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Roscovitine Triggers Excitotoxicity in Cultured Granule Neurons by Enhancing Glutamate Release

Edward A. Monaco III[1] and Mary Lou Vallano

Department of Neuroscience and Physiology, State University of New York, Upstate Medical University, Syracuse, New York

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ABSTRACT

Cerebellar granule neurons are highly susceptible to injury in vivo and in vitro, and primary cultures are widely used to characterize relevant receptors and signaling pathways. However, there are problems associated with their use. In particular, cultures are typically grown in medium supplemented with elevated KCl levels because it improves survival, but accumulating evidence indicates that this causes profound neuroadaptations. For example, growth in elevated KCl levels renders neurons electrically silent. Thus, they cannot be used to examine excitotoxicity of synaptic origins. On the other hand, cultures grown in physiological medium are rarely studied because a proportion undergoes apoptosis. Herein, we provide evidence that mature neurons cultured in physiological KCl develop spontaneous action potentials that support survival through *N*-methyl-D-aspartate (NMDA) receptor-mediated mechanisms. Furthermore, the cdk inhibitor roscovitine enhances the coupling between tetrodotoxin-sensitive action potentials and P/Q-type voltage-dependent calcium channels (VDCCs), thereby converting this survival program to excitotoxicity of synaptic origin. Therefore, roscovitine-triggered necrosis requires spontaneous Na^+-based action potentials (tetrodotoxin inhibits, (±)-2-amino-4-phosphonobutyric acid enhances), P/Q-type VDCC currents (ω-agatoxin-IVA and ω-conotoxin-MVIIC inhibit, but not ω-conotoxin-GVIA), intact vesicle fusion processes (tetanus toxin inhibits), and transmitter-filled vesicles (concanamycin and bafilomycin inhibit). From a postsynaptic standpoint, roscovitine-mediated excitotoxicity requires the functionally linked activation of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate/kainate (AMPA/KA) and NMDA receptors, which is consistent with evidence that activated AMPA/KA receptors relieve the voltage-dependent Mg^{2+} block of NMDA receptors, resulting in excitotoxic Ca^{2+} influx. In the end, NMDA receptor-linked pathways transduce excitotoxicity. On the other hand, L-type VDCC blockers are not protective. Further characterization of this new model is expected to provide important insights about excitotoxicity of synaptic origins and about roscovitine as a selective modulator of this process.

Glutamate-mediated excitotoxicity is extensively studied in cell cultures and animals to model key events underlying neuronal loss in human neurodegeneration (Aarts and Tymianski, 2004). Numerous experimental paradigms support the theory that sustained NMDA receptor activation leads to toxic intracellular Ca^{2+} deregulation and death by necrosis and/or apoptosis. Therefore, NMDA receptor antagonists afford protection and provide a rationale for their use in clinical trials in humans. Unfortunately, these trials were not correspondingly successful, prompting investigators to re-evaluate the predictive value of existing models and stimulating renewed efforts to identify novel therapeutic targets.

The "source-specificity hypothesis" of excitotoxicity posits that discrete Ca^{2+} microdomains, as opposed to alterations in bulk cytoplasmic Ca^{2+}, activate distinct signaling pathways and functional responses. For example, Ca^{2+} loading through L-type voltage-dependent calcium channels (VDCCs) is not harmful, whereas comparable loads gated by NMDA receptors are excitotoxic (Sattler et al., 1998). Furthermore, in certain contexts, activation of synaptic NMDA receptors promotes survival, whereas extrasynaptic NMDA receptor activation triggers death (Hardingham and Bading, 2002). Likewise, neuronal loss in humans can involve global increases in glutamate, accessing both synaptic and extrasynaptic receptors, or more discrete and localized disorders of synaptic overactivity, as observed in transient focal cerebral

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[1] Current address: Columbia University College of Physicians and Surgeons, New York, New York.
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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; VDCC, voltage-dependent calcium channel; MK801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5–10-imine maleate; CTx, ω-conotoxin; Aga, ω-agatoxin; TnTx, tetanus toxin; DNQX, 6,7-dinitroquinoxaline-2,3-dione; AP5, 2-amino-5-phosphonopentanoic acid; 4-AP, (±)-2-amino-4-phosphonobutyric acid; DIV, days in vitro; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; AMPA/KA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate/kainate; Rosco, roscovitine.

ischemia, stroke, aging, and epilepsy (Aarts and Tymianski, 2004).

Cerebellar granule neurons are often used to study signaling pathways underlying survival and excitotoxicity because highly enriched preparations are readily obtained from the cortex of neonatal rats or mice (Contestabile, 2002). In vivo, they demonstrate selective vulnerability to excitotoxins acting through NMDA receptors and glutamate-mediated ischemic necrosis (Esrefoglu et al., 2003; Fonnum and Lock, 2004). However, cultures are usually grown in media supplemented with elevated KCl because it enhances their long-term survival by activating L-type VDCCs (Gallo et al., 1987). Although these conditions purportedly mimic afferent mossy fiber activity in vivo, neurons do not typically experience long-term depolarization. Rather, the amplitude, duration, and route of Ca^{2+} influx profoundly influence cell fate (Sattler et al., 1998; Hardingham and Bading, 2002). Thus, unlike neurons grown in 5 mM KCl, continually depolarized cultures undergo numerous Ca^{2+}-mediated adaptations (Condorelli et al., 1993; Vallano et al., 1996; Moulder et al., 2003; Choi et al., 2004; Tremper-Wells and Vallano, 2005) and are electrically silent because of Na^{+}-channel inactivation (Mellor et al., 1998). Despite this, they continue to be a favored model, whereas cultures grown in 5 mM KCl are largely ignored because a proportion dies by apoptosis 3 to 5 days after plating, a process not unlike neuronal pruning in vivo.

To effectively model neurodegeneration of synaptic origin, it is critical to study electrically viable neurons that release endogenous glutamate from presynaptic boutons. Indeed, if cultured granule neurons grown in physiological medium develop spontaneous action potentials that support exocytosis, then specific agents that modify this process could yield novel information about the survival versus excitotoxic effects of synaptic glutamate and associated receptor-linked pathways. In a previous study, we demonstrated that 2,6,9-trisubstituted purines such as roscovitine induce apoptosis in immature granule neurons (1–2 days) through a mechanism believed to involve inhibition of the transcriptional cdk 7 (Monaco et al., 2004). This report built on earlier studies that had identified roscovitine as a potent inhibitor of cdks 1, 2, and 5 and a potential cancer chemotherapeutic agent (Meijer and Raymond, 2003). Distinct studies conducted in hippocampal neurons and synaptosomes showed that roscovitine potentiates Ca^{2+} currents through P/Q-type VDCCs, thereby increasing vesicular glutamate release upon application of depolarizing stimuli, possibly by inhibiting cdk 5, which is highly enriched in postmitotic neurons (Tomizawa et al., 2002; Yan et al., 2002). Taken together, these studies indicate that the effects of roscovitine are multifunctional and complex and depend on the cell type, maturation state, and experimental paradigm. In the context of neurotransmitter release, we were interested in exploring the actions of roscovitine in mature, synaptically competent granule neurons grown in physiological medium. Herein, we show that spontaneous bioelectrical activity supports survival in granule neurons through an NMDA receptor-mediated signaling pathway, and the addition of roscovitine converts this survival program to one of glutamate excitotoxicity by enhancing the process of exocytosis in a highly specific manner.

Materials and Methods

Materials. Roscovitine, MK801, ω-conotoxin GVIA (CTx-GVIA), ω-conotoxin MVIIC (CTx-MVIIC), ω-agatoxin IVA (Aga-IVA), tetanus toxin (TnTx), nimodipine, nifedipine, cyclosporin A, concanamycin A1, bafilomycin, and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were purchased from EMD Biosciences (San Diego, CA). 2-Amino-5-phosphonopentanoic acid (AP5), (±)-2-amino-4-phosphonobutyric acid (4-AP), and firefly lantern extract were purchased from Sigma-Aldrich (St. Louis, MO). Tetrodotoxin was obtained from Alomone Labs (Jerusalem, Israel). Polyclonal rabbit anti-c-Jun and polyclonal rabbit anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Polyclonal goat anti-synaptophysin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. Primary cultures of cerebellar granule neurons were prepared and grown in an atmosphere of 5% CO_2 as described previously by our laboratory (Vallano et al., 1996). The institutional review committee, in accordance with governmental guidelines, approved all procedures involving animal experimentation. Because of their unique postnatal pattern of migration compared with other neuronal types, highly enriched preparations of granule neurons (~90–95%) can be obtained for study. In brief, cerebella from postnatal day 8 Sprague-Dawley rat pups were minced, digested with trypsin, and triturated to dissociate the cells. Cells were plated on poly-L-lysine-coated (10 μg/ml) tissue culture dishes at a density of 1.25×10^6 cells/ml. Twenty-four hours after plating, serum-containing medium was replaced with chemically defined medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 200 μg/ml transferrin, 20 ng/ml triiodothyronine, 32 μg/ml putrescine, 10 ng/ml selenium, 4 ng/ml corticosterone, 10 μg/ml bovine serum albumin, 10 μg/ml insulin, and 1.25 ng/ml progesterone. Where indicated, the culture medium was supplemented with 20 mM KCl to obtain a final concentration of 25 mM. Cultures were grown for 8 to 10 days in vitro (DIV) before experimentation. In some cases, these were compared with younger cultures grown for 1 to 2 DIV.

