Published in final edited form as:

Mol Cell Neurosci. 2007 June ; 35(2): 328–338.

Differential effects of AMPA receptor activation on survival and neurite integrity during neuronal development

M. Chiara Manzini^{a,b,1}, Donald J. Joseph^{a,c}, Amy B. MacDermott^{a,c}, and Carol A. Mason^{a,b,*}

^aCenter for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, NY 10032

^bDepartment of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY 10032

^cDepartment of Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032

Abstract

While neuronal cultures are an established model for analyzing excitotoxic brain injury in the adult, *in vitro* systems have not been extensively employed to study how developing neurons respond to levels of excitatory compounds that are lethal to mature neurons. Recently, we reported that the *in vivo* differentiation programs of cerebellar granule cells (CGNs) are recapitulated in purified CGN cultures (Manzini et al, 2006). Here, we have used this model system to compare the response of immature and mature neurons to excitotoxic compounds. We found that immature CGNs are less sensitive to AMPA receptor (AMPA-R) activation than mature cells and that levels of AMPA-Rs expression on the plasma membrane are critical in regulating the balance between death and survival during maturation of these neurons. However, the majority of immature cells that survive excitotoxic treatment bear a degenerating neurite, suggesting that AMPA-R activation can still cause damage in the absence of cell death.

Introduction

Neonatal brain injury following acute neurological disorders such as status epilepticus, encephalopathy and stroke often leads to excitotoxicity and long-term neurological perturbations, including motor and mental deficits, learning disabilities, mental retardation and/or epilepsy (Huttenlocher and Hapke, 1990; Ferriero, 2004). While the effects of brain injury, especially from excitotoxicity, have been well studied in mature neurons *in vivo* and *in vitro* (Lipton, 1999; Sattler and Tymianski, 2001; Contestabile, 2002), the mechanisms mediating responses to excitotoxic challenge in immature neurons remain poorly understood.

During the perinatal period, spontaneous neuronal activity modulates cell differentiation, from proliferation to circuit formation (Volpe, 2001; Spitzer et al., 2002; Moody and Bosma, 2005). Nonetheless, despite increased spontaneous excitability, the immature brain undergoes less cell death than the mature brain after seizures of identical magnitude (Rennie, 1997; Sperber et al., 1997). It is unclear how immature neurons survive concentrations of excitatory

*Corresponding author: Dr. Carol Mason, Department of Pathology, 14-509 P&S Building, Columbia University, College of Physicians and Surgeons, 630 W 168th Street, New York, NY 10032, Tel: 212-305-2105, Fax: 212-305-5498, Email: cam4@columbia.edu

¹Present address: Dept. of Neurology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

compounds that are lethal to mature neurons, and whether in the absence of cell death, neuronal differentiation is impaired by the changes in the developmental patterns of activity, which could in turn result in later cognitive or motor neurological deficits (Huttenlocher and Hapke, 1990; Holmes et al., 2002; Ferriero, 2004).

The results of excitotoxic damage in the developing brain have been studied *in vivo* in rodents and rabbits to define long-term effects of neonatal brain injury on excitability, morphology, learning abilities and motor coordination (Jensen, 1999; Holmes et al., 2002; Olney et al., 2002; Mesples et al., 2005). Other studies have aimed to define how immature neurons respond to excessive excitation in cell culture (Choi et al., 1987; Gallo and Balazs, 1988; Didier et al., 1990), but the exact progression of neuronal development in those models was not known, preventing a direct comparison with the *in vivo* setting.

Using molecular and morphological criteria, we recently reported that the differentiation of purified CGNs *in vitro* mirrors the developmental transitions that CGNs undergo *in vivo* (Manzini et al., 2006). In such a controlled setting, proliferating, migrating and differentiated cell populations can be identified by cell marker expression and morphology, and thus can be separately analyzed. Here, we used this model of developing neurons to compare responses of immature and mature CGNs to excitotoxic glutamate receptor activation. Immature neurons displayed greatly reduced sensitivity to concentrations of the AMPA/KA receptor agonist, kainate (KA), that trigger apoptotic cell death in mature cells. This developmental increase in sensitivity to KA reflects an increase in the surface expression of functional AMPA-Rs. However, although most immature neurons survive KA treatment, the majority of KA-treated cells bear degenerating neurites, suggesting that AMPA-R activation can still cause damage in these cells.

Results

Immature CGNs survive treatment with concentrations of glutamate receptor agonists that are lethal to mature cells

Recent reports demonstrated that rodent CGN differentiation can be closely followed over the first week *in vitro* (Raetzman and Siegel, 1999; Diaz et al., 2002; Manzini et al., 2006). Only proliferating and immature CGNs survive the purification procedure, and transit from a proliferative population with characteristics of cells in the external germinal layer (EGL) at plating, to a population of differentiated CGNs over the first week in culture. At 1 day *in vitro* (div), half the cells have a flat amoeboid shape and express the outer-EGL marker Math-1, while the other half are post-mitotic CGNs. At 2 div, cultures are composed of a few proliferating EGL cells and mostly post-mitotic neurons extending processes, which express TAG-1, a marker of inner-EGL and migratory CGNs. By 4 div, all cells have downregulated TAG-1 expression and have become post-migratory, as shown by expression of the transcription factors, Zic1 and 2 and MEF2 (Manzini et al., 2006).

We used this *in vitro* model to compare immature and mature CGN cultures from P5 mouse pups at two time-points during differentiation: at 2 days div, when CGN cultures are still immature and are composed of a mixture of proliferating, post-mitotic and migrating cells, and at 4 div, when CGNs have differentiated to a mature population (Raetzman and Siegel, 1999; Manzini et al., 2006). Immature (2 div) and mature (4 div) CGN cultures were treated for 16 hours (overnight) using a classic excitotoxic paradigm using concentrations of glutamate (500 μ M) comparable to those found in the cortico-spinal fluid during an epileptic seizure or following ischemia. Immature neurons proved to be resistant to glutamate (Glu) treatment (live cells: 98 $\pm$ 4% of control (contr), N=4), while a third of mature cells died (69 $\pm$ 5% of contr, P_{compared to contr}<0.001, N=4; Fig. 1A, B). (Note: "N" indicates the number of experiments, "n" represents the number of cells, where applicable)

NMDA receptors (NMDA-Rs) are the main mediator of Glu-induced excitotoxicity in neurons (Garthwaite and Garthwaite, 1986; Hack and Balazs, 1995), but AMPA-R activation was also found to trigger cell death (Grooms et al., 2000; Noh et al., 2005). We investigated the contribution of the three types of ionotropic Glu receptors (iGluRs), AMPA-, KA- and NMDA-Rs, in Glu-mediated neuronal death in mature CGNs. Mature cultured CGNs were treated with 500 μ M Glu and activation of different iGluR subtypes was blocked with specific antagonists, 100 μ M 3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) for AMPA/KA-Rs, 100 μ M 2-amino-5-phosphonovalerate (APV) for NMDA-Rs, or with 3 μ M SYM2081, a fast-desensitizing agonist of KA-Rs. NBQX and APV each partially rescued cell death (Glu+NBQX: 90 $\pm$ 9% of contr, N=4; Glu+APV: 94 $\pm$ 7% of contr, N=4, $P_{\text{compared to Glu}} < 0.05$; Fig. 1C), while SYM2081 had highly variable effects (Glu+SYM: 82 $\pm$ 24% of contr, N=4; Fig. 1C). Glu-induced death could only be completely rescued if both AMPA/KA- and NMDA-Rs were blocked (Glu+NBQX+APV: 104 $\pm$ 4% of contr, N=4, $P_{\text{Glu}} < 0.01$; Fig. 1C). When tested alone NBQX, APV or SYM2081 had no effect on survival (data not shown). Thus, both NMDA- and non-NMDA-Rs are involved in Glu-induced excitotoxic death of mature CGNs.

