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Roscovitine, olomoucine, purvalanol: inducers of apoptosis in maturing cerebellar granule neurons

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Abstract

Cyclin-dependent kinases (CDKs) mediate proliferation and neuronal development, while aberrant CDK activity is associated with cancer and neurodegeneration. Consequently, pharmacologic inhibitors, such as 2,6,9-trisubstituted purines, which potently inhibit CDKs 1, 2, and 5, were developed to combat these pathologies. One agent, *R*-roscovitine (CYC202), has advanced to clinical trials as a potential cancer therapy. In primary neuronal cultures, these agents have been used to delineate the physiologic and pathologic functions of CDKs, and associated signaling pathways. Herein we demonstrate that three 2,6,9-trisubstituted purines: olomoucine, roscovitine, and purvalanol, used at concentrations ascribed by others to potently inhibit CDKs 1, 2, and 5, are powerful triggers of death in maturing cerebellar granule neurons, assessed by loss of mitochondrial reductive capacity and differential staining with fluorescent indicators of living/dead neurons. Based on several criteria, including delayed time course and establishment of an irreversible commitment point of death, pyknotic cell and nuclear morphology, and caspase-3 cleavage, the death process is apoptotic. However, pharmacological and biochemical data indicate that apoptosis is independent of CDK 1, 2, or 5 inhibition. This is based on the pattern of changes in *c-jun* mRNA, c-Jun protein, and Ca^{2+}/cAMP response element binding protein (CREB) phosphorylation, and also, the ineffectiveness of structurally distinct CDK 1, 2, and 5 inhibitors butyrolactone-1 and PNU112445A to induce apoptosis. Collectively, our results, and those of others, indicate that the CDK regulation of transcription (CDKs 7 and 9) should be examined as a target of these agents, and as an indirect mediator of neuronal fate.

Keywords: 2,6,9-Trisubstituted purines; Cyclin-dependent kinase inhibitors; Apoptosis; Cerebellum; CREB; c-Jun

1. Introduction

During maturation of the mammalian central nervous system (CNS), neuronal numbers are substantially reduced by apoptosis as a result of competition for trophic factors and electrical activity, thus optimizing inter-neuronal connectivity [1]. However, apoptosis is also a mode of death contributing to neuronal loss in some of our most crippling neurodegenerative diseases, such as Alzheimer's [2–4], Parkinson's [5–7], and amyotrophic lateral sclerosis [8,9]. Over the last decade, an emerging literature indicates that cell cycle proteins, in particular, cyclins and cyclin-dependent kinases (CDKs), are induced by apoptotic stimuli in post-mitotic neurons, and this aberrant cell cycle activity underlies neuronal death [10–12]. To combat pathological apoptosis, a number of approaches have been tested in the laboratory, with varying success. One approach utilizes cell-permeable pharmacologic agents that inhibit CDKs.

CDKs are serine/threonine kinases, inactive until their preferred binding partners become available. Best understood for their role in regulating cell cycle progression [13,14], they now are implicated in diverse cellular functions, including regulation of transcription and neuronal migration (for review see [15]). Accumulating evidence

Abbreviations: AraC, cytosine arabinoside; CAK, CDK-activating kinase; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; CDK, cyclin-dependent kinase; CNS, central nervous system; CREB, Ca^{2+}/cAMP response element binding protein; CTD, carboxy-terminal domain; DIV, days in vitro; DYRK, dual-specificity protein kinase; ERK, extracellular regulated kinase; FDA, fluorescein diacetate; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NFDM, non-fat dry milk; PBA, 1% BSA in PBS; PI, propidium iodide; *Taq*, *Thermus aquaticus*; TBS, Tris buffered saline; TTBS, 0.1% Tween-20 in TBS

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supports a number of hypotheses linking CDKs to neuronal degeneration; two are particularly relevant to the study of human neurodegeneration: (1) in post-mitotic neurons, inappropriate activation of cell cycle CDKs triggers apoptosis, and (2) dysregulation of CDK 5 follows calpain-dependent cleavage of its activating protein p35 to p25.

Over 50 CDK inhibitors have been identified to date, all demonstrating a competitive inhibitory interaction at the ATP-binding pocket (for review see [15]). Inhibitors fall into several chemical classes, including: staurosporines, flavonoids, paullones, polysulfates, indigoids, and purine analogues. They exhibit three primary selectivity profiles against CDKs: those that selectively inhibit CDKs 1, 2, 5, 7, and 9; those that selectively inhibit CDKs 4, 6, and 8; and those that are non-selective. Due to the highly conserved nature of the amino acid residues lining the ATP-binding pocket, inhibitors that target individual CDKs have remained elusive [16].

First identified for their ability to halt cellular proliferation, CDK inhibitors were developed as potential cancer chemotherapeutics. For example, *R*-roscovitine (CYC202) has advanced as far as phase 1 clinical trials [17]. Recent attention has focused on their potential to rescue neurons from apoptosis. Among the classes of inhibitors available, the 2,6,9-trisubstituted purine analogues are protective in post-mitotic neurons subjected to various apoptotic stimuli: trophic factor withdrawal [18–20], DNA damage [21,22], ischemic insult [23,24], nerve growth factor induced death [25], and axotomy [26]. Olomoucine and roscovitine, the two most frequently examined compounds, have several advantages, including: well characterized in vitro selectivity against CDKs (CDK 1, 2, 5, 7, and 9); existence of stereoisomers with different activities; high potency against CDKs; and commercial availability. In vitro kinase assays against a subset of cellular proteins originally indicated that olomoucine and roscovitine are selective for CDKs 1, 2, and 5 (IC_{50} = 7, 7, and 3 μM, and 0.65, 0.7, and 0.16 μM, respectively) [27,28]. Subsequently, two independent in vitro analyses demonstrated selectivity of olomoucine and roscovitine for CDK 7 (IC_{50} = 10 and 0.5 μM, respectively) [29–31]. In addition, CDK 9 is a potential target of roscovitine (in vitro $IC_{50} \sim 0.6$ μM) [31], but a formal analysis of olomoucine has not been conducted. The extracellular regulated kinases (ERKs) 1 and 2 are non-CDK targets with IC_{50} values under 70 μM (olomoucine = 50, 40 μM; roscovitine = 34, 14 μM, respectively) [27,28].

To study the effects of trisubstituted purine CDK inhibitors on neuronal physiology, cerebellar granule neurons, prepared from P8 rat cerebellar cortex, were chosen as a model system. Granule neurons are extensively used to study survival and apoptotic signaling in the context of the nervous system [32]. Paradoxically, trisubstituted purine CDK inhibitors are reported to rescue from [19,20] as well as induce [33] apoptosis in granule neurons. In these studies, drug effects on survival or apoptosis have been attributed to inhibition of CDKs 1, 2, and 5. Although these agents potently inhibit CDKs 1, 2, and 5, depending on the context they may also inhibit other kinases, such as CDKs 7, 9, and the ERKs, yielding complex results. As such, garnering a more complete understanding of their functional effects in different contexts is critical to their appropriate use in the laboratory or the clinic. In the present study, we provide evidence that 2,6,9-trisubstituted purines induce apoptosis in maturing cerebellar granule neurons, rather than preventing it, in a complex manner that we propose is likely due to inhibition of CDK 7, and appears to be independent of effects on CDKs 1, 2, 5, or 9.

2. Materials and methods

2.1. Materials

Sprague–Dawley neonatal rats were purchased from Taconic Farms. The institutional review committee, in accordance with governmental guidelines, approved all procedures involving animal experimentation. RNeasy® Mini Kit for RNA isolation was purchased from Qiagen. *Thermus aquaticus* polymerase (*Taq* polymerase) and 10× *Taq* buffer were purchased from Fermentas. dNTPs were purchased from Amersham Biosciences. Basal Eagle's medium with Earle's salts, Dulbecco's modified Eagle medium, and 1:1 Dulbecco's modified Eagle medium/Ham's F-12 were purchased from Invitrogen. DNA standards (100-bp) were obtained from New England Biolabs. Cleaved caspase-3 rabbit polyclonal antibody, phospho-c-Jun (Ser63) rabbit polyclonal antibody, c-Jun rabbit polyclonal antibody, Ca^{2+}/cAmp response element binding protein (CREB) rabbit polyclonal antibody, and phospho-CREB (Ser133) rabbit polyclonal antibody were purchased from Cell Signaling Technology. CDK 5 mouse monoclonal and p35 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. Calcium/calmodulin-dependent protein kinase IV (CaMKIV) mouse monoclonal antibody was purchased from Transduction Laboratories. 2-(2-Hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine), 6-benzylamino-2-(2-hydroxyethylamino)-7-methylpurine (iso-olomoucine), 2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (*R*-roscovitine), 2-(*S*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (*S*-roscovitine), 2-(1*R*-isopropyl-2-hydroxyethylamino)-6-(3-cholroanilino)-9-isopropylpurine (purvalanol), and N^4-(6-aminopyrimidin-4-yl)-sulfanilamide, HCl (PNU112455A) were purchased from Calbiochem. Butyrolactone-1 was purchased from Biomol. Texas Red and FITC labeled secondary antibodies of multiple-labeling grade were from Jackson ImmunoResearch Laboratories. Supersignal® West Pico and Micro BCA™ reagents were purchased from Pierce. Vectashield® fluorescence mounting medium was purchased from Vector Laboratories. Other reagents

for RT-PCR and cell culture were molecular biology grade and tissue culture grade, respectively, and were obtained from commercial sources. MCF-7 breast carcinoma cells were the generous gift of Dr. M.S. Sheikh at SUNY Upstate Medical University.

