Properties of Nicotinic Receptors Underlying Renshaw Cell Excitation by α-Motor Neurons in Neonatal Rat Spinal Cord

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Dourado, Michelle and Peter B. Sargent. Properties of Nicotinic Receptors Underlying Renshaw Cell Excitation by α-Motor Neurons in Neonatal Rat Spinal Cord. J Neurophysiol 87: 3117–3125, 2002; 10.1152/jn.00745.2001. We used anatomical and physiological approaches to characterize nicotinic receptors (AChRs) on Renshaw cells of the neonatal rat spinal cord. Confocal imaging of Renshaw cells, identified by their characteristic pattern of gephyrin immunoreactivity, revealed that these neurons are immuno-positive for the α4 and β2 AChR subunits but not for the α7 subunit. We used whole cell recording in spinal cord slices to characterize synaptic transmission from α-motor neurons to Renshaw cells, which could be identified pharmacologically by the sensitivity of transmission to d-tubocurarine. α-Motor neuron-to-Renshaw cell synapses were blocked by 10 μM dihydro-β-erythroidine (dHβE), but not 50 nM methyllycaconitine (MLA), a selective α7 antagonist. These findings support a role for αβ2-like AChRs, but not α7 AChRs, in rapid excitatory transmission between α-motor neurons and Renshaw cells in rat spinal cord.

INTRODUCTION

Nicotinic acetylcholine receptors (AChRs) are a diverse family of pentameric proteins assembled from more than a dozen different subunits. Individual neurons may express several types, and the extent of diversity in AChR types among neurons is substantial. This diversity has the potential of allowing AChRs to serve several functions. Currently, nicotinic receptors in the CNS are thought to be important for memory, cognition, regulation of excitability, plasticity, and development (reviewed in Broide and Leslie 1999; Clementi et al. 2000; Cordero-Erausquin et al. 2000; Dani 2001; Jones et al. 1999; Levin and Simon 1998; Paterson and Nordberg 2000; Vizi and Lendvai 1999).

Neuronal AChRs were identified very early as the ionotropic receptors underlying rapid excitatory transmission at ganglionic synapses in the peripheral nervous system. While neuronal AChRs are expressed widely in the brain and spinal cord, their chief functional role in the CNS is not as the principal excitatory transmitter. The discovery that central nicotinic AChRs are often located presynaptically has led to the suggestion that they modulate synaptic transmission (reviewed in Wonnacott 1997), and recently much interest has been focused on “nonclassical” roles for central nicotinic receptors. We have chosen to return to the first-recognized function for central nicotinic receptors as mediators of rapid excitatory synaptic transmission.

The first demonstration that central nicotinic receptors can mediate fast excitatory synaptic transmission was made on Renshaw cells of the cat spinal cord (Curtis and Ryall 1966a,b; Eccles et al. 1954; Ryall 1981). Renshaw cells are inhibitory interneurons that fire bursts of action potentials in response to motor neuron stimulation. Activation of Renshaw cells depresses α-motor neuron firing as a result of chloride-dependent inhibitory currents induced by both GABA and glycine (Schneider and Fyffe 1992) possibly released by the same cell (Jonas et al. 1998). Renshaw cells remain one of the few examples in the CNS where nicotinic AChRs are known to underlie rapid synaptic transmission (see also Alkondon et al. 1998; Frazier et al. 1998; Hefft et al. 1999; Nong et al. 1999; Roerig et al. 1997; Zhang et al. 1993). Most of the functional studies of nicotinic receptors on Renshaw cells were done 30–40 yr ago in the intact cat spinal cord using a combination of extracellular and intracellular (sharp electrode) recording techniques. We wished to extend these studies to rat spinal cord, where transmission is also nicotinic (Headley et al. 1975), by using whole cell recording in slices and by examining the sensitivity of α-motor neuron-Renshaw cell transmission with AChR subunit-specific antagonists.