The mechanisms of NMDA-R-mediated CGN death have been extensively explored *in vitro* (Didier et al., 1990; Sattler and Tymianski, 2001), but the molecular and cellular responses to excessive non-NMDA-R activation have not been well defined. We first asked whether selective activation of AMPA/KA-Rs with the AMPA/KA-R agonist KA had similar effects on neuronal survival to those of Glu. Immature and mature purified CGN cultures were treated with 100 μ M KA for 16 hrs. As observed during Glu treatment, most immature CGNs survived a 16 hr 100 μ M KA treatment (87 $\pm$ 11% of contr, N=5; Fig. 1D, E), while half of the mature cells that were exposed to KA died (52 $\pm$ 6% of contr, $P_{\text{contr}} < 0.01$, N=8; Fig. 1D, E). Death of mature CGNs was completely blocked with the AMPA/KA-R antagonist NBQX (100 $\pm$ 7% of contr, N=5). As KA treatment can lead to Glu release following AMPA/KA-R activation and action potential firing, cell death might be partially due to ensuing NMDA-R activity (Garthwaite and Wilkin, 1982). No additional involvement of neuronal activity or NMDA-Rs was observed with KA treatment, as neither the Na⁺ channel blocker, tetrodotoxin (TTX), or the NMDA-R antagonist, APV improved neuronal survival (TTX: 63 $\pm$ 13% of contr, $P_{\text{contr}} < 0.05$, N=4; APV: 52 $\pm$ 6% of contr, $P_{\text{contr}} < 0.001$, N=5; Fig. 1F).

In dose-response studies, KA concentrations that completely activated KA-Rs, but only partially activated AMPA-Rs (1-10 μ M KA) did not trigger cell death, while increasing KA concentrations to levels that fully activate AMPA-Rs (50-500 μ M KA) caused a gradual dose-dependent increase in neuronal death (Supplementary Fig. 1A). These findings, together with the inability of SYM2081 to rescue KA-induced cell death (SYM: 69 $\pm$ 10% of contr, $P_{\text{contr}} < 0.01$, N=4; Fig. 1F), indicated that death in mature CGNs was principally mediated by AMPA-R activation. These results reveal that immature CGNs are less sensitive than mature neurons to KA treatment and suggest a role for AMPA-Rs in mediating cell death of mature CGNs.

To analyze CGN survival in a more intact system, acute vermal and paravermal parasagittal slices were prepared from mouse cerebellum at P8-9. At this age the EGL is the thickest and the internal granule layer (IGL) is well defined (Altman and Bayer, 1997), so that responses of immature and mature CGNs can be studied in the same slice (Supplementary Fig. 2). Slices were incubated with 100 μ M KA for 7 hrs and cell death was analyzed via three independent methods: TUNEL labeling, propidium iodide (PI) counterstaining and PI live staining. Very little cell death was observed in control conditions (TUNEL labeling: Supplementary Fig. 2; PI live staining: not shown; PI counterstaining: Fig. 1G). Whereas no increase in cell death over basal levels was observed in the EGL after KA application, pyknotic cells were present among migratory CGNs (CGNs found between the EGL and the IGL) and among the IGL cells (Fig. 1G). Because TUNEL labeling has been shown to have limited sensitivity (Labat-Moleur

et al., 1998), we quantified cell death in slices by PI counterstaining. After KA treatment, approximately one quarter of migratory and IGL CGNs appeared shrunken and pyknotic (live cells: $73 \pm 2\%$ of contr migratory CGNs, $P_{\text{contr}} < 0.001$, $73 \pm 3\%$ of contr IGL CGNs, $P_{\text{contr}} < 0.001$, $N=9$ slices; Fig. 1H). In many KA-treated slices the molecular layer and the Purkinje cell layer was shrunken (Fig. 1G), possibly because Purkinje cells are more sensitive than CGNs to KA treatment and die shortly after the beginning of treatment (Garthwaite and Wilkin, 1982 and our observations).

Cell death was rescued by addition of $50 \mu\text{M}$ NBQX during treatment ($93 \pm 3\%$ of contr migratory CGNs, $94 \pm 3\%$ of contr IGL CGNs, $N=7$ slices). Treatment with $100 \mu\text{M}$ APV did not significantly prevent cell death ($81 \pm 3\%$ of contr migratory CGNs, $P_{\text{contr}} < 0.001$, $86 \pm 5\%$ of contr IGL CGNs, $P_{\text{contr}} < 0.05$, $N=4$ slices; Fig. 1H), but showed a trend towards improved survival possibly indicating an involvement of NMDA-R in KA-induced cell death in slices as found by Garthwaite and Wilkin (1982).

In summary, we found that immature CGNs are less sensitive to activation of iGluRs compared to migrating and mature cells in an intact acute slice system.

Developing CGNs survive exposure to KA concentrations that are lethal to more mature cells, because of low surface levels of AMPA-Rs

The amplitude of AMPA-R currents has been shown to increase during CGNs maturation in acute cerebellar slices (Smith et al., 2000). Therefore, the lack of KA toxicity in immature CGN cultures could be due to lower levels of AMPA-R activation compared to mature cells, so that receptor activation in immature neurons is not large enough to trigger apoptosis. We assessed AMPA-R function during CGN differentiation by voltage-clamp whole-cell recordings in immature and mature CGN cultures. 94% of cells in 2 div cultures (31/33 cells) responded to $100 \mu\text{M}$ KA application, while 100% of cells responded in 4 div cultures (41/41). As expected, the amplitude of the responses evoked by $100 \mu\text{M}$ KA increased with differentiation, from an average current amplitude of 32.7 ± 4.8 pA ($n=31$ 2 div cells) in immature CGNs to 121.7 ± 21.8 pA in mature cultures ($n=41$ 4 div cells) (Fig. 2A).

Based on the voltage-clamp recording results, we anticipated AMPA-R expression levels to also increase with neuronal differentiation, but when we analyzed total protein levels of GluR2 and GluR4, the AMPA-R subunits most expressed in CGNs, expression was similar throughout development (Fig. 2B). It is possible that even if a large pool of AMPA-R proteins is present in the cell, developmental regulation of trafficking of AMPA-R subunits may maintain surface expression at a minimum in immature CGNs. By surface biotinylation, we found that both GluR2 and GluR4 were expressed on the plasma membrane at low levels at 2 div and expression increased with time in culture (Fig. 2C). GluR4 surface expression doubled from $11 \pm 1\%$ of total GluR4 expressed on the surface at 2 div, to $24 \pm 4\%$ of total GluR4 expressed on the surface at 4 div ($N=4$, $p < 0.05$). GluR2 surface expression tripled from $8 \pm 10\%$ at 2 div to $26 \pm 12\%$ at 4 div ($N=3$) (Fig. 2D), but it was difficult to measure due to high variability of GluR2 detection/expression especially in immature cultures. Thus, increased sensitivity to KA treatment between immature and mature CGNs is mirrored by an increase in amplitude of KA-evoked currents due to surface targeting of AMPA-Rs.

