Gap Junctions Are Required for NMDA Receptor–Dependent Cell Death in Developing Neurons

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INTRODUCTION

In the developing nervous system, coupling of neurons by gap junctions is abundant and plays an important role in neurogenesis (Becker and Mobbs 1999), neuronal differentiation (Bani-Yaghoub et al. 1999), neuroblast migration (Lo Turco and Kriegstein 1991), synaptogenesis, and neural circuit formation (Kandler and Katz 1995; Peinado et al. 1993). Gap junction coupling decreases during later stages of development as chemical synapses mature (Arumugam et al. 2005; Connors et al. 1983; Kandler and Katz 1995) and the decrease is mediated by N-methyl-D-aspartate (NMDA) receptor activity through the Ca2+/cyclic adenosine monophosphate (cAMP) response element binding protein-dependent signaling pathway (Arumugam et al. 2005). In the mature nervous system, gap junction coupling increases during traumatic injury and ischemia (Chang et al. 2000; de Pina-Benabou et al. 2005; Frantseva et al. 2002a; Scemes and Spray 1998). The death of neurons also may occur during development, trauma, and ischemia (Buss and Oppenheim 2004; Choi 1996; Cole and Perez-Polo 2004), i.e., when gap junction coupling is high. However, whether gap junctions contribute to death of neural cells (neurons and glia) or are protective is still controversial (Perez Velazquez et al. 2003).

The NMDA receptor, a glutamate-gated ion channel, plays a critical role in neuronal development and has been extensively studied for its role in adult models of learning and memory (Contestabile 2000; Goodman and Shatz 1993; Nakazawa et al. 2004; Scheetz and Constantine-Paton 1994). In vivo and in vitro studies indicate that normal endogenous levels of NMDA receptor activity are required for neuronal survival, and too much or too little NMDA receptor function causes neuronal cell death (Adams et al. 2004; Arundine and Tymianski 2004; de Rivero Vaccari et al. 2006; Hardingham and Bading 2003; Waxman and Lynch 2005; Yoon et al. 2003). For example, the absence or blockade of NMDA receptors in vivo increases neuronal apoptosis in the developing brain during the peak of naturally occurring cell death and synaptogenesis (Adams et al. 2004; de Rivero Vaccari et al. 2006; Ikonomidou et al. 1999). On the other hand, in traumatic injuries and ischemia excessive amounts of glutamate are released, thus causing NMDA receptor hyperactivity and also neuronal cell death due to excitotoxicity (Arundine and Tymianski 2004; Biegon et al. 2004; Giza et al. 2006). Although some data regarding signaling mechanisms that underlie NMDA receptor–dependent regulation of cell death are available (Adams et al. 2004; Arundine and Tymianski 2004; de Rivero Vaccari et al. 2006; Waxman and Lynch 2005; Yoon et al. 2003), it is unknown whether gap junctions contribute to these mechanisms. Here we investigate the role of gap junctions in neuronal cell death that is caused by both NMDA receptor hypofunction and NMDA receptor hyperfunction in developing hypothalamic neurons. Our studies demonstrate that gap junctions, particularly those containing neuronal connexin 36 (Cx36), contribute to neuronal cell death caused by either increasing or decreasing NMDA receptor function.

METHODS

Animal care

The use of animal subjects in these experiments was approved by the Tulane University Animal Care and Use Committee. All experi-

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ments were conducted in accordance with guidelines issued by the US National Institutes of Health.

**Animals**

Sprague–Dawley rats and Cx36 knockout and wild-type mice were used in these experiments. The Cx36 knockout was originally created by Dr. David Paul (Harvard Medical School) and was donated to us by Dr. Marla Feller (University of California, San Diego). In the Cx36 knockout, the Cx36 coding sequence is replaced by a LacZ-IRES-PLAP reporter cassette (Deans et al. 2001); activation of the Cx36 promoter results in expression of the cytoplasmic protein β-galactosidase and not Cx36. Cx36 knockout and wild-type mice were kept as homozygous colonies. For mouse genotyping, tail samples were collected from each animal. Samples were lysed in buffer overnight at 55°C, then 100 μl of HPLC·H2O was added, and the material was boiled for 10 min. The extracted DNA was precipitated in 1 ml of isopropanol and resuspended in 150 μl of HPLC·H2O overnight at 65°C. PCR analysis was done using a set of primers for the Cx36 wild-type band (5′-AGG GGA GGG AGC AAA CGA GAAG-3′ and 5′-CTG CCG AAA TTT GGA ACA CTG AC-3′; 533 bp product) and the Cx36 knockout band (5′-TCC GCC TGT GGT GGA-3′ and 5′-CAG GTA GCC GGA TCA AGC GTA TG-3′; 360 bp product) to determine the genotype.