2.2. Cell culture

Primary cultures enriched in cerebellar granule neurons were prepared and grown in an atmosphere of 5% CO_2 as described [34]. Briefly, cerebella from postnatal day 8 Sprague–Dawley rats were minced, trypsinized, and triturated to dissociate the cells. Cells were plated at a density of 1.25×10^6 cells/ml on poly-L-lysine coated (10 μg/ml) tissue culture dishes. Twenty-four hours after plating, serum-containing media was replaced with chemically defined media [35] and, as indicated, was supplemented with KCl to a final concentration of 25 mM KCl. Also at this time, unless otherwise stated, cultures were supplemented with the anti-mitotic agent cytosine arabinoside (AraC, 10 μM) to prevent proliferation of non-neuronal cells and was continually present during drug treatments. In a limited number of comparative experiments, granule neurons were grown, in and examined in, serum-containing medium. In our laboratory, this method consistently generates cultures with >95% cerebellar granule neurons [36,37]. Culture purity was repeatedly verified by morphologic examination and immunostaining [38]. Unless otherwise stated, cultures were treated with CDK inhibitors at 1 day in vitro (DIV) after the change to chemically defined medium, and were subsequently processed for cell viability analysis, Western immunoblotting, immunocytochemistry, and RT-PCR at the times indicated. For roscovitine washout experiments, cultures were washed twice with drug-free 1:1 Dulbecco's modified Eagle medium/Ham's F-12. To protect against possible untoward effects of adding fresh medium to cultures, media from untreated cultures, prepared in parallel, were collected, centrifuged, and supernatants were added back to experimental cultures as 'conditioned' media. Cultures were subsequently returned to the incubator. In some experiments, the effects of *S*-roscovitine on granule neurons were also examined. *S*- and *R*-roscovitine yielded qualitatively similar results. For brevity, only data obtained with *R*-roscovitine is presented and it is denoted 'roscovitine'. MCF-7 cells were cultured as recommended by the American Type Culture Collection.

2.3. Neuronal viability/death

Cellular reductive capacity of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used as an indirect measure of neuronal viability as previously described [39,40]. Briefly, MTT (5 mg/ml) was added directly to culture medium at a final concentration of 0.5 mg/ml, and cultures were incubated at 37 °C for 10 min. Following incubation, culture medium was removed and replaced with an equal volume of dimethyl sulfoxide (DMSO). Absorbance measurements were then quantified using a Hitachi U-2000 spectrophotometer at a wavelength of 540 nm. Confirmation of MTT data was performed by comparison with fluorescein diacetate and propidium iodide (FDA and PI) double-labeling for live and dead cells, respectively, as described previously [35]. Additionally, morphologic analysis of the mechanism of cell death was made via phase-contrast and fluorescence microscopy. Finally, nuclear morphology was examined by staining CGNs with the DNA dye bisbenzimide (Hoescht 33298; 1 mg/ml).

2.4. Immunocytochemistry/western immunoblotting

For immunocytochemistry, cells grown on glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBA (1% BSA in PBS). Cells were incubated overnight with the following diluted primary antibodies: cleaved caspase-3 (1:100 in PBA), CaM-KIV (1:50 in PBA/2% donkey serum), or CDK 5 (1:100 in 1.5% serum/PBS). Texas Red or FITC conjugated donkey anti-rabbit or anti-mouse secondary antibody was diluted (1:400) in 2% donkey serum/PBA and incubated for 1–3 h at room temperature in the dark. Coverslips were mounted on slides using Vectashield®, with or without PI to label nuclei as an indicator of total cell number, and digitally photographed under confocal fluorescence microscopy (20–60× objectives, Nikon Eclipse E600).

For Western immunoblotting, cultures were harvested in SDS buffer (1 mM $NaVO_4$, 0.3 mM PMSF, 2% SDS, 62.5 mM Tris, 10% glycerol), sonicated, and equalized for protein content based on the Micro BCA™ assay. Proteins were resolved on 8% SDS–PAGE, transferred to nitrocellulose and blocked in 3–5% non-fat dry milk (NFDM)/0.1% Tween-20 in Tris buffered saline (TTBS). Blots were incubated at 4 °C overnight in diluted primary antibody as follows: c-Jun (1:1000 in 5% BSA/TTBS), cleaved caspase-3 (1:1000 in 5% NFDM/TTBS), p35 (1:400 in 5% NFDM/TTBS), total CREB (1:500 in 3% NFDM/PBS), or serine 133 phosphorylated CREB (1:500 in 3% NFDM/PBS). Blots were incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibodies (anti-rabbit, 1:1000), diluted in 5% NFDM/TTBS. Supersignal® West Pico and X-ray films were used to visualize immunoreactivity. Multiple protein concentrations were routinely analyzed to ensure linearity of the assays.

2.5. RNA isolation and RT

Whole-cell RNA was isolated from granule neurons (0–24 h post-treatment) using a RNeasy Mini Kit according to the manufacturer's protocol. Concentration and purity of RNA were assessed with a spectrophotometer using nucleotide absorption at 260 nm and a ratio at 260/280 nm

(nucleotide/protein), respectively. RNA was used in a RT reaction to produce cDNA for use as template in PCR as previously described [34]. As controls, a RT reaction was performed at least once for each set of samples in the absence of either reverse transcriptase or RNA.

2.6. PCR

PCR of *c-jun* mRNA was performed in a final volume of 100 μl containing 5 ng of input RNA (following RT) and the following components (final concentrations): *Taq* buffer (1X); *Taq* polymerase (0.025 U/μl); BSA (0.17 μg/μl); $MgCl_2$ (1.5 mM); the four dNTPs (0.05 mM); and *c-jun* oligonucleotide primers (0.25 pmole/μl each) upstream (5′-GCT TCT CTA GTG CTC CGT AA-3′; sense) and downstream (5′-TCT AGG AGT CGT CAG AAT CC-3′; antisense) [41]. The PCR protocol was as follows: 95 °C for 5 min, then 40 cycles of 95 °C for 1.5 min, 50 °C for 1.5 min, 72 °C for 1.5 min, followed by 72 °C for 5 min. Samples were resolved on 1.5% ethidium bromide-stained agarose gels to separate and visualize amplicons (expected size 774 bp) and photographed while illuminated with UV light.

PCR of β-actin mRNA was performed in a final volume of 100 μl containing 5 ng of input RNA (following RT) and the following components (final concentrations): *Taq* buffer (1×); *Taq* polymerase (0.025 U/μl); BSA (0.17 μg/μl); $MgCl_2$ (2 mM); the four dNTPs (0.05 mM); and β-actin oligonucleotide primers (0.25 pmole/μl each) upstream (5′-TCA TGA AGT GTG ACG TTG AC-3′; sense) and downstream (5′-CCT AGA AGC ATT TGC GGT GC-3′; antisense) [42]. The PCR protocol was as follows: 95 °C for 5 min, then 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 10 min. Samples were resolved on 8% ethidium bromide-stained polyacrylamide gels to separate and visualize amplicons (expected size 285 bp) and photographed while illuminated with UV light.

2.7. Statistical analysis

Values stated in the text are means ± S.E.M. and have been analyzed for their statistical significance using an ANOVA, followed by Fischer's test. Values of $P < 0.05$ are considered statistically significant.

3. Results

3.1. Purine analogue CDK inhibitors trigger apoptosis in maturing cerebellar granule neurons

To examine the effects of purine analogue CDK inhibitors on cerebellar granule neurons physiology, cultures were treated with the cell permeable agents: roscovitine, olomoucine, and its inactive isomer, iso-olomoucine. Neurons were grown to 1 DIV, then a purine analogue or vehicle (DMSO) was added. After 24-h, cell viability was assessed by: (1) MTT assay; (2) FDA/PI staining; (3) morphological examination; and (4) activation state of caspase-3. Fig. 1a demonstrates that olomoucine and roscovitine significantly decrease cell viability (MTT reduction as percent vehicle-treated control (set at 100%) = 60.4 ± 8.9% and 53.3 ± 0.5%, $N = 3$–5, $P < 0.05$, respectively). In contrast, iso-olomoucine treatment does not significantly alter viability (93.3 ± 4.0% of control, $N = 5$). The presence or absence of AraC (10 μM) in the culture medium during purine analogue treatment has no demonstrable affect on granule neuron viability (50 μM roscovitine: MTT reduction with AraC = 53.3 ± 0.5% versus without AraC = 52.6 ± 0.3%, $N = 3$, $P < 0.05$).

Investigators typically supplement the culture medium with KCl (final concentration 25 mM KCl) to promote long-term survival [43,44]. To determine whether maturing granule neurons grown in elevated KCl (25 mM) are protected from the detrimental effects of CDK inhibitors, compared to neurons grown in standard medium (5 mM KCl), granule neurons (1 DIV) were challenged with olomoucine or iso-olomoucine (100 μM), and MTT assays performed 24 h later. In both culture conditions olomoucine causes significant and comparable decreases in cell viability, whereas iso-olomoucine and vehicle are without significant effect (5 mM KCl, olomoucine: 50.4 ± 3.4% versus iso-olomoucine: 91.7 ± 3.8%, $N = 5$; 25 mM KCl, olomoucine: 49.9 ± 4.7% versus iso-olomoucine: 94.8 ± 1.8%, $N = 6$) (Fig. 1b). (Note that the inhibitory potency of olomoucine was not significantly different in granule neurons grown in defined versus serum-containing culture media (not shown).) Olomoucine also triggers a significant decrease in cell viability versus iso-olomoucine in cultures grown to 7 DIV and treated for 48 h (5 mM KCl, Olo versus Iso-olo: 61.7 ± 5.5% versus 101.7 ± 6.8%; 25 mM KCl, Olo versus Iso-olo: 71.0 ± 7.1% versus 99.3 ± 3.5, $N = 3$; Fig. 1c). These data indicate that granule neurons, grown in trophic medium containing elevated KCl, are not protected from the detrimental effects of purine analogue CDK inhibitors, even after extended maturation in culture.

Roscovitine, reportedly more potent and selective for CDKs than olomoucine [28], was used to test the concentration dependence of the effect. Following 24-h treatment of granule neurons (1 DIV), a concentration-dependent decrease in cell viability is observed (1 μM = 87.4 ± 4.8%, 10 μM = 74.9 ± 7.9%, 25 μM = 50.3 ± 4.9%, $N = 3$; Fig. 1d). As with 50 μM roscovitine (Fig. 1a), these decreases are statistically significant at 10 and 25 μM, compared to vehicle-treated cultures. As both 25 and 50 μM roscovitine produce similar decreases in MTT reduction after treatment for 24-h, the lower concentration was used for subsequent experiments.

