0026-895X/95/020248-10$03.00/0

MOLECULAR PHARMACOLOGY, 47:248-257, 1995.

Activation of Muscarinic Cholinergic Receptors Blocks Apoptosis of Cultured Cerebellar Granule Neurons

GUANG-MEI YAN, SUI-ZHEN LIN, ROBERT P. IRWIN, and STEVEN M. PAUL

Section on Molecular Pharmacology, Clinical Neuroscience Branch, National Institute of Mental Health, Bethesda, Maryland 20892 (G.-M.Y., S.-Z.L., R.P.I., S.M.P.); Departments of Pharmacology and Toxicology (G.-M.Y., S.-Z.L., R.P.I., S.M.P.), Medicine (R.P.I.), and Psychiatry (S.M.P.), Indiana University, School of Medicine, Indianapolis, Indiana 46202; and Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285 (G.-M.Y., S.M.P.)

Received August 17, 1994; Accepted November 10, 1994

SUMMARY

We have recently reported that the majority of cultured rat cerebellar granule neurons undergo apoptosis when maintained in the presence of physiological concentrations of K^+ (nondepolarizing conditions). We now report that exposure of cultured cerebellar granule neurons, maintained under nondepolarizing conditions, to the muscarinic cholinergic receptor (mAchR) agonists carbachol and muscarine results in a concentration- and time-dependent inhibition of apoptosis. The nicotinic cholinergic receptor agonist (−)-nicotine fails to mimic, and the nicotinic cholinergic receptor antagonist dihydro-β-erythroidine fails to antagonize, the survival-promoting effects of carbachol. In contrast, relatively low concentrations of atropine completely prevent the effects of carbachol in blocking apoptotic death of cultured granule neurons. Although the m1- and m2-preferring mAchR antagonists pirenzepine and gallamine, respectively, fail to reverse the effects of carbachol, the m3-preferring antagonist 4-diphenylacetoxyl-*N*-methylpiperidine methiodide completely blocks the survival-promoting effects of carbachol. These data demonstrate that activation of the mAchR (possibly of the m3 subtype) blocks apoptosis of cultured cerebellar granule neurons. The antiapoptotic effects of mAchR agonists are not indirectly mediated via glutamate release from granule neurons, because antagonists of either *N*-methyl-D-aspartate or non-*N*-methyl-D-aspartate glutamate receptors fail to affect the antiapoptotic effects of carbachol or muscarine. Moreover, exposure of cultured cerebellar granule neurons to antiapoptotic concentrations of carbachol, in contrast to high concentrations of K^+ or glutamate receptor agonists, results in only a small and transient elevation of the intracellular Ca^{2+} concentration, as measured by fura-2 microfluorimetry. Slow neurotransmitters such as acetylcholine, acting via their cognate G protein-coupled receptors, may prevent neuronal apoptosis in the developing (and perhaps adult) central nervous system.

Apoptosis is one type of programmed cell death responsible for the physiological elimination of various cell populations during development (1). It has been estimated that 50% or more of vertebrate neurons in the CNS die during embryonic development and/or early postnatal maturation, via programmed cell death (2). There is increasing evidence that apoptosis may also be triggered pathologically in the adult CNS and may mediate the nonphysiological death of neurons that is characteristic of various neurodegenerative disorders, such as Alzheimer's disease (3), and that which occurs after ischemic or traumatic injury of the CNS (4).

In the mammalian CNS, cell-cell interactions appear to play an important role in regulating the survival and phenotypic development of various populations of neurons (5–7). Because of its relatively simple cellular architecture and the relatively late postnatal development of its synaptic connections, the cerebellum has been a useful model for investigating such cell-cell interactions, especially as they affect the development of specific neuronal phenotypes (8). Cerebellar granule neurons, the principal interneurons of the cerebellum, are among the most abundant neuronal phenotype in the mammalian CNS. Granule neurons receive their afferent input from mossy fibers and project axons (parallel fibers) to form synapses with dendrites of Purkinje cells (8). There is a rather precise stoichiometry between the number of cerebellar granule neurons and the number of Purkinje cells (8).

Postmitotic granule neurons can be readily maintained in their fully differentiated state *in vitro* for several weeks if depolarized with high concentrations of K^+ (9) or by exposure to the excitatory amino acids glutamate or NMDA (10). We (11) and others (12) have recently shown that depolarizing concentrations of K^+ (11, 12) or subtoxic concentrations of

ABBREVIATIONS: CNS, central nervous system; Ach, acetylcholine; Ara-C, cytosine arabinoside; cHK medium, conditioned high-potassium (25 mM) medium; cLK medium, conditioned low-potassium (5 mM) medium; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; 4-DAMP, 4-diphenylacetoxyl-*N*-methylpiperidine methiodide; DHE, dihydro-β-erythroidine; DIV, days *in vitro*; mAchR, muscarinic cholinergic receptor; MK-801, (±)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; nAchR, nicotinic cholinergic receptor; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; ANOVA, analysis of variance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[K^+]_o$, extracellular potassium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration.

excitatory amino acids (11) promote the survival of cultured cerebellar granule neurons by blocking their programmed cell death via apoptosis. The latter occurs in >80% of cultured granule neurons when they are maintained in physiological concentrations of K^+ (11, 12). The death of cultured cerebellar granule neurons induced by nondepolarizing conditions is characterized by all of the morphological and biochemical features of apoptosis, including cytoplasmic blebbing, condensation and aggregation of nuclear chromatin, and internucleosomal DNA fragmentation (11, 12). Moreover, inhibitors of RNA or protein synthesis greatly attenuate neuronal death induced by nondepolarizing culture conditions (11, 12). Due to their relative homogeneity, primary cultures of cerebellar granule neurons represent an ideal *in vitro* model system for studying the cellular and molecular events underlying apoptosis of mammalian CNS neurons.

In both rodents and humans, it appears that mossy fiber axons from the dorsal pontine nuclei of the brainstem, which innervate cerebellar granule neurons, utilize Ach as a primary neurotransmitter (8, 13). In addition, cerebellar granule neurons express nAchRs (14) and mAchRs, mainly the m2 and m3 subtypes (15, 16). Given our recent findings that subtoxic concentrations of the fast neurotransmitters glutamate and NMDA, acting at ionotropic glutamate receptors, prevent apoptosis of cultured cerebellar granule neurons (11), it was reasonable to investigate whether Ach itself would affect the survival of cultured cerebellar granule neurons. Using cellular viability assessment, DNA fragmentation analysis, *in situ* labeling, and morphological methods, we demonstrate that activation of mAchRs blocks the apoptotic death of cultured cerebellar granule neurons.

Experimental Procedures

Materials. Carbachol (carbamylcholine chloride), (+)-muscarine chloride, oxotremorine methiodide, (+)-pilocarpine-HCl, atropine, (−)-nicotine, pirenzepine, gallamine, 4-DAMP, DHE, MK-801, and CNQX were from Research Biochemicals (Natick, MA). Fluorescein diacetate, Hoechst 33258, trypsin, soybean trypsin inhibitor, Ara-C, DNase, RNase A, diaminobenzidine, and carbonyl cyanide *m*-chlorophenylhydrazone were all obtained from Sigma Chemical Co. (St. Louis, MO). Apop Tag *in situ* apoptosis detection kit (peroxidase) was from Oncor (Gaithersburg, MD). Ethyleneimine polymer was obtained from Fluka Chem (Ronkonkona, NY). Fura-2/acetoxymethyl ester and EGTA/acetoxymethyl ester were from Molecular Probes (Eugene, OR), and ionomycin was from Calbiochem (La Jolla, CA).

Preparation of cerebellar granule neurons. Rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat pups (15–19 g; Taconic Farms) as described previously (17). Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase and were then plated in poly-L-lysine-coated 35-mm culture plates (Nunc). Cells were seeded at a density of 1.5–1.8 × 10^6 cells/ml (2 ml/dish) in basal modified Eagle medium containing 10% fetal bovine serum and 25 mM KCl. Ara-C (10 μM) was added to the culture medium after 24 hr to limit the growth of non-neuronal cells. Previous work by our laboratory and others have shown that these cultures contain ≥95% granule neurons (18, 19). D-Glucose (100 μl of a 100 mM solution prepared in sterile water) was added to the cultures at 7 DIV and every fourth day thereafter (11).