Cultures were treated with the *R*-isomer of roscovitine, designated herein as "roscovitine". To ascertain the mechanism of roscovitine-mediated effects, the following agents were used: MK801, CTx-GVIA, CTx-MVIIC, Aga-IVA, tetrodotoxin, TnTx, nimodipine, nifedipine, AP5, cyclosporin A, concanamycin A1, bafilomycin, 4-AP, and DNQX. Most stock solutions were prepared in dimethyl sulfoxide, and the toxins were diluted in water. The final concentration of dimethyl sulfoxide was 0.1%, which did not cause significant toxicity when added alone.

Cell Viability. To visualize changes in granule neuron morphology, random microscopic fields (four to six fields per neuronal preparation; 40× objective lens) were digitally captured on an Olympus inverted microscope under phase-contrast optics (Olympus, Tokyo, Japan). Two-diameter measurements were made per cell for 20 cells in each field. For simplicity, granule neurons were assumed to be spherical, and volumes were approximated by applying the averaged diameter measurements for each cell to the equation [volume = $(4/3)(\pi)(\text{radius})^3$]. The validity of this procedure was routinely determined by verifying that coefficients of variation became asymptotic for a given field (<0.25). All morphological analyses were verified by an observer blinded to the treatment condition.

Cellular reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was used as indirect measure of neuronal metabolism and viability as described previously (Monaco et al., 2004). In brief, MTT was added directly to culture medium for a final concentration of 0.5 mg/ml, and cultures were incubated at 37°C for 10 min. After incubation, culture medium was replaced with an equal volume of dimethyl sulfoxide to solubilize the precipitate. Absorbance measurements were then made using a spectrophotometer at a wavelength of 540 nm (U-2000; Hitachi Software Engineering, Yokohama, Japan). Linearity of the assay was routinely verified by measuring absolute absorbance values from cultures plated at 75

or 50% of the standard density. Cultures were also evaluated by phase-contrast microscopy immediately before MTT assay.

Immunochemistry. For Western immunoblotting, whole-culture homogenates were harvested in SDS buffer (1 mM $NaVO_4$, 0.3 mM phenylmethylsulfonyl fluoride, 2% SDS, 62.5 mM Tris, and 10% glycerol), sonicated, and equalized for protein content using a Micro BCA assay (Pierce Chemical, Rockford, IL). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (8% gels), transferred to nitrocellulose, and blocked in 5% nonfat dry milk. Blots were incubated at 4°C overnight in primary antibodies diluted as follows: polyclonal goat anti-synaptophysin (1:1000 in 5% nonfat dry milk), polyclonal rabbit c-Jun (1:1000 in 5% bovine serum albumin), or polyclonal rabbit cleaved caspase-3 (1:1000 in 5% nonfat dry milk). Thereafter, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (1:1000). Supersignal West Pico and X-ray films were used to visualize immunoreactivity (Pierce). Multiple protein concentrations were routinely analyzed to ensure linearity of the assay.

For immunofluorescence, cultures attached to coverslips were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 for 20 min, blocked with 10% donkey serum for 30 min, and then incubated overnight with antibody specific for synaptophysin (1:200) in 1.5% donkey serum in phosphate-buffered saline. Thereafter, coverslips were incubated with fluorescein isothiocyanate-labeled donkey anti-goat antibody (1:400 dilution, 1 h at room temperature), and then mounted on glass slides with Vectashield antifade containing propidium iodide (Vector Laboratories, Burlingame, CA). Photomicrographs were produced using a Zeiss Axioskop microscope (40× objective lens) with a digital camera (Carl Zeiss GmbH, Jena, Germany).

ATP Measurements. Cellular ATP content was determined by a luciferin/luciferase assay (Bihler and Jeanrenaud, 1970). The luciferin/luciferase solution was prepared by dissolving firefly lantern extract in a reaction buffer, pH 7.4, containing 0.33 M K_3AsO_4 and 0.02 M $MgSO_4$. Cultures were harvested in lysis buffer, pH 7.4, containing 25 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 15% glycerol. Samples were centrifuged at 14,000*g* for 10 min, and ATP was measured in the supernatant under linear rate conditions. Samples (100 μl) and luciferin/luciferase (20 μl) were added to 2.9 ml of reaction buffer and briefly mixed. Luciferase luminescence was measured after 30 s in a Beckman Coulter LS 6500 multipurpose scintillation counter set for counting ^3H (Beckman Coulter, Fullerton, CA). Under these conditions, the light emitted from the luciferase reaction is proportional to the square of the ATP concentration.

Statistical Analysis. Values stated in the text are given as means ± S.E.M. for results obtained from at least three separate cell preparations and have been analyzed for their statistical significance using an analysis of variance, followed by the Tukey-Kramer test. Results were considered statistically significant when $p < 0.05$.

Results

Spontaneous Action Potentials and NMDA Receptors Support Survival in Granule Neurons Grown in Physiological Medium. It is well established that media supplementation with elevated KCl, via activation of L-type VDCCs, enhances long-term survival in granule neurons prepared from rat cerebellum, whereas a substantial proportion of those grown without media KCl supplementation (i.e., in 5 mM KCl) undergo apoptosis beginning ~3 DIV (Gallo et al., 1987; Contestabile, 2002). This is exemplified in Fig. 1A, which shows that MTT reductive capacity, a reliable index of viability, is significantly reduced in cultures grown for 8 DIV in chemically defined medium containing 5 mM KCl compared with those grown in medium containing 25 mM KCl

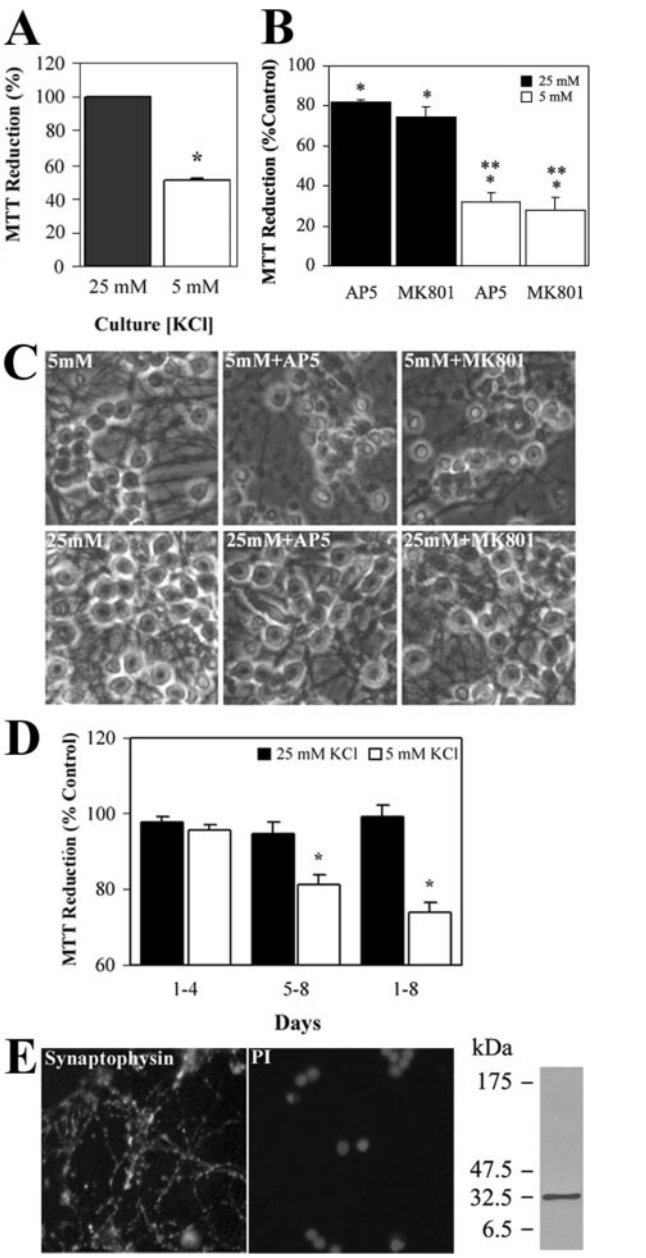

Fig. 1. NMDA receptor activation and spontaneous action potentials support survival in granule neurons grown in medium containing physiologic KCl. A, comparison of granule neuron viability, on the basis of MTT reductive capacity, in cultures grown for 8 days in medium containing 5 mM KCl (□) or 25 mM KCl (■). ⋆, significantly different from cultures grown in 25 mM KCl. B, summary of the effects of NMDA receptor antagonism on viability of granule neurons grown in medium containing 5 mM KCl (□) or 25 mM KCl (■). NMDA receptor antagonists (AP5, 300 μM; MK801, 10 μM) were added at 1 to 2 DIV, and viability was measured at 8 DIV by MTT assay. ⋆, significantly different from culture-condition–matched vehicle controls. ⋆⋆, significantly different from drug-treated cultures grown in 25 mM KCl. C, representative phase-contrast images of cultures grown for 8 DIV in medium containing 5 or 25 mM KCl alone and in the presence of 300 μM AP5 or 10 μM MK801. D, summary of the effect of tetrodotoxin on viability of granule neurons grown in medium containing 5 mM KCl (□) or 25 mM KCl (■). Tetrodotoxin (1 μM) was added at 1 or 5 DIV, and viability was measured by MTT assay at 4 or 8 DIV, as indicated. ⋆, significantly different from culture-condition–matched vehicle controls. E, granule neurons were cultured for 8 DIV in medium containing 5 mM KCl, fixed, and processed for immunocytochemistry. Antibody against synaptophysin was used, and cultures were stained with propidium iodide (PI) to label granule cell nuclei, as indicated. Western immunoblot shows that a single immunoreactive protein of apparent molecular mass of 38 kDa is recognized.