To confirm that the MTT assay is a valid index of granule neuron death, and not merely indicative of a metabolic disturbance, FDA and PI were used to stain living (FDA)

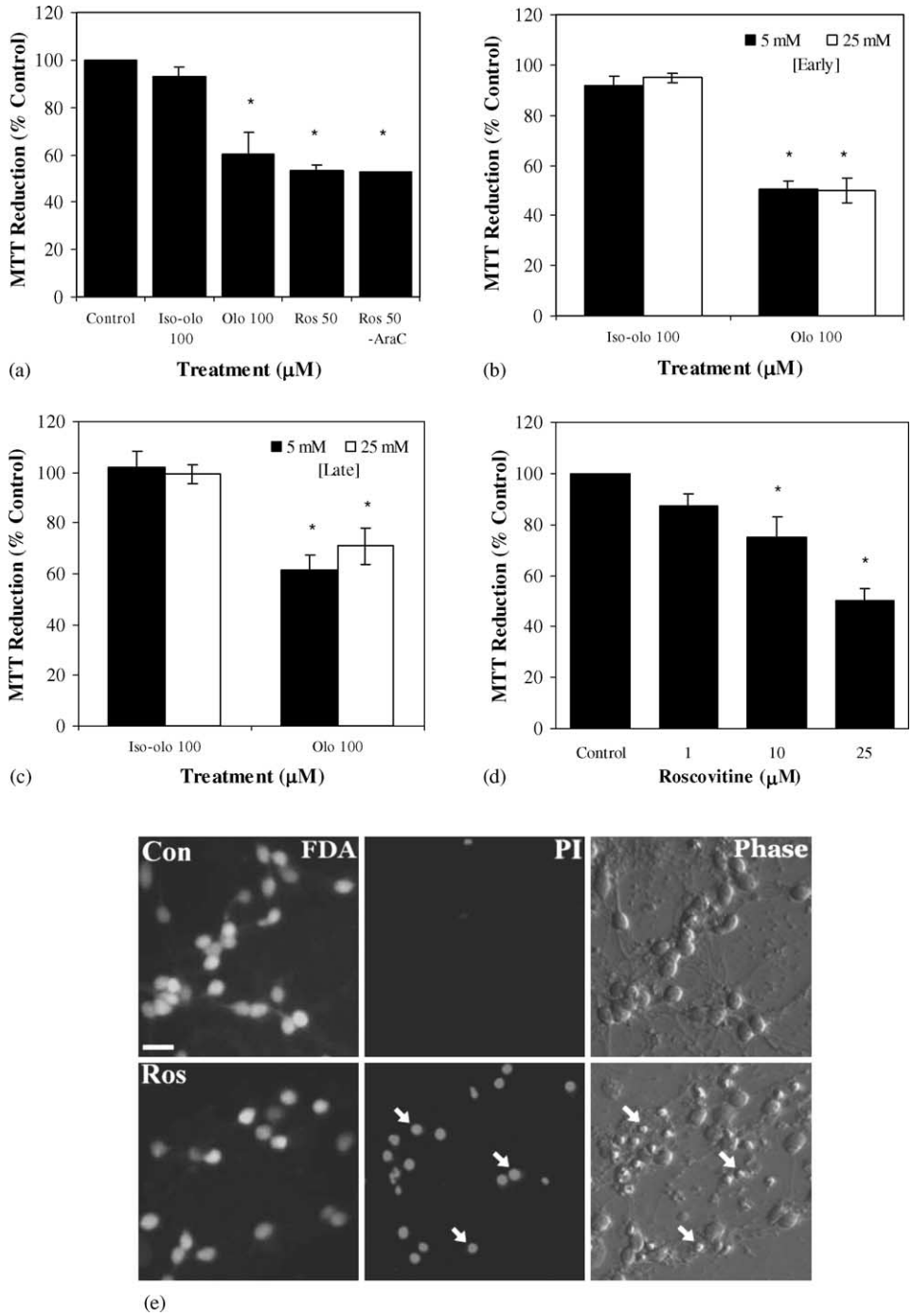

Fig. 1. Effects of purine analogue CDK inhibitors on neuronal viability. Cerebellar granule neurons were grown in media containing 5 or 25 mM KCl. (a) Histogram showing MTT reduction following 24-h exposure at 1 DIV to iso-olomoucine or olomoucine (Iso-olo or Olo, 100 μM, $N = 5$), roscovitine (Ros, 50 μM, $N = 3$), roscovitine in the absence of AraC (Ros − AraC, 50 μM, $N = 3$), or vehicle (DMSO, $N = 5$). (b) Histogram showing MTT reduction following 24-h exposure at 1 DIV ([early]) to iso-olomoucine and olomoucine (100 μM each, $N = 5$–6) or vehicle (control, $N = 5$–6). *Significantly different than vehicle-treated control. (c) Histogram showing MTT reduction following 48-h exposure at 7 DIV ([late]) to iso-olomoucine and olomoucine (100 μM each, $N = 3$). (d) Histogram showing MTT reduction following 24-h exposure at 1 DIV to increasing concentrations of roscovitine (1, 10, and 25 mM, $N = 3$). (e) Fluorescence micrographs showing FDA/PI stained (FDA, living; PI, dead) CGNs following 24-h exposure at 1 DIV to roscovitine (25 μM) or vehicle control. Corresponding phase contrast micrographs are also shown. Arrows depict some of the pyknotic CGNs in both images. Scale bar indicates ∼10 μm.

and dead (PI) neurons in the presence or absence of 25 μM roscovitine, added to cultures at 1 DIV, and examined 24 h later. Roscovitine treatment yields an impressive increase in the proportion of PI-stained, dead, neurons (arrows), in conjunction with a proportionate decrease in FDA-stained, living, neurons (Fig. 1d). In contrast, the majority of vehicle-treated neurons remain viable (i.e. stained with FDA and not PI).

Apoptosis and necrosis lie on two ends of a death continuum. Apoptosis is a highly ordered and tightly regulated physiological process, typically requiring transcription and translation, and yielding cell death without inflammation. It is morphologically and biochemically distinct from necrosis, and is characterized by cellular shrinkage and fragmentation, nuclear condensation, proteolysis, and internucleosomal DNA cleavage, among other things [45]. Conversely, necrosis is a pathological response to extreme stress. Ultimately, cells cannot maintain osmotic balance, they swell and lyse, releasing their intracellular contents, which induces an inflammatory response. Thus, if the CDK inhibitors interfere with a physiologically relevant survival pathway in granule neurons, then they should trigger a form of apoptotic neuronal death.

Phase contrast micrographs of maturing granule neurons, treated at 1 DIV, and assayed after 24-h roscovitine treatment (25 μM) show morphologic hallmarks of apoptosis. These include cellular shrinkage and fragmentation (Fig. 1e, phase panels), as well as neurite fragmentation and membrane blebbing (Fig. 2a). Granule neurons also demonstrate stereotypical apoptotic changes in nuclear morphology, including chromatin condensation and nuclear pyknosis (Fig. 2b, arrows) assessed by Hoescht 33298 staining. In contrast to the rapid cellular swelling and lysis associated with necrosis, these morphologic alterations develop after a delay of 12–24 h. Note also that roscovitine triggers a comparable degree of nuclear pyknosis in neurons grown in medium containing 25 mM KCl.

Activation of caspases, cysteine-containing aspartases, is a common biochemical feature of apoptosis. Numerous signal transduction pathways, including those governed by plasma membrane death receptors (i.e. Fas) and mitochondrial elements (i.e. cytochrome *c*), yield cleavage and activation of members of this protease family. Caspase-3 is a terminal effector of apoptosis, upon which upstream caspases converge (for reviews see [46,47]). To examine whether caspase-3 activity is associated with neuron death following CDK inhibitor treatment, a monoclonal antibody that recognizes only the cleaved/active form of caspase-3 was used. Granule neurons (1 DIV) were treated with roscovitine (25 μM), olomoucine (100 μM), or vehicle. After 24-h, cultures were fixed and processed for immunocytochemistry. PI was used to counter-stain granule cell nuclei. As shown in Fig. 2c, the majority of granule neurons treated with roscovitine and olomoucine, but not vehicle, express the cleaved form of caspase-3. To verify the functional activity of caspase-3 in response to roscovitine and olomoucine, cultures were treated overnight with roscovitine and olomoucine, fixed, and then double labeled with antibodies specific for cleaved caspase-3 and one of its targets, CaMKIV. CaMKIV is enriched in the nuclei of maturing cerebellar granule neurons [48] and is a substrate for caspase-3 [49]. In vehicle-treated controls, the granule neurons express CaMKIV with little or no active caspase-3. Treatment with roscovitine or olomoucine activates caspase-3 in many neurons, and the majority of these no longer contain detectable CaMKIV. However, occasionally, neurons were observed that expressed both cleaved caspase-3 and a lower, but still detectable level of CaMKIV (Fig. 2d, arrow). To ascertain the time course of caspase-3 activation following roscovitine treatment, Western immunoblotting was performed. As shown, cleaved caspase-3 becomes detectable only after a delay of ∼8 h following treatment (25 μM), and it continues to increase to 24 h (Fig. 2e). Taken together, the data indicate that these members of the purine analogue class of CDK inhibitors activate caspase-3 and trigger apoptosis in maturing granule neurons after a delay of several hours.