**Culture preparation**

Neuronal cultures were prepared as described (Belousov et al. 2001) from embryonic day 18–19 medial hypothalamus (obtained from rats or mice). Pregnant animals were anesthetized with Nembutal before embryos were removed. After disaggregation using papain, cells were resuspended in standard tissue culture medium, triturated to form a single cell suspension, and plated on glass coverslips (~250,000 of cells per coverslip). To ensure that the same number of cells was plated on each coverslip, the following procedures were done. First, the sample of cell suspension medium was diluted two times with Trypan Blue dye-exclusion medium. After such a treatment, live cells stay uncolored while the dead cells appear blue under the microscope. Second, the number of live cells was counted in five large squares in a hemacytometer (Hauser Scientific), in four corner squares and in the middle square. The number of cells was estimated using the following formula: the number of cells per coverslip (~250,000) = (total number of cells in 5 squares, ~1,250)/(chamber depth, 0.1 mm)/(the total surface area counted, 5 mm²) × (dilution factor, 2) × (volume of the cell suspension plated on a coverslip, 5 × 10⁻⁶ ml). Third, if the number of cells in suspension was too high or too low, the volume of the plated cells was adjusted. Cultures were raised in glutamate- and glutamine-free minimal essential medium (Invitrogen) with supplements (Belousov et al. 2001). Cytosine β-arabinofuranoside selectively inhibits DNA synthesis, but does not affect RNA synthesis (Parson et al. 1995). It does not kill dividing cells, but suppresses their proliferation. To suppress proliferation of glial cells, cytosine β-d-arabinofuranoside (1 μM) was added to the culture medium on day in vitro 1 (DIV1; in cultures treated/tested on DIV1–4 and in the corresponding controls) or DIV2 (in all other cultures).

**Pharmacological treatments**

Pharmacological treatments were done in sister cultures for 3, 13, 17, or 27 days as indicated herein. The following pharmacological agents were added to the culture medium (either alone or in combinations): dizocilpine maleate (MK-801, 20 μM), d,L-2-amino-5-phosphonovalerate (AP5, 100 μM), 6-cyano-7-nitroquinolinoxaline-2,3-dione (CNQX, 10 μM), glutamate (100 μM), NMDA (100 μM), bicuculline (Bic, 50 μM), picrotoxin (PiTX, 500 μM), carbenoxolone (CBX, 25 μM), and 18α-glycyrhrhetic acid (18-GA, 20 μM) (all obtained from Sigma-Aldrich). Some cultures were not treated and served as control. In all cultures, the culture medium was changed twice a week.

**Neuronal survival analysis**

Neuronal survival was analyzed at the end of the treatment period (e.g., on DIV17 for DIV14–17 treatment) using a toxicity assay as described (Belousov et al. 2001). In brief, cells on coverslips were washed in buffer and stained for 30 min with 1 μM calcein AM (Sigma–Aldrich); this staining labels only live cells. Coverslips were then washed and mounted on a glass slide. Photographs from 60 randomly chosen fields were taken in each coverslip using an Olympus BX51 microscope (×20 magnification), 490 nm (FITC) filter, and ORCA-285 digital camera (50 ms, camera exposure time). Because bleaching of calcein fluorescence was relatively fast (~15 s), this helped to prevent the double photographing of the same fields. The number of live neurons was counted in the obtained photographs using OpenLab software (Improvision). Neurons had the characteristic rounded cell body, small size (~20 μm), and multiple tiny processes and were easily distinguished from astrocytes that did not have a clear-cut cell body and processes, were spread, and had a bigger size (40–100 μm). Each test was done blindly on at least four coverslips from four independent cultures.