One useful approach to studying native AChRs is to compare their functional properties with AChRs of known subunit composition expressed heterologously. Expressed AChRs fall into two broad classes: hetero-oligomeric receptors containing both alpha (α2–α6) and beta (β2–β4) subunits, and homomorphic receptors (α7–α10) (see Yu and Role 1998), of which the α7 AChR is the only known member with widespread distribution in brain. α7 AChRs are functionally different from alpha-beta receptors; for example, they have a greater calcium permeability and can be gated either by ACh or by choline. cDNA hybridization studies (Wada et al. 1989) in rat CNS suggest that both α7- and non-α7-containing receptors are expressed in the spinal cord. The immediate purpose of this study was to determine whether nicotinic receptors underlying excitatory transmission onto Renshaw cells are α7 or non-α7. Our results show that Renshaw cells display α4-like immunoreactivity (α4-LI) but not α7-LI and that excitatory postsynaptic currents (EPSCs) evoked in Renshaw cells by antidiromic stimulation of α-motor neurons are sensitive to low concentrations of dHβE but not MLA. These findings suggest that excitatory synaptic transmission between α-motor neurons and Renshaw cells is mediated by a non-α7 AChR, possibly α4β2.
METHODS

Immunolabeling

Spinal tissue was prepared for immunocytochemistry using standard procedures (Alvarez et al. 1999). Briefly, 2-wk-old Sprague-Dawley rats were deeply anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused transcardially with warm (37°C) PBS followed by ice-cold PBS containing 1–2% formaldehyde. The spinal cord was dissected out and placed in freshly prepared 1% formaldehyde in PBS for 1 h at room temperature. The tissue was then washed in PBS, placed in 30% sucrose in PBS at 4°C overnight, and mounted in TissueTek (Triangle Biomedical Sciences, Durham, NC). Transverse sections (30 μm) were cut on a cryostat and mounted on gelatin-coated slides.

The following monoclonal antibodies (mAbs) were tested: 299 (anti-rat α4), 270 (anti-rat β2), 306 and 307 (anti-mouse-α7), 318 and 319 (anti-rat α7), and 35 and 210 (assumed to be anti-α1, α3, and α5). All of these antibodies stained sets of neurons in either rat brain or chicken ciliary ganglia. The following goat polyclonal anti-AChR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-α3 (A-20), anti-α4 (A-20), anti-α7 (SC-20), and anti-β2 (C-20). Only one of these four stained sets of neurons in slices of rat spinal cord or brain (anti-α4), and negative results from the remaining three are not considered to be meaningful. Anti-gephyrin antibodies were generously provided or were purchased from Alexis Biochemicals (San Diego, CA). Alexa 488-labeled α-bungarotoxin (α-BuTx) was purchased from Molecular Probes (Eugene, OR).

Tissue sections were preincubated in a blocking solution of 5% normal donkey serum in PBS for 1 h at room temperature. Primary antibodies were used at concentrations of 5–50 nM. When primary antibodies against rat subunits were tested, we preincubated the sections with goat anti-rat antibody IgG to block nonspecific labeling by anti-rat labeled secondary antibodies. When permeabilization was required, the blocking solution contained 0.1% (vol/vol) Triton X-100. Sections were washed with PBS following block and incubated with primary antibody in PBS overnight at 4°C (Fig. 1A) followed by Cy3- (Amersham Life Sciences, Pittsburgh, PA) or Alexa 488-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 1–2 h at room temperature. All labeled anti-rat and anti-mouse antibodies were obtained from Jackson Immunoresearch (West Grove, PA) and were predissorbed by the manufacturer to remove nonspecific cross-reactivity. Texas Red-avidin was purchased from Molecular Probes (Eugene, OR). Fluorescent labeling was visualized using a confocal imaging system (BioRad 1024), as described previously (Wilson Horch and Sargent 1995).