Our findings suggest that the developmental increase in AMPA-R activation levels is mediating the increased sensitivity of mature CGNs to excitotoxic death, but additional factors may be involved. Neuronal survival can also be controlled by the balance of expression and activation of pro-apoptotic and pro-survival signaling pathways (West et al., 2002; Hardingham and Bading, 2003), and immature neurons may have neuroprotective mechanisms that render them more resilient to excitation. We analyzed expression and activation of pro-survival transcription factors which have been shown to protect mature CGNs from Glu and KA

excitation, MEF2, CREB and NF- κ B (West et al., 2002; Scholzke et al., 2003). We found no selective activation of MEF2, CREB or NF- κ B in immature cells (Supplementary Fig. 3).

We then asked whether caspase-3 and its activator, caspase-9, which are molecular landmarks of apoptotic cell death of mature neurons following glutamate receptor activation (Marks et al., 1998), could be expressed at lower levels in immature neurons than mature cells. The zymogen form of caspase-3 and 9, procaspase-3 and 9, were expressed in all neurons throughout the cytoplasm (data not shown). Protein expression levels of procaspase 3 were identical throughout differentiation, while procaspase 9 levels were lower at 4 div than at 2 div (Fig. 3A).

To confirm that caspase activation was in fact involved in KA-induced apoptosis in mature CGN cultures, we used antibodies raised against the cleaved/active form of caspase-3 and 9 both in control and KA-treated mature CGN cultures. Most pyknotic nuclei in control and KA treated cultures were decorated by activated caspase-3 and 9 antibodies (Fig. 3B). Cells with a normal nuclear morphology were negative for active caspase-3 and 9, but displayed punctate cytochrome *c* staining, indicating that the mitochondria were intact (Fig. 3B, see inset). KA-induced cell death could be significantly rescued by adding an irreversible inhibitor of caspase function, DEVD-CHO to the KA treatment at 4 div (KA: $70 \pm 6\%$ of contr, $P_{\text{contr}} < 0.01$; KA + DEVD: $89 \pm 5\%$ of contr, $P_{\text{KA}} < 0.05$; $N=3$; Fig. 3C). DEVD-CHO alone had no effect on survival ($95 \pm 7\%$ of contr, $N=3$).

Since the analysis of pro-survival and pro-apoptotic factors did not indicate that immature neurons are better equipped than mature cells to withstand excitotoxic injury, it is possible that AMPA-R expression levels are the determining factor regulating excitotoxic sensitivity in these neurons. We should be able to activate apoptotic pathways in immature cells simply by increasing AMPA-R activation.

Instead of overexpressing recombinant AMPA-R subunits in CGN cultures, we chose to pharmacologically increase current amplitude blocking receptor desensitization with cyclothiazide (CTZ) to manipulate endogenous AMPA-Rs. We first confirmed the effect of CTZ on KA-evoked responses in immature CGNs by whole-cell voltage-clamp recordings. 75% of 2 div CGNs tested (15/20 2 div cells) showed an enhancement of the KA response in the presence of CTZ, with an average increase in response amplitude of $54 \pm 15\%$ (Fig. 4A). In the remaining 25% of neurons (5/20 2 div cells), KA responses were depressed by CTZ ($-47 \pm 15\%$ of control KA response).

While KA alone had little effect on survival of immature neurons (live cells: $93 \pm 4\%$ of contr, $N=7$), approximately 30% of cells underwent cell death when $100 \mu\text{M}$ KA was supplemented with $25 \mu\text{M}$ CTZ ($70 \pm 6\%$ of contr, $N=7$, $P_{\text{contr}} < 0.001$, $P_{\text{KA}} = 0.01$; Fig. 4B). KA+CTZ-induced cell death could be completely rescued with NBQX ($103 \pm 5\%$ of contr, $N=6$). To confirm that KA+CTZ treatment activated the same apoptotic mechanisms responsible for apoptotic cell death in mature CGNs, we investigated caspase-3 and 9 expression and function. Active caspase-3 and 9 were observed in pyknotic nuclei after KA+CTZ treatment in immature CGNs (Fig. 4C). As in mature cells, KA+CTZ-induced cell death could be rescued by addition of DEVD-CHO to KA treatment ($92 \pm 4\%$ of contr, $P_{\text{KA+CTZ}} < 0.05$, $N=4$) (Fig. 4D).

The effect of CTZ addition to KA treatment was also tested in cerebellar slices. KA+CTZ had no effect on the EGL (live cells: $100 \pm 3\%$ of contr EGL CGNs, $N=7$ slices; Fig. 4B), but CTZ enhanced KA-mediated CGN death in both migratory and IGL populations (KA+CTZ: $58 \pm 4\%$ of contr migratory CGNs, $P_{\text{contr}} < 0.001$; $43 \pm 4\%$ of contr IGL CGNs, $P_{\text{contr}} < 0.001$, $N=7$ slices; Fig. 4B). Cell death was completely prevented by NBQX treatment, indicating that CTZ action was mediated exclusively through AMPA-R activation (NBQX: $104 \pm 1\%$ of contr EGL CGNs; $96 \pm 4\%$ of contr migratory CGNs; $91 \pm 7\%$ of contr IGL CGNs, $N=4$ slices; Fig. 4B).

In summary, we found that immature CGNs display lower surface expression levels of functional AMPA-Rs resulting in reduced sensitivity to excitotoxic challenge. However, caspase-dependent apoptotic pathways are present in immature and mature neurons, and can be activated when AMPA-R activation levels are pharmacologically increased in a subpopulation of immature CGNs.

KA treatment causes neuritic degeneration in immature CGNs

Even in the absence of cell death, KA treatment could damage immature neurons and affect aspects of neuronal differentiation that are regulated by AMPA-R activation. AMPA/KA receptors have been shown to be present on filopodia and growth cones in developing axons of hippocampal cultures (Chang and De Camilli, 2001). Axons and growth cones of developing CGNs also express AMPA-Rs, as shown by immunocytochemistry (Fig. 5A). Therefore, we asked whether KA treatment on immature cells could affect axonal morphology and outgrowth.

To analyze morphology of individual cells, we mixed wild-type CGNs with 2-3% CGNs from a transgenic mouse where EGFP is expressed under the β -actin promoter. In immature cells fixed after 16 hr 100 μ M KA treatment average neurite length was not significantly affected (contr: 944 ± 66 μ m, $n=24$ cells; KA: 829 ± 54 μ m, $n=24$ cells). However, axonal morphology was severely perturbed in KA-treated cultures. Normal growth cone morphologies were not present and the distal one third of the axon was beaded and tipped by a club-like stump in $73 \pm 4\%$ of CGNs in culture ($N=5$, $P_{\text{contr}} < 0.001$) (Fig. 5 B). Similar axonal beading was also occasionally observed in control cultures in $30 \pm 2\%$ of CGNs ($N=5$). The remaining two thirds of the axon were not changed, and cell body morphology and nuclear chromatin were indistinguishable from cells bearing a normal growth cone.

Axonal beading and growth cone collapse are characteristic of axonal degeneration (Coleman, 2005; Luo and O'Leary, 2005), and while axonal degeneration is often an aspect of neuronal cell death, axonal and dendritic beading has been observed in mature neurons in response to sublethal excitotoxic stimuli (Ikegaya et al., 2001; Sun et al., 2001). To confirm that the immature neurons bearing degenerating axons were alive, we stained KA-treated GFP-wild type CGN cultures with the live dye, Cell Tracker Red CMTPX, which appears diffuse in the cytoplasm of live cells and is concentrated in apoptotic bodies (Fig. 5C). All cell bodies of KA-treated cells that bore a degraded distal axon showed diffuse staining characteristic of live cells (Fig. 5C). Thus, in immature cells 100 μ M KA treatment causes degeneration of the distal portion of the axon, which is not immediately associated with cell death.