Preparation of cerebellar astrocytes. Primary cerebellar astrocytes were prepared as described above, except that the final pellet was resuspended in basal Eagle medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and 100 μg/ml gentamicin. The cells were plated on uncoated Nunc plastic dishes without Ara-C and in low $[K^+]_o$ (5 mM). At 8 DIV, astrocytes were >95% pure, as determined by glial fibrillary acidic protein immunoreactivity (18).

Exposure of cerebellar granule neurons to drugs. All experiments, unless otherwise stated, were carried out with cerebellar granule neurons or cerebellar astrocytes at 8 DIV. For medium exchange, cLK medium was collected from sister cultures (cultures derived from the same initial tissue preparation and maintained under identical conditions), centrifuged at 1500 rpm for 5 min, and stored at 4°. Drugs, unless otherwise stated, were prepared as 100× stock solutions in distilled water and the pH was adjusted to 7.2–7.4. All drugs were diluted to their desired final concentration with warmed cLK medium prepared as described above.

Assessment of neuronal viability. Viable granule neurons were quantified after staining with fluorescein formed from fluorescein diacetate, which is deesterified only by living cells (19). Briefly, after incubation with fluorescein diacetate (10 μg/ml) neurons were examined and for each culture at least three randomly selected fields were photographed, using UV light microscopy. The numbers of neurons per representative low-power field were counted from the photomicrographs by an observer blind to the treatments. Values are generally expressed as the percentage protection or percentage of control cHK culture values in each experiment.

Detection of DNA fragmentation. Granule neurons (3 × 10^7) were plated in poly-L-lysine-coated 150- × 20-mm tissue culture dishes (Falcon 3003). After 8 DIV, the neurons were switched into cLK medium with or without drugs, as described in the figure legends, and then collected in cold PBS, pH 7.2, after removal of the medium and one wash with cold PBS. The neurons were centrifuged at 5000 rpm (Beckman JA-20 rotor) for 5 min. The pellet was lysed in 600 μl of a buffer consisting of 10 mM Tris·HCl, 10 mM EDTA, and 0.2% Triton X-100, pH 7.5. After 15 min on ice, the lysate was centrifuged at 12,000 rpm for 10 min at 4°. The supernatant (containing RNA and fragmented DNA but not intact chromatin) was extracted first with phenol and then with phenol-chloroform/isoamyl alcohol (24:1). The aqueous phase was adjusted to 300 mM with sodium acetate and nucleic acids were precipitated overnight with 1 volume of isopropanol. The pellet was washed with 70% ethanol, air-dried, and dissolved in 15 μl of 10 mM Tris·HCl, 1 mM EDTA, pH 7.5. After digestion of RNA with RNase A (0.6 mg/ml, at 37° for 30 min), the sample was electrophoresed in a 2% agarose gel with TBE buffer. DNA was visualized by ethidium bromide staining (11).

Morphological methods. Cerebellar granule neurons were cultured in 35-mm tissue culture dishes as described above. After removal of the medium, the neurons were rinsed once with cold PBS, pH 7.2, fixed for 10 min with 4% formaldehyde in PBS at 4°, washed with distilled water, and dried at room temperature. Cells were stained with Hoechst 33258 (5 μg/ml) for 5 min, washed, and dried (11). Photographs were randomly obtained, by an observer blind to the treatments, with a Zeiss Axiophot microscope.

***In situ* labeling of apoptotic neurons.** Cerebellar granule neurons were cultured on poly-L-lysine-coated 13-mm coverslips with the same protocol as described above. At 8 DIV, the neurons were switched to cLK medium, with or without various drugs as indicated, for 24 hr. After removal of the medium, the neurons were rinsed once with cold PBS, pH 7.2, fixed for 10 min with 4% formaldehyde in PBS at room temperature, washed with distilled water, and dried at room temperature. The coverslips were fixed onto glass slides and dehydrated to 70% ethanol at 4°. Staining was carried out according to the protocol provided by the supplier (Oncor). Photomicrographs were randomly obtained, by an observer blind to the treatments, with a Zeiss Axiophot microscope.

Measurement of $[Ca^{2+}]_i$. Measurement of $[Ca^{2+}]_i$ was carried out as described previously, using microspectrofluorimetry and the Ca^{2+}-sensitive indicator fura-2 (20). In brief, cultures (8–9 DIV) were gently washed three times with buffer containing 143 mM NaCl, 25 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, and 5.6 mM glucose (adjusted to pH 7.4 with NaOH at 37°) and were then

incubated with fura-2/acetoxymethyl ester (2.5 μM) for 30–45 min in the dark at 37°. After the incubation period, the cells were gently washed three times with buffer and were allowed to remain undisturbed for ≥15 min, to permit complete hydrolysis of the ester (20). Neurons grown on ethyleneimine polymer-coated, glass-bottomed, 35-mm culture dishes (MatTek, Ashland, MA) were illuminated on an inverted microscope (Nikon Diaphot). The cells were continuously perfused with buffer alone or with added drug at 37°. Excitation of fura-2 was at 340 and 380 nm, with emitted light being monitored at 510 nm. Cell-derived fluorescent images were visualized using a 40×, 1.3-numerical aperture, oil-immersion objective and were intensified with a Videoscope KS1381 intensifier (Videoscope International, Washington, D. C.) before entering the camera (Dage-MTI, Michigan City, IN). Images were digitized and stored for subsequent analysis (Universal Imaging Co., West Chester, PA). *In situ* calcium calibration was determined using a buffer containing 130 mM KCl, 17 mM NaCl, 10 mM HEPES, 10 mM glucose, 0.015 mM ionomycin, and 0.01 mM carbonyl cyanide *m*-chlorophenylhydrazone, pH 7.2 (37°). For maximum and minimum calcium responses, 3 mM $CaCl_2$ or 5 mM EGTA plus 120 μM EGTA/acetoxymethyl ester, respectively, was added to this buffer. Calculation of $[Ca^{2+}]_i$ was carried out as described by Grynkiewicz *et al.* (21), using an apparent dissociation constant of 285 nM (22).

Statistical analysis. Data are presented as the mean ± standard error. Unless otherwise stated, statistical comparisons were made using ANOVA, followed by the Bonferroni-Dunn test.

Results

Block of apoptosis of cerebellar granule neurons by carbachol and muscarine. Initially we determined the effects of carbachol on cultured cerebellar granule neuron viability over a broad range of concentrations. As shown in Fig. 1, *upper*, exposure to carbachol of cerebellar granule neurons that were first switched to physiological concentrations of K^+ (5 mM) results in a concentration-dependent increase in neuronal survival (EC_{50}, ~204 μM). The mAchR agonist muscarine also blocks apoptosis of cultured cerebellar granule neurons induced by nondepolarizing conditions, in a concentration-dependent manner (Fig. 1, *lower*). Consistent with their relative potencies in activating the mAchR, muscarine is more potent than carbachol in blocking apoptosis of cerebellar granule neurons, with an EC_{50} value of ~74 μM (Fig. 1, *lower*).

Given that apoptosis of cerebellar granule neurons induced by cLK medium displays internucleosomal DNA fragmentation, resulting in nucleosome-sized DNA and multiples thereof (11), we examined the effects of carbachol treatment on the DNA fragmentation pattern induced by nondepolarizing conditions. Cerebellar granule neurons switched from cHK to cLK medium were exposed to different concentrations of carbachol for 12 hr, and DNA was extracted as described above. Agarose gel electrophoresis of granule neuron DNA reveals that cLK medium induces typical apoptotic DNA fragmentation characterized by oligonucleosome-length DNA (~185 base pairs). Exposure of cultured cerebellar granule neurons to carbachol prevents the DNA fragmentation induced by cLK medium, in a concentration-dependent manner (Fig. 2).