**Confocal microscopy and dye transfer**

DIV15 untreated (control) cultures were loaded for 30 min at 37°C with calcein AM (125 μM) in a standard perfusion solution containing: 137 mM NaCl, 25 mM glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, and 1 mM CaCl₂. The medium was aspirated, and the coverslips were washed in buffer and stained for 30 min with 1 μM calcein AM (Sigma–Aldrich) in a standard perfusion solution. Coverslips were washed in buffer and mounted on a glass slide. Photographs from 60 randomly chosen fields were taken in each coverslip using an Olympus BX51 microscope (×20 magnification), 490 nm (FITC) filter, and ORCA-285 digital camera (50 ms, camera exposure time). Because bleaching of calcein fluorescence was relatively fast (~15 s), this helped to prevent the double photographing of the same fields. The number of live neurons was counted in the obtained photographs using OpenLab software (Improvision). Neurons had the characteristic rounded cell body, small size (~20 μm), and multiple tiny processes and were easily distinguished from astrocytes that did not have a clear-cut cell body and processes, were spread, and had a bigger size (40–100 μm). Each test was done blindly on at least four coverslips from four independent cultures.

**FIG. 1.** Change in the number of neurons in rat hypothalamic cultures over time. A and B: live neurons that are fluorescently stained with calcein AM (green color, A) and the corresponding bright field image (B) are shown [images from day in vitro (DIV) 14 control cultures are demonstrated]. C: graph shows the number of live neurons per microscope field (p.m.f.) in control cultures over time. Data are presented as mean ± SE (n = 6 or more for each test). Statistical significance was estimated using one-way ANOVA with post hoc Tukey–Kramer.
mM MgCl₂, 3 mM CaCl₂, 1 μM glycine (aerated with 95% O₂-5% CO₂ at pH 7.4). The coverslip was then washed in a perfusion solution and held in a laminar-style chamber (Warner Instrument, Hamden, CT) that allows for rapid (5–10 s) and complete change in the medium. Cells were continuously perfused, using a flow pipe perfusion system (Belousov et al. 2001), initially with the perfusion solution and then for 25 min with solutions containing NMDA (100 μM) or AP5 (100 μM) (all solutions were aerated and applied at a constant perfusion rate of 1.5 ml/min at room temperature, 20–22°C). Laser scanning confocal microscopy was performed using a Nikon C1 Plus microscope, Nikon ×60 oil objective, and Nikon EZ-C1 software. Calcein fluorescence was visualized by excitation at 488 nm with the argon laser and emission fluorescence was collected at 505–525 nm. Images were taken before and 25 min after the beginning of drug application. Intensity of calcein fluorescence was expressed as absolute fluorescence intensity in arbitrary units (0–4,096); in different neurons the fluorescence intensity varied between 950 and 3,000. In each group, the analysis was done in six coverslips (in one microscope field per coverslip) obtained from three independent culture preparations. Each microscope field contained 17–22 neurons that were easily distinguished from astrocytes as described earlier. The fluorescence intensity was measured in a neuronal cell body. The concentration of calcein AM chosen for these experiments (125 μM) was in the same range as in the previous similar study in neuronal cultures (Thompson et al. 2006); in that study, 25 min also was more than sufficient to detect the ischemia-induced hemichannel opening.

Statistical analysis

Data were analyzed using ANOVA with post hoc Tukey test or paired Student’s t-test and InStat software. The data are presented as means ± SE. The numerical values are expressed according to this formula: percentage of live neurons per microscope field (p.m.f.) = (mean number of live neurons in 60 microscope fields in a coverslip) × 100/(mean number of live neurons in 60 fields in a coverslip with the control culture).

RESULTS

Neuronal death caused by NMDA receptor hypofunction in developing neurons

We studied the role of gap junctions in neuronal cell death caused by NMDA receptor hypofunction in rat hypothalamic neuronal cultures. To visualize living neurons we used calcein AM, the fluorescent dye that labels live cells (Fig. 1, A and B). In untreated (control) cultures, the number of live neurons p.m.f. decreased between DIV1 and DIV14, but it did not significantly change between DIV14 and DIV31 (Fig. 1 C). Previous experiments showed that, in these cultures, gap junction coupling is high on DIV15–19 and is absent or minimal on DIV1–4 and DIV28–31 as estimated by dye coupling (neurobiotin) and expression of the neuronal connexin, Cx36 (Aru mugam et al. 2005). A 3-day treatment with the NMDA receptor antagonists AP5 or MK-801 was performed in cultures on DIV1–4, DIV14–17, and DIV28–31. NMDA receptor blockade reduced the number of live neurons p.m.f. on DIV14–17 (Fig. 2 B), but did not affect neuronal survival on

