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Neuronal gap junction coupling as the primary determinant of the extent of glutamate-mediated excitotoxicity

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Abstract

In the mammalian central nervous system (CNS), coupling of neurons by gap junctions (electrical synapses) increases during early postnatal development, then decreases, but increases in the mature CNS following neuronal injury, such as ischemia, traumatic brain injury and epilepsy. Glutamate-dependent neuronal death also occurs in the CNS during development and neuronal injury, i.e., at the time when neuronal gap junction coupling is increased. Here, we review our recent studies on regulation of neuronal gap junction coupling by glutamate in developing and injured neurons and on the role of gap junctions in neuronal cell death. A modified model of the mechanisms of glutamate-dependent neuronal death is discussed, which includes neuronal gap junction coupling as a critical part of these mechanisms.

Keywords

Gap junctions; Connexin 36; Neuronal death; Glutamate; Excitotoxicity

Introduction

Gap junctions include intercellular channels and allow communication between neighboring cells via direct diffusion of ions and small molecules (Bennett and Zukin 2004). Six subunits of the integral membrane proteins known as connexins combine to form hemichannels, that, when joined between adjacent cells, form gap junction channels. Connexins are encoded by a family with 20 and 21 genes in the mouse and human genomes, respectively (Beyer and Berthoud 2009). Gap junctions are expressed by a variety of cell types, perhaps with exception of the blood cells. Aside from gap junctions, mammalian cells also may express unopposed connexin hemichannels (Giaume and Theis 2010; Saez et al. 2010) and unopposed channels consisting of pannexins (which are vertebrate homologues of

invertebrate gap junction proteins, innexins) (MacVicar and Thompson 2010; Saez et al. 2010; Sosinsky et al. 2011).

In the mammalian central nervous system (CNS), 11 connexins are expressed (Condorelli et al. 2003; Nagy et al. 2004; Sohl et al. 2005) and most of them are localized to glial cells (Rash et al. 2012; Giaume and Theis 2010). Connexin 36 (Cx36) is the major (though not exclusive) neuronal connexin (Belluardo et al. 2000; Venance et al. 2000; Rash et al. 2012). Cx36 knockout in mice results in almost complete (~95%) loss of neuronal gap junction coupling (De Zeeuw et al. 2003; Deans et al. 2001; Hormuzdi et al. 2001). During development of the mammalian CNS, the expression of Cx36 increases during the first two postnatal weeks with concomitant coupling of neurons by gap junctions (as documented for the hypothalamus, cortex, hippocampus and other regions) (Belluardo et al. 2000; Arumugam et al. 2005; Park et al. 2011). In some areas of the brain and in the spinal cord, however, the increase presumably occurs during embryonic development (Mentis et al. 2002; Pastor et al. 2003; Lee et al. 2010). It has been hypothesized that this developmental increase in the coupling contributes to a number of developmental events, including neuronal differentiation (Hartfield et al. 2011), synaptogenesis (Personius et al. 2007), cell migration (Cina et al. 2007; Elias et al. 2007) and neural circuit formation (Yuste et al. 1995; Dupont et al. 2006; Hanganu et al. 2009; Maher et al. 2009). This contribution presumably is via the passage of Ca^{2+} , IP_3 , cAMP and small molecules between the coupled cells and coordination of metabolic and transcriptional activities in developing neurons (Kandler and Katz 1998a). In addition, generation of synchronized, network-driven electrical activity (that is a critical feature in the developing brain) also depends upon the presence of gap junctions (Garaschuk et al. 2000; Ben-Ari 2001; Dupont et al. 2006).

During later stages of postnatal development, where chemical synaptic transmission increases (Kandler and Katz 1998b), the general level of neuronal gap junction coupling and Cx36 expression declines: usually during weeks 3–4; or during the first two postnatal weeks, e.g., in the spinal cord (Arumugam et al. 2005; Belluardo et al. 2000; Park et al. 2011). In addition, Cx36-containing gap junctions become restricted primarily to homogeneous neuronal subpopulations, e.g., to subtypes of GABAergic interneurons (Deans et al. 2001; Galarreta and Hestrin 2001; Hestrin and Galarreta 2005).