(50.6 ± 1.7% of 25 mM condition, set at 100%; $n = 8$). Consistent with this, phase-contrast microscopy indicates that many neurons grown in 5 mM KCl demonstrate apoptotic features such as cell shrinkage and appearance of inclusion bodies. Nevertheless, ~50% of neurons grown in defined media containing 5 mM KCl remain viable after 8 DIV. The contribution of glutamate-mediated activation of NMDA receptor channels to cell survival was also examined. To test this, an NMDA receptor antagonist (AP5, 300 μM; or MK801, 10 μM) was added at 1 to 2 DIV to cultures grown in medium containing 5 or 25 mM KCl, and viability was measured at 8 DIV by MTT assay. Figure 1B shows that blockade of NMDA receptor channels results in a modest but significant decrease in MTT reductive capacity in cultures grown in 25 mM KCl (AP5, 81.7 ± 1.7%, and MK801, 74.6 ± 4.9% of vehicle-treated control, set at 100%; $n = 7$ or 5, respectively). In contrast, MTT reductive capacity in these cultures was substantially decreased by the addition of the L-type VDCC antagonist nimodipine (21.4 ± 2.5% of vehicle-treated control, set at 100%, $n = 8$, data not shown). It is noteworthy that NMDA receptor blockade leads to a significantly greater decrease in MTT reductive capacity in cultures grown in 5 mM KCl (AP5, 32.2 ± 4.4%; MK801, 27.8 ± 6.4% of vehicle-treated control, set at 100%; $n = 8$ or 5, respectively). Note that this decrease is above and beyond that which occurs naturally in 5 mM cultures (Fig. 1A). In all cases, microscopic examination of cultures was in good agreement with MTT data (Fig. 1C). Thus, NMDA receptor channels are active in granule neurons cultured in medium containing 5 mM KCl and contribute substantially to their survival.

Vesicular release of neurotransmitter can occur in the presence (evoked) and in the absence (miniature synaptic potentials) of an action potential. To assess the dependence of granule neurons on spontaneous action potentials for their survival, cultures were grown in medium containing 5 or 25 mM KCl in the presence or absence of tetrodotoxin to block Na^+ channels. Cultures were treated with tetrodotoxin (1 μM) or vehicle beginning at 1 until 4 DIV, beginning at 5 until 8 DIV, or from 1 until 8 DIV, and neuronal viability was assessed by MTT assay. As shown in Fig. 1D, the addition of tetrodotoxin to immature neurons has no effect (5 mM, 97.9 ± 1.2%; 25 mM, 95.8 ± 1.2%; $n = 3$) compared with vehicle-treated controls (set at 100%). However, the addition of tetrodotoxin after a critical period of ~4 days, in parallel with synapse formation (Trenkner and Sidman, 1977), leads to a significant decrease in MTT reductive capacity in neurons cultured in 5 mM KCl (5–8 DIV, 81.4 ± 2.6%; 1–8 DIV, 73.9 ± 2.6%; $n = 3$) but not 25 mM KCl (5–8 DIV, 94.6 ± 3.1%; 1–8 DIV, 99.2 ± 3.1%; $n = 3$). The integral synaptic vesicle protein synaptophysin is the most widely used immunohisotchemical marker of synapse density (Valtorta et al., 2004). Figure 1E shows that mature cultures grown in 5 mM KCl express immunoreactive synaptophysin in a punctate manner at numerous regions of contact between neurons. Taken together, these data indicate that a significant proportion of mature granule neurons grown in medium containing 5 mM KCl require tetrodotoxin-sensitive currents and, correspondingly, action potentials for their survival. Furthermore, an even greater proportion requires NMDA receptor activation (Fig. 1B), presumably because of action potential-independent spontaneous vesicular glutamate release (Mellor et al., 1998). In contrast, long-term growth of granule neurons in medium containing 25 mM KCl perturbs these requirements, which underscores the need to use nonadapted cultures if the goal is to develop improved models of glutamate excito-toxicity.

Roscovitine Triggers Rapid Cell Swelling and Necrosis in Mature Granule Neurons Grown in Physiological Medium. At concentrations of 5 to 50 μM, roscovitine enhances transmitter release in cultured hippocampal neurons and synaptosomes (Tomizawa et al., 2002; Yan et al., 2002). However, these studies used an "evoked" release paradigm, and excitotoxicity was not observed. Because our tetrodotoxin data suggest that mature granule neurons grown in medium containing 5 mM KCl develop spontaneous action potentials that contribute to survival, we examined whether the application of roscovitine would further enhance glutamate-mediated survival or lead to excitotoxic death. To test this, cultures were grown for 8 to 10 DIV and then treated overnight (~16 h) with a range of concentrations of roscovitine (1–50 μM) or vehicle, and viability was assessed by MTT assay (Fig. 2A). As shown, roscovitine treatment decreases MTT reductive capacity in a concentration-dependent manner, with 25 and 50 μM triggering significant death (25 μM, 46.3 ± 8.3%; 50 μM, 30.0 ± 7.3%, compared with vehicle, set at 100%; $n = 3$). To examine time dependence, the lowest effective concentration of roscovitine (25 μM) was applied to cultures, and viability was assessed by MTT assay over the following 1 to 24 h. Figure 2B shows that MTT reductive capacity is significantly decreased at 4 and 12 h (81.1 ± 4.0% and 59.1 ± 3.7%, respectively) compared with vehicle controls (set at 100%). By 24 h, it is almost completely lost (11.3 ± 5.4%), and the residual MTT reductive capacity is probably contributed by glia, because viable neurons are not apparent upon microscopic examination of the cultures (data not shown). These data indicate that roscovitine produces a time- and concentration-dependent excitotoxicity in mature, spontaneously active granule neurons.

Glutamate is trophic when added to the media bathing immature cerebellar granule neurons but, when applied after 7 to 8 DIV, triggers death by necrosis or apoptosis, depending on the concentration (Ankarcrona et al., 1995). These data suggest that alterations in NMDA receptor coupling to downstream signaling pathways may occur during maturation. To observe the morphology of the death process and the development of vulnerability to drug, neurons grown in 5 mM KCl were treated with roscovitine (25 μM) at an early (1–2 days, before development of tetrodotoxin-sensitivity) or late (8–10 days, after development of synapses and tetrodotoxin sensitivity) stage in culture. Within hours after roscovitine administration, 8- to 10-day neurons demonstrate a remarkable increase in size (Fig. 2C, panel B), indicative of cellular swelling. Compared with the vehicle-treated controls (Fig. 2C, panel A), the volume of roscovitine-treated neurons increases by more than 2-fold (Fig. 2C, panel B, Late Rosco, 259.8 ± 4.5%, $n = 3$) when measured after 2 to 4 h. In contrast, roscovitine does not provoke volume changes in neurons cultured for 1 to 2 days (Fig. 2C, panel D, and 2D, Early Rosco, 100.0 ± 0.8; $n = 3$), in which cell shrinkage and apoptosis, but not swelling, eventually occur over a more prolonged time course (Monaco et al., 2004). It has long been established that rapid swelling is the product of Na^+ and Cl^- ionic dyshomeostasis followed by the influx of water (Choi, 1987), leading to necrotic death. Thus, the absence of delayed

cell shrinkage, coupled with rapid cellular swelling, suggests that roscovitine triggers necrotic rather than apoptotic death in mature granule neurons.

Rapid induction of c-Jun followed by delayed activation of caspase-3 are prominent features of apoptosis triggered by KCl and serum withdrawal from mature, neuroadapted granule neurons that have been grown for extended periods in media containing 25 mM KCl (Watson et al., 1998), and in immature granule neurons (1–2 DIV) treated with roscovitine (Monaco et al., 2004). To further distinguish the mode of roscovitine-mediated death in mature granule neurons, its effects on immunoreactive c-Jun and cleaved caspase-3 were determined. Therefore, cultures were grown in medium containing 5 mM KCl for 8 to 10 days, roscovitine (25 μM) or vehicle was added, and whole-cell homogenates were harvested and processed for Western immunoblotting at the indicated times (Fig. 2E). In mature roscovitine-treated neurons, total c-Jun protein decreases by 4 h and remains depressed, compared with vehicle controls. In addition, the amount of cleaved (i.e., active) caspase-3 does not change appreciably over a 24-h period (late; Fig. 2E, top). In contrast, roscovitine-treated immature neurons (early; Fig. 2E, bottom) demonstrate substantial increases in c-Jun protein (peak at 8 h) and, later, in cleaved caspase-3 (peak at 24 h). Together, these data indicate that roscovitine is a potent trigger of cell death in both developing and phenotypically more mature granule neurons grown in 5 mM KCl, but by distinct mechanisms: it rapidly triggers necrosis in mature neurons but causes a delayed form of apoptosis associated with activation of c-Jun and caspase-3 in immature neurons.