3.2. Following roscovitine treatment, granule neurons become irreversibly committed to an apoptotic death program

A temporal feature of apoptosis is the development of a commitment point, due to initiation of transcriptional events. Thus, the death process becomes irreversible and gradually proceeds over time, irrespective of the continued presence of the triggering agent, in this case, roscovitine. The presence of cleaved caspase-3 in the majority of granule neurons exposed to roscovitine and olomoucine for 24 h is consistent with this notion (Fig. 2c). We, therefore, examined if a majority of granule neurons are irreversibly committed to apoptosis after 24-h exposure to roscovitine, followed by roscovitine washout. Granule neurons (1 DIV; 5 and 25 mM KCl containing media) were treated for 24 h with roscovitine (10 or 25 μM), then half of the cultures were immediately processed for MTT assay (2 DIV) and the other half were washed with, and switched to, drug-free media, and returned to the incubator. At 7 DIV, these cultures were analyzed for the extent of neuronal death compared to their vehicle-treated controls, also grown for 7 DIV. Consistent with the data in Fig. 1d, granule neuron viability (5 mM KCl condition, ◆), when assayed at 2 DIV following 24-h exposure to 10 and 25 μM roscovitine, is $79.1 \pm 1.9\%$ and $50.3 \pm 4.2\%$ ($N = 3$, $P < 0.05$), respectively. On the other hand, a significantly greater proportion of granule neurons ultimately undergo apoptosis following 24-h exposure to roscovitine, drug washout, and subsequent assay at 7 DIV (5 mM KCl, ●: $46.4 \pm 4.4\%$ and $12.2 \pm 5.9\%$, respectively, for 10 and 25 μM roscovitine, versus vehicle control (set at 100%), $N = 3$, $P < 0.05$) (Fig. 3a). (Note that in experiments conducted in 5 mM KCl at 7 DIV, some neurons died through naturally occurring apoptosis. However, all drug-treated samples are directly compared to their vehicle-treated controls of the same age.) Also, as shown previously (Fig. 2b), supplementing the media with 25 mM KCl does not protect granule neurons from roscovitine-induced death, and a greater degree of death is observed

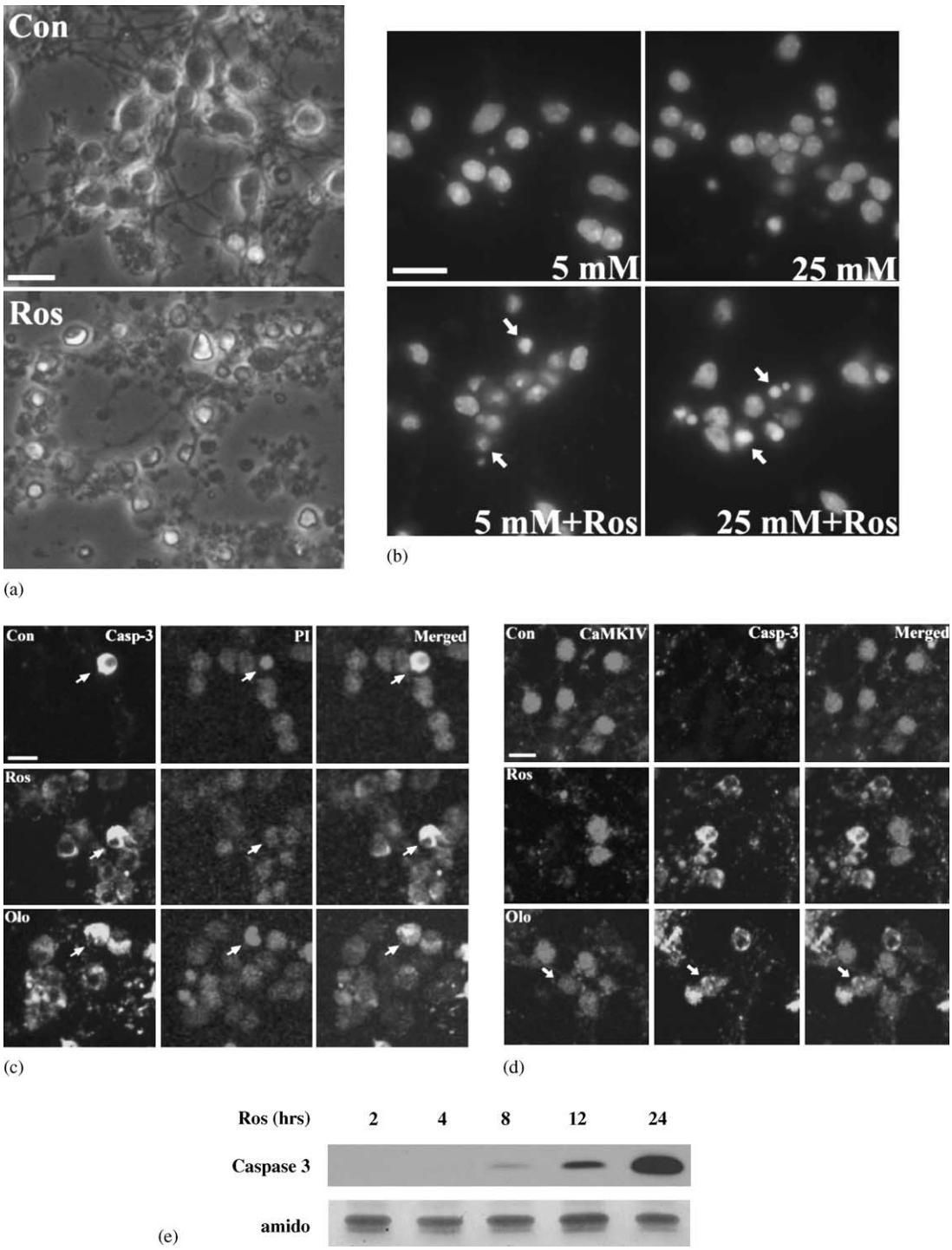

Fig. 2. Purine analogue CDK inhibitors trigger morphological and biochemical changes indicative of apoptosis. (a) Phase contrast micrographs showing granule neurons (5 mM KCl) after 24-h exposure at 1 DIV to roscovitine (25 μM) or vehicle control. (b) Fluorescence micrograph showing Hoescht 33258 stained CGN nuclei (5 and 25 mM KCl) following 24-h exposure at 1 DIV to roscovitine (+Ros, 25 μM). Arrows depict some of the fragmented apoptotic nuclei. (c) Fluorescence micrograph showing cleaved caspase-3 staining in granule neurons (5 mM KCl) following 24-h exposure at 1 DIV to roscovitine (Ros, 25 μM), olomoucine (Olo, 100 μM), or vehicle (Con). PI staining is used to show all cell nuclei. (d) Fluorescence micrograph showing CaMKIV and cleaved caspase-3 staining in granule neurons (5 mM KCl) following 24-h exposure at 1 DIV to roscovitine (Ros, 25 μM), olomoucine (Olo, 100 μM), or vehicle (Con). (e) Western immunoblot comparing cleaved caspase-3 in granule neurons (5 mM KCl) following 2, 4, 8, 12, and 24-h exposure at 1 DIV to roscovitine (25 μM). Forty micrograms protein were loaded per well. Scale bars indicate ~10 μm.

after drug washout and assay at 7 DIV (○), compared to assay at 2 DIV (◇).

As shown (Fig. 2e), roscovitine does not induce a detectable amount of active immunoreactive caspase-3 until ~8 h post-treatment. Since caspase-3 is a terminal executioner of apoptotic cell death (for reviews see [46,47]), its activation in granule neurons may indicate the arrival of an irreversible commitment point. The next

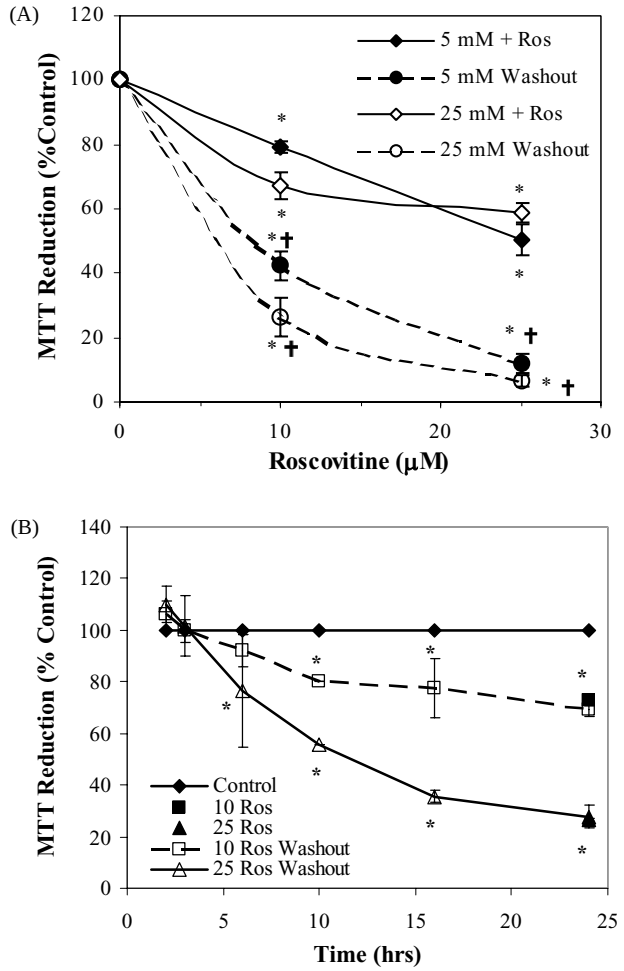

Fig. 3. Presence of an irreversible commitment point following roscovitine treatment. (a) Line graph showing MTT reduction immediately after 24-h roscovitine exposure at 1 DIV (10 and 25 μM; ◆ and ◇) and after 24-h exposure at 1 DIV, followed by drug washout, continued culture, and assay at 7 DIV (● and ○). *Significantly different than respective vehicle-treated control. †Significantly different than respective treatment condition assayed at 2 DIV. (b) Line graph showing MTT reduction following drug washout after 2, 3, 6, 10, 16, and 24-h exposure to roscovitine at 1 DIV (10 and 25 μM; □ and △) compared to continuous roscovitine exposure for 7 DIV (■ and ▲). *Significantly different than respective vehicle-treated control.

series of experiments was designed to determine if granule neurons can be rescued from apoptosis if roscovitine is "washed out" prior to detectable activation of caspase-3 (i.e. ~8 h). To test this, granule neurons (1 DIV) were treated with roscovitine (10 or 25 μM) for periods ranging from 2 to 24 h, followed by drug washout. All cultures were then returned to the incubator and assayed by MTT at 7 DIV. When granule neurons are exposed to roscovitine (10 or 25 μM) for 3 h or less, then washed free of drug and assayed at 7 DIV, there are no significant alterations in MTT reductive capacity, compared to vehicle-treated control (Fig. 3b). Moreover, exposure for 6 h, followed by washout and analysis at 7 DIV, produces only a marginal decrease in MTT reductive capacity in granule neurons receiving the higher roscovitine concentration (25 μM). In contrast, after 10-h treatment or longer, followed by washout of roscovitine, a significant proportion of neurons are committed to apoptosis (10 μM, □; 25 μM, △), and the extent of cell death approaches values obtained at 7 DIV with constant exposure to roscovitine (■ and ▲). These data indicate that signaling events associated with roscovitine-mediated apoptosis are reversible prior to 6 h of drug exposure. However, after 10 or more hours, the majority of granule neurons reach an irreversible commitment point.