Spinal cord slice preparation

Six- to 10-day-old Sprague-Dawley rats were anesthetized with 50 mg/kg sodium pentobarbital (ip) and decapitated. A section of the lumbar spinal cord was dissected in ice cold low-sodium Ringer containing (in mM) 240 sucrose, 2.5 KCl, 1 CaCl₂, 3 MgCl₂, 10 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄ at pH 7.4 when bubbled with carbogen gas (95% O₂/5% CO₂). The dissected lumbar cord was immobilized in low-melting point agarose and immersed in ice-cold Ringer solution for sectioning. Slices of 300–350 μm thickness were obtained by using a Lancer Vibratome Series 1000 and were incubated in extracellular recording solution at room temperature while being continuously bubbled with carbogen gas. Extracellular recording saline contained (in mM) 125 NaCl, 2.5 KCl, 3 MgCl₂, 2 CaCl₂, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄ at pH 7.4 when bubbled with carbogen gas. Cells in the slices maintained in this condition remained viable for approximately 5–6 h.

Electrophysiological recording

Lumbar L3-L5 slices were immobilized in a recording chamber by using a fine nylon mesh and were continuously perfused with extracellular recording solution at 2 ml/min. Neurons in the slice were visualized using infrared differential interference contrast optics produced by an Olympus BX50WI microscope with a 40× water-immersion lens. Patch pipettes for whole cell recording were pulled from 1.2 mm diameter borosilicate glass using a P-87 Flaming-Brown micropipette puller (Sutter Instrument Co., Novato, CA). Initial pipette resistances were 3–5 MΩ.

Whole cell patch clamp recordings were made at room temperature (21–23°C). A drug cocktail consisting of 10 μM 6-cyano-7-nitroquinolinole-2,3-dione (CNQX), 10 μM D,L-2-amino-5-phosphonovaleric acid (APV), 5 μM bicuculline and 1 μM atropine was used to isolate nicotinic currents. All drugs were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Chloride currents were minimized by using a low-chloride intracellular saline, consisting of (in mM) 125 Cs-gluc onate, 15 CsCl, 10 HEPES, 2 EGTA, 2 ATP, 0.2 GTP, and 0.1 glucose, pH 7.3. QX-314 at 0.5 mM was added to the pipette solution to decrease voltage-activated sodium channel activity. Cells were selected for recording from lumbar interneurons in the ventral part of Rexed’s lamina VII, where Renshaw cell density is highest (Fyffe 1990; Geiman et al. 2000; Thomas and Wilson 1965). Excitatory postsynaptic currents (EPSCs) were evoked in interneurons by means of a 0.05 ms stimulus which was delivered through a bipolar electrode (FHC Inc., Bowdoinham, ME) that was placed over the clearly visible motor nerve tracts at the ventral margin of the cord. While the intent of this procedure was to stimulate selectively α-motor neuron axons, we clearly stimulated other white matter axons as well, since the majority of neurons responded to stimulation with glutamatergic EPSCs, not nicotinic ones (see RESULTS).

Currents were filtered at 2 kHz (Axopatch 200B) and digitized at 10 kHz. Data acquisition and analysis was performed using pClamp 7 (Axon Instruments, Union City, CA) and Origin (OriginLab, Northampton, MA) software.

RESULTS

Renshaw cells display α4-like immunoreactivity

To distinguish Renshaw cells from other neurons, we used an antibody to glycine, a glycinergic receptor clustering protein (Kneussel and Betz 2000). Renshaw cells in rats display stronger glycine immunoreactivity than other interneurons with a distinctive staining pattern (Alvarez et al. 1997). Figure 1A1 shows a low power view of the ventral horn of the spinal cord visualized for glycine. Glycinergic immunoreactivity is present widely but is especially pronounced on two neurons, identified as Renshaw cells, whose cell bodies and proximal dendrites are outlined by the label. Both of these neurons are stained by an antibody to neuronal AChR subunits (Fig. 1, A2 and A3).