An important exception to this general decline in Cx36 expression is following neuronal injury, which results in a dramatic increase in both expression of Cx36 and formation of gap junctions. This occurs independently of the nature of the insult, having been observed following spinal cord and traumatic brain injury (TBI) (Chang et al. 2000; Frantseva et al. 2002; Ohsumi et al. 2006), ischemia (Oguro et al. 2001; de Pina-Benabou et al. 2005; Wang et al. 2012), inflammation (Garrett and Durham 2008), retinal injury (Striedinger et al. 2005) and epilepsy (Perez Velazquez et al. 1994; Gajda et al. 2003).

N-methyl-D-aspartate (NMDA) receptor (NMDAR) activity is a critical determinant of the programmed cell death that, in the developing CNS, contributes to the distribution of various cell classes, neuronal circuit formation as well as to the regulation of the overall number of neurons (Nijhawan et al. 2000). NMDAR activity above or below a specific level results in neuronal cell death (Johnston et al. 2002; Adams et al. 2004). Following injury in the mature

CNS, hyperactivity of glutamate receptors (mostly NMDARs) also plays a critical role in neuronal death (Choi 1988; Arundine and Tymianski 2004; Hazell 2007). However, whether during development, NMDAR excitotoxicity or neuronal injury, gap junctions play a role in neurodegeneration or neuroprotection is controversial as their contribution to both neural cell death and survival has been reported (reviewed in Perez Velazquez et al. 2003; Decrock et al. 2009). Further, until recently, the mechanisms controlling changes in neuronal gap junction coupling during development and neuronal injury were not understood. In this paper, we will review our recent studies that have addressed these critical issues. Particularly, we will discuss a modified model of the mechanisms of glutamate-mediated neuronal death, where neuronal gap junctions play critical role.

Regulation of neuronal gap junction coupling in the developing CNS

Our recent work established key aspects of the mechanisms underlying the developmental increases and decreases in the expression of Cx36 and neuronal gap junctional coupling. The coupling and Cx36 expression increase in the rodent hypothalamus and cortex between postnatal day 1 (P1)-P15, followed by a significant decrease between P15–30 (Arumugam et al. 2005; Park et al. 2011). These changes are consistent in timing with those that we observed for neuronal coupling and Cx36 expression in neuronal cultures (Arumugam et al. 2005; Park et al. 2011; Wang et al. 2012). The developmental increase in the coupling is regulated by the interplay between group II metabotropic glutamate receptors (mGluR) and GABA_A receptors (GABA_AR) (schematically illustrated in Figure 1a) (Park et al. 2011). This is supported by our observation that long-term (14 day) activation of group II mGluRs amplifies the normal developmental increase in neuronal gap junction coupling and Cx36 expression. A long-term blockade of group II mGluRs prevents the increase. Conversely, pharmacologic activation or inactivation of GABA_ARs have the opposite effects. However, activation or inactivation of other neurotransmitter receptors (acetylcholine, GABA_B and other glutamate receptors) do not affect the developmental increases in Cx36 or neuronal coupling.

Regulation of Cx36 and gap junctional coupling by group II mGluRs requires the cAMP/protein kinase A-dependent signaling pathway. The regulation by GABA_ARs is via depolarization of neurons (observed during development; Stein and Nicoll 2003) that results in Ca²⁺ influx via voltage-gated Ca²⁺ channels with subsequent activation of protein kinase C. The receptor-mediated up-regulation of Cx36 expression (that occurs in response to the group II mGluR activation or GABA_AR inactivation) is primarily transcriptional, requiring a neuron-restrictive silencer element in the Cx36 gene promoter. In contrast, the down-regulation of Cx36 (induced by inactivation of group II mGluRs or activation of GABA_ARs) is not associated with changes in mRNA levels. The down-regulation likely involves post-transcriptional mechanisms as depends upon sequences within the 3'-untranslated region of Cx36 mRNA (Park et al. 2011). Furthermore, the mechanisms responsible and the kinetics of alterations in Cx36 expression are dependent upon duration of the receptor activation. For example, acute (one hour) activation of group II mGluRs in developing (day *in vitro*; DIV3) cortical neurons results in only a transient increase in Cx36 protein expression (peaking at three hours post stimulation) without any change in mRNA levels (Song et al. 2012). Presumably, this initial post-transcriptional mechanism in response to short-term activation