3.3. Roscovitine induced apoptosis is not due to disinhibition of a CDK 5/JNK3/c-Jun signaling cascade

Unlike other members of the AP-1 family of transcription factors, c-Jun initiates transcriptional death programs within hours after stress, or trophic factor withdrawal, in several types of neurons [50,51], including cerebellar granule neurons [52–54]. Specifically, c-Jun becomes phosphorylated on serines 63 and 73 retarding its electrophoretic mobility, then *c-jun* mRNA and c-Jun protein increase in abundance [52–54]. Importantly, induction of c-Jun lies upstream of caspase activation, as caspase inhibitors protect neurons from apoptosis after stressful insult inducing c-Jun [55,56]. A recent report demonstrated that CDK 5 negatively regulates c-Jun activity [57], thereby serving directly as a pro-survival protein. In this model, constitutive phosphorylation of c-Jun N-terminal kinase 3 (JNK3) renders it catalytically inactive, preventing it from activating c-Jun (via phosphorylation on serines 63 and 73; Fig. 4a, left). In this context, inhibition of CDK 5 with roscovitine promotes activation of JNK3, leading to phosphorylation of c-Jun and pro-apoptotic gene expression (Fig. 4a, right).

Based on the selectivity profiles of olomoucine and roscovitine, one substrate for which they are highly selective is CDK 5 [28]. Although CDK 5 is expressed ubiquitously, its catalytic activity is largely restricted to a few cell types, including post-mitotic neurons, based on the co-expression of its activator p35 (or p39) [58–60]. To verify previous findings and show that CDK 5 and p35 are co-expressed in the cultures used herein, granule neurons were grown for 2 DIV (5 and 25 mM KCl-containing medium), then fixed and processed for immunocytochemistry or immunoblotting using CDK 5- and p35-specific antibodies, respectively. As expected, CDK 5 is readily detectable in the majority of granule neurons and appears to be concentrated at the base of developing neurites (Fig. 4b). Importantly, p35 is co-expressed in granule neurons, but not in the human breast carcinoma cell line MCF-7 where CDK 5 is expected to be catalytically inactive (Fig. 4b; Western immunoblot). These data confirm previous findings indicating that a possible target (CDK 5/p35) for the apoptosis-triggering effect of roscovitine and olomoucine is present in maturing cerebellar granule neurons.

To examine potential effects on the CDK 5/JNK3/c-Jun signaling cascade, we determined the time course of roscovitine-mediated alterations in *c-jun* mRNA and

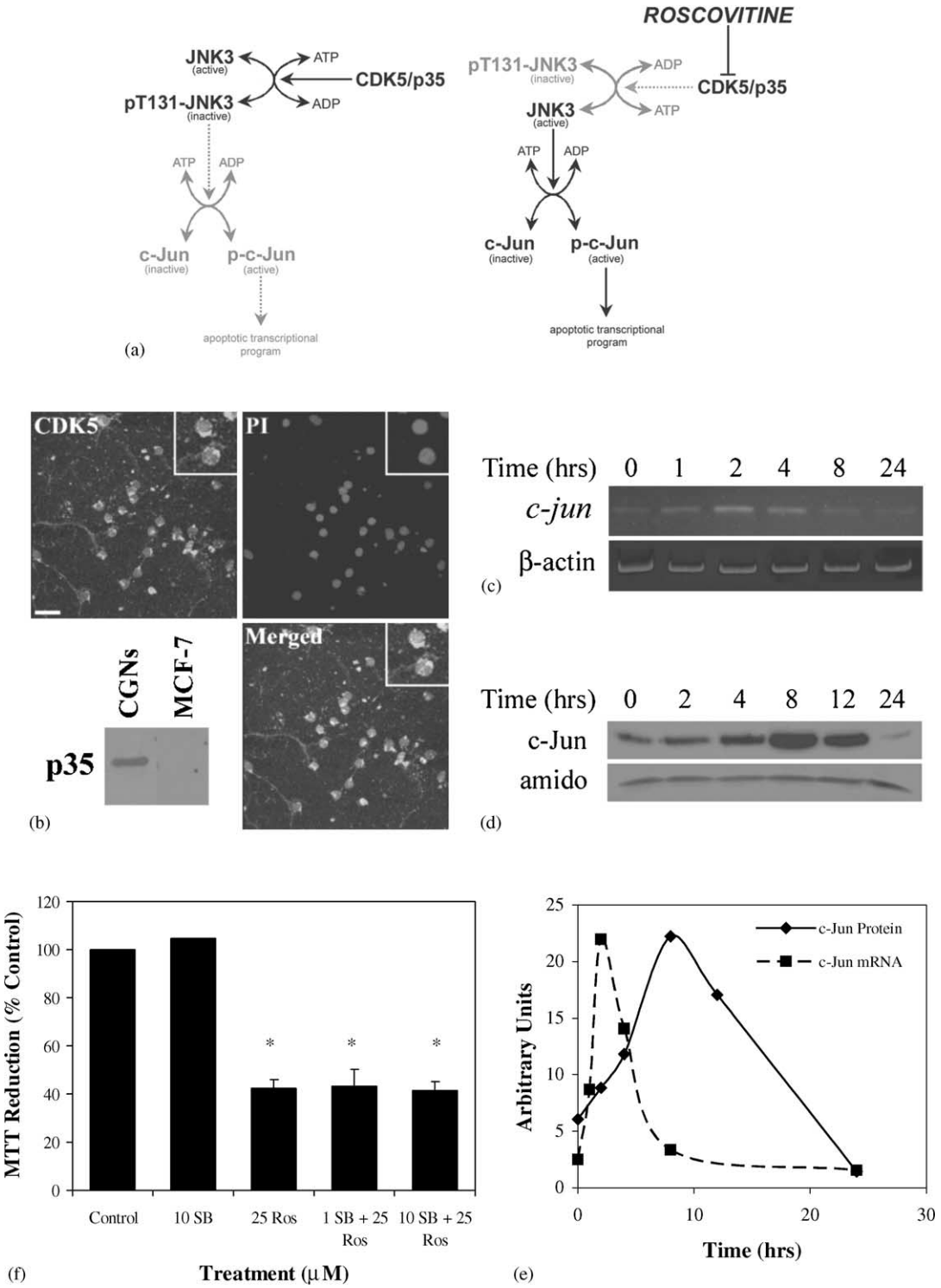

Fig. 4. A CDK 5/JNK3/c-Jun signaling cascade does not mediate granule neuron death following roscovitine treatment. (a) A model depicting regulation of JNK3/c-Jun by CDK 5 and the putative effects of roscovitine on this pathway. CDK 5 constitutively phosphorylates JNK3 on threonine-131, thereby suppressing its activity and preventing it from phosphorylating (serines 63 and 73) c-Jun and activating a c-Jun-mediated apoptotic transcriptional program (left). Following roscovitine treatment, CDK 5 is inhibited, resulting in JNK3 phosphorylation/activation of c-Jun and apoptosis (right). Gray text depicts inactive segments of pathway. (b) Fluorescence micrographs showing immunoreactive CDK 5 in maturing granule neurons. PI staining was used to show all cell nuclei. Scale bar indicates ~10 μm. Western immunoblot showing p35 protein expression in granule neurons and MCF7 breast carcinoma cells (40 μg protein loaded). (c) Ethidium bromide stained gel showing amplicons corresponding to *c-jun* and *β-actin* mRNA following exposure to roscovitine (25 μM) for 1, 2, 4, 8, and 24 h at 1 DIV. (d) Western immunoblot showing changes in c-Jun protein following roscovitine (25 μM) exposure for 2, 4, 8, 12, and 24 h at 1 DIV (40 μg protein loaded). (e) Graphical representation of alterations in the levels of c-Jun mRNA and total immunoreactive protein following roscovitine treatment. Analyses were performed on three separate cell preparations. (f) Histogram showing MTT reduction in granule neurons after 24-h exposure to roscovitine (Ros, 25 μM) alone, SB203580 alone (SB, 10 μM), and roscovitine plus SB203580 (SB + Ros, 1 and 10 μM; added 1 h prior to roscovitine) at 1 DIV. *Significantly different than vehicle-treated control ($N = 3$–6).

c-Jun protein. Granule neurons were treated with roscovitine (25 μM) for 0–24 h, then protein or RNA was extracted and analyzed. Fig. 4c and e show that roscovitine induces a detectable increase in *c-jun* mRNA expression by 1 h, peaking at 2 h, and gradually diminishing by 24 h. In the same samples, no changes in amounts of *β-actin* mRNA are observed (Fig. 4c). Later, roscovitine induces an increase in immunoreactive c-Jun protein, first detectable at 2 h, peaking at 8 h, and decreasing to control levels by 24 h (Fig. 4d and e). However, a retardation in the electrophoretic mobility of immunoreactive c-Jun, indicative of phosphorylation, was not observed in three of three cell preparations treated with roscovitine. Moreover, using an antibody that recognizes only serine 63 phosphorylated c-Jun, we were unable to detect appreciable changes in immunoreactive phosphorylated c-Jun (data not shown). These data suggest that the roscovitine-mediated increases in *c-jun* mRNA and c-Jun protein are not a consequence of c-Jun phosphorylation. Thus, although roscovitine induces an early increase in *c-jun* mRNA (peak at 2 h), which precedes an increase in immunoreactive protein (peak at 8 h) and an increase in immunoreactive cleaved caspase-3 (peaks to 24 h), the lack of phosphorylated c-Jun is inconsistent with involvement of a c-Jun-mediated death program.

To further test this, SB203580 was used. At lower concentrations (1 μM), SB203580 blocks the activity of the p38α/β isoforms, while higher concentrations (10 μM) inhibit JNK 2/3 isoforms [54,61,62]. Accordingly, pretreatment of mature granule neurons with 10 μM, but not 1 μM, SB203580 followed by withdrawal of 25 mM KCl (an apoptotic stimulus requiring activation of JNK2/3 and c-Jun [52–54]), is protective [54]. In the cultures used herein, we verified the protective effect of 10 μM SB203580 against apoptosis due to KCl withdrawal, after growth for 7 DIV in medium containing 25 mM KCl (data not shown). To determine whether SB203580 protects immature granule neurons from roscovitine-mediated apoptosis, cultures (1 DIV, 5 mM KCl) were pretreated with SB203580 (1 or 10 μM) prior to challenge with roscovitine (25 μM). Twenty-four hours later, neuronal viability was assayed by MTT. Fig. 4f demonstrates that SB203580 does not protect granule neurons from roscovitine-mediated apoptosis (MTT reduction = 42.3 ± 3.9%, 43.1 ± 7.2%, and 41.5 ± 3.5% for 25 μM roscovitine, roscovitine + 1 μM SB203580, and roscovitine + 10 μM SB203580, respectively, $N = 3$). Taken together, these data indicate that purine analogue-mediated apoptosis in developing granule neurons is not attributable to a CDK 5/JNK3/c-Jun-mediated death program.