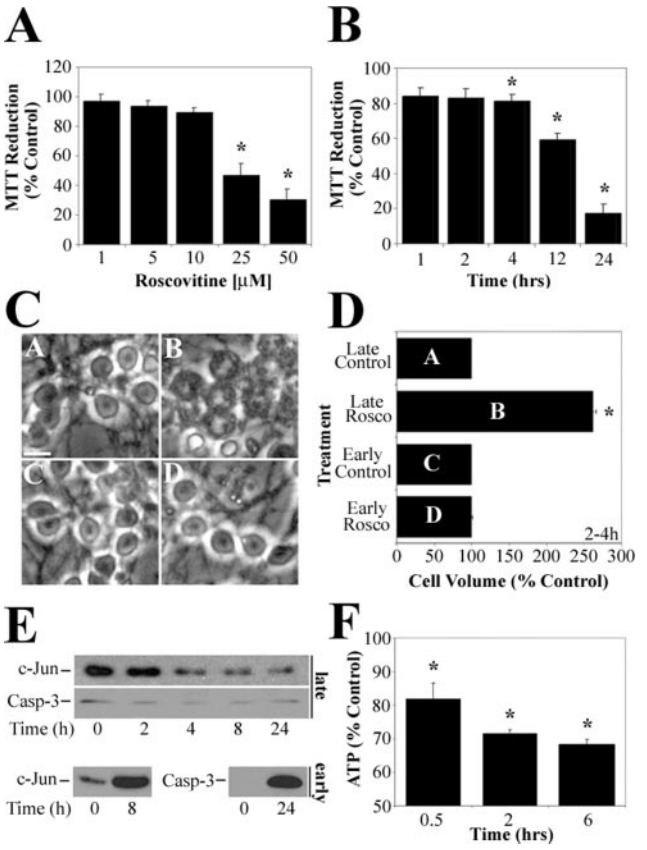

Fig. 2. Roscovitine triggers distinct death programs in immature versus mature granule neurons. A, summary of effects on neuronal viability of overnight treatment of cultures with increasing concentrations of roscovitine. Cultures were grown for 8 to 10 DIV in medium containing 5 mM KCl and assayed for viability by MTT. ⋆, significantly different from vehicle-treated controls. B, summary of the time-dependent effect of roscovitine (25 μM) on the viability of mature granule neurons (8–10 DIV), assessed by MTT assay. ⋆, significantly different from vehicle-treated controls. C, representative photomicrographs of granule neurons grown in medium containing 5 mM KCl and treated with vehicle (A and C) or roscovitine (25 μM; B and D) at either 8 to 10 DIV (A and B) or 1 to 2 DIV (C and D). Scale bar, ~10 μm. D, summary of the effects of roscovitine (25 μM) on neuronal volume when applied overnight to mature neurons (i.e., late, 8–10 DIV) versus immature neurons (i.e., early, 1–2 DIV). ⋆, significantly different from age-matched vehicle-treated control. E, representative Western immunoblot of c-Jun and cleaved/active caspase-3 in cultures treated overnight with roscovitine at 8 to 10 DIV (late) or 1–2 DIV (early); 40 μg of protein was loaded per lane. F, summary of the time-dependent effect of roscovitine (25 μM) on the cellular ATP content in cultures grown in medium containing 5 mM KCl. ⋆, significantly different from vehicle-treated controls.

Mitochondrial injury is understood to have a critical role in excitotoxic neuronal death and, in this context, perturbation of mitochondrial function compromises cellular energetics (Ankarcrona et al., 1995; Ward et al., 2000). As an index of this, the effect of roscovitine (25 μM) on the cellular ATP contents of mature cultures was determined. Figure 2F shows that ATP is significantly decreased at 30 min, 2 h, and 6 h (81.8 ± 4.7, 71.5 ± 1.1, and 68.3 ± 1.4%, respectively), compared with vehicle-treated controls (set at 100%, $n = 3$). The early decline in ATP levels precedes morphological evidence of cell damage and is consistent with the notion that the neurons possess insufficient energetic support to maintain ionic homeostasis. In this regard, it is noteworthy that the earliest decreases in ATP levels do not occur in conjunction with significant decreases in MTT reductive capacity (Fig. 2B). This is in contrast to excitotoxicity triggered by bath application of exogenous glutamate to neuroadapted granule neurons in which significant decreases in ATP and MTT reductive capacity occur in parallel (Ankarcrona et al., 1995), thus hinting at differences between the two excitotoxicity paradigms.

Roscovitine-Mediated Necrosis Requires Ionotrophic Glutamate Receptors but Not L-Type VDCCs. There is ample evidence that glutamate excitotoxicity in granule neurons grown under continually depolarizing conditions requires activation of Ca^{2+}-permeable NMDA receptor channels (Lysko et al., 1989; Schramm et al., 1990). Similar effects are obtained with NMDA, if applied in Mg^{2+}-free buffer, to eliminate the voltage-dependent blockade of NMDA receptors. In contrast, L-type VDCCs mediate the survival-promoting effects of elevated KCl (Gallo et al., 1987) and do not have a significant role in neuronal glutamate excitotoxicity (Sattler et al., 1998) or in the regulation of presynaptic neurotransmitter release (Harrold et al., 1997). To test the involvement of ionotropic glutamate receptors and L-type VDCCs in our model, mature cultures (8–10 days) grown in medium containing 5 mM KCl were treated with roscovitine (25 μM) or vehicle alone or were cotreated with roscovitine and one of the following: the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate/kainate (AMPA/KA) receptor antagonist DNQX (100 μM); the open-channel NMDA receptor antagonist MK801 (10 μM); or the L-type VDCC antagonist nimodipine (10 μM). Within a few hours, neurons treated with roscovitine alone (Fig. 3A, panel B), or roscovitine plus nimodipine (panel E), are enlarged and show a necrotic phenotype compared with vehicle controls (panel A), whereas cotreatment with DNQX or MK801 is neuroprotec-