FIG. 2. A 3-day blockade of gap junctions in rat hypothalamic cultures prevents neuronal cell death caused by inactivation of N-methyl-D-aspartate (NMDA) receptors. A and C: treating cultures with d,l-2-amino-5-phosphonovalerate (AP5) or dizocilpine maleate (MK-801) on DIV1–4 (A) or DIV28–31 (C) does not induce neuronal cell death. B: treating cultures with AP5, MK-801, or AP5/6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on DIV14–17 induces cell death, which is prevented by coinoculation with the gap junction blockers carbenoxolone (CBX) and 18β-glycyrrhetinic acid (18-GA). CNQX added on DIV14–17 does not affect neuronal survival. CBX and 18-GA applied alone do not affect neuronal survival under all conditions (A–C). Here and in Figs. 3–5: the data are presented as means ± SE (n = 4 or more for each test); statistical significance (one-way ANOVA with post hoc Tukey–Kramer) is shown relative to the control (*) or the primary treatment (#) [e.g., AP5, MK-801, and AP5/CNQX in Figs. 2, 3, 5; or glutamate, NMDA, and bicuculline/picrotoxin (Bic/PiTX) in Figs. 4 and 5].
either DIV1–4 or DIV28–31 (Fig. 2, A and C). The cell death caused by either AP5 or MK-801 was prevented by the gap junction blockers CBX and 18-GA (Fig. 2B). Adding AP5 plus CNQX (a non-NMDA glutamate receptor antagonist) to cultures yielded results similar to those obtained with AP5 or MK-801 (Fig. 2B). Treatment of neurons with CNQX, CBX, and 18-GA (alone or in combinations) did not affect neuronal survival (Fig. 2B). A more prolonged blockade of NMDA receptors on DIV4–17 or DIV14–31 did not affect neuronal survival (Fig. 3, A and B), although survival increased during NMDA receptor blockade on DIV4–31 (Fig. 3C). Further, during these prolonged treatments, the survival of neurons was not affected by CBX under any of the conditions tested (Fig. 3, A–C). The results indicate that gap junctions are not necessary for neuronal survival, that NMDA receptor hypofunction induces cell death during a specific critical period (in this case DIV14–17), and that this increase in cell death requires gap junctions.

Neuronal death caused by NMDA receptor hyperfunction in developing neurons

We also studied the role of gap junctions in neuronal cell death induced by glutamate and NMDA receptor hyperfunction. Rat hypothalamic neuronal cultures were treated with glutamate to activate all glutamate receptors, NMDA to activate NMDA receptors only, or a combination of Bic and PiTX to block γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors, thus to increase glutamate-mediated synaptic transmission and the activity of synaptic glutamate receptors (Hardingham and Badding 2002). None of these manipulations affected survival of the neurons when the drugs were present in the cultures on DIV1–4 (Fig. 4A). Administration of these drugs on DIV14–17, however, induced cell death that was prevented by the gap junction blockers CBX and 18-GA (Fig. 4B). When added to cultures on DIV28–31, glutamate induced cell death, but the effects of NMDA and Bic/PiTX were either undetectable (Bic/PiTX) or statistically insignificant (NMDA) (Fig. 4C). In contrast to the results obtained for DIV14–17, inactivation of gap junctions with CBX at DIV28–31 did not inhibit the excitotoxic effect of glutamate. As expected, the effects of glutamate, NMDA, and Bic/PiTX were also prevented by NMDA receptor antagonists alone (AP5 or MK-801) or in combination with a non-NMDA receptor antagonist (CNQX) (Fig. 4D). In addition, neither CBX nor 18-GA affected cell survival when added to cultures alone (Fig. 4, A–C). The results confirm that NMDA receptor hyperfunction is the critical element in glutamate-dependent excitotoxicity in developing neurons and indicate that NMDA receptor–mediated cell death requires gap junctions.