Axonal beading was observed in immature CGN cultures after only 1 hour of KA-treatment, and degeneration was induced exclusively by AMPA-R activation as it could be blocked with NBQX and was not observed following 100 μ M NMDA treatment (cells with degenerating axon: $35 \pm 2\%$ of KA+NBQX treated cells, $N=3$; NMDA; $34 \pm 5\%$ of NMDA treated cells, $N=3$) (Fig. 5B). If CGN axonal outgrowth is affected during cerebellar circuit formation *in vivo*, connections with the CGN targets, the Purkinje cells, could form abnormally.

Discussion

Purified CGNs as a model to study excitotoxic challenge during neuronal differentiation

The rodent brain displays similar responses to the human brain with regard to excitability and cell death (Holmes et al., 2002), and several forms of hypoxic-ischemic and epileptic injury have been modeled in infant mice and rats (Holmes and Ben-Ari, 1998; Sanchez and Jensen, 2001; Holmes et al., 2002). *In vivo* models of neonatal excitotoxic and ischemic trauma are necessary tools to investigate the physiological and behavioral consequences of neonatal brain injury (Holmes and Ben-Ari, 1998; Jensen et al., 1998; Kubova et al., 2000; Sanchez and

Jensen, 2001). Nonetheless, the cellular and molecular mechanisms involved in the development of neurological defects following perinatal brain injury remain unclear, especially in cases where cell death is not observed.

Primary cell cultures from adult rodents, hippocampal and cerebellar preparations in particular, have been widely employed to explore cellular and molecular responses to excitation in the adult (Jensen, 1999; Lipton, 1999; Holmes et al., 2002). While previous attempts have aimed at defining responses to pro-convulsant agents in immature cells (Choi et al., 1987; Gallo and Balazs, 1988; Didier et al., 1990), the lack of knowledge of the differentiation state of the neurons in the culture settings used in those studies did not allow a clear comparison with developing neurons *in vivo*.

Previous analyses of CGNs in culture demonstrated that only proliferating and post-mitotic immature neurons from the EGL survive the harvesting procedure, since cells having mature properties are not seen at plating or shortly thereafter (Raetzman and Siegel, 1999; Diaz et al., 2002). By combining morphological and molecular approaches to analyze CGN differentiation, we found that during the first week in culture, purified CGNs are indeed immature upon plating, and mature almost synchronously, transitioning through the same differentiation stages as *in vivo* over a 5 day culture period (Manzini et al., 2006).

Immature CGNs represent an ideal model to study pathological responses, because of the wealth of extant data on excitotoxic responses in mature CGNs (Contestabile, 2002). Here, for the first time, identified population of CGNs at different developmental stages were individually analyzed and compared, to establish whether and how immature and mature neurons differ in their molecular responses to excitotoxic challenge. In addition, CGN survival was tested in an acute cerebellar slice preparation in which CGNs at the different developmental stages identified in culture were analyzed together in a more intact setting to confirm the reduced sensitivity of immature CGNs to cell death in high levels of KA. In these systems, molecular and physiological responses of immature CGNs can be studied in detail and the mechanisms regulating cell death and survival identified.

Mechanisms of survival and cell death in immature and mature CGNs

We found that immature CGNs display lower surface expression levels of functional AMPA-Rs resulting in reduced sensitivity to excitotoxic challenge.

Developmental regulation of surface expression and trafficking of AMPA-R subunits can modulate receptor function (Dingledine et al., 1999; Zhu et al., 2000). Even if a large pool of AMPA-R proteins exists within the cell, as was found in this study, surface expression may be maintained at a minimum to protect immature CGNs from excessive activation in the developing brain. Multiple mechanisms determining differential surface expression of AMPA-Rs during neuronal development and plasticity have been identified, including 1) changes in expression of post-synaptic scaffolding proteins (Bredt and Nicoll, 2003; Malenka, 2003), 2) regulation of receptor trafficking via phosphorylation (Krapivinsky et al., 2004), and 3) expression of specific splice-variants of AMPA-R subunits with different retention times in the endoplasmic reticulum to regulate the speed of receptor insertion (Vandenberghe and Bredt, 2004). In order to maintain lower surface expression of AMPA-Rs and achieve lower AMPA-R activation levels, immature CGNs may express specific AMPA-R subunits with trafficking properties that are different than AMPA-R subunits in mature cells. In cortical and hippocampal pyramidal neuron survival, higher expression of Ca²⁺-permeable vs. Ca²⁺-impermeable AMPA-Rs increases sensitivity to excitotoxicity (Pellegrini-Giampietro et al., 1997; Sanchez et al., 2001; Kumar et al., 2002). In our experiments the GluR2 subunit, which confers Ca²⁺ impermeability to the receptor complex, was expressed at very low levels in immature CGNs. However, GluR2 expression increased during differentiation in parallel with GluR4,

suggesting that the ratio between Ca^{2+} -permeable and impermeable AMPA-Rs is not as critical for CGN survival as for cortical and hippocampal neurons. Alternatively, the subcellular distribution of AMPA-Rs may be important to regulate cellular responses, as shown for NMDA-Rs in hippocampal neurons where activation of extrasynaptic NMDA-Rs triggers apoptotic pathways, while activation of synaptic NMDA-Rs is anti-apoptotic (Hardingham et al., 2002).

Neuronal survival upon excitotoxicity is also controlled by the balance of pro-apoptotic and pro-survival signaling pathways (West et al., 2002; Hardingham and Bading, 2003). Reduced expression of caspases or increased activation of pro-survival pathways in immature CGNs could lead to reduced sensitivity to excitotoxicity. To test this hypothesis we explored the expression of caspase-3 and 9 and the expression and activation of pro-survival transcription factors, such as MEF2, CREB and NF- κ B following KA-treatment at different stages of CGN differentiation. We found no evidence consistent with a role for pro-apoptotic and pro-survival factors in selectively protecting immature cells from excessive excitation. Rather, immature CGNs could be induced to undergo caspase-mediated apoptosis when the amplitude of AMPA-R responses was pharmacologically increased by CTZ treatment to levels similar to those in mature CGNs. Our results suggest that the amplitude of AMPA-R responses is a principle determinant in the triggering of cell death.

It is nonetheless clear from our results that at the CGN developmental stages analyzed in this study, only a subset of CGNs in culture are affected by KA treatment, and only a subset of immature CGNs undergo apoptosis during the KA+CTZ treatment. This is not surprising. Even though only cells from the EGL survive dissociation and purification, and are the major component of the CGN cultures at 0-1 days in vitro, we have found that both the proliferating outer-EGL population and the post-mitotic inner EGL neurons are represented (Manzini et al., 2006). These cells differentiate in parallel, yielding at 2 div a combination of immature neurons in the post-mitotic premigratory and migratory stage (Manzini et al., 2006). It is plausible that only the most differentiated of these cells will become sensitive to KA+CTZ treatment, since voltage-clamp recordings show that CTZ can induce a subset of neurons to be activated at “mature” levels. Results in the slice setting, where EGL neurons are impervious to KA or KA+CTZ treatment, while more mature cells are increasingly affected as differentiation progresses, supports this hypothesis. In fact, recordings in a similar cerebellar slice system, detected no AMPA-R activation in the EGL and increasing AMPA-R current amplitudes as recordings were made in migratory CGNs into the IGL (Smith et al., 2000).