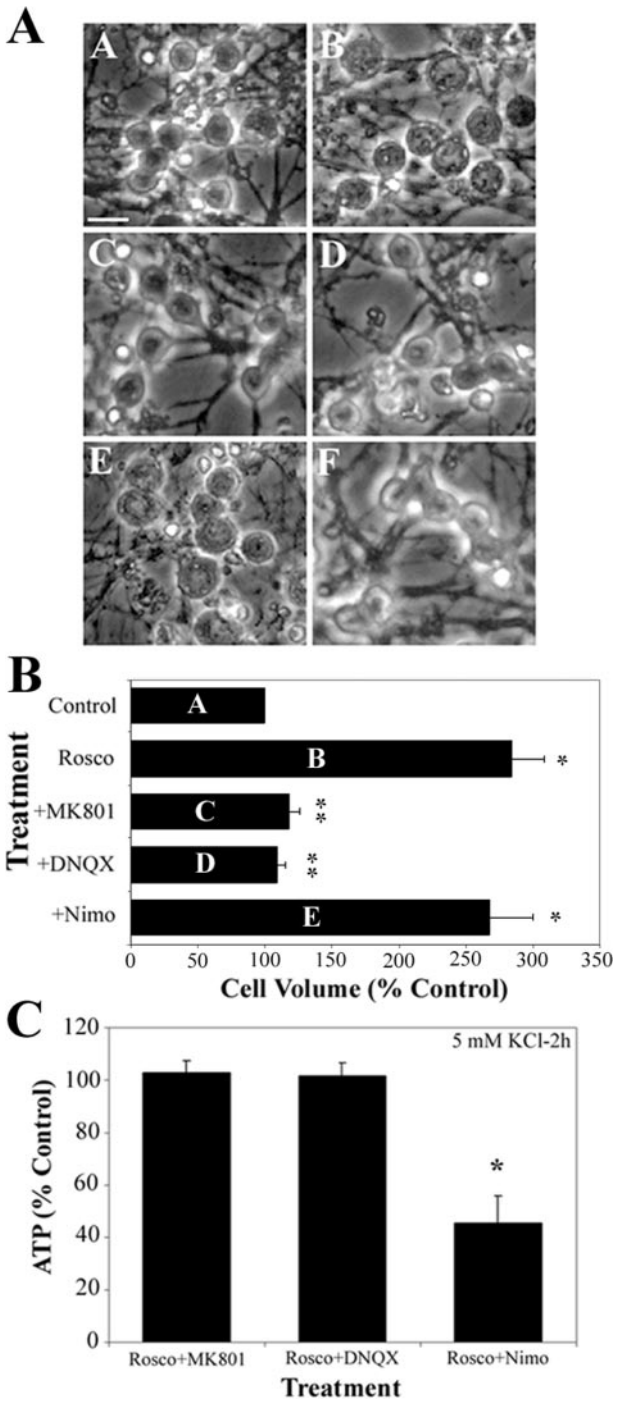

Fig. 3. Roscovitine-mediated excitotoxicity requires activation of ionotropic glutamate receptors. In all cases, granule neurons were grown for 8 to 10 DIV in medium containing 5 mM KCl, and 25 μM roscovitine was used. A, representative photomicrographs of granule neurons 2 to 4 h after treatment with vehicle (A), roscovitine (B), roscovitine plus MK801 (10 μM, C), roscovitine plus DNQX (100 μM, D), roscovitine plus nimodipine (10 μM, E), or MK801 alone (10 μM, F). Scale bar, ~10 μm. B, summary of effects on neuronal volume of roscovitine treatment for 2 to 4 h with vehicle (Control), roscovitine (Rosco), roscovitine plus MK801 (10 μM, +MK801), roscovitine plus DNQX (100 μM, +DNQX), or roscovitine plus nimodipine (10 μM, +Nimo). ⋆, significantly different from vehicle-treated controls. ⋆⋆, significantly different from roscovitine alone. C, summary of drug effects on cellular ATP content after 2-h treatment with roscovitine plus MK801 (10 μM, Rosco+MK801), roscovitine plus DNQX (100 μM, Rosco+DNQX), or roscovitine plus nimodipine (50 μM, Rosco+Nimo). ⋆, significantly different from vehicle-treated controls (set at 100%).

tive (C and D). MK801 (F), DNQX (data not shown), or nimodipine (data not shown), when applied to cultures individually, results in no obvious untoward effects during this relatively short treatment period. In addition, when Cd^{2+} (20 μM) was added just before roscovitine challenge to block divalent cation-permeable channels, neurons are protected (data not shown). Consistent with these morphological data, cultures receiving DNQX or MK801 plus roscovitine do not demonstrate significant changes in cell volume, compared with vehicle controls (Fig. 3B; +DNQX, 109.7 ± 5.7%; +MK801, 118.1 ± 7.8%; $n = 6$), whereas roscovitine alone or roscovitine plus nimodipine causes impressive swelling (Rosco, 284.8 ± 24%; +Nimo, 268.1 ± 32; $n = 4$). Moreover, both glutamate receptor antagonists also prevent the decrease in ATP concentration associated with roscovitine treatment (Fig. 3C; Rosco + MK801, 102.7 ± 4.8%; Rosco + DNQX, 101.8 ± 4.6%, versus vehicle controls; $n = 3$), whereas inhibition of L-type VDCCs by nimodipine does not (Rosco + Nimo, 45.3 ± 10.5%; $n = 3$). This lack of effect of nimodipine is in good agreement with observations that, despite comparable increases in intracellular Ca^{2+}, it is the NMDA receptor and not the L-type VDCC that mediates excitotoxic Ca^{2+} influx (Sattler et al., 1998; Aarts and Tymianski, 2004). The observation that DNQX and MK801 are equally neuroprotective suggests that excessive glutamate release, triggered by the addition of roscovitine to spontaneously active granule neurons, produces sufficient postsynaptic depolarization through the activation of AMPA/KA receptors to relieve the Mg^{2+} blockade of NMDA receptors. It is noteworthy that these data expand the role of functionally linked AMPA/KA and NMDA receptor activation to a model of excitotoxicity.

Roscovitine-Mediated Necrosis Requires Spontaneous Action Potentials, P/Q-Type VDCCs, and Vesicular Fusion. In cerebellar granule neurons, N-, P-, and Q-type Ca^{2+} currents can be discerned pharmacologically by the use of selective antagonists: CTx-GVIA (N-type), CTx-MVIIC (P/Q- and N-types), and Aga-IVA (P/Q-type) (Randall and Tsien, 1995). These VDCCs contribute differently to evoked transmitter release (Harrold et al., 1997), with major contributions by P- and Q-type channels, and a minor contribution by the N-types. In the following series of experiments, we examined the hypothesis that roscovitine-mediated necrosis requires spontaneous action potentials, P/Q-type VDCCs, and an intact vesicular fusion apparatus. To test this, cultures were grown for 8 to 10 days in medium containing 5 mM KCl and treated with roscovitine (25 μM) or vehicle alone, or cotreated with roscovitine plus distinct VDCC antagonists. Unlike cultures receiving roscovitine alone, those cotreated with roscovitine plus P/Q-type channel antagonists are protected (Fig. 4A; + 1 μM CTx-MVIIC, panel C; + 1 μM Aga-IVA, panel D). On the other hand, those cotreated with an N-type-selective agent seem necrotic (Fig. 4A; +1 μM CTx-GVIA, panel E). Corresponding volume determinations indicate that, after only a few hours, neurons cotreated with roscovitine plus CTx-MVIIC (1 μM) or Aga-IVA (1 μM) are not significantly different from vehicle controls (Fig. 4B; 121.2 ± 0.5 and 124.8 ± 3.7%, respectively; $n = 3$), whereas those receiving roscovitine plus CTx-GVIA (1 μM) are significantly swollen, with volumes indistinguishable from roscovitine treatment alone (Fig. 5B; + CTx-GVIA, 278.4 ± 31.8%; Rosco, 284.8 ± 24%; $n = 3$). These data indicate that rosco-

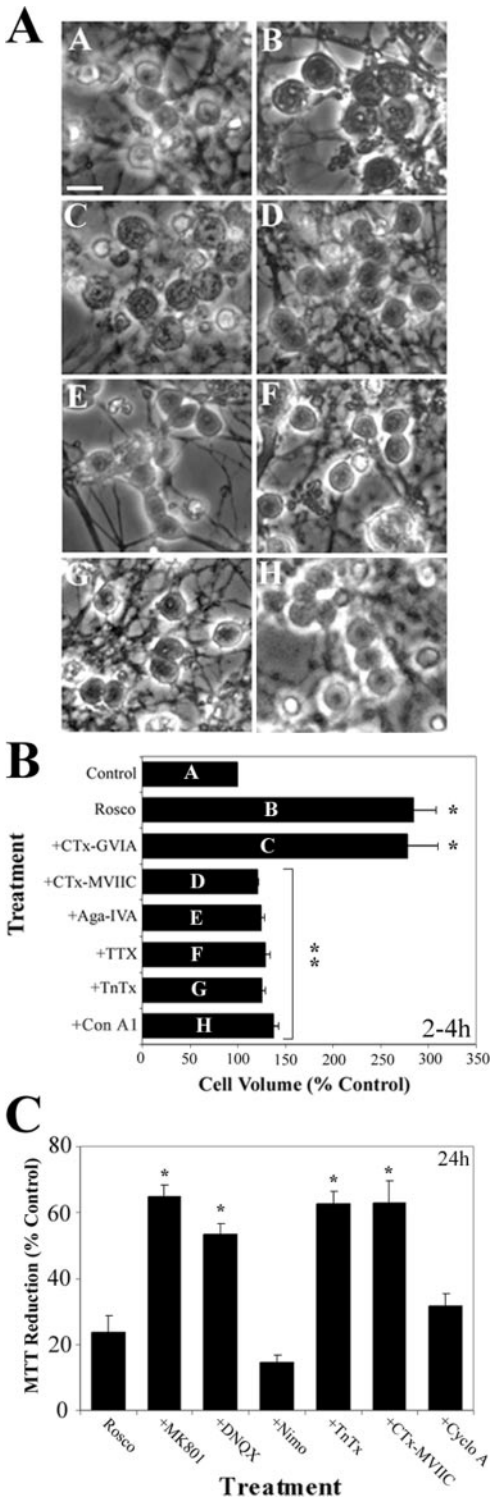

Fig. 4. Inhibition of P/Q-type VDCCs, action potentials, and neurotransmitter vesicle fusion prevents rapid neuronal swelling and provides protection from roscovitine. In all cases, granule neurons were grown for 8 to 10 DIV in medium containing 5 mM KCl, and 25 μM roscovitine was used. A, representative photomicrographs of granule neurons 2 to 4 h after treatment with vehicle (A), roscovitine (B), roscovitine plus CTx-MVIIC (1 μM, C), roscovitine plus Aga-IVA (1 μM, D), roscovitine plus CTx-GVIA (1 μM, E), roscovitine plus tetrodotoxin (10 μM, F), roscovitine plus TnTx (5 μg/ml, G), or roscovitine plus concanamycin A1 (1 μM, H). Scale bar, ~10 μm. B, summary of effects on neuronal volume by treatment for 2 to 4 h with vehicle (Control), roscovitine (Rosco), roscovitine plus CTx-GVIA (1 μM, +CTx-GVIA), roscovitine plus CTx-MVIIC (1 μM, +CTx-MVIIC), roscovitine plus Aga-IVA (1 μM, +Aga-IVA), roscovitine plus tetrodotoxin (10 μM, +TTX), roscovitine plus TnTx (5 μg/ml, +TnTx), or roscovitine plus concanamycin A1 (1 μM, +Con A1). ⋆, significantly different from vehicle-treated controls. ⋆⋆, significantly different from roscovitine alone. C, summary of effects on neuronal viability 24 h after treatment with roscovitine, roscovitine plus MK801 (10 μM, +MK801), roscovitine plus DNQX (100 μM, +DNQX), roscovitine plus TnTx (5 μg/ml, +TnTx), roscovitine plus CTx-MVIIC (1 μM, +CTx-MVIIC), or roscovitine plus cyclosporin A (1 μM, +Cyclo A). ⋆, significantly different from roscovitine alone.

vitine-mediated toxicity requires activation of P/Q-type but not N-type VDCCs.

