PERSPECTIVE

Role of neuronal gap junctions in NMDA receptor-mediated excitotoxicity and ischemic neuronal death

In the mammalian central nervous system (CNS) coupling of neurons by gap junctions and the expression of neuronal gap junction protein, connexin 36 (Cx36) rapidly increases (usually during 1–2 hours) following a wide range of neuronal injuries, including ischemia, traumatic brain injury (TBI), spinal cord injury and epilepsy (reviewed in Belousov and Fontes, 2013). Pharmacological blockade or genetic elimination of Cx36-containing gap junctions dramatically reduce neuronal death in animal models of ischemia, TBI and epilepsy and prevent NMDA receptor (NMDAR)-mediated excitotoxicity (Belousov and Fontes, 2014). This suggests a critical role for neuronal gap junctions in the mechanisms of neuronal death. In our recent study, we addressed the following three questions (Fontes et al., 2015). Are changes in the expression of Cx36 directly affect neuronal death? Is the contribution of Cx36 to neuronal death depends on channel activity of gap junctional complexes among neurons or it is channel-independent? Do neuronal hemichannels contribute to neuronal death?

To address the first question, we produced the following lentiviral vectors to manipulate Cx36 expression in cultured neurons: pCDH-Cx36 (contains cDNA for induction of Cx36), pCDH-LUC (control; contains cDNA for *Cypridina* luciferase), two shRNA-Cx36 vectors (containing a small hairpin RNA [shRNA] targeting the Cx36 cDNA in positions at 611 [shRNA1] and 1270 [shRNA2]) and shRNA-LUC (control; containing shRNA against *Cypridina* luciferase). All lentiviruses also expressed green fluorescent protein (GFP). These viral vectors were characterized in neuronal cortical cultures prepared from wild-type (WT) mice, Cx36 knockout (Cx36-KO) mice and in HeLa cells. Immunostaining for a neuron-specific marker NeuN was conducted and showed the effective transduction of cultured neurons with each of the lentiviruses (> 90% of neurons were transduced). Methyl thiazolyl tetrazolium (MTT) assay was performed in neuronal cultures and showed that the transductions with any of the lentiviruses only slightly reduced neuronal survival over a 3 day period, and the effect is independent of the cDNA or siRNA expressed. Western blotting showed induction and down-regulation of Cx36, respectively, in Cx36-KO neurons transduced with pCDH-Cx36 and in WT neurons transduced with either shRNA-Cx36. In HeLa cells stably-transduced with pCDH-Cx36, immunostaining and scrape-loading dye transfer assay revealed that Cx36 is present at cell plasma membranes and forms channel-permeable gap junctions.

The lentiviruses were singly transduced into WT mouse purified neuronal cortical cultures that, 48 hours later, were subjected to administration of NMDA (10 μM for 30 minutes; a model of NMDAR excitotoxicity *in vitro*) or oxygen-glucose deprivation (OGD; for 30 minutes; a model of cortical ischemia *in vitro*). The analysis of neuronal death was conducted using MTT assay 24 hours post-insult and revealed that over-expression and knockdown of Cx36 augmented and prevented, respectively, neuronal death. This suggested that neuronal death following injury is directly influenced by the level of expression of Cx36 in neurons.

To address the second question, on whether the contribution of Cx36 to neuronal death depends on channel activity of gap junctional complexes among neurons or is channel independent, we produced a number of other lentiviral vectors. This included the lentiviruses expressing two non-neuronal connexins, pCDH-Cx43 and pCDH-Cx31 (containing cDNA for WT Cx43 or Cx31), in addition to the vectors inducing three mutant Cx36 and one mutant Cx43 proteins: pCDH-Cx36(AA), L10A/Q15A mutation; pCDH-Cx36(PP), L10P/Q15P mutation; pCDH-Cx36(I22R); and pCDH-Cx43(G21R). These lentiviruses also were characterized in