Sublethal activation of AMPA-Rs causes neuritic degeneration in immature CGNs

A central issue in understanding excitotoxic responses during development is the identification of morphological and functional changes following excessive excitation in the absence of cell death. Since learning and motor deficits are often a long-term outcome of neonatal brain injury (Rennie, 1997; Volpe, 2001; Ferriero, 2004), it is vital to define how excitotoxic compounds lead to these defects. AMPA/KA-R activation has been shown to regulate motility of filopodia in growing axons of hippocampal cultures (Chang and De Camilli, 2001), suggesting that AMPA-Rs are involved in controlling axonal extension. We found that activation of these axonal receptors during excitotoxic treatment interferes with normal axonal development leading to axonal beading and fragmentation. Interestingly, NMDA treatment has no effect on immature axons, indicating that AMPA-R activation is specifically involved in this phenomenon.

Process beading with no change in length indicates that neuritic degeneration but not retraction is taking place in immature CGNs within the time-frame of these experiments (Luo and O'Leary, 2005). We tested the involvement of the proteasome and activated caspases, which have been frequently implicated in neurite degeneration and remodeling (El-Khodori and Burke,

2002; Coleman, 2005; Verma et al., 2005). However, the proteasome inhibitors lactacystin and MG-132, or the caspase inhibitor DEVD-CHO, did not rescue axonal blebbing during KA treatment (M.C. Manzini and C.A. Mason, unpublished), suggesting that a different mechanism must be involved in immature CGNs. It is possible that bleb formation is localized at sites of Na^+ and Ca^{2+} influx, as shown during KA treatment in Purkinje cell axons in culture (Bindokas and Miller, 1995) and NMDA treatment in dendrites of hippocampal neurons (Ikegaya et al., 2001). Local entry of ions could be mediated through AMPA-Rs directly or through voltage sensitive Ca^{2+} channels activated following membrane depolarization.

The very long-term consequences of axonal degeneration on developing CGNs also must be determined, as it is unclear whether the loss of neuronal integrity corresponds to the initiation of a slow cell death program, or whether the neuron is able to recover from the injury and reextend its processes. Reorganization of afferent pathways, such as axonal sprouting in the hippocampal mossy fibers following KA injections, is often observed in models of epilepsy, (Represa and Ben-Ari, 1992; Buckmaster and Dudek, 1997), leading to changes in synapse number and efficacy (Villeneuve et al., 2000; Cossart et al., 2001; Sanchez and Jensen, 2001; Smith and Dudek, 2002). It is possible that if CGN axonal outgrowth is affected during cerebellar circuit formation *in vivo*, connections with the CGN targets, the Purkinje cells, could form abnormally, leading to motor and cognitive impairments, such as those seen in autism spectrum disorders (Folstein and Rosen-Sheidley, 2001).

Conclusions

Increasing evidence suggests that immature neurons differ from mature neurons in their reactions to pathological conditions (Rennie, 1997; Olney et al., 2004). To understand which cellular responses to trauma are different and which are similar, immature and mature neurons must be analyzed in comparison. In this model of developing neurons, CGN cultures at different stages of development can be used in parallel to study molecular and physiological responses to excitotoxic treatment. These data are an inroad to understand how the infant cerebellum is affected by excessive excitation. Future analyses of the regulation of AMPA-R targeting and of the mechanism of neurite degeneration following AMPA-R activation in immature CGNs will help define how developing cerebellar neurons respond to concentrations of excitatory neurotransmitters that are lethal to mature cells, and how excitotoxic insults can lead to neurodevelopmental disorders.

Experimental Methods

Animals

Experiments were carried out with C57BL/6J mice or transgenic mice expressing EGFP under a β -actin promoter (Okabe et al., 1997) on a C57BL/6J background. Animals were derived from a timed pregnancy breeding colony under our direction at Columbia University, with the day the pups were born designated as postnatal day (P)0.

Cerebellar granule neuron (CGN) purification

Methods for isolation and purification of murine CGNs were as previously described (Hatten et al., 1998). CGNs were purified from P5 mouse pups, resuspended in serum-free medium (SFM) and plated at a density of 2.5×10^5 cells/cm² on 16-well LabTek™ chamber slides (Nunc) coated with poly-L-ornithine (100 $\mu\text{g}/\text{ml}$; Sigma) and mouse laminin (20 $\mu\text{g}/\text{ml}$; Invitrogen). SFM was composed of Eagle's basal medium with Earle's salts (BME; Gibco) supplemented with insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), selenite (5 ng/ml), bovine serum albumin (10 mg/ml), all from Sigma, and glutamine (2 mM; Gibco), glucose (0.5%) and penicillin/streptomycin (20 U/ μl ; Gibco).

Parasagittal and frontal cerebellar slice preparation

P8-9 wild-type mice were decapitated and their brains rapidly removed and placed in ice-cold modified ACSF of composition (mM), NaCl (86), NaH₂PO₄ (1.2), KCl (2.5), NaHCO₃ (25), glucose (25), CaCl₂ (0.5), MgCl₂ (7) and sucrose (75). 150µm sections were cut with a Leica vibratome under cold modified ACSF, then transferred to a storage container filled with standard ACSF at 37 °C for 45-60 min. Composition of standard ACSF (mM), NaCl (124), NaH₂PO₄ (1.2), KCl (2.5), NaHCO₃ (25), glucose (20), CaCl₂ (2), MgCl₂ (1). For treatment, slices were incubated in 5% CO₂ at 37 °C for 6-7 hours on Millicell culture plate inserts (Millipore) in SFM containing the indicated concentrations of glutamate receptor agonists and antagonists.

Drug treatments and chemicals

Kainate (KA; Tocris) was diluted in SFM and added to the cells by completely substituting the culture medium at set time points. CGNs were usually treated for 16 hrs starting at 2 and 4 div unless otherwise stated and fixed with 4% paraformaldehyde at end of treatment. Additional drugs were used when indicated: NBQX (Tocris), APV (Tocris), NMDA (Sigma), glutamate (Sigma), SYM2081 (Tocris). To test for caspase-dependency of excitotoxic cell death, CGN cultures were pretreated for 1 hr with the membrane-permeable caspase inhibitor DEVD-CHO (50µM; Calbiochem), then cotreated with 100µM KA and 50µM DEVD-CHO for 16 hrs.

Cell death assays in culture

At the beginning of our study, the Live/Dead Reduced Biohazard Cell Viability Kit #1 from Invitrogen was used to differentiate live and dead CGNs according to instructions from the manufacturer. When compared with the Live/Dead kit, Hoechst 33258 (Invitrogen), a very common method used for visualization of pyknotic cells, was found to yield identical results (MC Manzini, unpublished data). All experiments in the current study were performed using Hoechst counterstaining after fixation to label chromatin and identify pyknotic cells. To analyze survival in mixed wild type-GFP CGN cultures, Cell Tracker Red CMTPX (Invitrogen) was used according to instructions from the manufacturer.

Cell death assays in slices

Live/Dead slice staining—To compare the amount of neuronal death and survival, a 10µM solution of the SYTO®10 green live dye in conjunction with 5µg/ml Propidium Iodide (both from Invitrogen) was added to living slices for 30 min at RT in the dark, before fixation with 4% paraformaldehyde.

TUNEL staining—In Situ Cell Death Detection Kit, Fluorescein (Roche) was used according to instructions from the manufacturer.

PI counterstain—After fixation, slices were immersed in a solution of 5µg/ml Propidium Iodide (Invitrogen) in PBS for 30 min at RT.