Fig. 3 shows the time course of the effects of carbachol or muscarine in blocking apoptosis of cerebellar granule neurons induced by cLK medium. Switching cultured cerebellar granule neurons from cHK to cLK medium for 12 hr results in a time-dependent increase in neuronal death. Both carbachol and muscarine (at concentrations of 2.5 mM and 0.25 mM, respectively) significantly block this apoptotic death at all time points from 12 to 96 hr. Thus, the nonselective mAchR agonists

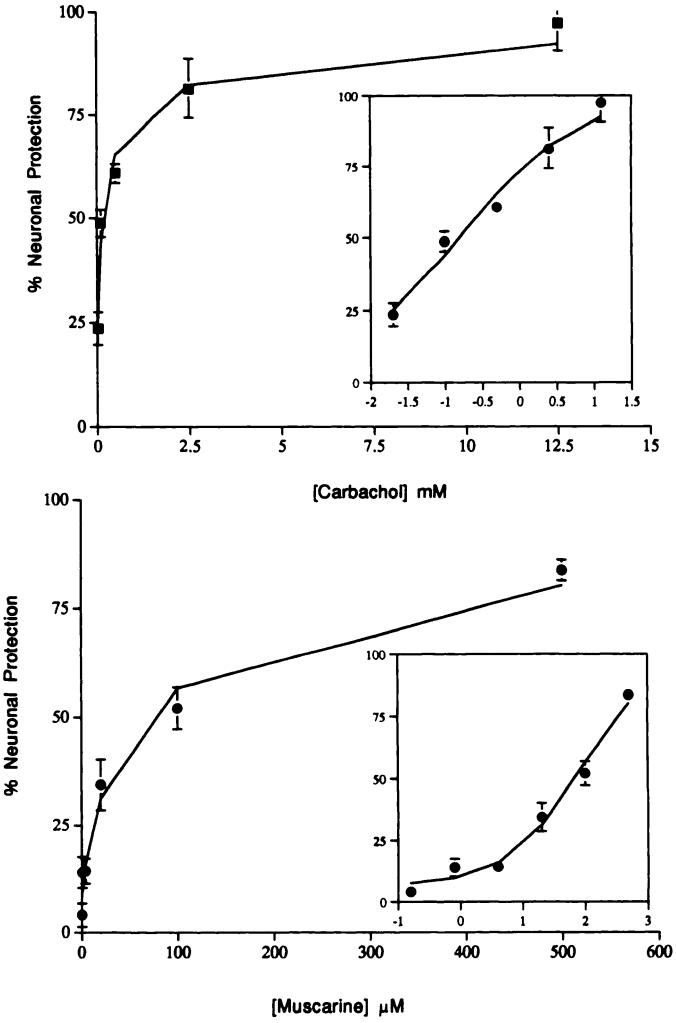


Fig. 1. Carbachol and muscarine block apoptosis of cultured cerebellar granule neurons induced by nondepolarizing conditions. *Upper*, cerebellar granule neurons were exposed at 8 DIV to different concentrations of carbachol (0.02–12.5 mM) in cLK medium for 24 hr (see text for details). Neuronal viability was determined by fluorescein diacetate staining of living neurons, followed by visual counting by an observer blind to the experimental groups (see Experimental Procedures). The data shown represent the mean ± standard error of triplicate determinations from a representative experiment, which was repeated three times with similar results. Neuronal protection was calculated as percentage neuronal protection = (surviving neurons with carbachol in cLK medium − surviving neurons in cLK medium alone)/(surviving neurons in cHK medium − surviving neurons in cLK medium alone) × 100. *Inset*, semilogarithmic plot of the data; the *x* values represent log [carbachol] (in mM). *Lower*, cerebellar granule neurons were exposed at 8 DIV to different concentrations of muscarine (1.6–500 μM) and cLK medium for 24 hr. The percentage protection was calculated and presented as in *upper*.

carbachol and muscarine block apoptosis of cultured cerebellar granule neurons induced by nondepolarizing conditions.

Effects of mAchR agonists and antagonists in blocking apoptosis of cultured cerebellar granule neurons. To test whether the effects of carbachol and muscarine in blocking apoptosis of cerebellar granule neurons are mediated via a specific mAchR, we examined the effects of various mAchR agonists and antagonists on cerebellar granule neuron viability under nondepolarizing conditions. We first confirmed that nAchRs are not involved in the survival-promoting effects of carbachol. DHE, a specific neuronal nAchR antagonist (23) at

Fig. 2. Carbachol prevents internucleosomal DNA fragmentation of cerebellar granule neurons induced by cLK medium. Cerebellar granule neurons were exposed at 8 DIV to different concentrations of carbachol and cLK medium for 12 hr (see text for details). Agarose gel electrophoresis and ethidium bromide staining were used to visualize DNA extracted from cerebellar granule neurons after exposure to cLK medium with or without different concentrations of carbachol. *Lane 1*, DNA size marker ladder; *lane 2*, cHK medium control; *lane 3*, cLK medium alone; *lanes 4-9*, cLK medium plus carbachol at 12.5, 2.5, 0.5, 0.1, 0.02, and 0.004 mM, respectively. Note that the 185-base pair ladder characteristic of the DNA degradation that occurs in apoptotic cells is detected in cLK medium-treated neurons but not in cHK medium-treated (control) neurons or cLK medium- plus K^+-treated neurons. Carbachol prevents the DNA fragmentation of cerebellar granule neurons induced by cLK medium in a concentration-dependent manner. This experiment was repeated two times with similar results.

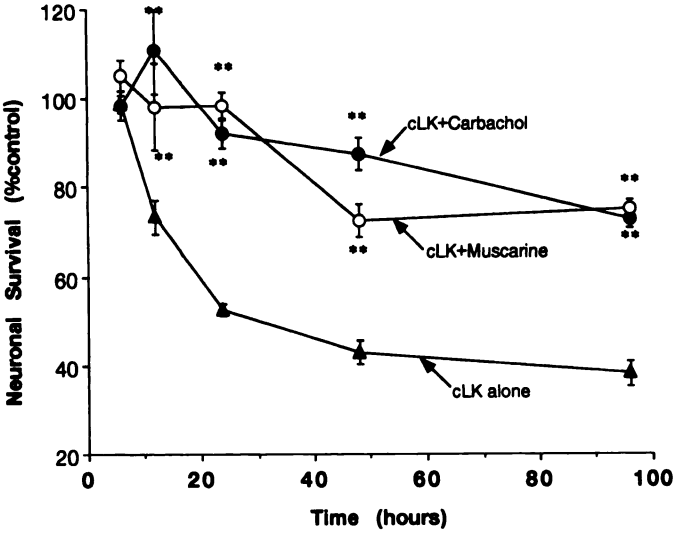


Fig. 3. Time course of antiapoptotic actions of carbachol and muscarine. Cerebellar granule neurons were exposed at 8 DIV to carbachol (2.5 mM) or muscarine (0.25 mM) in cLK medium, for different lengths of time, and at each time point an independent control (cHK medium-treated) group was assessed for neuronal viability as described in Experimental Procedures and in the legend to Fig. 1. Note the substantial fraction of granule neurons that can be rescued by carbachol or muscarine at all time points examined. The data shown represent the mean ± standard error of triplicate determinations from a representative experiment, which was repeated three times with similar results. **, $p < 0.001$, compared with the cLK medium-treated group alone at same time point (Student *t* test, two tails).

concentrations of ≤100 μM, does not alter the effects of carbachol in blocking apoptosis of cerebellar granule neurons in cLK medium. DHE also fails to induce apoptosis in neurons maintained in cHK medium (data not shown). Moreover, (−)-nicotine (at concentrations of ≤100 μM) does not mimic the effects of carbachol in blocking apoptosis (data not shown). In contrast, relatively low concentrations of atropine (1–10 μM) completely block the effects of carbachol and muscarine in preventing apoptosis of cerebellar granule neurons exposed to cLK medium (Fig. 4a and data not shown). Moreover, the m1- and m2-preferring antagonists pirenzepine and gallamine, respectively (each tested at concentrations of 10 μM), fail to