Role of Cx36 in NMDA receptor–dependent cell death

Cx36 is a gap junction protein that is neuron-specific and is essential for functional gap junction coupling between hypothalamic neurons (Belluardo et al. 2000; Long et al. 2005; Rash et al. 2000). We used hypothalamic cultures obtained from wild-type and Cx36 knockout mice to test whether elimination of the neuronal connexin affects cell death caused by decreased or increased NMDA receptor function. As in rat hypothalamic cultures (Figs. 2 and 4), DIV14–17 treatment of wild-type mouse hypothalamic cultures with AP5, NMDA, or Bic/PiTX all caused neuronal cell death that was prevented by the blockade of gap junctions with CBX (Fig. 5). Moreover,
neither NMDA receptor hypofunction nor NMDA receptor hyperfunction affected neuronal survival in cultures prepared using Cx36 knockout mice (Fig. 5). The results confirm a critical role for gap junctions, in particular Cx36-containing, in NMDA receptor–dependent regulation of survival in developing neurons.

**Are hemichannels involved?**

It has been reported that open hemichannels may play a role in mediating neuronal cell death. For example, ischemic-like conditions open pannexin hemichannels, and it has been suggested that this contributes to cell death during ischemia (Thompson et al. 2006). Moreover, connexin 32 hemichannels open in a Ca²⁺-dependent manner that results in release of ATP (De Vuyst et al. 2006). Although there is no evidence that mammalian Cx36 forms hemichannels, fish connexin 35 (Cx35), which is a member of the Cx35/Cx36 subgroup of connexins, does form hemichannels (Al-Ubaidi et al. 2000; Valiunas et al. 2004). We tested the possibility that hemichannels open during activation or inactivation of NMDA receptors in developing neurons, thus contributing to NMDA receptor–dependent regulation of neuronal cell death during development. We loaded DIV15 untreated (control) neurons with calcein AM, a fluorescent dye that is known to pass through hemichannels (Thompson et al. 2006), to measure calcein efflux from neurons. No change in calcein fluorescence was detected in neurons after administration of NMDA (Fig. 6, A, B, and E) or AP5 (Fig. 6, C–E). This suggests that neuronal hemichannels are unlikely to contribute to NMDA receptor–regulated cell death in developing hypothalamic neurons.

**DISCUSSION**

**Role of gap junctions in NMDA receptor–dependent cell death**

NMDA receptors play a critical role in regulating neuronal survival and either too much or too little of NMDA receptor activity leads to increased neuronal cell death: the neurodegeneration caused by NMDA receptor excitotoxicity is well documented (Arundine and Tymianski 2004; Waxman and Lynch 2005); the absence or inactivation of NMDA receptors also increases neuronal cell death in the developing brain (Adams et al. 2004; de Rivero Vaccari et al. 2006; Yoon et al. 2003). Here we address the role of gap junctions in the NMDA receptor–regulated cell death caused by either increased or decreased NMDA receptor function in developing hypothalamic neurons. We demonstrate that both inactivation and hyperactivation of NMDA receptors induce neuronal cell death in hypothalamic cultures and this occurs at the time when neuronal gap junction coupling is high (i.e., DIV14–17; Arumugam et al. 2005). The neuronal cell death caused by inactivation of NMDA receptors is completely prevented by pharmacological blockade of gap junctions. Additionally, such NMDA receptor hypofunction-mediated cell death does not

![FIG. 4. A 3-day blockade of gap junctions in rat hypothalamic cultures prevents neurodegeneration caused by hyperactivation of NMDA receptors. A: treating cultures with glutamate, NMDA, or Bic/PiTX on DIV1–4 does not induce neuronal cell death. B: treating cultures with these agents on DIV14–17 induces neurodegeneration, which is prevented by the gap junction blockers CBX and 18-GA. C: only application of glutamate induces neurodegeneration on DIV28–31 and this is not affected by CBX. CBX or 18-GA applied alone does not affect neuronal survival under all conditions (A–C). D: on DIV14–17, NMDA receptor antagonists prevent neuronal cell death caused by glutamate, NMDA, and Bic/PiTX. Sample size: n ≥ 4 for each test.](image-url)
occur at the time when gap junction coupling is absent or minimal (on DIV1–4 or DIV28–31; Arumugam et al. 2005). Thus the results suggest that gap junctions play a major role in neuronal cell death caused by inactivation of NMDA receptors.