of group II mGluRs in young neurons converts to adaptive changes in Cx36 gene transcription, if the increased level of receptor activity sustains (Song et al. 2012; Park et al. 2011).

We also characterized mechanisms responsible for the uncoupling of neuronal gap junctions that occurs during development. Glutamate (Figure 1c) via its activation of NMDARs and subsequent CREB (Ca^{2+} /cAMP response element binding protein)-dependent down-regulation of Cx36, is a key determinant of developmental uncoupling in the rat hypothalamus (Arumugam et al. 2005). The blockade of NMDARs or inhibition CREB prevents Cx36 down-regulation (and, therefore, uncoupling) during development. In contrast, CREB overexpression accelerates these processes (Arumugam et al. 2005). A previous study (Mentis et al. 2002), that identified a role for NMDARs in the developmental uncoupling of motoneurons in the rat spinal cord, is consistent with our findings.

Our results lead to the formulation of a model outlining the mechanisms responsible for regulation of neuronal gap junction coupling during development (Park et al. 2011). We believe that in early postnatal development, GABA_AR-dependent excitation maintains minimal Cx36 expression in neurons, an effect dependent on Ca^{2+} /protein kinase C-dependent signaling and the 3'-UTR of Cx36 mRNA. With the transition of GABA_AR responses from excitatory to inhibitory, in conjunction with the increased activity of group II mGluRs, Cx36 expression increases; this is a transcriptional effect mapped to the neuron-restrictive silencer element in the Cx36 gene. As a result, neuronal gap junction coupling also increases (Figure 1a). Subsequently, the developmental increase in NMDARs activity leads to down-regulation of Cx36 expression (via Ca^{2+} -dependent signaling, including CREB) and associated neuronal uncoupling (Figure 1c). It is likely that these mechanisms are universal in the CNS given that they were observed operating in different brain regions (hypothalamus, cortex and spinal cord) and species (rats and mice). However, additional, region-specific factors and/or neurotransmitter systems presumably may contribute to developmental regulation of Cx36 gap junctions, e.g., to acute regulation (reviewed in Belousov and Fontes 2013).

Role of gap junctions in glutamate-dependent neuronal death in the developing CNS

One of the striking features of neuronal gap junctions we have explored is their role in death and survival of developing neurons (Figure 1b) (de Rivero Vaccari et al. 2007). In rat and mouse hypothalamic neuronal cultures, inactivation or hyperactivation of NMDARs for three days both induced neuronal death. This occurred specifically during the peak of developmental gap junction coupling (i.e., on DIV14–17). Consistent with this finding, increasing or blocking NMDAR activity at a later (DIV28–31) or earlier (DIV1–4) time point, when neuronal gap junction coupling is low, had no or greatly reduced impact on cell survival. Pharmacological inactivation of gap junctions and genetic knockout of Cx36 prevented neuronal death caused by NMDAR hypofunction or hyperfunction in DIV14–17 cultures. These data support the conclusion that during development, Cx36-containing gap junctions are a major determinant of neuronal death resulting from increased or decreased NMDAR function (de Rivero Vaccari et al. 2007).