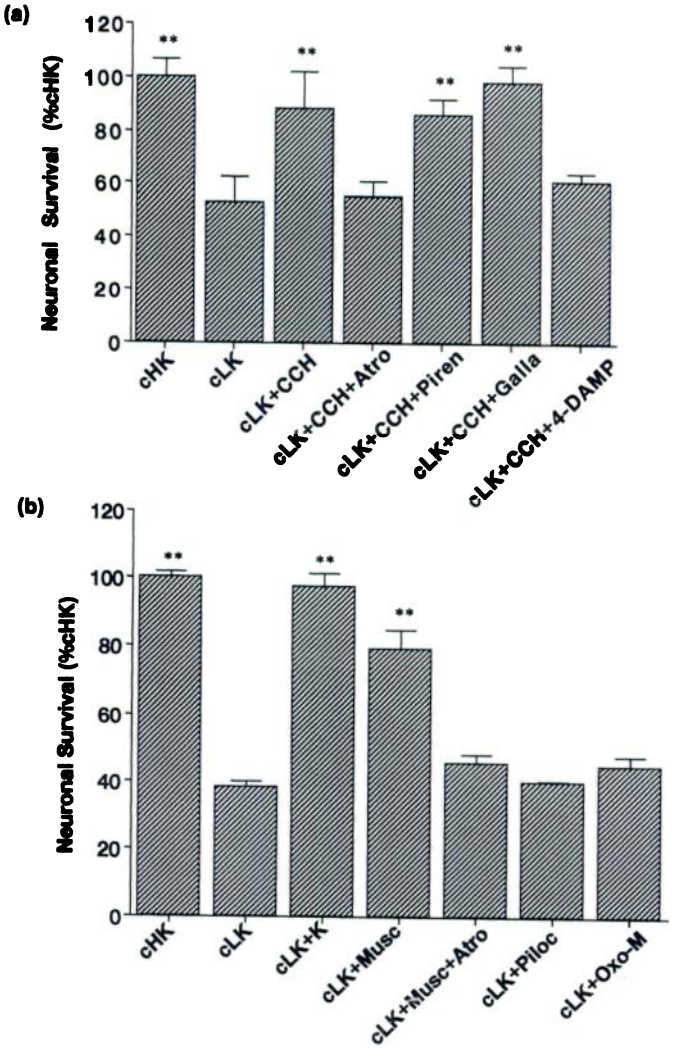
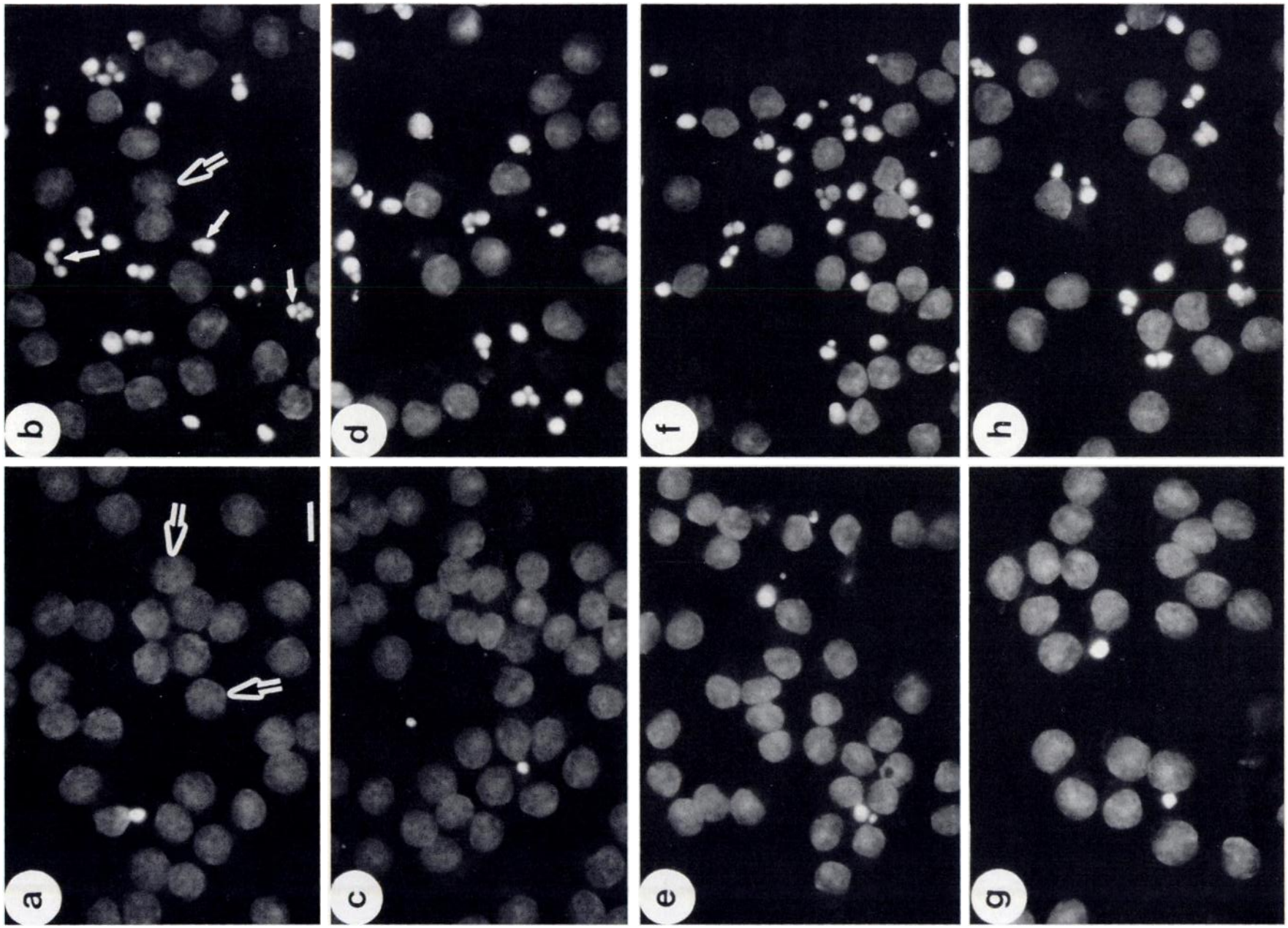


Fig. 4. Effects of various mAchR agonists and antagonists on apoptosis of cerebellar granule neurons induced by nondepolarizing conditions. a, Cerebellar granule neurons were exposed at 8 DIV to carbachol (*CCH*) (1 mM), under nondepolarizing conditions, with or without the following mAchR antagonists: atropine (*Atro*) (10 μM), pirenzepine (*Piren*) (10 μM), gallamine (*Galla*) (10 μM), and 4-DAMP (10 μM). After 24 hr, neuronal viability was determined as described in the legend to Fig. 1. The data shown represent the mean ± standard error of the percentage of three cHK medium-treated (control) cultures and are from a representative experiment, which was repeated three times with similar results. **, $p < 0.001$, compared with the cLK medium-treated cultures alone (ANOVA and then Bonferroni-Dunn test). b, Effects of various mAchR agonists on apoptosis of cerebellar granule neurons maintained under nonpolarizing conditions are shown. Cerebellar granule neurons were exposed at 8 DIV to cLK medium with or without the following mAchR agonists: muscarine (*Musc*) (250 μM), muscarine (250 μM) plus atropine (10 μM) (*Musc+Atro*), pilocarpine (*Piloc*) (100 μM), and oxotremorine methiodide (*Oxo-M*) (100 μM). After 24 hr, neuronal viability was determined as described in a. The data shown represent the mean ± standard error of the percentage of the cHK medium-treated (control) cultures and are from a representative experiment, which was repeated three times with similar results. **, $p < 0.001$, compared with cultures in cLK medium alone (ANOVA and then Bonferroni-Dunn test).



reverse the effects of carbachol in blocking apoptosis of cerebellar granule neurons. On the other hand, 4-DAMP, an m3-preferring antagonist, completely blocks the antiapoptotic effects of carbachol (Fig. 4a). These data suggest that the m3 subtype of mAchR may mediate the antiapoptotic effects of carbachol in cultured cerebellar granule neurons. Consistent with these observations, we have also found that mAchR agonists with relative m1 or m2 subtype selectivity, i.e., oxotremorine-M and pilocarpine, respectively (each at a concentration of 100 μM), fail to show any survival-promoting effects in cultured cerebellar granule neurons switched to cLK medium (Fig. 4b).

Morphological and *in situ* studies. As shown in Fig. 5, we examined the morphological changes in cultured cerebellar granule neurons induced by cLK medium by using Hoechst 33258 staining, which specifically labels nuclear chromatin. Switching cerebellar granule neurons from cHK medium to cLK medium results in typical apoptotic changes in neuronal nuclei, including a characteristic condensation of nuclear chromatin and heterochromatic clumping (Fig. 5, a and b) (11). Addition of carbachol (2.5 mM) or muscarine (0.25 mM) to cLK medium rescues most of the neurons and maintains normal nuclear morphology. The antiapoptotic effects of carbachol and muscarine are reversed by atropine (10 μM) (Fig. 5, c-f). The m3-preferring antagonist 4-DAMP also blocks the effects of carbachol (Fig. 5h), but the m2-preferring antagonist gallamine does not (Fig. 5g).