Antibodies for immunocytochemistry

Primary antibodies were: rabbit anti-caspase-3; rabbit anti-cleaved caspase-3; rabbit anti-caspase-9; rabbit anti-cleaved caspase-9, all from Cell Signaling Technology, and mouse IgG anti-cytochrome c (BD Biosciences), mouse IgG anti-calbindin (Sigma), rabbit anti-Zic2 (generous gift of Dr. Steve Brown, Columbia University, New York, NY). Secondary antibodies were conjugated to either Cy3 (Jackson ImmunoResearch) or Alexa488 (Invitrogen).

Microscopy and image analysis

All images, apart from those used for analysis of acute cerebellar slices, were collected at 63X on a Zeiss Axioplan 2 microscope via a black and white Axiocam digital camera (Zeiss) and Openlab acquisition software (Improvision Inc.). Images for analysis of acute cerebellar slices were collected at 40X on a Zeiss LSM 510 META scanning confocal microscope using Zeiss MetaView software.

Cell death analysis in culture—For an accurate and unbiased representation of cell distribution in the culture well, 9 independent fields were photographed at fixed positions. To assess survival, CGNs that were alive at the time of fixation, identified as Hoechst 33258-positive cells with diffuse chromatin and evident nucleoli, were counted. Results from the 9 fields were averaged for each independent culture well.

Cell death analysis in slices—Images were collected by confocal microscopy at a fixed distance from the upper surface of the slice in four fixed positions: sulcus between folium I and II, sulcus between folium V and VI, top of folium VI, sulcus between folium IX and X. All PI labeled CGNs were counted and Purkinje cells and Golgi cells, identified by their large nuclei, were excluded. Nuclei with diffuse chromatin staining were considered alive and pyknotic nuclei were counted as dead cells. As there was no variability in the amount of cell death at different locations in the slice, the four counts obtained for each slice were averaged.

Analyses of axonal morphology—The number of growth cones vs. axonal stumps following KA treatment was quantified, by choosing 9 fixed positions in the culture well, as described above, and assigning all axon endings visible in the field to one of two categories: growth cone or stump. Axon length was measured on the first ten GFP positive CGNs starting from the upper left-end side of the culture well, using Openlab image software (Improvision Inc.).

Whole-cell voltage clamp recordings

CGN cultures at 2 and 4 div were transferred to the stage of a Nikon microscope and bathed in an external solution containing (mM) NaCl (145), KCl (5), CaCl₂ (2), MgCl₂ (2), HEPES (10) and glucose (5.5), pH 7.3 and 325 mOsm. The pipette solution consisted in (mM) Cs-methanesulfonate (120), HEPES (10), EGTA (10), NaCl (10), CaCl₂ (1), QX-314 (5), MgATP (5), NaGTP (0.5); pH was adjusted to 7.1 with CsOH, and osmolarity adjusted to 310 mOsm with sucrose. Pipette resistances of 6 to 8 MΩ were used for all recordings. CGNs were held at -70 mV and currents evoked by 100μM KA were recorded in the voltage clamp mode with an Axopatch 200A amplifier (Axon Instruments). All KA-evoked current were recorded in the presence of 0.5 μM tetrodotoxin (TTX) to block any unclamped action potentials. Membrane currents were filtered at 2 KHz, and digitized at 2-5 kHz with pClamp 9-acquisition software (Axon Instruments). Drug solutions were applied to cells by local perfusion. The speed of drug application was measured at around 20-50 ms. KA-evoked currents were analyzed using Clampfit (Axon Instruments). Peak amplitudes were calculated as the difference between baselines measured before drug application and the peak current amplitude during the last second of the drug application. For calculations of percent potentiation by 25μM cyclothiazide (CTZ) (Tocris), end-of-drug amplitudes of KA-evoked currents in the presence of CTZ were compared with the control values and expressed as a percentage.

Western blotting

Protein lysates were collected from CGN cultures grown on glass coverslips at set time points in vitro in 0.5% SDS, 1% Triton X-100, 2mM DTT, 5mM EDTA and Complete Mini protein inhibitor cocktail (Roche) in PBS. For all biochemical studies cell density was the same as in

survival assays: 2.5×10^5 cells/cm². Western blots were probed with the following antibodies: rabbit anti-caspase-3 (Cell Signaling Technology), rabbit anti-caspase-9 (Cell Signaling Technology), mouse IgG anti-GluR2 (Chemicon), rabbit anti-GluR4 (Upstate), or mouse IgG anti- β III-tubulin (Sigma). Secondaries conjugated to infrared dyes (Rockland Immunochemicals) were detected using an Odyssey system (LI-COR Biosciences), which also performed densitometric measurements.

Surface biotinylation

CGN cultures were grown in 6-well culture vessels as at least 3×10^6 cells were necessary to obtain sufficient material for biotinylation. Cultures were incubated with 1 mM sulfo-NHS-LC-biotin (Pierce Biotechnology) in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ at pH 8.0 for 30 min on ice. After unbound biotin was quenched with a solution of TBS and 0.3% BSA at pH 7.2, cells were lysed as described above. Cell lysates were incubated with ImmunoPure immobilized-streptavidin agarose beads (Pierce Biotechnology) overnight at 4 °C. Unbound protein was removed from the beads with 4 washes in 0.2% Triton X-100 and 0.2% SDS in PBS and samples were processed for Western blotting analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants NS15961 and EY015290 (CAM) and NS029797 (ABM). We thank Chris Henderson, Carol Troy, Peter Scheiffele, Lorna Role and Lloyd Greene for helpful discussion and comments on the manuscript, David MacLeod and Molly Cahill for help with the experiments, and Hranush Melikyan and Rich Blazeski for invaluable technical assistance.

References

- Altman, J.; Bayer, SA. Development of the cerebellar system: In relation to its evolution, structure and functions. CRC Press; New York: 1997.
- Bindokas VP, Miller RJ. Excitotoxic degeneration is initiated at non-random sites in cultured rat cerebellar neurons. *J Neurosci* 1995;15:6999–7011. [PubMed: 7472456]
- Bredt DS, Nicoll RA. AMPA receptor trafficking at excitatory synapses. *Neuron* 2003;40:361–379. [PubMed: 14556714]
- Buckmaster PS, Dudek FE. Neuron loss, granule cell axon reorganization, and functional changes in the dentate gyrus of epileptic kainate-treated rats. *J Comp Neurol* 1997;385:385–404. [PubMed: 9300766]
- Chang S, De Camilli P. Glutamate regulates actin-based motility in axonal filopodia. *Nat Neurosci* 2001;4:787–793. [PubMed: 11477424]
- Choi DW, Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 1987;7:357–368. [PubMed: 2880937]
- Coleman M. Axon degeneration mechanisms: commonality amid diversity. *Nature Reviews Neuroscience* 2005;6:889–898.
- Contestabile A. Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. *Cerebellum* 2002;1:41–55. [PubMed: 12879973]
- Cossart R, Dinocourt C, Hirsch JC, Merchán-Pérez A, De Felipe J, Ben-Ari Y, Esclapez M, Bernard C. Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. *Nat Neurosci* 2001;4:52–62. [PubMed: 11135645]
- Diaz E, Ge Y, Yang YH, Loh KC, Serafini TA, Okazaki Y, Hayashizaki Y, Speed TP, Ngai J, Scheiffele P. Molecular analysis of gene expression in the developing pontocerebellar projection system. *Neuron* 2002;36:417–434. [PubMed: 12408845]