As discussed above, we observed that chronic (two week) activation of group II mGluR in developing neurons augments the developmental increase in neuronal gap junction coupling (Park et al. 2011). Importantly, we found that this group II mGluR receptor activation also results in the increased susceptibility of neurons to NMDAR-mediated excitotoxicity (Park et al. 2011). Moreover, chronic blockade of group II mGluRs prevents the developmental increase in neuronal gap junction coupling. In addition, it also prevents NMDAR-mediated death in developing neurons. We interpreted these observations as that the mechanisms underlying developmental changes in neuronal gap junction coupling (Figure 1a) also influence death and survival in developing neurons (Figure 1b) and, via this influence, they regulate formation of neuronal circuits (Park et al. 2011).

Regulation of neuronal gap junction coupling during neuronal injury

Our recent work also involved exploring the regulation of neuronal gap junction coupling following neuronal injury (Wang et al. 2012). We used photothrombotic focal cerebral ischemia in adult mice *in vivo* and oxygen-glucose deprivation (OGD) in mouse mature cortical neuronal cultures as an *in vitro* ischemic model. In addition, three other *in vitro* injury models in mouse mature cortical cultures were used: hyposmotic shock as a model of cytotoxic and osmotic edemas that occur during stroke and TBI (Unterberg et al. 2004); hydrostatic pressure injury that represents mechanical aspects of TBI (Morrison et al. 1998); and administration of 4-aminopyridine to cultures as a model of epileptic seizures (Wong and Yamada 2001). In these models, neuronal gap junction coupling and/or Cx36 expression were studied and showed a significant increase 2 hours post-injury (schematically illustrated in Figure 1d) (Wang et al. 2012).

As stated earlier, activation of group II mGluRs leads to increased neuronal gap junction coupling during development (Park et al. 2011). Since neuronal injury results in extensive and rapid release of glutamate from cells (Lobner and Choi 1994; De Cristobal et al. 2001; Guyot et al. 2001), we tested whether an activation of group II mGluRs by glutamate also produces up-regulation of neuronal gap junction coupling following injury. Indeed, blockade of group II mGluRs prevented any injury-mediated increases in neuronal gap junction coupling and expression of Cx36. Consistent with this, activation of group II mGluRs rapidly (within 1–3 hrs) increased the coupling and Cx36 expression in mature neuronal cultures and in the mouse cortex *in vivo*. The mechanism responsible for this up-regulation is through post-transcriptional control of Cx36 expression and is specific for neuronal (Cx36-containing), but not astrocytic (connexin 43-containing) gap junctions. Pharmacological experiments demonstrated that other neurotransmitter receptors were not involved directly in these regulatory mechanisms, including NMDARs, AMPA receptors, group I mGluRs, group III mGluRs, GABA_ARs or GABA_B receptors.

Role of gap junctions in cell death following neuronal injury

In experiments described in the previous section, regarding the group II mGluR-dependent regulation of Cx36 expression and gap junction coupling following injury, perhaps the most significant observation was that blockade of group II mGluRs, that prevents the injury-mediated increase in neuronal gap junction coupling, also dramatically reduces injury-

mediated neuronal death. Additionally, genetic knockout of Cx36 and/or pharmacological blockade of gap junctions also showed substantial neuroprotection following injury. This observation was independent of the model of neuronal injury used, since the dependence of cell death on the activity of group II mGluRs and the expression of Cx36 was seen in all five injury models outlined above (Wang et al. 2012). Essentially similar results were obtained using an *in vivo* model of TBI in mice, utilizing controlled cortical impact (Belousov et al. 2012). We concluded that neuronal gap junction coupling plays critical role in the injury-mediated neuronal death. We also suggested that group II mGluRs not only control the injury-mediated increase in neuronal gap junction coupling (Figure 1d), but via this regulation, they also control the death/survival mechanisms in injured neurons (Figure 1e) (Wang et al. 2012; Belousov et al. 2012).