We used *in situ* labeling of the 3′-OH end of fragmented DNA to further examine and quantitate the presence of neurons undergoing apoptosis in cultures of cerebellar granule neurons exposed to cLK medium and various drugs. As is evident in Fig. 6, a and b, the number of positively staining neurons is greatly increased in cultures switched from cHK to cLK medium. Exposure to carbachol or muscarine of granule neurons maintained in cLK medium blocks apoptosis, as indicated by the marked decrease in positively staining neurons (Fig. 6, c and e). Atropine (10 μM), again, reverses the effects of both carbachol and muscarine (Fig. 5, d and f). Similarly, the m3-preferring antagonist 4-DAMP also prevents the effects of carbachol and muscarine in blocking apoptotic neuronal death (data not shown).

Direct block of apoptosis by carbachol and muscarine via the mAchR. Neither the selective NMDA receptor antagonist MK-801 nor the non-NMDA receptor antagonist CNQX (at concentrations of 1 and 10 μM, respectively), when added to neurons in cLK medium, affects the neuroprotective effects of carbachol or muscarine (Fig. 7a). We have previously reported that these concentrations of glutamate receptor antagonists effectively block the ability of glutamate to prevent apoptosis of cultured cerebellar granule neurons exposed to cLK medium (11). Moreover, neither antagonist alone has any effect on neuronal survival in cHK or cLK medium (data not shown).

Because primary cultures of cerebellar granule neurons contain small numbers (≤5%) of glia, we examined whether glial cells are involved in the observed survival-promoting effects of mAchR agonists. Glia-enriched cultures were prepared in low-K^+ medium, in the absence of antimitotics. After 8 DIV these cultures contain ≥95% glia, primarily astrocytes (18). Glia-enriched cultures were exposed to carbachol or muscarine for 24 hr, and the culture medium was collected as described above. As shown in Fig. 7b, cLK medium from cerebellar glial cell cultures (Fig. 7b, *cLK-glia*), like cLK medium from cerebellar granule neuron cultures (Fig. 7b, *cLK*), results in neuronal death of the same magnitude and time course. The neuronal death observed in cLK medium from glia-enriched cultures can be blocked by increasing the $[K^+]_e$ to 25 mM. After incubation of cerebellar astrocytes with carbachol (2.5 mM) or muscarine (0.25 mM) for 24 hr, the media show significant survival-promoting effects [Fig. 7b, *(cLK+CCH)-glia* and *(cLK+Musc)-glia*] ($p < 0.001$ or < 0.05, compared with cLK medium from glial cell cultures alone, respectively). However, atropine (10 μM) completely antagonizes the survival-promoting effects of media from carbachol- or muscarine-treated glial cell cultures. These results clearly show that mAchR agonists exert their antiapoptotic actions directly, via activation of mAchRs on cerebellar granule neurons, rather than by stimulating glial elements to release neurotrophic factors.

Effects of carbachol on $[Ca^{2+}]_i$ in cultured cerebellar granule neurons. To determine whether the antiapoptotic effects of carbachol in cultured cerebellar granule neurons are due to an elevation in $[Ca^{2+}]_i$ (such as is observed in high-K^+ medium), we examined the immediate effects of carbachol on $[Ca^{2+}]_i$, as well as the $[Ca^{2+}]_i$ after 24 hr of exposure to carbachol. To mimic as closely as possible the experimental conditions used in our cytotoxicity experiments, we added carbachol (2.5 or 12.5 mM) simultaneously with the change from high-$[K^+]_e$ to low-$[K^+]_e$ conditions (Fig. 8, A and B). As expected, the rapid lowering of $[K^+]_e$ from 25 to 5 mM results in a rapid and dramatic lowering of $[Ca^{2+}]_i$ (Fig. 8, A and C). The addition of carbachol (2.5 mM) to the low-K^+ buffer, however, has no effect on this reduction in $[Ca^{2+}]_i$ (Fig. 8, B and C). Although the addition of a high concentration of carbachol (12.5 mM) results in a small elevation of $[Ca^{2+}]_i$ in some neurons, compared with the level observed in low-K^+ buffer alone (Fig. 8C), this elevation in $[Ca^{2+}]_i$ disappears within 20 min of continuous carbachol exposure (data not shown). Neither concentration of carbachol elevates $[Ca^{2+}]_i$ after 24 hr of continuous exposure (Fig. 8D).

Discussion

In the present study we have demonstrated that the mAchR agonists carbachol and muscarine prevent apoptosis of cultured cerebellar granule neurons induced by nondepolarizing conditions, in a concentration- and time-dependent manner (Fig. 1). The survival-promoting effects of these mAchR agonists cor-

Fig. 5. Morphological features characteristic of apoptosis are induced in cultured cerebellar granule neurons by cLK medium and are blocked by mAchR agonists. Cerebellar granule neurons were prepared as described in Experimental Procedures. At 8 DIV, the cultures were switched from cHK medium to cLK medium, with drugs at the concentrations indicated, for 24 hr. a, cHK medium control; b, cLk medium alone; c, cLK medium plus carbachol (2.5 mM); d, cLK medium plus carbachol (2.5 mM) plus atropine (10 μM); e, cLK medium plus muscarine (0.25 mM); f, cLK medium plus muscarine (0.25 mM) plus atropine (10 μM); g, cLK medium plus carbachol (2.5 mM) plus gallamine (10 μM); h, cLK medium plus carbachol (2.5 mM) plus 4-DAMP (10 μM). Cells were stained with the fluorescent dye Hoechst 33258 as described in the text. Note the typical apoptotic morphology (nuclear condensation and heterochromatic clumping) in neurons in b, d, f, and h but not in a, c, e, and g. *Black arrows*, neurons with normal morphology; *white arrows*, apoptotic neurons. *Bar*, 10 μm.