- Didier M, Heaulme M, Soubrie P, Bockaert J, Pin JP. Rapid, sensitive, and simple method for quantification of both neurotoxic and neurotrophic effects of NMDA on cultured cerebellar granule cells. *J Neurosci Res* 1990;27:25–35. [PubMed: 1979352]
- Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. *Pharmacol Rev* 1999;51:7–61. [PubMed: 10049997]
- El-Khodori BF, Burke RE. Medial forebrain bundle axotomy during development induces apoptosis in dopamine neurons of the substantia nigra and activation of caspases in their degenerating axons. *J Comp Neurol* 2002;452:65–79. [PubMed: 12205710]
- Ferriero DM. Neonatal brain injury. *N Engl J Med* 2004;351:1985–1995. [PubMed: 15525724]
- Folstein SE, Rosen-Sheidley B. Genetics of autism: complex aetiology for a heterogeneous disorder. *Nat Rev Genet* 2001;2:943–955. [PubMed: 11733747]
- Gallo V, Balazs R. Effect of depolarization on the maturation of cerebellar granule cells in culture. *Brain Res* 1988;448:46–52. [PubMed: 3390716]
- Garthwaite G, Garthwaite J. Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci Lett* 1986;66:193–198. [PubMed: 3014386]
- Garthwaite J, Wilkin GP. Kainic acid receptors and neurotoxicity in adult and immature rat cerebellar slices. *Neuroscience* 1982;7:2499–2514. [PubMed: 6294559]
- Grooms SY, Opitz T, Bennett MV, Zukin RS. Status epilepticus decreases glutamate receptor 2 mRNA and protein expression in hippocampal pyramidal cells before neuronal death. *Proc Natl Acad Sci U S A* 2000;97:3631–3636. [PubMed: 10725374]
- Hack N, Balazs R. Properties of AMPA receptors expressed in rat cerebellar granule cell cultures: Ca²⁺ + influx studies. *J Neurochem* 1995;65:1077–1084. [PubMed: 7543928]
- Hardingham GE, Fukunaga Y, Bading H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* 2002;5:405–414. [PubMed: 11953750]
- Hardingham GE, Bading H. The Yin and Yang of NMDA receptor signalling. *Trends Neurosci* 2003;26:81–89. [PubMed: 12536131]
- Hatten, ME.; Gao, W-Q.; Morrison, ME.; Mason, CA. The cerebellum: Purification and coculture of identified cell populations. In: Banker, G.; Goslin, K., editors. *Culturing Nerve Cells*. MIT Press; Cambridge, Mass.: 1998. p. 419-459.
- Holmes GL, Ben-Ari Y. Seizures in the developing brain: perhaps not so benign after all. *Neuron* 1998;21:1231–1234. [PubMed: 9883716]
- Holmes GL, Khazipov R, Ben-Ari Y. Seizure-induced damage in the developing human: relevance of experimental models. *Progr Brain Res* 2002;135:321–334.
- Huttenlocher PR, Hapke RJ. A follow-up study of intractable seizures in childhood. *Ann Neurol* 1990;28:699–705. [PubMed: 2260857]
- Ikegaya Y, Kim JA, Baba M, Iwatsubo T, Nishiyama N, Matsuki N. Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA receptor activation: implication for a cellular defense against excitotoxicity. *J Cell Sci* 2001;114:4083–4093. [PubMed: 11739640]
- Jensen FE, Wang C, Stafstrom CE, Liu Z, Geary C, Stevens MC. Acute and chronic increases in excitability in rat hippocampal slices after perinatal hypoxia *In vivo*. *J Neurophysiol* 1998;79:73–81. [PubMed: 9425178]
- Jensen FE. Acute and chronic effects of seizures in the developing brain: experimental models. *Epilepsia* 1999;40(Suppl 1):S51–58. [PubMed: 10421561] discussion S64-56.
- Krapivinsky G, Medina I, Krapivinsky L, Gapon S, Clapham DE. SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. *Neuron* 2004;43:563–574. [PubMed: 15312654]
- Kubova H, Haugvicova R, Suchomelova L, Mares P. Does status epilepticus influence the motor development of immature rats? *Epilepsia* 2000;41(Suppl 6):S64–69. [PubMed: 10999522]
- Kumar SS, Bacci A, Kharazia V, Huguenard JR. A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci* 2002;22:3005–3015. [PubMed: 11943803]

- Labat-Moleur F, Guillermet C, Lorimier P, Robert C, Lantuejoul S, Brambilla E, Negoescu A. TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement critical evaluation and improvement. *J Histochem Cytochem* 1998;46:327–334. [PubMed: 9487114]
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999;79:1431–1568. [PubMed: 10508238]
- Luo L, O'Leary DD. Axon retraction and degeneration in development and disease. *Annu Rev Neurosci* 2005;28:127–156. [PubMed: 16022592]
- Malenka RC. Synaptic plasticity and AMPA receptor trafficking. *Ann NY Acad Sci* 2003;1003:1–11. [PubMed: 14684431]
- Manzini MC, Ward MS, Zhang Q, Lieberman MD, Mason CA. The stop-signal revised: immature cerebellar granule neurons in the external germinal layer arrest pontine mossy fiber growth. *J Neurosci* 2006;26:6040–6051. [PubMed: 16738247]
- Marks N, Berg MJ, Guidotti A, Saito M. Activation of caspase-3 and apoptosis in cerebellar granule cells. *J Neurosci Res* 1998;52:334–341. [PubMed: 9590441]
- Mesples B, Plaisant F, Fontaine RH, Gressens P. Pathophysiology of neonatal brain lesions: lessons from animal models of excitotoxicity. *Acta Paediatrica* 2005;94:185–190. [PubMed: 15981752]
- Moody WJ, Bosma MM. Ion channel development, spontaneous activity, and activity-dependent development in nerve and muscle cells. *Physiol Rev* 2005;85:883–941. [PubMed: 15987798]
- Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MV. Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. *Proc Natl Acad Sci U S A* 2005;102:12230–12235. [PubMed: 16093311]
- Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Letters* 1997;407:313–319. [PubMed: 9175875]
- Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. Drug-induced apoptotic neurodegeneration in the developing brain. *Brain Pathology* 2002;12:488–498. [PubMed: 12408236]
- Olney JW, Young C, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C. Do pediatric drugs cause developing neurons to commit suicide? *Trends Pharmacol Sci* 2004;25:135–139. [PubMed: 15019268]
- Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS. The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. *Trends Neurosci* 1997;20:464–470. [PubMed: 9347614]
- Raetzman LT, Siegel RE. Immature granule neurons from cerebella of different ages exhibit distinct developmental potentials. *J Neurobiol* 1999;38:559–570. [PubMed: 10084689]
- Rennie JM. Neonatal seizures. *Br J Ob Gyn* 1997;104:1341–1350.
- Represa A, Ben-Ari Y. Kindling is associated with the formation of novel mossy fibre synapses in the CA3 region. *Exp Brain Res* 1992;92:69–78. [PubMed: 1486956]
- Sanchez RM, Jensen FE. Maturation aspects of epilepsy mechanisms and consequences for the immature brain. *Epilepsia* 2001;42:577–585. [PubMed: 11380563]
- Sanchez RM, Koh S, Rio C, Wang C, Lamperti ED, Sharma D, Corfas G, Jensen FE. Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced seizures. *J Neurosci* 2001;21:8154–8163. [PubMed: 11588188]
- Sattler R, Tymianski M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol* 2001;24:107–129. [PubMed: 11831548]
- Scholzke MN, Potrovita I, Subramaniam S, Prinz S, Schwaninger M. Glutamate activates NF-kappaB through calpain in neurons. *Eur J Neurosci* 2003;18:3305–3310. [PubMed: 14686903]
- Smith BN, Dudek FE. Network interactions mediated by new excitatory connections between CA1 pyramidal cells in rats with kainate-induced epilepsy. *J Neurophys* 2002;87:1655–1658.
- Smith TC, Wang LY, Howe JR. Heterogeneous conductance levels of native AMPA receptors. *J Neurosci* 2000;20:2073–2085. [PubMed: 10704481]
- Sperber EF, Moshe SL, Bennett MV, Zukin RS. The relationship between seizures and damage in the maturing brain. *Dev Neurosci* 1997;19:529–542. [PubMed: 9445090]
- Spitzer NC, Kingston PA, Manning TJ, Conklin MW. Outside and in: development of neuronal excitability. *Curr Opin Neurobiol* 2002;12:315–323. [PubMed: 12049939]