Similarly, the neuronal death caused on DIV14–17 by hyperactivation of NMDA receptors is prevented by the pharmacological blockade of gap junctions. The NMDA receptor hyperactivity-induced neurotoxicity is not observed in cultures when gap junction coupling is absent or minimal (i.e., DIV1–4 and DIV28–31; Arumugam et al. 2005). Interestingly, similar effects are observed when synaptic glutamate receptors are hyperactivated during neuronal disinhibition with the GABA_A receptor antagonists: such a treatment causes neurodegeneration in cultures only at the time when gap junction coupling is high (DIV14–17) and the neurodegeneration is prevented by pharmacological gap junction coupling blockade. As for the neurodegenerative effect caused by the application of glutamate, it progresses over the age of cultures: it is not observed in young cultures (DIV1–4) and is detected after DIV14–17 and DIV28–31 treatments, although the effect is lower when gap junction coupling is also low (DIV28–31). Thus our results suggest that gap junctions play an important role in neuronal cell death caused by hyperactivation of synaptic glutamate receptors, and NMDA receptors specifically.

Cx36 is the major connexin in neurons, including the hypothalamic neurons (Belluardo et al. 2000; Long et al. 2005; Rash et al. 2000; Sohl et al. 1998). The expression of Cx36 in the CNS is developmentally regulated and correlates to the amount of neuronal gap junction coupling (Arumugam et al. 2005; Sohl et al. 1998). Cx36 expression and gap junction coupling increase in the rat hypothalamus during early postnatal development, peaking on postnatal day 15, and then decrease during later developmental stages (Arumugam et al. 2005; Gulisano et al. 2000). Similar changes are observed in hypothalamic cultures prepared from rats and wild-type mice (Arumugam et al. 2005). Neuronal gap junction coupling is absent in the hypothalamus of Cx36 knockout mice (Long et al. 2005). In our study, the prolonged blockade of NMDA receptors on DIV4– DIV31 does not induce neuronal cell death, but, instead, increases the neuronal survival (Fig. 3C). This suggests an adaptation of neurons to the chronic change in the level of NMDA receptor–dependent excitatory activity in a neuronal network. These results agree with the data reported by Obrietan and van den Pol (1995) showing that the chronic blockade of ionotopic glutamate receptors (with AP5 and CNQX) increases neuronal survival in hypothalamic cultures. Our results in hypothalamic cultures also correlate with the previous observations indicating that inactivation of NMDA receptors in neuronal cortical cultures induces cell death, but inactivation of non-NMDA receptors does not have an effect on neuronal survival (Yoon et al. 2003).

What is the nature of neurodegenerative signals?

Gap junctions are channels between cells that allow the passage of molecules and ions <1–1.5 kDa (Bennett and Zukin 2004; Simpson et al. 1977). If gap junctions contribute to the cell death caused by increased or decreased NMDA receptor function then the critical components that induce (or propagate) such cell death must be able to pass through the gap junction channels. It is of particular interest that cell death...
and 25 min after (\textit{signaling molecules} that are generated during the NMDA studies will be to identify the specific gap junction–permeable traumatic injury (Frantseva et al. 2002a,b). A focus of future during NMDA receptor–dependent excitotoxicity and serve as factors. In our experimental conditions gap junctions are critical for the induction of two different types of NMDA receptor–dependent neuronal cell death. Although gap junction coupling and the expression of Cx36 are high during early postnatal development and increase in the nervous system during ischemia and traumatic injury (Aru-mugam et al. 2005; de Pina-Benabou et al. 2005; Gulisano et al. 2000; Oguro et al. 2001), whether gap junctions contribute to neural cell death or are neuroprotective is controversial (Perez Velazquez et al. 2003). Some reports indicate that blocking gap junctions during development, ischemia, or traumatic brain injury rescues cells from apoptosis or necrosis, suggesting that gap junctions contribute to neural cell death (Cusato et al. 2003, 2006; de Pina-Benabou et al. 2005; Frantseva et al. 2002a,b; Nomine et al. 2005; Perez Velazquez et al. 2006; Rami et al. 2001; Rawanduzy et al. 1997; Udawatte and Rippes 2005); others demonstrate that gap junctions are neuroprotective (Blanc et al. 1998; Nakase et al. 2003; Oguro et al. 2001; Ozog et al. 2002; Siushansian et al. 2001; Striedinger et al. 2005). Apparently, gap junctions may contribute to both neurodegeneration and neuroprotection depending on the type of connexins, cells coupled, region of the nervous system, stage of development, type of injury, and other factors. In our experimental conditions gap junctions are critical for the induction of two different types of NMDA receptor–dependent neuronal cell death. Ischemic and NMDA mediated toxicity models in vivo show that the damage is greater in younger than that in adult brains (Ikonomidou et al. 1989). Farahani and colleagues (2005) suggested that the abundance of gap junction coupling in the immature brain is the reason that after hypoxic-ischemic attacks the damage progresses faster in immature than in mature brains. Our data in cultures correlate with this idea and suggest that there is a window of opportunity for NMDA receptor–dependent neurodegenerative signals to kill neurons, which is during the periods when gap junction coupling is high.