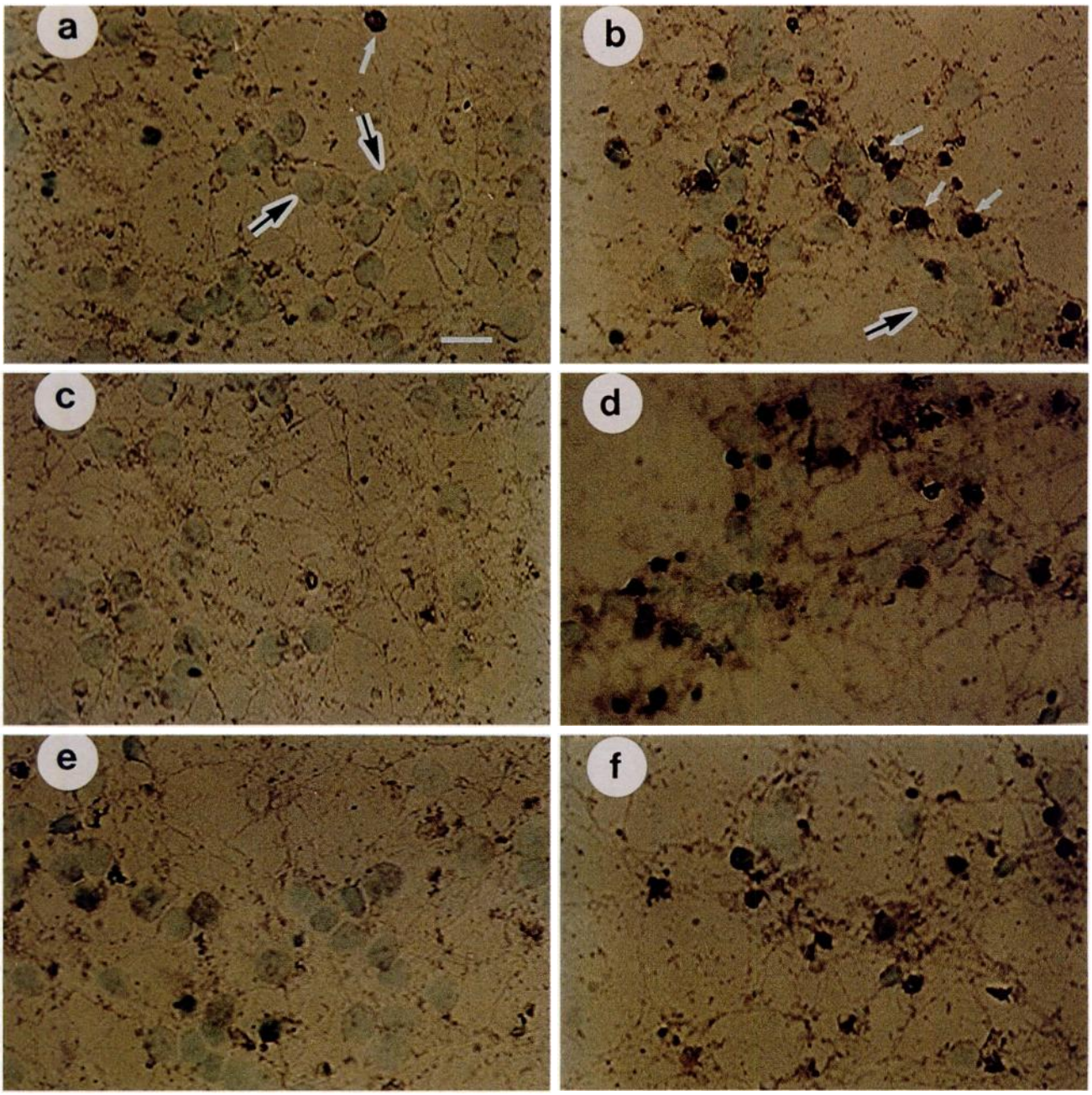


Fig. 6. *In situ* labeling of apoptotic neurons reveals antiapoptotic actions of mAchR agonists. Cerebellar granule neurons were cultured on poly-L-lysine-coated coverslips as described in Experimental Procedures. At 8 DIV, the neurons were switched to cLK medium, with or without drugs at the concentrations indicated, for 24 hr. a, cHK medium control; b, cLk medium alone; c, cLK medium plus carbachol (2.5 mM); d, cLK medium plus carbachol (2.5 mM) plus atropine (10 μM); e, cLK medium plus muscarine (0.25 mM); f, cLK medium plus muscarine (0.25 mM) plus atropine (10 μM). Note the positive staining (*dark brown*) of apoptotic neurons (*white arrows*) that are abundant in cLK medium-treated cultures and the normal neurons that weakly counterstain *green* (*black arrows*). *Bar*, 25 μm.

relate with their potencies in activating mAchRs, i.e., the EC_{50} value for muscarine in blocking apoptosis of cerebellar granule neurons is almost 3 times lower than that of carbachol. The ability of these mAchR agonists to prevent apoptosis of cerebellar granule neurons is supported by morphological, biochemical, and *in situ* labeling data (Figs. 2, 5, and 6). The antiapoptotic actions of carbachol and muscarine were completely blocked by atropine and 4-DAMP but not by pirenzipine or gallamine. These data, coupled with the observations that neither pilocarpine nor oxotremorine could mimic the effects of carbachol or muscarine, suggest (but do not prove) that the m3 subtype of mAchRs may mediate the antiapoptotic actions of mAchR agonists (see below).

Cerebellar granule neurons are innervated *in vivo* by mossy fibers that originate from different populations of brainstem nuclei that may use different neurotransmitters. There is considerable evidence, however, that at least some mossy fibers, especially those from the dorsal pontine nuclei, are cholinergic

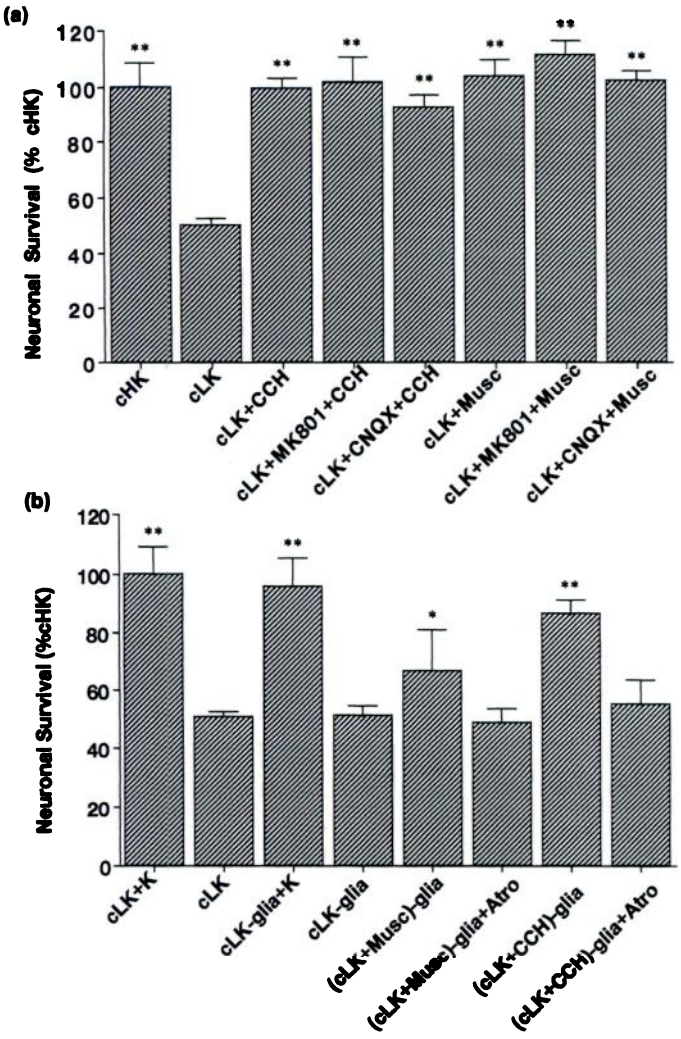


Fig. 7. Antiapoptotic actions of mAchR agonists in cerebellar granule neurons do not involve ionotropic glutamate receptors or release of a glial growth factor. a, Cerebellar granule neurons were exposed at 8 DIV to carbachol (*CCH*) (1 mM) or muscarine (*Musc*), under nondepolarizing conditions, with or without the NMDA antagonist MK-801 (1 μM) or the non-NMDA antagonist CNQX (10 μM). After 24 hr, neuronal viability was determined as described in the legend to Fig. 1. The data shown represent the mean ± standard error of the percentage of cHK medium-treated control cultures and are from a representative experiment, which was repeated three times with similar results. **, $p < 0.001$, compared with the cLK medium-treated group alone (ANOVA and then Bonferroni-Dunn test). b, Cerebellar granule neurons at 8 DIV were switched from cHK medium to cLK medium or sister culture glial cell cLK medium pretreated with carbachol or muscarine (see text for details). The data represent the mean ± standard error of the percentage of cHK medium-treated control cultures and are from a representative experiment, which was repeated three times with similar results. **, $p < 0.001$, compared with the cLK medium-treated group alone (ANOVA and then Bonferroni-Dunn test). Note that, compared with cLK medium prepared from cerebellar granule neuron cultures (*cLK*), cLK medium from cerebellar glial cell cultures (*cLK-glia*) results in neuronal death of the same magnitude. The latter is completely blocked by raising the $[K^+]_o$ to 25 mM (*cLK-glia+K*). After pretreatment of cerebellar astrocytes with carbachol (2.5 mM) or muscarine (0.25 mM) for 24 hr, the cLK media [*(cLK+CCH)-glia* or *(cLK+Musc)-glia*, respectively] show significant effects on neuronal survival. However, the latter is completely blocked by atropine (*Atro*) (10 μM), indicating that the observed rescue of neurons by media from carbachol- or muscarine-treated cerebellar astrocytes is due to residual carbachol or muscarine present in the media and is not due to the production of a noncholinergic glial growth factor.