3.4. Butyrolactone-1 and PNU112455A do not trigger apoptosis in granule neurons

An additional pharmacologic strategy was undertaken to assess the likelihood that roscovitine and olomoucine trigger granule neuron apoptosis by inhibiting CDKs 1, 2, or 5. The effects of butyrolactone-1, a structurally distinct CDK 5 inhibitor, on granule neuron physiology were examined. Butyrolactone-1 was chosen because it displays a selectivity profile similar to the purine analogues (e.g. inhibits CDKs 1, 2, and 5), and potently inhibits CDK 5 with an in vitro IC_{50} of 0.49 μM [63]. It has been used with success in elucidating CDK 5-mediated processes in other neuronal types [63–65]. Granule neurons (5 and 25 mM KCl) were grown to 1 DIV, and then treated with butyrolactone-1 (10, 25, and 50 μM) or vehicle. Note that these concentrations accord with, or are greater than those described by others to elicit CDK 5-specific effects [63–65]. Following 24-h exposure, viability was assayed by MTT reduction. Distinct from roscovitine and olomoucine, butyrolactone-1 fails to trigger death in granule neurons (5 mM KCl = 99.9 ± 4.6%, 98.0 ± 3.3%, and 101.2 ± 2.0% for 10, 25, and 50 μM, respectively; 25 mM KCl = 102.0 ± 0.2% and 104.1 ± 2.0%, for 25 and 50 mM, respectively, $N = 6$, compared to control; Fig. 5a). Recently, the aminopyrimidine compound PNU112455A was identified as a potent CDK 2/5 inhibitor (K_i = 2.0 μM for CDKs 2 and 5) [66]. PNU112455A is selective and does not inhibit c-Met, the insulin-like growth factor-1 receptor tyrosine kinases, c-AMP-depen-

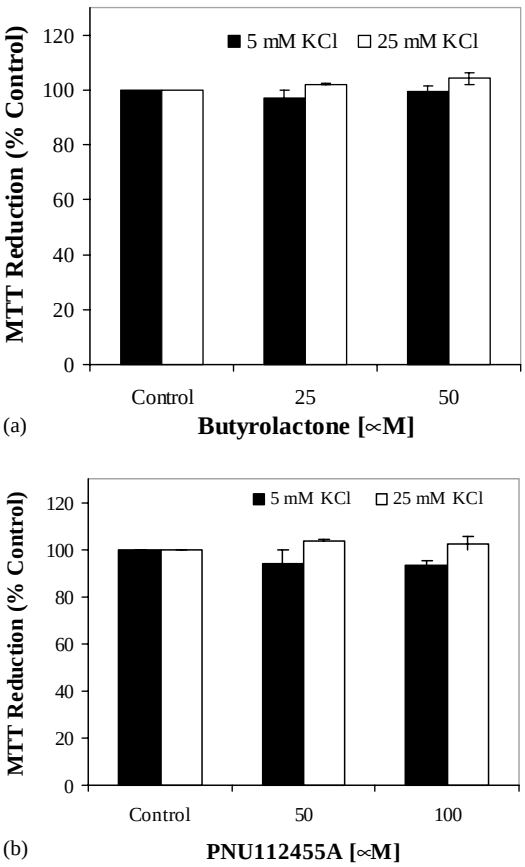

Fig. 5. Butyrolactone-1 or PNU112455A do not reduce granule neuron viability. (a) Histogram showing MTT reduction in granule neurons (5 and 25 mM KCl) following 24-h exposure to butyrolactone (25 and 50 μM) at 1 DIV. (b) Histogram showing MTT reduction in granule neurons (5 and 25 μM KCl) following 24-h exposure to PNU112455A (50 and 100 μM) at 1 DIV.

dent kinase, or ERK2 even at 100 μM [66]. Again, granule neurons (5 and 25 mM KCl) were grown to 1 DIV, then treated with PNU112455A (50 and 100 μM) for 24-h. Like butyrolactone-1, PNU112455A fails to significantly reduce neuronal viability, determined by MTT assay (5 mM KCl = 94.1 ± 5.8% and 93.3 ± 2.2% for 50 and 100 μM, respectively; 25 mM KCl = 103.9 ± 0.9% and 102.5 ± 2.8% for 50 and 100 mM, respectively, $N = 3$; Fig. 5b). Consistent with these findings, neurons treated with butyrolactone-1 or PNU112455A do not demonstrate any morphologic hallmarks of apoptosis when analyzed by phase contrast microscopy (data not shown). These data argue against a role for CDKs 1, 2, or 5 in apoptosis, triggered by the purine analogue inhibitors.

3.5. Purvalanol induces apoptosis in maturing granule neurons and inhibits CREB phosphorylation

The purine analogue CDK inhibitor purvalanol, like olomoucine and roscovitine, demonstrates selectivity for CDKs 1, 2, 5, 7, and 9, and is the most potent member of this class to date. However, its ability to rescue or induce apoptosis in neurons has yet to be explored. Examination of ~25 cellular proteins indicates that purvalanol inhibits CDKs 1, 2, and 5 in the low nanomolar range (in vitro IC_{50} = 4–6, 6–70, and 6–75 nM, respectively) [67], but similar data regarding inhibition of CDKs 7 and 9 are unavailable. Purvalanol also inhibits ERKs 1 and 2 in the low micromolar range (in vitro IC_{50} = 9 and 3.3, respectively) [67]. To test its effects on granule neuron viability, cultures (5 and 25 mM KCl) were grown to 1 DIV, treated with purvalanol (1, 10, 25, and 50 μM) or vehicle for 24 h, and analyzed by MTT assay. As shown in Fig. 6a, purvalanol induced a concentration-dependent decrease in cell viability (5 mM KCl = 98.9 ± 4.9%, 50.5 ± 8.1%, 36.8 ± 6.9%, and 34.7 ± 12.1%, respectively, for 1, 10, 25, and 50 μM; 25 mM KCl = 95.1 ± 7.9%, 52.3 ± 9.6%, 34.2 ± 7.6%, and 30.4 ± 8.6%, respectively, for 1, 10, 25, and 50 μM, $N = 6$). Consistent with this, purvalanol treatment (25 μM) induced the morphological hallmarks of apoptosis (Fig. 6b), as well as immunoreactive c-Jun protein (data not shown). Collectively, these data indicate that purvalanol potently induces apoptosis in granule neurons via a mechanism similar to rosocovitine and olomoucine.

CREB, a nuclear transcription factor, is a common convergence point for several intracellular kinase cascades, including the ERKs and CaMKIV [68]. Loss-of-function mutations in mice show that CREB family members are critical for promoting neuronal survival and development in the mammalian brain [69]. In cerebellar granule neurons, nuclear kinase CaMKIV phosphorylates CREB on serine 133, inducing changes in gene expression that favor neuronal survival and maturation [70]. Consistent with this, our laboratory has shown that inhibition of CaMKIV activity by the selective pharmacologic inhibitor KN-62

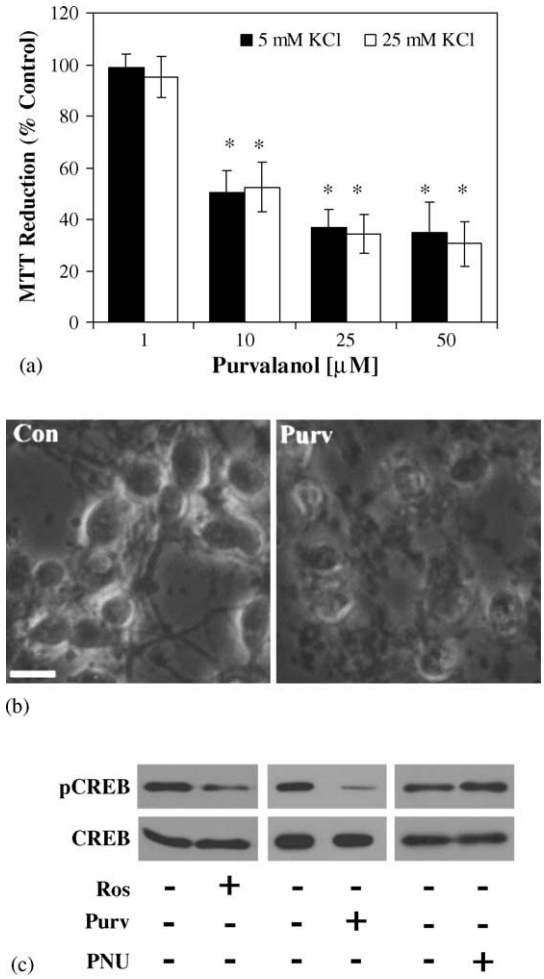

Fig. 6. Purvalanol and roscovitine exert similar effects on granule neuron viability and serine 133 CREB phosphorylation. (a) Histogram showing MTT reduction in granule neurons (5 and 25 mM KCl) following 24-h exposure to purvalanol (1, 10, 25, and 50 μM) at 1 DIV. *Significantly different than respective vehicle-treated control ($N = 6$). (b) Phase contrast micrographs showing granule neurons (5 mM KCl) after 24-h exposure at 1 DIV to purvalanol (25 μM) or vehicle control. Scale bar indicates ~10 μm. (c) Western immunoblots showing serine 133 phosphorylated CREB and total CREB, after 24-h exposure to roscovitine (25 μM), purvalanol (25 μM), or PNU112455A (PNU, 100 μM) compared to vehicle-treated controls (40 μg protein were loaded). Blots shown are representative of at least three separate cell preparations.

inhibits serine 133 CREB phosphorylation and induces granule neuron apoptosis [38]. To test whether the purine analogue CDK inhibitors interfere with serine 133 CREB phosphorylation, cultures (1 DIV) were treated for 2 h with roscovitine (25 μM), purvalanol (25 μM), or vehicle, then whole cell lysates were harvested and compared by Western immunoblotting using antibodies selective for phosphorylated CREB and total CREB protein. PNU112455A (100 μM) was also tested for comparison. As shown in Fig. 6c, exposure to roscovitine or purvalanol, but not PNU112455A, substantially diminishes the amount of phospho-CREB, compared to the control samples. Total CREB protein is comparable in all cases. Note that the observed reductions in CREB phosphorylation are not due

to caspase-3-mediated proteolysis of CaMKIV because cultures were treated with the purine analogues for only 2-h, when caspase activation is not yet detectable (Fig. 2d and e). Taken together, these data indicate that the purine analogue CDK inhibitors, roscovitine and purvalanol, affect signaling upstream of CREB activation, and this occurs prior to attainment of an apoptotic commitment point.