The central role of neuronal gap junctions in neuronal death extends to a model of NMDAR-mediated excitotoxicity in adult mice *in vivo* (Wang et al. 2010). A single intraperitoneal administration of NMDA to wild-type (WT) mice induced 24 hrs later a substantial neuronal death in three regions of the forebrain: hippocampus (particularly, rostral dentate gyrus), hypothalamus and medial habenula (Figure 2a,b). This neuronal death was dramatically reduced by co-administration of mefloquine, a relatively selective blocker for Cx36-containing gap junctions, and was statistically insignificant in Cx36 knockout mice (Figure 2c,d). The expression of NR1 subunit of the NMDAR and the amplitude of NMDAR-mediated neuronal Ca^{2+} responses were not lower (but even higher) in Cx36 knockout than in WT mice, suggesting that the reduced level of NMDAR-mediated neuronal death in Cx36 knockout animals is not due to the reduced expression or activity of NMDARs. In addition, NMDA permeability of the blood-brain barrier (BBB) in the whole brain was not different between WT and Cx36 knockout mice, suggesting that the reduced neuronal death in Cx36 knockout mice is not due to the reduced permeability of the BBB to NMDA. Finally, mefloquine did not have any additional neuroprotective effects in Cx36 knockout mice (Figure 2e), indicating that the neuroprotective mechanism of mefloquine likely is based upon blockade of Cx36-containing neuronal gap junctions.

An interesting aspect in that study (Wang et al. 2010) was, as indicated above, that the NMDAR-mediated neuronal death was found in the forebrain only in three regions (Wang et al. 2010). This was observed in both WT (substantial neuronal death) and Cx36 knockout mice (statistically non-significant, but still detectable neuronal death). Although, permeability of the BBB in the whole brain to NMDA was not different between WT and Cx36 knockout mice, we believe that the only logical explanation for neuronal death only in three forebrain regions is that, somehow, permeability of the BBB to NMDA in those three regions is higher than in the rest of the forebrain.

Together, these data supported a critical role for neuronal gap junctions in neuronal death following injury. They also suggested that the coupling of neurons by gap junctions is required for NMDAR-mediated excitotoxicity in the mature CNS (Wang et al. 2010).

Modified model for mechanisms of glutamate-dependent excitotoxicity

Following many types of brain injury, the resulting brain damage has a core area where the loss of neurons is essentially complete, e.g., the area subject to direct physical impact in TBI or to anoxia in ischemic stroke. The surrounding neural tissue suffers from the secondary effects characterized by apoptosis (Guyot et al. 2001; Stoffel et al. 2002). A critical determinant of cell death in the penumbra is thought to be excessive release of glutamate from injured cells and the attendant glutamate-dependent excitotoxicity of “bystander” cells (Arundine and Tymianski 2004; Choi 1988; Hazell 2007). The excitotoxic mechanisms of glutamate include a hyperactivation of glutamate receptors (mainly NMDARs), massive influx of Ca^{2+} ions, overactivation of Ca^{2+} -dependent signaling pathways and, ultimately, neuronal death (Arundine and Tymianski 2004; Choi 1988; Hazell 2007) (Figure 3a). Thus, the use of NMDAR antagonists appears to be sound strategy to minimize neuronal loss following injury. However, clinical trials for the majority of NMDAR antagonists showed significant limitations of this approach and did not result in development of the effective neuroprotective agent (Ikonomidou and Turski 2002).

The results of our studies allowed us to refine the model of glutamate-dependent excitotoxicity, centered on Cx36-containing gap junctions, which offers an alternative strategy to minimizing cell death following injury (Figure 3b) (Wang et al. 2012; Belousov 2012; Belousov and Fontes 2013). We propose that, during neuronal injury, the primary determinant of glutamate-dependent neuronal death is not the hyperactivation of NMDARs *per se*, but rather the presence of neuronal gap junctions. Although all CNS neurons express NMDARs (as all of them respond to NMDA; Belousov et al. 2001) and NMDAR hyperactivity likely triggers neuronal death, in the absence of neuronal gap junctions this death is limited to a relatively small group of neurons, which for various reasons may be especially sensitive to excitotoxicity (as we discussed in de Rivero Vaccari et al. 2007) (Figure 3b{i}, which is the same as 3a{i}). However, in the presence of neuronal gap junctions, the amount of NMDAR-mediated neuronal death is greatly multiplied and death also occurs in nearly all (or all) coupled neurons (Figures 3b{ii} and 3b{iii}).