Activation of P/Q channels and subsequent Ca^{2+} entry into presynaptic terminals is a voltage-dependent process that requires depolarization of the presynaptic membrane. In the mature central nervous system, electrical activity in the form of Na^+-driven action potentials initiates this process and can be inhibited by tetrodotoxin. To determine whether electrical activity is a requirement for roscovitine-mediated toxicity, cultures were grown in medium containing 5 mM KCl for 8 to 10 days and were treated with roscovitine in the presence or absence of tetrodotoxin. As shown in Fig. 4A, tetrodotoxin rescues neurons from roscovitine-mediated swelling (Fig. 4A, panel F), and these cultures are morphologically indistinguishable from vehicle-treated controls (Fig 4A, panel A). Consistent with this, tetrodotoxin attenuates roscovitine-induced increases in cell volume compared with controls (Fig. 4B; + tetrodotoxin, 128.8 ± 5.2%; $n = 7$). These data support a functional link between action potential-dependent Na^+-channel currents, activation of voltage-dependent P/Q channels, and roscovitine-mediated excitotoxicity in these cultures.

Synaptobrevin is believed to bind plasma membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins to form a core complex, which couples N- and P/Q-channel activity to vesicular docking and fusion. TnTx blocks Ca^{2+}-dependent neurotransmitter release from presynaptic terminals by selectively cleaving synaptobrevin (Schiavo et al., 1992). We tested whether intact synaptobrevin is required for roscovitine-mediated excitotoxicity by treating cultures with roscovitine (25 μM) or vehicle alone, or cotreating cultures with roscovitine plus TnTx. Applied just before the addition of roscovitine, TnTx attenuates roscovi-

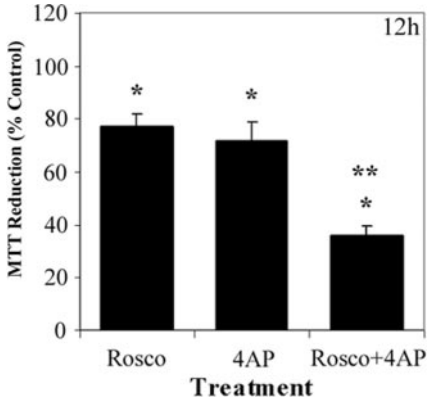

Fig. 5. Cotreatment with 4-aminopyridine triggers death and exacerbates roscovitine-mediated necrosis. Granule neurons were grown for 8 to 10 DIV in medium containing 5 mM KCl. Summary of drug effects on neuronal viability in neurons treated for 12 h with vehicle (set at 100%), roscovitine (25 μM, Rosco), 4-AP (0.5 mM, 4AP), or roscovitine plus 4-AP (Rosco + 4AP). ⋆, significantly different from vehicle-treated control. ⋆⋆, significantly different from roscovitine alone.

tine-mediated swelling of granule neurons (Fig. 4A, panel G), and volume measurements in neurons receiving roscovitine plus TnTx are not significantly different from those of vehicle-treated controls (Fig. 4B; + TnTx, 125.7 ± 3.1%; $n = 3$). Thus, upon roscovitine addition, mature granule neurons grown under nondepolarizing conditions release glutamate from presynaptic terminals in a manner that requires coupling of Ca^{2+} currents to the vesicle fusion apparatus.

The V-type ATPase in synaptic vesicle membranes regulates the transmembrane proton and electrochemical gradients essential for the accumulation and retention of glutamate (Maycox et al., 1990). The antibiotics bafilomycin A1 and concanamycin A are potent and selective inhibitors of the V-ATPase (Drose et al., 1993) and, as a consequence, they interfere with synaptic transmission by depleting vesicles of a neurotransmitter. By this line of reasoning, if presynaptic vesicles containing glutamate are required for roscovitine-induced necrosis in our model, then preincubation of cultures with V-ATPase inhibitors should be neuroprotective. To test this, cultures grown for 8 to 10 days in medium containing 5 mM KCl were preincubated with either concanamycin A1 (1 μM) or bafilomycin A1 (1 μM) for 1 h, treated with roscovitine (25 μM), and examined for evidence of swelling and necrosis. As shown, neurons cotreated with roscovitine and concanamycin A1 do not demonstrate a necrotic morphology, when examined after 2 to 4 h (Fig. 4A, panel H), an observation verified by the lack of a significant increase in cell volume (Fig. 4B, + Con A1, 138 ± 4.8%; $n = 3$), compared with vehicle-treated controls (set at 100%). A similar result is obtained with bafilomycin A1 (data not shown). These data provide additional evidence that the excitotoxic action of roscovitine is caused by enhanced release of presynaptic glutamate in spontaneously active granule neurons.

To examine whether the observed neuroprotection is enduring, cultures were assayed for viability by MTT 24 h after the addition of roscovitine alone or after combinations of roscovitine and neuroprotective agents (Fig. 4C). Consistent with previous data (Fig. 2B), roscovitine greatly decreases MTT reductive capacity to 22.8 ± 5.9% of that of vehicle-treated controls ($n = 3$), whereas blockade of NMDA (+ MK801, 64.9 ± 3.5%) or AMPA/KA (+ DNQX, 53.5 ± 2.9%) receptor activity significantly attenuates this effect ($n = 3$; Fig. 4C). Likewise, inhibiting vesicle fusion (+ TnTx, 62.6 ± 3.8%) or P/Q-type VDCC activity (+ CTx-MVIIC, 62.8 ± 6.7%) is similarly neuroprotective ($n = 3$). In contrast, no protection is observed in cultures cotreated with roscovitine and nimodipine (14.5 ± 2.1%; $n = 3$). In agreement with MTT measurements, microscopic examination of cultures treated with roscovitine alone or roscovitine plus nimodipine revealed massive cell lysis (data not shown). It is noteworthy that perturbing glutamate release and subsequent downstream signaling pathways, in and of themselves, have deleterious effects on survival (Fig. 1, A–C). Thus, it is not surprising that the neurons are not completely rescued by agents that interfere with the action of roscovitine.

Finally, cyclosporin A significantly protects neuroadapted granule neurons from excitotoxicity in response to bath application of exogenous glutamate (Ankarcrona et al., 1995). We confirmed the protective effect of cyclosporin A in cultures grown in physiological medium and receiving bath-applied glutamate (data not shown). The mode of protection is believed to be limited to glutamatergic insults of lesser intensities (Brustovetsky and Dubinsky, 2000). Because necrotic death is believed to result from more intense insults, we examined the effect of cyclosporin A in our model. Figure 4C shows that cotreatment of neurons with roscovitine (25 μM) and cyclosporin A (1 μM) fails to attenuate roscovitine-induced glutamate toxicity, assessed by MTT reductive capacity (29.1 ± 3.0%, $n = 3$). These data suggest that modulation of exocytosis by roscovitine produces a form of excitotoxicity that is more highly intense or mechanistically distinct than bath application of exogenous glutamate.

Potassium Channel Blockade Triggers Excitotoxicity and Exacerbates the Effect of Roscovitine. If conversion of a glutamate-mediated survival program to an excitotoxicity program requires increased presynaptic glutamate release, then other pharmacological agents that enhance this process should also trigger excitotoxicity. For example, the nonselective potassium-channel antagonist 4-AP will lengthen the duration of an action potential by slowing membrane repolarization (Mathie et al., 1998) and should therefore enhance exocytosis when given alone and perhaps may exacerbate the effect of roscovitine. To test this, granule neurons were grown for 8 to 10 days in medium containing 5 mM KCl and were challenged with vehicle, roscovitine alone (25 μM), 4-AP alone (0.5 mM), or roscovitine plus 4-AP. Viability of cultures was measured by MTT assay after 12 h, because longer exposure to roscovitine alone produces extensive neuronal death (Figs. 2B and 4C). As shown in Fig. 5, treatment with roscovitine or 4-AP significantly decreases MTT reductive capacity (77 ± 4.7 and 71.5 ± 7.3%, respectively) compared with vehicle (set at 100%, $n = 4$). Moreover, cotreatment with 4-AP significantly exacerbates the roscovitine-mediated decrease in MTT reductive capacity (35.9 ± 3.6%). Consistent with this, cell swelling was apparent in cultures receiving roscovitine plus 4-AP when examined microscopically 2 to 4 h after treatment (data not shown). These data indicate that, in addition to roscovitine, a structurally and functionally distinct agent that promotes exocytosis triggers excitotoxicity in mature granule neurons.