4. Discussion

In the present study, we show that the 2,6,9-trisubstituted purine CDK inhibitors roscovitine, olomoucine, and purvalanol potently trigger death in maturing cerebellar granule neurons. Morphological and biochemical examination of the death process indicates these agents induce a form of death characteristic of apoptosis. These findings are consistent with some aspects of an earlier report in which roscovitine triggered granule neuron death [33], and are in contrast with reports that roscovitine is neuroprotective [19,20]. However, this report preceded recent work revealing a wider diversity of CDK function and CDK inhibitor specificities. Specifically, data interpretation focused upon inhibition of the cell cycle functions of CDKs. In this context, we sought to examine several of the most studied targets of these drugs and their potential contribution to the observed granule neuron apoptosis.

4.1. CDKs 1, 2, 4, and 6

Inappropriate reactivation of cell cycle CDKs (1, 2, 4, and 6) in post-mitotic neurons triggers apoptosis in response to stressful stimuli [10–12], and pharmacological inhibitors of CDKs are protective [18,19,22,71]. For example, when post-mitotic cerebellar granule neurons are grown for 8 DIV in media containing 25 mM KCl, and KCl is subsequently withdrawn to trigger apoptosis, flavopiridol (1 μM), roscovitine (50 μM), or olomoucine (200 μM) are protective [19]. Furthermore, these agents prevent associated changes in several components of the cell cycle machinery (e.g. cyclin D and E induction, increases in cyclin D- and E-associated kinase activities, pRb phosphorylation). CDK activity has also been implicated in naturally occurring neuronal apoptosis, resulting from failed competition for synaptic contacts and/or trophic factors. For example, in post-mitotic cerebellar granule neuron cultures deprived of neuronal activity, CDC2 (a.k.a. CDK 1) activates the pro-apoptotic Bcl-2 family member BAD, leading to apoptosis [20]. Roscovitine rescues granule neurons, and inhibits an associated CDK 1/BAD signaling pathway. In each of these paradigms, protection of cultured neurons from apoptosis was attributed to the pharmacologic inhibition of CDKs 1, 2, 4, and 6. Taken together, such findings suggest that these CDK activities are important components of the apoptotic process in post-mitotic neurons. In contrast, we demonstrate that purine analogue CDK inhibitors trigger, rather than inhibit, apoptosis in granule neurons. Several points are noteworthy to explain this discrepancy.

The primary difference between our study and those reporting neuro-protection is the experimental model. In the present study, granule neurons are grown in a physiologic medium, and addition of CDK inhibitors is used to determine the importance of constitutive CDK activity in survival and development. Alternatively, a commonly used experimental paradigm is growth of granule neurons for several days in media supplemented with 25 mM KCl, followed days later by abrupt withdrawal of this trophic support (e.g. [19,20,53,54]). While this growth paradigm has been extraordinarily useful in characterizing the existence of important components of neuronal signaling pathways, we [34,38,72–74] as well as others (e.g. [75,76]), have demonstrated that granule neurons chronically exposed to medium containing 25 mM KCl undergo several Ca^{2+}-dependent neuroadaptive alterations resulting in phenotypic abnormalities not observed in vivo or in cultures grown in medium containing near physiologic concentration of KCl (i.e. 5 mM). For example, inositol 1,4,5-trisphosphate receptor mRNA and protein, and neurofilament mRNAs and proteins are profoundly increased [72,74], whereas these proteins are barely detectable in vivo or in cultures grown in medium containing 5 mM KCl. Moreover, granule neurons grown under chronically depolarizing conditions are electrically silent, as they do not exhibit action potentials [75] and they down-regulate Ca^{2+} influx into presynaptic terminals [76], indicating that the 25 mM KCl growth paradigm does not mimic the activity-dependent phenomena observed in vivo. These are but a few well studied examples of the numerous phenotypic alterations that occur, however, they underscore the fundamental differences between cultures grown in 5 mM versus 25 mM KCl. Acknowledging this, it becomes quite difficult to compare these completely distinct paradigms, and it is thus not surprising that CDK inhibitor treatment yields paradoxical results.

As mentioned, the abundance of evidence regarding cell cycle CDK activity in post-mitotic neurons indicates a role in promoting apoptosis. Thus, we originally reasoned: if excessive CDK activity (i.e. from CDKs 1, 2, 4, or 6) is a critical component of apoptosis in post-mitotic granule neurons, then CDK inhibitors should prevent apoptosis in 5 mM KCl-containing cultures and enhance their long-term survival. However, our data suggest that neuronal death following purine analogue treatment is not attributable to inhibition of CDKs 1, 2, 4, or 6 for the following reasons. First, the purine analogue CDK inhibitors do not inhibit CDKs 4 or 6 in vitro at the concentrations utilized for these experiments [27,28,67]. Second, our cultures consist of post-mitotic, maturing cerebellar granule neurons. This is supported by the expression of several proteins characteristic of non-proliferating neurons: CDK 5,

p35, and CaMKIV [48,49,58–60]. Although not examined herein, freshly plated granule neurons can express CDKs 1, 2, and 4, but they are rapidly down-regulated 1–32 h after plating [33]. We demonstrated that olomoucine triggers neuronal death when added shortly after plating (1–2 DIV), but also when added several days later at 7 DIV, when CDKs 1 and 2 are no longer detectably expressed. Consistent with this, the structurally distinct, but similarly selective CDK inhibitors butyrolactone-1 and PNU11245A, did not trigger significant neuronal death compared to the purine analogues. Taken together, our data suggest that CDKs 1, 2, 4, and 6 are unlikely to be the targets that trigger granule neuron death. Nevertheless, this argument could be strengthened further by studies using non-pharmacologic approaches.

4.2. CDK 5

The non-cell-cycle CDK 5, which is abundant in post-mitotic neurons and important for neuronal survival and maturation [77,78] has been implicated in the neuronal degeneration that characterizes Alzheimers' disease (for review see [79]). In particular, a stressful stimulus such as β-amyloid promotes cleavage of p35 to p25 [80]. Like p35, p25 activates CDK 5, but has a longer half-life and lacks the myristoylation site that targets p35 to membranes, resulting in both sustained and mislocalized CDK 5 activity and tau hyperphosphorylation [81]. Using this β-amyloid toxicity model, CDK inhibitors were shown to be protective against CDK 5-mediated neuronal death [64,82,83]. As such, one could predict that inhibitors of CDK 5 will protect granule neurons if CDK 5 activity triggers death. However, in the paradigm used herein, no stimulus is applied to induce p35 cleavage, no p35 cleavage is detectable, and the purine analogue CDK inhibitors trigger granule neuron death.

Alternatively, Li et al. [57], in cortical neurons and HEK293T cells, demonstrated that a constitutive CDK 5 activity directly promotes neuronal survival by inhibiting a pro-apoptotic signaling pathway. Specifically, CDK 5 phosphorylates and inhibits JNK3, thereby preventing its catalytic activation of a c-Jun-mediated death program (Fig. 4a). Until now, the relevance of this CDK 5-regulated pathway in granule neurons has not been examined. However, JNK3/c-Jun signaling has been extensively studied in granule neurons, and several reports support its primary role in apoptosis triggered by trophic factor withdrawal [52–54]. According to this model, JNKs become activated and phosphorylate c-Jun on serines 63 and 73 to potentiate its transcriptional activity, and *c-jun* mRNA and c-Jun protein become more abundant, consistent with the theory that c-Jun regulates its own transcription [52–54,57]. Thus, pharmacologic inhibition of CDK 5 in this paradigm should induce apoptosis, via dysinhibition of JNK3 activity, resulting in c-Jun phosphorylation and transcriptional activation. Indeed, we observed apoptosis following treatment with the purine analog inhibitors, all potent CDK 5 inhibitors, but there was no evidence for activation of a JNK3/c-Jun signaling cascade. In particular, within an hour of roscovitine treatment, *c-jun* mRNA and c-Jun protein became more abundant, but phosphorylated c-Jun was not detectable by either an electrophoretic mobility shift or a serine 63 phospho-c-Jun specific antibody. Moreover, SB203580, at a concentration (10 μM) that prevents both JNK2/3 phosphorylation of c-Jun and apoptosis in granule neurons (7 DIV) in the aforementioned trophic factor withdrawal paradigm (note: drug effectiveness was verified herein), failed to rescue maturing granule neurons (1–2 DIV) following roscovitine addition. It is noteworthy that these findings are distinct from those of Courtney and Coffey [33], who, during AraC-mediated death, observed no changes in c-Jun regulation (roscovitine was not examined). Thus, their conclusion that Ara-C- and roscovitine-mediated death share a common CDK 5 signaling pathway is unlikely. Further supporting our argument, treatment of granule neurons with two structurally distinct, yet potent CDK 5 inhibitors, butyrolactone-1 and PNU112455A, were without effect on granule neuron viability. Taken together, these findings suggest that inhibition of a constitutive CDK 5 activity, at least via a direct JNK3/c-Jun signaling cascade, does not have a dominant role in granule neuron death in response to roscovitine, olomoucine, or purvalanol.

4.3. ERKs and DYRK

To date, all known cellular effects of the purine analogue CDK inhibitors can thus far be attributed to CDK inhibition (for reviews [84–86]). Nonetheless, the early decreases in nuclear CREB phosphorylation (on serine 133) following purine analogue treatment warrant consideration of kinases that mediate activation of CREB and can be inhibited by the purine analogues as potential targets of drug action.