The extent of coupling is, therefore, the major determinant of the severity of overall damage caused by the initiating insult. Thus, the background level of neuronal gap junction coupling, normally found in many mature brain regions (Bennett and Zukin 2004), is critical for NMDAR-mediated excitotoxicity to occur (Figure 3b{ii}). In addition, injury-mediated release of glutamate rapidly induces, via activation of group II mGluRs, new neuronal gap junctions and these new gap junctions enhance the amount of neuronal death (Figure 3b{iii}).

A critical implication of this model is that an intervention, following the initiating event (e.g., TBI or ischemic stroke), in the form of neuronal gap junction blockade, may limit the extent of neuronal death and reduce the functional consequences (Figure 3c{iv}). Further, the induction of Cx36 by group II mGluR occurs within 2–3 hours of the initial injury (Wang et al. 2012). It is during this critical time window, where therapeutic intervention would be most effective in limiting the extent of injury-mediated cell death and functional deficits. Accordingly, inactivation of group II mGluRs also should be considered as an

approach aimed in reduction of the extent of neuronal loss. We are aware of the presence of information that agonists of group II mGluRs can also be neuroprotective via their action on presynaptic receptors, an activation of which decreases synaptic glutamate release (Beraudi et al. 2007; Yoshioka et al. 2009). However, as we demonstrated (Wang et al. 2012), the injury-mediated up-regulation of Cx36 and associated gap junction coupling presumably occurs due to activation of postsynaptic group II mGluRs. Therefore, a detailed investigation of neuroprotective properties of both agonists and antagonists of group II mGluRs still is needed.

A very important question is on the mechanisms by which neuronal gap junctions contribute to cell death. One possibility is through propagation of gap junction-permeable neurodegenerative signals between the coupled neurons (Figure 3b{ii}). Our most recent results indicate that the neuronal death by Cx36 indeed is dependent solely upon the channel properties of the gap junctions (unpublished). The identity of the death signals passing through gap junctions have not been uncovered, but potential candidates may include NMDAR-, AMPA receptor-, kainate receptor-, inflammation- and apoptosis-dependent intracellular signals such as IP₃, Ca²⁺ and reactive oxygen and nitrogen species (Cusato et al. 2006; Decrock et al. 2009; Decrock et al. 2013). Identification of such gap junction-permeable death signals may offer additional ideas for development of new neuroprotective agents.

Other implications of the proposed model

Our model provides insight into the observation that the developing brain has greater susceptibility to NMDAR-mediated excitotoxicity than the adult brain. Administration of NMDA induces significant neuronal death in acute hippocampal slices from juvenile (1–3 week-old) rats, but not adult (3 month-old) rats in contrast to OGD which causes neuronal loss in slices from both age groups (Zhou and Baudry 2006). It was proposed that age-dependent changes in NMDAR subunit composition might explain this phenomenon (Zhou and Baudry 2006). We propose an alternative explanation: the age-related difference in the amount of neuronal gap junction coupling (high in the juvenile brain and low in the adult brain) is the factor that leads to the differential cell death by NMDA; increased neuronal gap junction coupling, due to OGD-induced glutamate release and activation of group II mGluRs, likely contributes to the ischemic neuronal death in slices from adult animals. The question is still open on why the neurons demonstrate different vulnerability following NMDAR overactivation, i.e., some of them are more susceptible to excitotoxicity than others (see the previous section). This selective sensitivity presumably is determined by the intrinsic vulnerabilities of neuronal subpopulations to stressors, as discussed previously in relation to neurodegenerative disorders (Saxena and Caroni 2011).