Roscovitine Does Not Induce Necrosis in Cerebellar Granule Neurons Grown in Continually Depolarizing Medium. As indicated, the growth of rat granule neurons in depolarizing KCl depresses multiple classes of VDCCs and renders them vulnerable to death upon KCl withdrawal (Moulder et al., 2003). Nonetheless, an elegant and comprehensive literature by Nicholls and colleagues describes the coupling of VDCCs to neurotransmitter exocytosis in granule neurons grown in medium supplemented with elevated KCl. In these paradigms, exocytosis was examined after cultures were equilibrated in medium containing physiological concentrations of KCl (Harrold et al., 1997; Ward et al., 2000), presumably to promote some recovery of the presynaptic fusion apparatus. Consistent with this, mouse granule neurons recover spontaneous miniature inhibitory postsynaptic potentials and miniature excitatory postsynaptic potentials after overnight equilibration in medium containing 5 mM KCl to replenish vesicles (Mellor et al., 1998; Urakubo et al., 2003), suggesting that particularly dynamic processes are able to revert to a state that resembles granule neurons in vivo when examined over a relatively limited time frame. It is well established, however, that withdrawal of elevated KCl from neuroadapted cultures is itself a potent trigger of neuronal death and is used extensively as a model of apoptosis

(Miller and Johnson, 1996; Watson et al., 1998; Contestabile, 2002). In fact, within 15 min of K^+ withdrawal, the activator protein-1 transcription factor c-Jun becomes phosphorylated and activated by c-Jun N-terminal kinase 3, an event required for apoptosis (Watson et al., 1998). Glucose uptake decreases by 70% and protein synthesis by 40% after only 2 h (Miller and Johnson, 1996). After an additional hour, the transcriptional events regulating cell death are no longer reversible (Watson et al., 1998). Thus, not only are continually depolarized neurons extensively neuroadapted, but an additional layer of complexity must be accounted for in experimental paradigms involving their equilibration in media lacking elevated KCl because of the induction of apoptosis. For comparative purposes, we examined the effects of roscovitine in mature granule neurons grown in medium supplemented with elevated KCl using a paradigm that supports evoked glutamate release. Cultures were grown for 8 to 10 days in medium containing 25 mM KCl and were switched to conditioned medium containing 5 mM KCl before the addition of roscovitine (25 μM) or vehicle. For comparison, cultures that were switched to conditioned medium containing 25 mM KCl were also included. When examined 2 to 4 h after treatment, all cultures seemed morphologically similar, with no evidence of necrotic injury (Fig. 6A). Consistent with this, roscovitine treatment for 2 to 4 h did not increase neuronal volumes (Fig. 6B). However, by 24 h, MTT reductive capacity is significantly and substantially decreased in cultures that were switched from medium containing 25 to 5 mM KCl (Fig. 6C), which is consistent with their reliance on activity for survival maintenance. Taken together, these data indicate that roscovitine does not trigger the release of excitotoxic levels of glutamate in neuroadapted cultures (i.e., those grown in medium containing 25 mM KCl), presumably because they are not spontaneously active and do not recover sufficient capacity for exocytosis even after equilibration in physiological media.

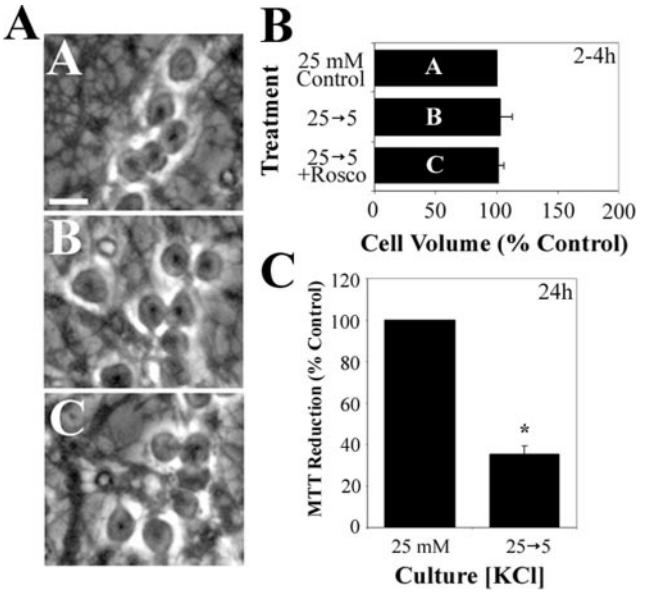

Fig. 6. Medium supplementation with 25 mM KCl prevents roscovitine-mediated granule cell death. In all cases, granule neurons were initially grown for 8 to 10 DIV in medium containing 25 mM KCl, and 25 μM roscovitine was used. A, representative photomicrographs of granule neurons were grown in medium containing 25 mM KCl and then treated overnight with vehicle (A) or switched to medium containing 5 mM KCl in the absence (B) or presence (C) of roscovitine. Scale bar, ~10 μm. B, summary of the effects of roscovitine on neuronal volume when applied for 2 to 4 h to neurons grown in medium containing 25 mM KCl (25 mM, Control) or switched to medium containing 5 mM KCl in the absence (25→5) or presence (25→5+Rosco) of roscovitine. C, comparison of viability, assessed by MTT assay in granule neurons grown in medium containing 25 mM KCl or switched to medium containing 5 mM KCl for 24 h before assay. ⋆, significantly different from 25 mM KCl.

Discussion

The cerebellum, extensively studied as a coordinator of motor tasks, has also been implicated in normal cognition and neuropsychiatric disorders of cognition (Middleton and Strick, 1994; Stanley, 2002; Schmahmann, 2004). Furthermore, because it continues to grow through adolescence (Giedd et al., 1999), it is at increased risk for irreversible damage in youth who consume drugs of abuse. Granule cells, the most abundant neurons in the brain, are vulnerable to insults involving NMDA receptor activation, including ischemia and various toxins (Esrefoglu et al., 2003), perhaps because they maintain relatively low amounts of Ca^{2+} binding proteins and DNA repair enzymes (Fonnum and Lock, 2004). As our appreciation of the cerebellum in cognition and neuropathology has evolved, so also has the need to understand more completely the signaling pathways contributing to injury and protection in a bona fide model of granule neurons in vivo.

Highly enriched cultures of granule cells are readily prepared from neonatal rodent, develop glutamatergic synapses and receptors, and have long been used to model neuronal physiology and pathology (Contestabile, 2002). Almost without exception, however, cultures are grown in media containing elevated KCl (or NMDA) because it enhances long-term survival compared with those grown in physiological media (Gallo et al., 1987). This is despite mounting evidence that continually depolarized cultures undergo numerous adaptations, including perturbed patterns of AMPA (Condorelli et al., 1993), NMDA (Vallano et al., 1996), and GABA (Mellor et al., 1998) receptor subunit expression; abnormal induction of inositol 1,4,5-trisphosphate receptor (Choi et al., 2004); calpain-mediated down-regulation of Ca^{2+}/calmodulin-dependent protein kinase type IV (Tremper-Wells and Vallano, 2005), a net decrease in synaptic vesicles (Urakubo et al., 2003); suppression of spontaneous synaptic activity and action potentials (Mellor et al., 1998); reduced VDCC currents; and increased susceptibility to death upon activity reduction (Moulder et al., 2003). On the other hand, a significant proportion of granule neurons grown in medium containing 5 mM KCl require both tetrodotoxin-sensitive Na^+ currents and active NMDA receptors for their survival (Fig. 1). Tetrodotoxin sensitivity emerges in a manner consistent with evolution of functional synapses (~3–5 days) (Trenkner and Sidman, 1977) and coincides with a wave of programmed cell death in these cultures (Gallo et al., 1987). The observation that some neurons are vulnerable to tetrodotoxin, and even more to ionotropic glutamate receptor antagonists, suggests that glutamate is released by action potential-dependent and -independent modes. Surviving neurons develop a robust neuritic network and demonstrate maturation-dependent alterations, also seen in acutely prepared slices, including a gradual switch in the expression of NMDA receptors containing NR2B to those containing NR2A (Vallano et al., 1996);

delayed expression of NR2C and a splice variant of NR1 containing exon 5 (Vallano et al., 1996); a developmentally appropriate $GABA_A$ receptor subunit expression program (Mellor et al., 1998); and a greatly enhanced degree of synaptic connectivity (Randall and Tsien, 1995). Moreover, they exhibit spontaneous action potentials, miniature inhibitory postsynaptic potentials, and miniature excitatory postsynaptic potentials (Mellor et al., 1998). In contrast, cultures grown in medium containing elevated KCl do not require tetrodotoxin-sensitive action potentials for survival (Fig. 1D), and their reliance for survival on NMDA receptor activation is markedly reduced (Fig. 1, B and C).