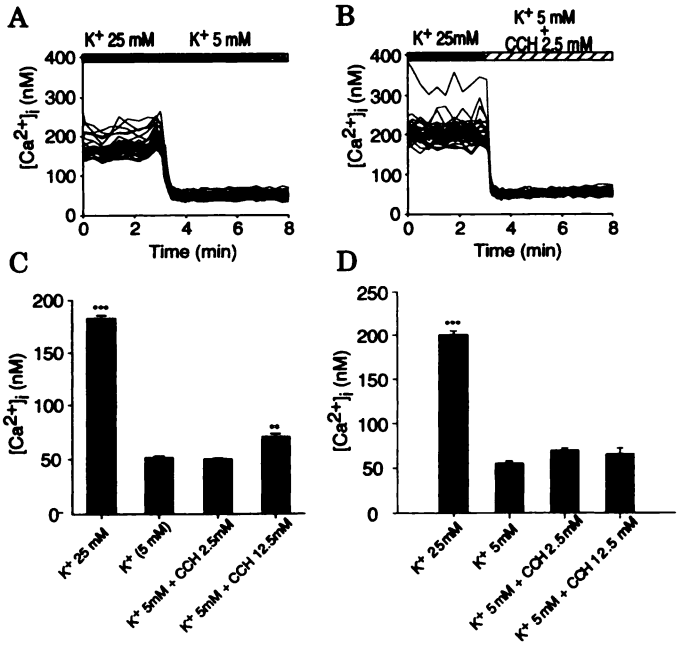


Fig. 8. Carbachol has little or no effect on $[Ca^{2+}]_i$ in cerebellar granule neurons exposed to low $[K^+]_o$. $[Ca^{2+}]_i$ was measured with the Ca^{2+}-sensitive fluorescent indicator fura-2 (19). A, Experiment showing the change of $[Ca^{2+}]_i$ when $[K^+]_o$ is rapidly changed from high (25 mM) to low (5 mM) values. Each *line* represents the calculated $[Ca^{2+}]_i$ of one cell (see Experimental Procedures for details). B, As in A but with the simultaneous application of carbachol (*CCH*) (2.5 mM) as shown. C, Summary of data (including A and B) showing the $[Ca^{2+}]_i$ under basal high-$[K^+]_o$ conditions and after 5 min of simultaneous exposure to low $[K^+]_o$ plus carbachol (2.5 and 12.5 mM). Data represent the mean ± standard error of two experiments, with 29 or 30 neurons at each concentration of carbachol. High-$[K^+]_o$ data represent the mean ± standard error of all six experiments (179 neurons total). D, $[Ca^{2+}]_i$ of cerebellar granule neurons under high-$[K^+]_o$ conditions and after 24-hr exposure to cLK medium and various concentrations of carbachol. Conditions of $[K^+]_o$ and carbachol exposure for each group were maintained during and after loading of the cells with fura-2/acetoxymethyl ester. Data represent the mean ± standard error of four experiments (116–120 neurons) for each condition (493 neurons total). **, $p \leq 0.01$; ***, $p \leq 0.001$ (ANOVA and Bonferroni *t* test). Note that a carbachol concentration of 2.5 mM, which results in a robust survival-promoting (antiapoptotic) effect (Figs. 1 and 3), fails to increase $[Ca^{2+}]_i$ after brief (5-min) or prolonged (24-hr) exposure. A very high concentration of carbachol (12.5 mM) results in a small transient elevation of $[Ca^{2+}]_i$ in some neurons (C and data not shown). Neither concentration of carbachol has any effect on $[Ca^{2+}]_i$ when cerebellar granule neurons are continuously exposed for 24 hr.

(13). Ach is present in relatively high concentrations in mossy fiber terminals (24). In addition, the Ach biosynthetic and degradative enzymes choline acetyltransferase and acetylcholinesterase, respectively, have both been localized to synaptic membranes of mossy fibers and glomeruli by immunohistochemistry (25–27). Furthermore, transection of the cerebellar peduncles markedly decreases choline acetyltransferase activity in the cerebellum (28).

The mAchR belongs to a large family of structurally related G protein-coupled receptors linked to several effector systems. Molecular cloning studies have demonstrated the existence of at least five different mAchR subtypes, designated m1–m5 (29). These receptor subtypes have distinct pharmacological properties, tissue distribution, and second messenger coupling (29). It has been shown that m1, m3, and m5 mAchRs are predominantly coupled to phosphoinositide hydrolysis, arachidonic acid release, cAMP elevation, and M-current inhibition, whereas

m2 and m4 mAchRs mediate the inhibition of adenylate cyclase activity (29). Consistent with our observations, Chuang and colleagues (16) previously reported that the m3 mAchR subtype is the predominant mAchR subtype expressed in cultured cerebellar granule neurons.

Cerebellar granule neurons contain glutamate as their primary neurotransmitter, as well as both NMDA and non-NMDA glutamate receptors on their cell surface (30, 31). We have previously demonstrated that subtoxic concentrations of excitatory amino acids, including glutamate and NMDA, can also block apoptosis of cultured cerebellar granule neurons (11). However, the effect of mAchR agonists in preventing apoptosis of cerebellar granule neurons does not appear to be mediated via stimulation of glutamate release, because neither NMDA or non-NMDA glutamate receptor antagonists influence the effects of carbachol or muscarine in blocking apoptosis of cerebellar granule neurons induced by nondepolarizing conditions (Fig. 7a). Moreover, despite the rather profound rescue of cultured cerebellar granule neurons by carbachol (Figs. 1, *upper*, 3, 4, 5c, 6c, and 7a) in cLK medium, carbachol exposure has only a small transient effect on $[Ca^{2+}]_i$, as assessed by microfluorimetry with fura-2 (Fig. 8) (see also Ref. 32). The latter contrasts with the well known effects of either elevated $[K^+]_e$, glutamate, or NMDA in elevating $[Ca^{2+}]_i$ in these same neurons (32). Thus, the ability of mAchR agonists to block apoptosis of these neurons does not appear to be due to maintenance of elevated $[Ca^{2+}]_i$, as occurs with depolarizing $[K^+]_e$ or after exposure to excitatory amino acids (32).

Conceivably, mAchR agonists could release glial growth or trophic factors capable of preventing apoptosis of neurons (33). Our findings do not support this hypothesis, however, because media derived from cerebellar astrocyte cultures incubated with carbachol or muscarine fail to prevent apoptotic death in the presence of the mAchR antagonist atropine. It is difficult to directly exclude the possibility that astrocytes enhance granule neuron survival by a direct action (34); however, it should be emphasized that cultured cerebellar granule neurons, such as those used in our experiments, are populated by ≤5% glial cells (18).

There is increasing evidence that cell-cell interactions between synaptic neurons can determine the survival or death of neurons that occurs during normal CNS development (5–7). For example, Baptista *et al.* (35) reported that the differentiation of purified cerebellar Purkinje cells *in vitro* is regulated by their interactions with granule neurons, but not by mossy fibers. Studies in Gunn rats, however, suggest a relationship between the development of granule neurons and synapse formation with mossy fibers (36). Recently, Wood *et al.* (37), using an *in situ* labeling method for detecting DNA fragmentation, have shown that granule neuron loss during the first 2 weeks of postnatal cerebellar development (but not during the third to fifth postnatal weeks) occurs via apoptosis. Because mossy fibers from dorsal pontine brainstem nuclei contain Ach as a primary neurotransmitter, these data suggest that innervation of postmigratory granule neurons by mossy fiber axons may prevent the elimination of these neurons by blocking their programmed death. Such afferent innervation by cholinergic mossy fiber axons would thus serve to stabilize synaptic contacts, resulting in mossy fiber-granule neuron-Purkinje cell circuits. In contrast, erroneous axonal projections or synaptic contacts would be eliminated as the granule interneuron degenerates via apoptosis. Although it has long been known that various aspects of neuronal development are regulated by afferent input, the possibility that such afferent stimuli might also influence neuronal survival *per se* has, until recently, received little attention. Several examples now exist, however, where it appears that the trophic (survival) influences of neurotransmitters released from presynaptic neurons are as important as the trophic factors released from target neurons (6, 7).

Our findings demonstrate that Ach acting via mAchRs blocks the programmed death of cultured cerebellar granule neurons that occurs by apoptosis. Significantly, activation of several other neurotransmitter receptors present on cerebellar granule neurons (e.g., 5-hydroxytryptamine type 2 receptors) fails to block apoptosis.[1] Neurotransmitters like Ach, therefore, may perform selective trophic functions during CNS development. Finally, the ability of neurotransmitters like Ach to block neuronal apoptosis may also be important in the adult CNS. The latter could, in part, be related to the pathogenesis of Alzheimer's disease, which is characterized by cholinergic denervation of the forebrain and subsequent degeneration of cortical and hippocampal neurons (38).