In the mature CNS, neuronal gap junctional coupling occurs primarily between neurons of the same type. For example, under non-pathological conditions in the brain of adult rodents, Cx36-containing gap junctions are restricted largely to subtypes of GABAergic interneurons, allowing formation of gap junction-coupled interneuronal networks (Deans et al. 2001; Galarreta and Hestrin 2001; Hestrin and Galarreta 2005). We predict that the networks of coupled interneurons would be particularly susceptible to the initial gap

junction-dependent cell death, e.g., if some of these interneurons die directly due to NMDAR excitotoxicity (shown in blue, Figure 3c{i–iv}) and then kill others via gap junction-dependent mechanisms (shown in red, Figure 3c{ii–iii}). Successive waves of gap junction-dependent cell death are supported by newly synthesized Cx36-containing gap junctions and could potentially affect both interneurons and other neuronal types (shown in green, Figure 3c{iii}). This potentially may include pyramidal neurons, that normally do not express Cx36 gap junctions (Connors and Long 2004), but demonstrate the increase in gap junction coupling rapidly (within 1–2 hrs) following neuronal injury (Frantseva et al. 2002).

Multiple lines of evidence also indicate participation of hemichannels, non-Cx36-containing gap junctions, and pannexin-containing channels in injury-induced neuronal death (Bargiotas et al. 2011; Davidson et al. 2012; Frantseva et al. 2002). However, given the substantial neuroprotection provided by pharmacological blockade and knockout of Cx36 gap junctions (Figure 3c{iv}), or by inactivation of the mechanisms controlling the injury-dependent increase in the coupling (de Rivero Vaccari et al. 2007; Wang et al. 2010; Wang et al. 2012; Belousov et al. 2012), Cx36-containing gap junctions seem to be a primary determinant of the extent of injury-mediated neuronal death.

Are gap junctions pro-death or pro-survival?

It must be noted, that the contribution of gap junctions (whether neuronal or non-neuronal) to cell death still remains somewhat controversial, given that reports of gap junctions contributing to both cell death and to survival exist. For example, specifically for neuronal gap junctions, it has been reported that genetic Cx36 knockout and blockade of gap junctions enhance injury- and glutamate-induced neuronal death (Ozog et al. 2002; Striedinger et al. 2005). However, as indicated above, under our experimental conditions with multiple *in vivo* and *in vitro* neuronal injury models and NMDAR-mediated excitotoxicity, we unvaryingly observed a neuroprotective effect following blockade of neuronal gap junctions (de Rivero Vaccari et al. 2007; Wang et al. 2010; Park et al. 2011; Wang et al. 2012).

Possible reasons for these disparate results have been discussed (Perez Velazquez et al. 2003; Decrock et al. 2009; Belousov and Fontes 2013). These reasons include the use of various cell death models and cell types, poor specificity of gap junction blockers, as well as the presence of connexin channels/hemichannels and pannexin channels. A more nuanced model (Belousov and Fontes 2013), that takes into account the apparently conflicting reports of the pro-death and pro-survival roles of gap junction coupling, is that the magnitude of injury is the key determinant of the pathologic outcomes of gap junctions. That is, whether an increase in coupling is beneficial or detrimental to the organism's survival depends on the severity of neuronal injury. At low, sub-lethal levels of injury, the coupling may support neuronal survival by promoting the spread of pro-survival signals. However, with severe neuronal injury, gap junctions mediate the spread of pro-death signals that results in widespread neuronal demise. Therefore, the contribution of Cx36 gap junctions to cell death, documented in multiple models of brain injury, may simply be an unfortunate by-product of a generally pro-survival mechanism that evolved to cope with moderate injury. Experiments are underway to test whether this is the case.

Conclusion

In conclusion, our studies suggest the new mechanism for glutamate-mediated excitotoxicity beyond ionotropic glutamate receptors. They show that neuronal gap junctions are the primary determinants of the extent of glutamate excitotoxicity and play critical role in cell death following neuronal injury. The studies identify group II mGluRs as regulators of the injury-mediated increase in neuronal gap junction coupling. They also suggest that the blockade of neuronal gap junctions and group II mGluRs should be considered as potential therapeutic approaches for neuroprotection following neuronal injury.