We provide novel evidence that granule neurons grown in 5 mM KCl, when treated with roscovitine, undergo a rapid glutamate-mediated excitotoxic necrosis that depends on the functionality of the presynaptic machinery and activation of AMPA and NMDA receptors (Fig. 7). Exposed to roscovitine for only a few hours, mature granule neurons undergo measurable swelling. Unlike studies in hippocampal cultures (in which excitotoxicity was not observed), a depolarizing stimulus is not required, consistent with evidence that cultures are spontaneously active. It is also noteworthy that roscovitine induces a distinct mode of death in synaptically incompetent granule neurons (1–2 days), characterized by activation of caspase-3 and delayed apoptosis (Fig. 2) (Monaco et al., 2004). In mature cultures (8–10 days), the toxic effect of roscovitine is caused by enhancement of action potential-dependent exocytosis. For that reason, it requires that the cultures support spontaneous Na^+-based action potentials (inhibited by tetrodotoxin, enhanced by 4-AP), activated P/Q-type VDCCs (inhibited by Aga-IVA or CTx-MVIIC, not CTx-GVIA), an intact vesicle fusion apparatus (inhibited by TnTx), and vesicular accumulation of glutamate (inhibited by concanamycin and bafilomycin). The population of neurons that survives in 5 mM KCl seems to be selectively sensitive to roscovitine-mediated necrosis. The observation that distinct and sequential components of the presynaptic regulatory apparatus can be inhibited to provide equivalent and nearly complete protection from roscovitine-mediated necrosis indicates that roscovitine's effect is highly selective in this paradigm. From a postsynaptic standpoint, antagonists of AMPA and NMDA receptors are neuroprotective. Moreover, in agreement with studies in other neuronal types (Sattler et al., 1998; Aarts and Tymianski, 2004), Ca^{2+} entry through L-type VDCCs does not contribute to roscovitine-induced excitotoxicity. Together, the data indicate that granule neurons grown in 5 mM KCl have a responsive exocytotic apparatus that can be modified by roscovitine, 4-AP, and probably other agents that influence the presynaptic machinery. In contrast, the growth of cultures in depolarizing medium perturbs these processes, rendering the cells unresponsive to roscovitine after brief equilibration in medium containing 5 mM KCl.

Roscovitine was first identified as a potent and selective inhibitor of the cell-cycle cdks 1 and 2 and cdk 5, which is expressed primarily in postmitotic neurons (Meijer and Raymond, 2003). In immature granule neurons grown in medium containing 5 or 25 mM KCl, roscovitine induces a delayed form of apoptosis that is independent of cdks 1, 2, and 5 and is probably dependent on inhibition of the transcriptional cdk 7 (Monaco et al., 2004). In whole brain synaptosomes, hippocampal slices and cultured hippocampal neurons, roscovitine enhances glutamate release via potentiation of Ca^{2+} influx through P/Q-type VDCCs, and evidence supports a direct interaction with the channel itself (Yan et al., 2002), as well as inhibition of cdk 5-mediated phosphorylation of intracellular loop domains of the channel's α_{1A} subunit (Tomizawa et al., 2002). In some contexts, cdk 5 phosphorylates NR2A subunits, thereby enhancing synaptic transmission on the postsynaptic side. In hippocampal CA1 neurons, for example, roscovitine-mediated inhibition of cdk 5 blocks long-term potentiation and NMDA-evoked currents (Li et al., 2001). It is noteworthy that this effect was observed at a lower concentration of roscovitine (5 μM). If cdk 5 similarly modulates NMDA receptors through a postsynaptic mechanism in spontaneously active granule neurons, then roscovitine should be neuroprotective, which is not observed. There are at least two possible explanations. First, the presynaptic action of roscovitine may supercede a potential postsynaptic effect on NMDA receptor conductance. This could be related to the fact that 25 μM roscovitine is used in the excitotoxicity

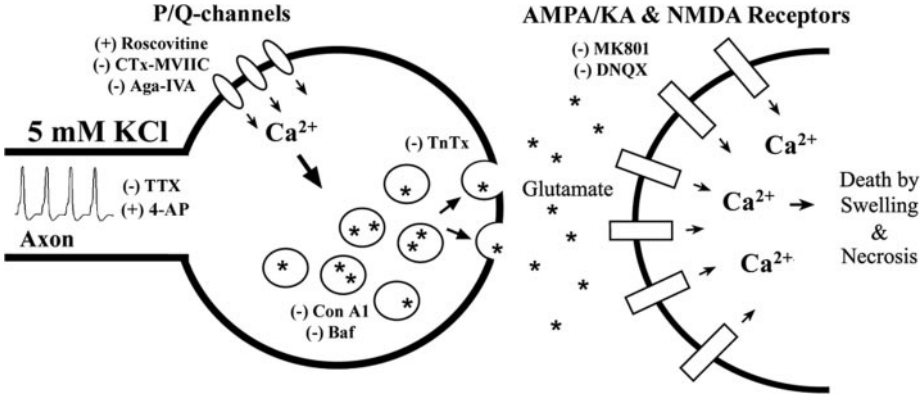

Fig. 7. Schematic diagram depicting the effects of roscovitine in granule neurons cultured in physiological medium. In the absence of roscovitine, spontaneous action potentials depolarize the presynaptic membrane, activating P/Q-type VDCCs, Ca^{2+} influx, and glutamate exocytosis. Synaptic glutamate activates AMPA/KA-receptor channels, causing depolarization of the postsynaptic membrane, followed by NMDA receptor-mediated Ca^{2+} influx, supporting survival. In the presence of roscovitine (or 4-AP), there is an increase in the coupling between action potentials and P-Q-type VDCC activity, leading to enhanced glutamate release. In the postsynaptic neuron, this additional synaptic glutamate triggers NMDA receptor-mediated excitotoxicity, characterized by rapid cell swelling and necrosis. This sequence of events is consistent with inhibition of roscovitine-mediated excitotoxic death by presynaptic agents that specifically interfere with action potential generation (TTX), P/Q channel activation (CTx-MVIIC, Aga-IVA), synaptic vesicle loading (Con A1, Baf), synaptic vesicle fusion (TnTx), and by agents that antagonize postsynaptic ionotropic glutamate receptors (DNQX, MK801).

paradigm, which is 5-fold higher than that used by Li and associates (2001) to inhibit cdk 5 and enhance NMDA receptor function in hippocampal cultures. It would be interesting to test the effect of lower, nontoxic concentrations of roscovitine on postsynaptic cdk 5 activity and NMDA receptor function in granule neurons. Second, unlike hippocampal pyramidal neurons, cdk 5 does not regulate NMDA receptor sensitivity in mature granule neurons. In future studies, such possibilities could be distinguished by characterizing the phosphorylation state of NMDA receptors in the presence and absence of roscovitine.

Our data unequivocally indicate that roscovitine-mediated excitotoxicity in granule neurons is mediated by presynaptic glutamate release and the linked activation of AMPA/KA and NMDA receptors, which gate excitotoxic Ca^{2+} influx. The downstream death pathway is transduced by NMDA receptor channels, because neuroprotection by MK801 is equivalent to that afforded by DNQX (Fig. 3). However, it is not known from these studies whether glutamate also reaches extrasynaptic NMDA receptors or whether it is limited only to those in the synapse. The site of NMDA receptor activation in our model is relevant because synaptic and extrasynaptic receptors are coupled to distinct intracellular effector molecules that can profoundly influence their functions, although controversy remains over their roles in excitotoxicity (Aarts and Tymianski, 2004). For example, in hippocampal cultures, extrasynaptic NMDA receptors are linked to a cAMP response element-binding protein shut-off pathway that leads to death, and activation of synaptic NMDA receptors opposes this effect (Hardingham and Bading, 2002). However, in another study, both synaptic and extrasynaptic NMDA receptors mediate death in response to bath-applied glutamate, whereas only synaptic NMDA receptors mediate death resulting from ischemia and glucose deprivation (Aarts and Tymianski, 2004). Likewise, excitotoxic neuronal loss in humans involves global increases in glutamate, which are predicted to activate both synaptic and extrasynaptic receptors, or more discrete disorders of synaptic overactivity (Aarts and Tymianski, 2004). It is clear that the rational design of effective therapies must be derived from knowledge of the relevant receptors and their downstream effectors, which can differ substantially depending on the insult, brain region, and neuronal type, underscoring the importance of the model systems being used. Further characterization of the granule cell model described herein is expected to address the role of synaptic versus extrasynaptic NMDA receptors in roscovitine-mediated excitotoxic necrosis.

In summary, we describe a new model of excitotoxicity in mature granule neurons cultured in medium containing physiological concentrations of KCl. Although roscovitine, like other small-molecule inhibitors, has the potential to target multiple effectors, it is highly selective in the paradigm characterized herein and provides a model of excitotoxicity that is derived from enhanced coupling between action potentials and VDCCs in these spontaneously active neurons. Further experimentation is expected to reveal valuable information about postsynaptic effectors lying downstream of NMDA receptor channels and additional insights into the precise mechanism of action of roscovitine and similar agents on VDCCs. All together, the data make a compelling argument to adopt granule neurons grown in 5 mM KCl for future experimentation of excitoxicity of synaptic origins and other models of granule cell physiology.

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Address correspondence to: Dr. M. L. Vallano, Department of Neuroscience and Physiology, SUNY Upstate Medical University, 750 East Adams St, Syracuse, NY 13210. E-mail: vallanom@upstate.edu