within the cerebellar cortex (Crepel and Dhanjal, 1982; Konnerth et al., 1990; Barbour, 1993; D'Angelo et al., 1993; Rossi et al., 1995). However, cholinergic mossy fibre responses may have been missed out because only a small proportion of mossy fibres is cholinergic. Our data show that in a reasonable proportion of granule cells at an early developmental stage, ACh interacts with glutamatergic transmission enhancing glutamate release. The TTX sensitivity of this effect suggests that the nicotinic receptors involved (likely α_7 receptors, from our study) are located either preterminally in mossy fibre terminals or on somato/dendritic areas of interacting cells. One possibility is that nicotinic receptors are present on the UBC, and their activation leads to action potential generation and Glu release on the postsynaptic granule cell. UBCs in acute slices have been reported to respond to ACh application with an inward current (Rossi et al., 1995), the identity of which has not been pharmacologically tested. Furthermore, [^{125}I]αBTX binding and ChAT reactivity distribution have been shown to correspond to the distribution of UBCs at least in the vestibulocerebellum (Jaarsma et al., 1997). Alternatively, α_7 receptors could be located at preterminal levels along the axon. ACh could be co-released with Glu at the mossy fibre terminal and activate autoreceptors. In fact, various studies suggest that ACh is co-localised with amino acid neurotransmitters (Caffé et al., 1996). ChAT-positive fibres, distinct from mossy fibres, could also be an alternative source of ACh.

Nicotinic receptor-mediated modulation of release in the cerebellar system represents an intriguing field for future studies. The highest proportion of ChAT-positive fibres is detected in the vestibulocerebellum; this part of the cerebellum receives large vestibular primary and secondary afferent inputs. Therefore, some authors have already proposed that the cholinergic pathway might be particularly important in regulating the sensitivity to vestibular information (Barmack et al., 1992a,b). However, ChAT fibres with

different morphologies have been found, even if in lower density, throughout the cerebellar cortex. It is possible that different morphologies reflect different anatomical origins or different postsynaptic receptors in the target neurone. Thus, cholinergic modulation of transmitter release could represent a fine mechanism for processing the information coming from different specific pathways.

Moreover, the developmental appearance and localisation of αBTX binding sites in the vestibulocerebellum have been shown to be correlated in time with the arrival of the mossy fibres originating in the vestibular nuclei and the VIIIth nerve (Frostholm and Rotter, 1986). Within the same time window, the rat becomes able to walk forward and air-righting reflexes appear. These observations led the authors to hypothesise that αBTX-sensitive receptors could have a role in the formation of stable vestibulocerebellar connections during development.

At the age covered by our study, the majority of the cells show immature electrophysiological properties (D'Angelo et al., 1994, 1997). The enhancement of Glu release by presynaptic nicotinic receptor activation could be a mechanism for synapse consolidation. The effect of nAChR activation on the cell excitability and of ACh-induced Glu release enhancement on synaptic transmission is currently under investigation.

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