Renshaw cells were immunoreactive for the α4 AChR subunit but not for the α7 AChR subunit. In Fig. 1B, a Renshaw cell, having characteristic glycine immunoreactivity (Fig. 1B1), also stains with anti-AChR mAb 299 (Fig. 1, B2 and B3), which is selective for rat α4 subunits (Whiting and Lindstrom 1988). The immunoreactivity is distributed widely within the cell body with no obvious clustering on the surface and with considerably less staining in the dendrites than in the cell body. In Fig. 1C a Renshaw cell from a different section is shown not to react with anti-AChR mAb 319 (Fig. 1C2), which is selective for the rat α7 subunit (Schoepfer et al. 1990). In parallel experiments, mAb 319 did label subsets of neurons...
within the brain (not shown; see Dominguez del Toro et al. 1994). Anti-α7 mAbs 307 and 318 (Schoepfer et al. 1990) also failed to label Renshaw cells or any other cells within spinal cord slices. However, anti-α7 mAb 306 (Schoepfer et al. 1990) labeled all cell bodies within the gray matter and, as well, cell bodies and fibers in the white matter (not shown). The finding that only one of four anti-α7 mAbs was reactive in the spinal cord raises the possibility that the reactivity of mAb 306 does not result from its binding to an α7-like component. We also examined staining of spinal cord sections with α-BuTx, which is specific among known CNS AChR subunits for α7. We found no detectable staining with Alexa 488-α-BuTx to either fixed or unfixed spinal cord slices (not shown). In parallel experiments, Alexa 488-α-BuTx labeled endplates in frozen sections of chick or rat muscle and also selected populations of neurons in fixed slices from rat brain (not shown). We were therefore unable to confirm the suggestion, based on the pattern of mAb 306 staining, that α7-LI is present widely in neonatal rat spinal cord. Finally, we found that neither mAb 35 (Tzartos et al. 1982) nor mAb 210 (Whiting and Lindstrom 1986), which recognize both α3 and α5 subunits in chicken (Conroy et al. 1992) and human (Wang et al. 1996), labeled Renshaw cells in the spinal cord, but they did label chicken ciliary ganglion neurons in sections run in parallel (not shown, Wilson Horch and Sargent 1995).

The presence of α4-like immunoreactivity (α4-LI) within Renshaw cells was confirmed by showing that these cells were stained by two anti-α4 antibodies with different specifics: mAb 299 (Whiting and Lindstrom 1988), which is directed against an extracellular epitope, and polyclonal antibody A-20, which is immunoreactive for α4. Scale bars represent 50 μm (A) and 17 μm (B and C). No modification was made in look-up tables during processing and printing.
**Renshaw cells display nicotinic EPSCs**

We recorded from interneurons located along the ventral margin of the ventral horn in search of Renshaw cells, which should display nicotinic currents in response to ventral root stimulation. The majority of neurons from which we recorded, responded to stimulation with short latency EPSCs, but most of these neurons (>90%) displayed EPSCs that were glutamatergic in character (Fig. 3). These currents appeared reliably and with short latency, had fast rise and decay times, and were reversibly blocked by 10 μM CNQX (Fig. 3). CNQX (10 μM) did not partially block EPSCs in any of the cells we examined: it either blocked currents nearly completely or not at all.