- Sun H, Hashino E, Ding DL, Salvi RJ. Reversible and irreversible damage to cochlear afferent neurons by kainic acid excitotoxicity. *J Comp Neurol* 2001;430:172–181. [PubMed: 11135254]
- Vandenberghe W, Bredt DS. Early events in glutamate receptor trafficking. *Curr Opin Cell Biol* 2004;16:134–139. [PubMed: 15196555]
- Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, Fawcett JW. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci* 2005;25:331–342. [PubMed: 15647476]
- Villeneuve N, Ben-Ari Y, Holmes GL, Gaiarsa JL. Neonatal seizures induced persistent changes in intrinsic properties of CA1 rat hippocampal cells. *Ann Neurol* 2000;47:729–738. [PubMed: 10852538]
- Volpe, JJ. *Neurology of the Newborn*. 4th. Edition. W.B. Saunders; Philadelphia: 2001.
- West AE, Griffith EC, Greenberg ME. Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci* 2002;3:921–931. [PubMed: 12461549]
- Zhu JJ, Esteban JA, Hayashi Y, Malinow R. Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat Neurosci* 2000;3:1098–1106. [PubMed: 11036266]

Figure 1. Immature CGNs survive treatments with concentrations of glutamate receptor agonists that are lethal to mature cells

CGNs purified from P5 mouse pups were maintained in culture for 2 or 4 days, then treated overnight (16 hrs) as indicated below and fixed. Survival was measured following Hoechst 33258 staining to visualize chromatin condensation: cells with nuclei presenting diffuse chromatin and evident nucleoli were counted as alive, while cells with pyknotic nuclei were counted as dead. (A) Increase in pyknotic cells (arrowheads) is only observed after 16-h 500 μ M Glu treatment of mature CGNs, which have differentiated in culture for 4 div (lower right-hand panel). Scale bar for A and D: 20 μ m. (B) Quantification of CGN survival after Glu treatment starting at 2 and 4 div. Average $\pm$ s.e.m. (C) Blockade of all subtypes of glutamate receptors rescues Glu-induced cell death. Specific antagonists were 100 μ M NBQX (NB) for AMPA/KA receptors, 100 μ M APV for NMDA receptors, 3 μ M SYM2081 (SYM) for KA receptors. (D-E) Increase in pyknotic/dead cells is also observed after 16-h 100 μ M KA treatment of mature CGNs starting at 4 div (lower right-hand panel). (F) Blockade of AMPA receptor produced the only significant rescue of KA-induced cell death. Cell death was not rescued by blocking other glutamate receptor subtypes or Na⁺ channels. (G-H) 100 μ M KA treatment in parasagittal cerebellar slices from P8 mouse pups affects migratory neurons and granule cells in the IGL, but not EGL cells. (G) CGN death is analyzed by PI counterstaining: pyknotic/dead CGNs are evident among the migratory cells and in the IGL, but not in the EGL (PI in red, CaBP to reveal Purkinje cells in blue). Scale bar: 100 μ m. Sensitivity of CGNs in

cerebellar slices to KA treatment is quantified in (H). The data are expressed as a ratio of pyknotic cells to total cells in each counted region. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Manzini et al, Figure 2

Figure 2. Both KA-evoked response amplitude and surface expression of AMPA receptors increase during CGN differentiation

(A) 100 μ M KA application generates larger response in mature (4 div) than in immature (2 div) CGNs in culture, as shown by the average increase in current amplitude recorded by whole-cell voltage-clamp. Representative currents are shown above the graph. (B) Total expression levels of the GluR2 and GluR4 AMPA receptor subunits in CGN cultures at 2 and 4 div. (C) Surface expression levels of GluR2 and GluR4, detected by surface biotinylation, increase with time in vitro. (+) biotinylated fraction; (-) no biotin controls; 'sup' cytosolic protein fraction.

Figure 3. Cell death following KA treatment in mature CGNs is mediated via caspase-3 activation (A) Protein expression levels of procaspase-3 are constant and procaspase-9 decreases during CGN differentiation in vitro. (B) Activated (cleaved) caspase-3 and caspase-9 (red) decorate pyknotic nuclei following KA treatment in mature CGNs. Live cells with normal nuclear morphology, express cytochrome c (green) in a punctate pattern in mitochondria, see inset. Hoechst counterstaining (blue) reveals nuclear morphology. Scale bar: 20 μ m. (C) KA-mediated cell death is rescued by treatment with the caspase inhibitor, DEVD-CHO, and NBQX.

Figure 4. KA treatment leads to cell death of immature CGNs when AMPA receptor activation levels are increased

(A) Coapplication of 100 μM KA and 25 μM CTZ increased KA-evoked response amplitude in 75% of CGNs at 2 div. Representative currents are shown on top and average increase in current amplitude is plotted in the graph on the lower left. Changes in current amplitude for individual cells are shown on the lower right. (B) CTZ treatment increases the effect of KA treatment inducing cell death in immature CGN cultures (i) and increasing cell death in migratory and IGL CGN populations in slices (ii). Death is completely rescued by addition of NBQX. Bi. Top: In cell cultures, pyknotic cells are revealed by Hoechst counterstaining (arrowheads in KA+CTZ box). Scale bar: 40 μm. Bottom: Quantification of survival. Bii. In slice preparations, EGL cells are not affected by KA or KA+CTZ treatment, while the CTZ-mediated enhancement of KA-induced cell death increases with differentiation. (C-D) Cell death induced by KA+CTZ treatment in immature CGNs is apoptotic. (C) Active caspase-3 is found in dying CGNs following KA+CTZ treatment at 2 div. Scale bar: 20 μm. (D) Apoptotic death is rescued by the caspase inhibitor, DEVD-CHO.

Manzini et al, Figure 5

Figure 5. Neurites of immature CGNs undergo degeneration during KA treatment

Few CGNs from EGFP transgenic mice are mixed to wild-type CGNs to clearly identify neuritic morphology. (A) AMPA receptors revealed by immunostaining for AMPA-R subunits, GluR2/3 (red) are visible in growth cone and filopodia of EGFP-positive CGNs (green). Scale bar: 5 μ m. (B) The distal portion of the axon of the majority of immature granule cells becomes beaded with a club-like tip after KA treatment. Scale bar: 10 μ m. Axonal degeneration is reduced to control levels when KA is applied with NBQX or in the presence of NMDA. (C) Granule neurons bearing degenerating axons (arrowhead) are alive as shown by uptake of the live dye CellTracker. Live cells display faint diffuse staining, while dye uptake in dead cells leads to a more intense signal (see cells indicated by the arrows in the inset). Scale bar: 20 μ m.