A common feature of olomoucine, roscovitine, and purvalanol is their selectivity for the mitogen activated protein kinases (MAPKs), ERKs 1 and 2. In vitro kinase assays indicate that olomoucine and roscovitine inhibit ERKs 1/2 with IC_{50} values of 50/40 and 34/14 μM, respectively [27,28]. Note, however, that roscovitine is 10–20-fold more potent than olomoucine against CDKs, but only marginally more so against ERKs 1/2. Purvalanol is more potent against CDKs than olomoucine or roscovitine (IC_{50} = 4–6, 6–70, and 6–75 nM, respectively, for CDKs 1, 2, and 5), and is also more potent against the ERKs (IC_{50} = 9 and 3.3 μM, respectively, for ERKs 1 and 2) [67]. In addition, purvalanol directly interacts with both ERKs 1 and 2 [30,87]. In contrast, neither butyrolactone-1 nor PNU112455A appreciably inhibit the ERKs when tested in vitro [66,88]. Although some controversy exists regarding the functions of ERKs 1/2 in neurons [89], they appear to contribute to granule neuron survival by virtue of their activity towards CREB (for review [68]).

Thus, inhibition of MAPK activity in granule neurons with the selective pharmacologic agent PD98059 reduces viability [90], though PD98059 was not as potent as the purine analogues in this respect (Monaco III, unpublished observation). PD98059 treatment also inhibits MAPK phosphorylation of CREB in granule neurons ([91]; Monaco III, unpublished observation). These findings are consistent with our observation that roscovitine and purvalanol, but not PNU112455A, reduce serine 133 phosphorylation of CREB. Inconsistent with the notion that the ERKs as relevant targets of the purine analogues, we observed significant granule neuron death using as little as 10 μM roscovitine (e.g. Fig. 1d), which is well below its in vitro IC_{50} values for these kinases. Moreover, it is predictable that concentrations several fold in excess of IC_{50} values would be required to achieve physiological inhibition.

An alternative, less known, potential drug target is DYRK1a. DYRK family kinases are distantly related to ERKs, and structurally similar to CDKs. Thus, it would not be surprising if pharmacologic CDK inhibitors also inhibited DYRKs (for review see [92]). The physiological functions of DYRKs are largely unknown, but evidence regarding the *Drosophila* homolog "*minibrain*" indicates that it is critical for nervous system development [93]. Such a role is also supported by the observation that the human DYRK1a gene maps to the "Down's Syndrome critical region" of chromosome 21, resulting in speculation that it may contribute to the Down's Syndrome phenotype [94]. Consistent with our results, in a hippocampal neuron cell line, DYRK1 binds, phosphorylates, and activates the nuclear transcription factor CREB on serine 133, promoting CREB-mediated gene transcription [95]. Interestingly, both roscovitine and purvalanol inhibit DYRK1a in vitro (IC_{50} = 3.1 and 0.3 μM, respectively). However, in preliminary experimentation using epigallocatechin (1–50 μM), one of the main polyphenolic constituents in green tea and a potent in vitro DYRK1a inhibitor (IC_{50} = 0.33 μM; [96]), we failed to detect significant granule neuron death at 1–2 DIV (Monaco III, unpublished observation). Taken together, these findings suggest that inhibition of the non-CDKs ERKs 1 and 2, as well as DYRK1a, are unlikely to play a role in granule neuron apoptosis triggered by the purine analogues.

4.4. CDKs 7 and 9

CDKs 7 and 9 are worthy of discussion considering their known functions, while CDK 8 can be eliminated as a drug target because it is insensitive to the purine analogues [30]. CDK 7 is unique because it has a role in both the cell cycle and transcriptional regulation. CDK 7/cyclin H is a CDK-activating kinase (CAK), important for phosphorylation and activation of other CDKs (i.e. CDKs 1, 2, and 4; for review see [97]). These complexes also comprise one of at least nine components of the RNA polymerase II general transcription factor TFIIH. Phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II by TFIIH is attributable to CDK 7/cyclin H and important for initiation and elongation (for review see [98]). However, this point requires refinement as work by Tirode et al. [99] and Makela et al. [100] indicates that the CTD phosphorylating activity of CDK 7 may not be uniformly required for gene transcription. Indeed, accumulating evidence suggests that a transcriptional requirement for CDK 7 activity depends upon a gene's promotor [99–102]. Like CDK 7, CDK 9 activity is required for gene transcription. Unlike CDK 7, it facilitates transcriptional elongation as part of the elongation factor P-TEFb (CDK 9/cyclin T), again via phosphorylation of the CTD of RNA polymerase II [103]. CDK 9 also does not possess any known CAK activity. Although it is clear that CDK 7 and cyclin H are expressed in post-mitotic neurons [104–106], we argue against CAK activity as critical to granule neuron survival, as inhibition would prevent activation of cell cycle CDKs, a pro-apoptotic phenomenon in post-mitotic neurons subjected to stressful stimuli. The roles of CDKs 7 and 9 in regulating transcription, thereby serving as targets of CDK inhibitors, have only recently begun to be explored.

In their study of the mechanism of granule neuron death in response to high concentrations of AraC, Courtney and Coffey [33] reported that roscovitine triggered apoptosis in a manner that was inhibited by flavopiridol (and cycloheximide), which they considered to be a selective CDK 4/6 inhibitor. Subsequently, Lam et al. [107], using DNA microarray analysis, demonstrated that it potently and globally suppresses gene transcription in a lymphoma cell line. Further, its effects were comparable to the established transcriptional inhibitors actinomycin D and 5,6-dichloro-1-*b*-D-ribofuranosyl-benzimidazole (DRB). As a consequence, the effects of flavopiridol were attributed to inhibition of CDKs regulating transcription, most likely CDK 9. Unlike all other known CDK inhibitor/CDK interactions, the mechanism by which flavopiridol inhibits CDK 9 is non-competitive [108]. Moreover, the flavopiridol concentrations that suppress transcription are too low to inhibit CDKs 1 and 2 in the presence of physiological ATP concentrations [107,108]. As such, one must be aware of the possibility, when using flavopiridol as a cell cycle research tool, that the results observed are likely the product of transcriptional suppression rather than inhibition of cell cycle CDK activity (e.g. [19,33]).

Interestingly, both flavopiridol and roscovitine rescue granule neurons from trophic factor withdrawal (i.e. [19]), suggesting that their effects can overlap. Accordingly, both flavopiridol and roscovitine inhibit the expression of some genes [19,107,109], although the magnitude (i.e. number of genes affected) of these effects are likely context dependent and are still in question. Current data indicate that inhibition of flavopiridol's targets may globally suppress transcription, whereas inhibition of roscovitine's targets likely does not [107]. Indeed, our data demonstrate that roscovitine treatment of maturing granule neurons

actually induces *c-jun* mRNA and has no detectable effect on *β-actin* mRNA (Fig. 4c–e). As the abundance of evidence indicates that CDK 9 is the most likely physiologic target of flavopiridol, these findings suggest that inhibition of CDK 9 may be the site of overlap with the purine analogues. However, flavopiridol also rescues granule neurons from roscovitine-mediated apoptosis [33], suggesting that the anti-apoptotic effects of flavopiridol supercede the apoptotic effects of roscovitine, and underscores their differences, and the context-dependent nature of their effects. Also, their effects, at least in part, do not overlap. In support of this statement, while the purine analogues inhibit CDK 9 in vitro [31], it does not appear likely that the purine analogues bind CDK 9 in vivo [30].

From these data, we propose the following hypothesis: the purine analogue CDK inhibitors trigger granule neuron death, observed herein, by inhibiting the expression of genes under the control of CDK 7 activity. Specifically, the purine analogues inhibit the expression of pro-survival genes that require phosphorylation of RNA polymerase II by CDK 7. In a different context, both roscovitine and flavopiridol would rescue granule neurons in a trophic factor withdrawal paradigm by inhibition of pro-apoptotic genes (i.e. cyclins D and E [19]). Moreover, the effects of flavopiridol are predicted to supercede those of the purine analogues in granule neurons by inhibiting the expression of genes required for purine-analogue-mediated death. To test this hypothesis, one should focus on the apparent promotor-based dependence of CDK 7-mediated gene expression [99,100]. Support for this hypothesis could come in the form of: (i) the identification of a subset of genes commonly influenced by the purine analogues; (ii) identification of common promotor characteristics critical for CDK 7 activity dependence; and (iii) knowledge of whether these critical promotor characteristics are present among the subset of genes that are repressed in the presence of the purine analogues.

To date, two reports have examined the role of CDK 7 in determining the survival/death balance in post-mitotic neurons. Zhu et al. [106] observed that the level of CDK 7 protein was more abundant in susceptible hippocampal neurons from Alzheimers disease patients versus age-matched controls, consistent with the notion that it has a role in aberrant cell cycle activation, a pro-apoptotic stimulus in post-mitotic neurons. It is noteworthy, however, that following global cerebral ischemia Jin et al. [104] observed that expression of cyclin H, the regulatory partner for CDK 7, is increased in surviving neurons, in contrast to cyclins B1 and D1. They proposed that cyclin H, and by inference CDK 7, may contribute to neuronal survival by virtue of its transcriptional functions. Interestingly then, CDK 7 may have a dual role in determining neuronal fate: the CAK activity of CDK 7 could serve a pro-apoptotic function, whereas its role in regulating transcription could be both pro- and anti-apoptotic, depending on which genes are affected and in what context.

Overall, our findings suggest a number of important conclusions. First, the purine analogue CDK inhibitors olomoucine, roscovitine, and purvalanol trigger apoptosis in maturing cerebellar granule neurons via a shared mechanism. In contrast to a previous report, our findings, together with those of others, indicate that roscovitine and AraC do not share common cell cycle CDK signaling pathways as targets. Although repeatedly used as tools for the study of CDKs 1, 2, and 5, caution must be observed when attributing their effects to inhibition of these CDKs, especially considering that their effects on CDK 7 have yet to be fully explored. For example, the data presented herein, together with an emerging literature on the subject, suggest that maturing cerebellar granule neurons undergo apoptosis in response to purine analogue CDK inhibitors, but this is not attributable to inhibition of CDKs 1, 2, 5, or 9. As such, it must be acknowledged that alternative targets exist in these neurons that, when inhibited, trigger apoptosis. Though as yet unknown targets cannot be formally excluded, the data provide a rationale for further study of CDK 7. A combination of non-pharmacological and molecular biological approaches should help to clarify its functions. Finally, this work serves as a caution to investigators using pharmacologic CDK inhibitors to target a specific pathway. Although inhibition of multiple targets may be beneficial in some circumstances (i.e. cancer chemotherapies), a more complete understanding will better serve our quest to study human physiology and develop pharmacologic therapies.

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