A small fraction of the cells from which we recorded EPSCs (8 of 150, 6%) displayed currents that were insensitive to CNQX but were sensitive instead to the nicotinic antagonist d-tubocurarine (10 μM d-TC, Fig. 4A). These currents had a latency of 2.2 ± 0.6 ms and a 10–90% rise time of 1.0 ± 0.2 ms (n = 8 cells). The averaged current obtained from control traces in Fig. 4A decayed with a single exponential function having a time constant of 4.9 ms (Fig. 4B). Overall, nicotinic
Nicotinic EPSCs elicited from Renshaw cells were typically small, on the order of 100 pA in amplitude, and displayed noticeable amplitude fluctuations when elicited at low frequency (0.2 Hz) and fixed stimulus strength. This fluctuation is illustrated in Fig. 5A (left) for a set of EPSCs to a stimulus just sufficient to elicit a response. At a stimulus intensity of 31 or 40 V, about 20% of the responses were “failures” and the average response, including failures, was about −25 pA. The failures might represent instances where no quanta were released from an afferent having a single release site; alternatively, they might represent instances where an action potential failed to reach the terminal. At stimulus intensities of 50 V and 70 V, the average response was larger and amplitude fluctuation was pronounced (Fig. 5A). Overall, the relationship between stimulus intensity and peak response amplitude (Fig. 5B) suggests that Renshaw cells are multiply innervated, which is consistent with the findings of Ryall (1981) for innervation of Renshaw cells in cat. At constant stimulus strength, we noted fluctuations in response latency as well as in amplitude (Fig. 5A, inset). This fluctuation cannot be explained by trial-to-trial variations in the number of released quanta; it may instead result from trial-to-trial variations in the number of afferents that fire. Within a set of responses at constant stimulus intensity, larger responses had a briefer latency ($P < 0.05$ by Pearson product moment correlation, $n = 4$), which suggests that individual afferents elicit responses with different latencies and that the latency for the response to activation of a population of these afferents will be briefer, on average, if more afferents are activated. If this interpretation is correct, then the average response latency should be briefer when we recruit more afferents by raising the stimulus strength, and this was observed (Fig. 5C). These results suggest that at least one source of fluctuation in response amplitude and latency is the trial-to-trial variation in the number of α-motor neuron collaterals that “fire” in response to stimulation.

We examined the sensitivity of α-motor neuron-Renshaw cell synaptic currents to nicotinic antagonists to make inferences about the subunit composition of nicotinic receptors that underlie these currents. $d$-TC, while useful as a diagnostic tool...
for detecting nicotinic currents, is not sufficiently selective to discriminate among different AChRs. However, we found clear results from two more selective antagonists, dHβE and MLA. dHβE at 10 μM reversibly blocked most of the α-motor neuron-Renshaw cell EPSC, as is illustrated for one cell in Fig. 6A. Overall, 10 μM dHβE reduced peak EPSCs by 87 ± 11% (n = 5 cells, P < 0.0001). We were unable to block significantly more of the EPSC with a 10-fold higher concentration of dHβE or with 10 μM d-TC (P > 0.5 by ANOVA). dHβE is relatively selective among expressed AChRs for those containing α4 subunits (Gopalakrishnan et al. 1996), which suggests that α4-containing receptors mediate Renshaw cell responses to α-motor neuron stimulation. MLA at a concentration of 50 nM, which selectively blocks α7 containing receptors (Palma et al. 1996), had no effect on α-motor neuron-Renshaw EPSCs (Fig. 6B, P > 0.5 by students’ t-test; similar results were obtained in two other experiments).

We studied the morphology of cells having nicotinic EPSCs by filling recording pipettes with neurobiotin, and visualizing neurobiotin-filled cells with Texas Red avidin after fixation. Cells displaying dHβE-sensitive currents were situated in layer VII of the lumbar spinal cord and had fusiform- or stellate-shaped cell bodies 15–25 μm in diameter with large dendrites that ran along the boundary between the gray and white matter orthogonal to motor axons as they leave the ventral horn (Fig. 7). These features are characteristic of Renshaw cells (Fyffe 1990; Lagerback and Kellerth 1985). We attempted to visualize gephyrin immunoreactivity in these cells, to confirm that they display the staining pattern characteristic of Renshaw cells (Alvarez et al. 1997). Unfortunately, gephyrin staining was not successful on cells from which whole cell recordings were made, even though neighboring cells in the slice were often gephyrin-immunopositive (not shown; see also Oleskevich et al. 1999).