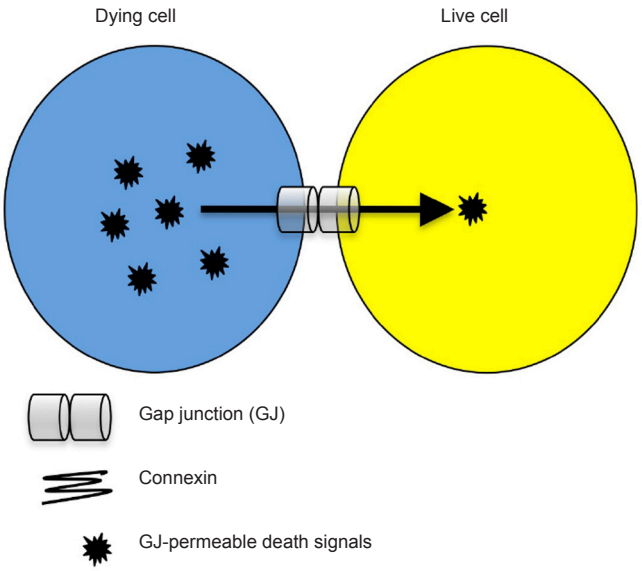

Figure 1 Schematic drawing on the contribution of gap junctions to cell death *via* channel dependent mechanisms.
The "bystander cell death" model is illustrated (Cusato et al., 2006; Decrock et al., 2009). According to this model, the contribution of neuronal gap junctions to cell death is through propagation of gap junction-permeable neurodegenerative signals between the coupled neurons. These may potentially include N-Methyl-D-aspartate (NMDA) receptor-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-, kainate receptor-, inflammation- and apoptosis-dependent signals such as inositol-1,4,5-trisphosphate (IP_3), reactive oxygen and nitrogen species and Ca^{2+}. The present study shows the important role for the main neuronal gap junction protein, connexin 36, in cell death that presumably involves the bystander cell death mechanism.

Cx36-KO neuronal cultures and stably-transduced HeLa cells. They efficiently transduced cultured neurons (> 90% transduction), did not affect dramatically neuronal survival, induced the corresponding proteins (either WT or mutant) at the cell plasma membrane and induced channel-permeable (containing WT Cx43 or Cx31) and channel-impermeable (containing either of four mutant connexins) gap junctions.

These lentiviruses and pCDH-Cx36 were singly transduced into Cx36-KO mouse neuronal cortical cultures. The analysis of neuronal death using MTT assay was conducted in these cultures 72 hours post-transduction, including 24 hours post-insult with NMDA or OGD. The data showed that neuronal expression of functional, channel-permeable gap junctions that contain Cx36 or either of two non-neuronal connexins (Cx43 or Cx31), resulted in increased neuronal death following insult. In contrast, the expression of dysfunctional, channel-impermeable gap junctions (containing any of the four mutated connexins) did not have this effect. These data suggested that the role of gap junctions in neuronal death is connexin type-independent. They also implied that a patent, conducting channel likely is necessary for neuronal gap junctions to induce cell death following injury.

Finally, to address the third question on whether neuronal hemichannels contribute to neuronal death, we analyzed ethidium bromide uptake in non-transduced WT mouse neuronal cortical cultures. The analysis was done two hours following induction of NMDAR excitotoxicity or ischemia, *i.e.*, at the time when expression of Cx36 is drastically increased following ischemic injury *in vivo* and *in vitro*, prior to the gradual decrease over next several hours (Wang et al., 2012). No neuronal dye uptake was detected in these cultures. This suggested the absence of active endogenous (either Cx36- or pannexin-containing) hemichannels in neurons two hours post-insult.

The simplest mechanistic explanation for the contribution of gap junction channels to cell death is that they allow propagation of pro-death signals between the coupled cells (**Figure 1**). Some of those death signals have been suggested based on studies with non-neuronal gap junctions. These include inositol-1,4,5-trisphosphate (IP3), reactive oxygen and nitrogen species and presumably Ca^{2+} (Cusato et al., 2006; Decrock et al., 2009). Such death signals have not been yet identified for neurons. Future studies will address this issue.

In conclusion, this study provided insight into the mechanisms of contribution of neuronal gap junctions in NMDAR-dependent excitotoxicity and ischemic neuronal death. Because clinical trials for NMDAR antagonists as neuroprotective agents largely failed (Ikonomidou and Turski, 2002), the study suggested that another important therapeutic target for the development of new neuroprotective agents can be neuronal gap junction coupling.

Addendum to: *Fontes, J.D., J. Ramsey, J.M. Polk, A. Koop, J.V. Denisova, and A.B. Belousov, Death of neurons following injury requires conductive neuronal gap junction channels but not a specific connexin. PLoS One, 10 (5): e0125395, 2015.*
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