**Functional implications**

Although gap junction coupling and the expression of Cx36 are high during early postnatal development and increase in the nervous system during ischemia and traumatic injury (Aru-mugam et al. 2005; de Pina-Benabou et al. 2005; Gulisano et al. 2000; Oguro et al. 2001), whether gap junctions contribute to neural cell death or are neuroprotective is controversial (Perez Velazquez et al. 2003). Some reports indicate that blocking gap junctions during development, ischemia, or traumatic brain injury rescues cells from apoptosis or necrosis, suggesting that gap junctions contribute to neural cell death (Cusato et al. 2003, 2006; de Pina-Benabou et al. 2005; Frantseva et al. 2002a,b; Nomine et al. 2005; Perez Velazquez et al. 2006; Rami et al. 2001; Rawanduzy et al. 1997; Udawatte and Rippes 2005); others demonstrate that gap junctions are neuroprotective (Blanc et al. 1998; Nakase et al. 2003; Oguro et al. 2001; Ozog et al. 2002; Siushansian et al. 2001; Striedinger et al. 2005). Apparently, gap junctions may contribute to both neurodegeneration and neuroprotection depending on the type of connexins, cells coupled, region of the nervous system, stage of development, type of injury, and other factors. In our experimental conditions gap junctions are critical for the induction of two different types of NMDA receptor–dependent neuronal cell death. Ischemic and NMDA mediated toxicity models in vivo show that the damage is greater in younger than that in adult brains (Ikonomidou et al. 1989). Farahani and colleagues (2005) suggested that the abundance of gap junction coupling in the immature brain is the reason that after hypoxic-ischemic attacks the damage progresses faster in immature than in mature brains. Our data in cultures correlate with this idea and suggest that there is a window of opportunity for NMDA receptor–dependent neurodegenerative signals to kill neurons, which is during the periods when gap junction coupling is high.
In neuronal cultures prepared from embryonic day 18–19 rats, DIV 14–17 approximately correlates with the 1st to 2nd wk of postnatal development in vivo (given some delay in the development caused by the cell culture preparation). This time in vivo in the hypothalamus is the period of naturally occurring cell death (Davis et al. 1996; Yoshida et al. 2000) and when the gap junction coupling peaks (Arumugam et al. 2005). In vitro conditions, this also is the period when NMDA receptor hypoactivity and hyperactivity induce neuronal cell death (present results). We hypothesize that the contribution of gap junctions in the NMDA receptor–dependent cell death that is revealed in this study in cultures on DIV 14–17, may reflect two possibilities. The first possibility is that gap junctions and NMDA receptors work in concert to regulate survival versus cell death decisions during neuronal development and, as a result, they contribute to the regulation of the hypothalamic network development. If this is the case, then the “critical period” for NMDA receptor–dependent neurodegenerative signals to kill neurons exists during the short period of postnatal development—i.e., at the time when neuronal gap junction coupling is high. During this time, NMDA receptors regulate cell death by gap junctions, whether the cell death is induced by increased or decreased NMDA receptor function. As development proceeds to later stages, NMDA receptors down-regulate neuronal gap junctions (Arumugam et al. 2005). It is possible that the cooperation of NMDA receptors and gap junctions depends on cofactors that determine the timing and amount of gap junction up- and down-regulation. In the mature nervous system, traumatic injury and ischemia may reactivate neuronal susceptibility by increasing gap junction coupling to higher levels than normally found among mature neurons. The second possibility is that the presence of gap junctions simply provides the general channel for propagation of death/survival signals independently of the nature of these signals. In this case, the signals will be propagated at any time when the gap junction coupling is high. A better understanding of how gap junctions and NMDA receptors interact in the regulation of cell death/survival will allow for the development of treatments that safeguard neuronal survival following traumatic injuries, ischemic attacks, or exposure to teratogens that disrupt NMDA receptor function.

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