**DISCUSSION**

With the aid of infra-red differential interference contrast optics in spinal cord slices, we have confirmed earlier findings, based largely on extracellular recordings, that Renshaw cell activation by α-motor neurons is nicotinic. We found also that the synaptic currents elicited from Renshaw cells by stimulation of α-motor neurons are reversibly blocked by dHβE, but not MLA, and that Renshaw cells display AChR α4 and β2 immunoreactivity. These results are consistent with the hypothesis that an α4β2-containing AChR underlies transmission between α-motor neuron collaterals and Renshaw cells.

Double labeling with anti-gephyrin antibodies, which produce a highly characteristic pattern and intensity of staining on Renshaw cells, shows that α4-LI is present there but also on other interneurons in the ventral horn and on α-motor neurons as well. Since we used two different antibodies with different specificity, one against the C-terminus and one an extracellular epitope, we suspect strongly that the staining is evidence for the presence of α4-containing nAChRs. Staining with the anti-β2 mAb 270 suggests that β2-LI is present on the same wide spectrum of neurons that express α4-LI. Because β2 subunits form functional AChRs with α4 subunits (Flores et al. 1992; Whiting and Lindstrom 1986), we accept mAb 270 staining as evidence for the presence of β2 subunits on these neurons, although the result could not be verified with a second antibody to β2. Relatively little staining was noted at the neuronal surface, where AChRs must be located if they are to underlie synaptic transmission from motor neuron collaterals. This lack of significant staining may be a consequence of low sensitivity of the fluorescent technique used to visualize AChRs and/or to steric hindrance resulting from the presence of AChR-associated proteins (see Sorenson et al. 1998). The presence of α4-LI and β2-LI on α-motor neurons is consistent with recent results demonstrating that α-motor neurons express functional AChRs and that they are contacted by cholinergic boutons (Ferreira et al. 2001; Messi et al. 1997; Zaninetti et al. 1999).

A majority of interneurons along the ventral margin of the ventral horn responded to stimulation at the ventral surface of the spinal cord with short latency EPSCs. Of these, only 6% had currents with nicotinic pharmacology. This frequency is
similar to the proportion of ventral horn interneurons found by Oleskevich et al. (1999) that had nicotinic mEPSCs (2 of 26 neurons). Carr et al. (1998) have reported that in adult rat there are only about 20 Renshaw cells for every 100 μm of rat lumbar spinal cord. The density of Renshaw cells is greater in neonatal rat than in adult (F. J. Alvarez, personal communication), but Renshaw cells nonetheless account for only a modest fraction of the interneurons in the ventral horn. It is striking that virtually all ventral horn neurons that we encountered had inward currents either largely glutamatergic or nicotinic in character: this finding suggests that Renshaw cells receive few glutamatergic inputs that can be activated by stimulation at the ventral margin of the cord.

The pharmacological sensitivity of the nicotinic synaptic currents recorded from Renshaw cells allows us to postulate which AChR(s) underlie these currents. dHβE at 10 μM blocked nearly 90% of the synaptic current, and the fraction of current blocked at 100 μM was not significantly different. Moreover, we were unable to block significantly more of the current than this with 10 μM d-TC. Thus 10 μM dHβE blocks virtually all of the nicotinic current elicited from Renshaw cells, which suggests that the IC_{50} for dHβE is likely to be at or below 1 μM. dHβE is a competitive antagonist of nicotinic receptors whose specificity has been tested on a number of expressed and native AChRs. Among expressed human AChRs, Chavez-Noriega et al. (1997) found that sensitivity to dHβE was greatest for α4β4 (K_d ~ 0.1 μM), and αβ2 receptors (0.1 μM; see also Sabey et al. 1999), less so for α2β2 and α3β2 receptors (K_d ~ 1 μM; see also Harvey and Luette 1996), and least for α2β4 and α3β4 receptors (K_d > 1 μM; see also Harvey and Luette 1996). We cannot say whether the native AChR(s) that underlie synaptic responses of Renshaw cells correspond to expressed AChRs tested for sensitivity that they attribute to native AChR(s) that underlie synaptic responses of Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells. We cannot say whether the IC_{50} for dHβE virtually all of the nicotinic current elicited from Renshaw cells.

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