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Figure 1. Neuronal gap junction coupling in the CNS

This figure schematically illustrates the summary of conclusions from our recent studies on the role of neurotransmitter receptors in changes in neuronal gap junction coupling (**a, c, d**) and the role of neuronal gap junctions in neuronal death (**b, e**) during development (**a–c**) and neuronal injury (**d, e**) (see text for details, abbreviations and references). Activation of group II mGluRs increases neuronal gap junction coupling and Cx36 expression during both development and neuronal injury (**a, d**: red arrows upward); however, note the difference in duration of developmental (i.e., 2 weeks) and injury-mediated (i.e., 2 hours) increases in the coupling. Activation of GABA_ARs counteracts to the developmental increase in coupling (**a**: red arrow downward). Activation of NMDARs causes the developmental uncoupling of neuronal gap junctions and down-regulation of Cx36 (**c**: red arrow downward). GJ, gap junctions; P1 and P30, postnatal days 1 and 30. This figure is adapted with permission from (Belousov 2012).

Figure 2. Inactivation of neuronal gap junctions prevents NMDAR-mediated neuronal death
Data for the hippocampus are illustrated. (a–d) Shown in these pictures are representative images of Fluoro-Jade B staining (that detects neuronal death) in brain sections from control wild-type mice (WT; a), mice that received a single intraperitoneal injection of NMDA (100 mg/kg; b), mice that received an injection of NMDA plus mefloquine (30 mg/kg; c) and NMDA-treated Cx36 knockout mice (Cx36 KO; d). Administration of NMDA induces neuronal death in the rostral dentate gyrus (b) that is prevented by co-treatment with mefloquine (c) or Cx36 knockout (d). (e) Graph presents statistical analysis of the number of Fluoro-Jade B-positive neurons in the hippocampus (ANOVA; n=4–7 mice per group; ***P<0.001; NS, non-significant; mean ± s.e.m.). The analysis was done 24 hrs after saline (control) or drug administrations. CA3, CA3 hippocampus; cc, corpus callosum; D3V, dorsal 3rd ventricle; GrDG, granule cell layer of dentate gyrus. This figure is adapted with permission from (Wang et al. 2010).

Figure 3. Glutamate-dependent excitotoxicity during neuronal injury

(a) Traditional model of the mechanisms for glutamate-dependent excitotoxicity (Choi 1988; Arundine and Tymianski 2004; Hazell 2007). (b) Alternative model of the mechanisms of glutamate-dependent excitotoxicity (Wang et al. 2012; Belousov 2012; Belousov and Fontes 2013). (c) This figure illustrates key points of the alternative model. In all figures: {i}, Neuronal death caused directly by overactivation of NMDARs; {ii}, Existing neuronal gap junctions (GJs) contribute substantially to neuronal death caused by overactivation of NMDARs (Wang et al. 2010; de Rivero Vaccari et al. 2007); {iii}, New neuronal gap junctions are induced by activation of group II mGluRs and also contribute to glutamate-dependent neuronal death (Wang et al. 2012); {iv}, Pharmacological (Wang et al. 2010) or genetic (Wang et al. 2010; Wang et al. 2012) blockade of neuronal gap junction coupling reduces glutamate-dependent neuronal death. In a, b: the sign (+) indicates the increase in receptor activity or expression of Cx36. In b: a possibility is illustrated that some Ca²⁺-dependent molecules serve as gap junction-permeable death signals (b{ii}) (Cusato et al. 2006; Decrock et al. 2009; Decrock et al. 2013); Ca²⁺ overload also directly induces neuronal death (b{i}), however, this is not the main driver of neuronal death, rather the key determinant for expansion of cell death is neuronal gap junction coupling (b{ii, iii}). This figure is adapted with permission from (Belousov and Fontes 2013).