Acknowledgments

We thank Drs. H. Gershenfeld, M. Weller, H. Shannon, F. Bymaster, and D. Nelson for invaluable discussions, helpful suggestions, and data analysis. We particularly acknowledge the technical assistance of Mr. Robert Long. Ms. Pamela Edmonds provided excellent editorial assistance.

References

1. Wyllie, A. H. Apoptosis. *ISI Atlas of Science: Immunology*. 192–196 (1988).
2. Raff, M. C., B. A. Barres, J. F. Burne, H. S. Coles, Y. Ishizaki, and M. D. Jacobson. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science (Washington D. C.)* **262**:695–700 (1993).
3. Barinaga, M. Death gives birth to the nervous system, but how? *Science (Washington D. C.)* **259**:762–763 (1993).
4. Yoshitatsu, S., D. K. J. E. Von Lubitz, A. S. Basile, M. M. Borner, R. C.-S. Lin, P. Skolnick, and L. H. Fossom. Internucleosomal DNA fragmentation in gerbil hippocampus following forebrain ischemia. *Neurosci. Lett.* **171**:179–182 (1994).
5. Gao, W.-Q., N. Heintz, and M. E. Hatten. Cerebellar granule cell neurogenesis is regulated by cell-cell interactions *in vitro*. *Neuron* **6**:705–715 (1991).
6. Okado, N., and R. W. Oppenheim. Cell death of motoneurons in the chick embryo spinal cord. IX. The loss of motoneurons following removal of afferent input. *J. Neurosci.* **4**:1639–1652 (1984).
7. Linden, R., and L. P. Ponon. Dual control by targets and afferents of developmental neuronal death in the mammalian central nervous system: a study in the parabigeminal nucleus of the rat. *J. Comp. Neurol.* **266**:141–149 (1987).
8. Ito, M. Granule cells, in *The Cerebellum and Neural Control* (M. Ito, ed.). Raven Press, New York, 74–85 (1984).
9. Gallo, V., A. Kingsbury, R. Balázs, and O. S. Jorgensen. The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* **7**:2203–2213 (1987).
10. Balázs, R., O. S. Jorgensen, and N. Hack. *N*-Methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* **27**:437–451 (1988).
11. Yan, G.-M., B. Ni, M. Weller, K. A. Wood, and S. M. Paul. Depolarization or glutamate receptor activation blocks apoptotic cell death of cultured cerebellar granule neurons. *Brain Res.* **656**:43–51 (1994).
12. D'Mello, S. R., C. Gall, T. Ciotti, and P. Calissono. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition by insulin-like growth factor I and cAMP. *Proc. Natl. Acad. Sci. USA* **90**:10989–10993 (1993).
13. Ito, M. Mossy fiber system: reticular and pontine nuclei, in *The Cerebellum and Neural Control* (M. Ito, ed.). Raven Press, New York, 235–255 (1984).
14. Pabreza, L. A., S. Dhawan, and K. J. Kellar. [^{3}H]Cytisine binding to nicotinic cholinergic receptors in brain. *Mol. Pharmacol.* **39**:9–12 (1991).
15. Aloso, R., M. Didier, and P. Soubrie. [^{3}H]*N*-Methylscopolamine binding studies reveal M2 and M3 muscarinic receptor subtypes on cerebellar granule cells in primary culture. *J. Neurochem.* **55**:334–337 (1990).
16. Fukamauchi, F., P. L. Saunders, C. Hough, and D.-M. Chuang. Agonist-induced down-regulation and antagonist-induced up-regulation of m2 and

[1] G.-M. Yan and S. M. Paul, unpublished observations.

m3 muscarinic acetylcholine receptor mRNA and protein in cultured cerebellar granule cells. *Mol. Pharmacol.* **44**:940–949 (1993).
17. Novelli, A., J. A. Reilly, P. G. Lysko, and R. C. Henneberry. Glutamate becomes neurotoxic via the *N*-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res.* **451**:205–212 (1988).
18. Marini, A. M., J. P. Schwartz, and I. J. Kopin. The neurotoxity of 1-methyl-4-phenypyridinium in cultured cerebellar granule cells. *J. Neurosci.* **9**:3665–3672 (1989).
19. Marini, A. M., and S. M. Paul. *N*-Methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis. *Proc. Natl. Acad. Sci. USA* **89**:6555–6559 (1992).
20. Irwin, R. P., S.-Z. Lin, R. T. Long, and S. M. Paul. *N*-Methyl-D-aspartate induces a rapid, reversible, and calcium-dependent intracellular acidosis in cultured fetal rat hippocampal neurons. *J. Neurosci.* **14**:1352–1357 (1994).
21. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
22. Groden, D. L., Z. Guan, and B. T. Stokes. Determination of fura-2 dissociation constants following adjustment of the apparent Ca-EGTA association constant for temperature and ionic strength. *Cell Calcium* **12**:279–287 (1991).
23. Wang, Y. T., R. S. Neuman, and D. Bieger. Nicotinic cholinoceptor-mediated excitation in ambigual motoneurons of the rat. *Neuroscience* **40**:759–767 (1991).
24. Israel, M., and V. P. Whittaker. The isolation of mossy fiber endings from the granular layer of the cerebellar cortex. *Experientia (Basel)* **21**:325–326 (1965).
25. Altman, J. Postnatal development of the cerebellar cortex in the rat. III. Maturation of the components of the granule layer. *J. Comp. Neurol.* **145**:465–513 (1972).
26. Brown, W. J., and S. L. Palay. Acetylcholinesterase activity in certain glomeruli and Golgi cells of the granular layer of the rat cerebellar cortex. *Z. Anat. Entwicklungsgesch.* **137**:317–334 (1972).
27. Kan, K.-S. K., L.-P. Chao, and L. S. Forno. Immunohistochemical localization of choline acetyltransferase in the human cerebellum. *Brain Res.* **193**:165–171 (1980).
28. Fonnum, F. Application of microchemical analysis and subcellular fractionation techniques to the study of neurotransmitters in discrete areas of mammalian brain. *Biochem. Psychopharmacol.* **6**:75–88 (1972).
29. Hosey, M. M. Diversity of structure, signaling and regulation within the family of muscarinic cholinergic receptors. *FASEB J.* **6**:845–852 (1992).
30. Burgoyne, R. D., and M. A. Cambray-Deakin. The cellular neurobiology of neuronal development: the cerebellar granule cell. *Brain Res.* **472**:77–101 (1988).
31. Chuang, D.-M., X. M. Gao, and S. M. Paul. *N*-Methyl-D-aspartate exposure blocks glutamate toxicity in cultured cerebellar granule cells. *Mol. Pharmacol.* **42**:210–216 (1992).
32. Fohrman, E. B., G. deErausquin, E. Costa, and W. J. Wojcik. Muscarinic m3 receptors and dynamics of intracellular Ca^{2+} in cerebellar granule neurons. *Eur. J. Pharmacol.* **245**:263–271 (1993).
33. Schwartz, J. P., N. Nishiyama, D. Wilson, and T. Taniwaki. Receptor-mediated regulation of neuropeptide gene expression in astrocytes. *Glia* **11**:185–190 (1994).
34. Nedergaad, M. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science (Washington D. C.)* **263**:1768–1771 (1994).
35. Baptista, C. A., M. E. Hatten, R. Blazeski, and C. A. Mason. Cell-cell interactions influence survival and differentiation of purified Purkinje cells *in vitro*. *Neuron* **12**:243–260 (1994).
36. Takagishi, Y., and H. Yamamura. Granule cells and the development of synapses with mossy fibers in the cerebellum of the jaundiced Gunn rat. *Neuropathol. Appl. Neurobiol.* **19**:305–312 (1993).
37. Wood, K. A., B. Dipasquale, and R. J. Youle. *In situ* labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron* **11**:621–632 (1993).
38. Coyle, J. T., D. L. Price, and M. R. DeLong. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science (Washington D. C.)* **219**:1184–1190 (1983).

Send reprint requests to: Steven M. Paul, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285.