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Neuronal gap junctions play a role in the secondary neuronal death following controlled cortical impact

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Abstract

In the mammalian CNS, excessive release of glutamate and overactivation of glutamate receptors are responsible for the secondary (delayed) neuronal death following neuronal injury, including ischemia, traumatic brain injury (TBI) and epilepsy. Recent studies in mice showed a critical role for neuronal gap junctions in NMDA receptor-mediated excitotoxicity and ischemia-mediated neuronal death. Here, using controlled cortical impact (CCI) in adult mice, as a model of TBI, and Fluoro-Jade B staining for analysis of neuronal death, we set to determine whether neuronal gap junctions play a role in the CCI-mediated secondary neuronal death. We report that 24 hrs post-CCI, substantial neuronal death is detected in a number of brain regions outside the injury core, including the striatum. The striatal neuronal death is reduced both in wild-type mice by systemic administration of mefloquine (a relatively selective blocker of neuronal gap junctions) and in knockout mice lacking connexin 36 (neuronal gap junction protein). It is also reduced by inactivation of group II metabotropic glutamate receptors (with LY341495) which, as reported previously, control the rapid increase in neuronal gap junction coupling following different types of neuronal injury. The results suggest that neuronal gap junctions play a critical role in the CCI-induced secondary neuronal death.

Keywords

Gap junctions; connexin 36; traumatic brain injury; glutamate; neuronal death

1. Introduction

In the mammalian central nervous system (CNS), direct intercellular communication between neighboring neurons occurs through electrical synapses (gap junctions). The coupling of neurons by gap junctions and the expression of the neuron-specific gap junction

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protein, connexin 36 (Cx36), increase transiently during early postnatal development and play critical role in death/survival mechanisms in developing neurons [7,15]. The coupling and Cx36 expression subsequently decrease [1]. However, they rapidly increase (often within 1-2 hrs) in the mature CNS following neuronal injury, such as ischemia [6,12,19], spinal cord and traumatic brain injury (TBI) [3,8,13], retinal injury [17], epilepsy [9,16] and inflammation [10].

It has been suggested previously that excessive release of glutamate and overactivation of glutamate receptors (mainly *N*-methyl-D-aspartate receptors; NMDAR) are primarily responsible for the secondary (delayed) neuronal death that occurs following neuronal injury [2,4,11]. We established recently that neuronal gap junctions play a critical role in NMDAR-mediated excitotoxicity and in neuronal death caused by focal cortical ischemia. Specifically, we showed [18] that in adult mice NMDAR-mediated neuronal death is prevented, and ischemic neuronal death is dramatically reduced, by both injection of mefloquine (a relatively selective blocker for Cx36-containing gap junctions [5]) and genetic knockout of Cx36. In addition, using *in vivo* and *in vitro* models of ischemia and *in vitro* models of TBI and epilepsy, we found [18] that group II metabotropic glutamate receptors (mGluRs) control the injury-mediated increases in neuronal gap junction coupling and expression of Cx36. We also showed that inactivation of group II mGluRs not only prevents these increases, but dramatically reduces injury-mediated neuronal death too. Based on these and other our findings [7,15,18,19], we proposed a novel model for the mechanisms of glutamate-mediated excitotoxicity [19]. We suggested that, during neuronal injury, the major determinant of glutamate-dependent neuronal death is the coupling of neurons by Cx36-containing gap junctions. We proposed that the presence of neuronal gap junctions determines the extent of NMDAR-mediated neuronal death. In addition, activation of group II mGluRs (caused by injury-mediated release of glutamate) induces synthesis of new neuronal gap junctions which enhance the extent of neuronal death [19]. Here, we used a model of TBI in adult mice to determine whether the TBI-induced secondary neuronal death is reduced by selective blockade of Cx36-containing gap junctions or by inactivation of group II mGluRs.

2. Materials and methods

The experiments were performed on 2.5-3 month old wild-type (WT; C57bl/6 strain) and Cx36 knockout (C57bl/6 background strain) male mice in accordance with NIH guidelines. The Cx36 knockout was originally created by Dr. David Paul (Harvard Medical School). Mice were genotyped as described [7].

Controlled cortical impact (CCI) was conducted as described earlier [14]. Briefly, after anesthesia, a 3.5 mm diameter circular craniotomy was performed; centered at anteroposterior (AP) = 0 mm and lateral to midline (ML) = 2.0 mm from bregma. The dural surface was exposed. To induce CCI, the CCI impact device was used: impact tip, 3 mm in diameter; strike velocity, 1.5 m/s; strike depth, 1 mm; contact time, 85 ms. The surgical field then was sutured closed. Ten minutes post-CCI, mice received a single intraperitoneal injection of sterile saline (100 μ L; control), mefloquine hydrochloride (a blocker for Cx36-containing gap junctions [5,18]; Sigma-Aldrich; 30 mg/kg in 100 μ L of saline) or LY341495 (a group II mGluR antagonist; (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; Sigma-Aldrich; 5 mg/kg in 100 μ L of saline) (i.e., we used the same drug doses as in our previous ischemic experiments in mice [18,19]). In all experiments, an analgesic was used post-surgery to reduce possible pain (buprenex, 2 mg/kg).

Animals were sacrificed under anesthesia 24 hrs post-CCI. Coronal brain sections were cut (50 μ m-thick) and Fluoro-Jade B staining (that is selective and highly sensitive for identification of degenerating neurons [20]) was conducted as we described earlier [18]. The sections were incubated with Fluoro-Jade B (0.001%) for 30 min. The staining was visualized using a Nikon Eclipse 80i fluorescent microscope, FITC filter and Photometrics ES2 digital camera. The staining was analyzed in the striatum, where the region containing staining was outlined. We measured size of the outlined region (that represents area of the secondary neuronal death; see Results) and the total number stained pixels in this region (that represents the amount of neuronal death). The latter was measured using a densitometric thresholding technique (ImageJ software, National Institutes of Health, USA). The threshold was set at a level just above that which would have counted background and non-specific staining in areas outside the outlined region. In each brain, the analysis was done in 8 striatal sections (between 1.2 and 0 mm from bregma) and data were averaged for 8 sections. Since Fluoro-Jade B has an excellent sensitivity and selectivity for identification of degenerating neurons [20], no additional co-staining with neuronal markers was necessary. The staining analysis was done by a person blinded to experimental groups. Data were analyzed using the one-tailed unpaired Student's *t*-test (with InStat software; GraphPad Software, USA) and are reported as mean $\pm$ S.E.M. for the number of samples indicated.

3. Results

CCI induced lesion cavity in the cortex. The cavity was seen 24 hrs post-CCI in all animal groups and included primary and secondary motor and primary somatosensory cortical regions (Fig.1a,b and 1d-f). In saline-treated WT mice, 24 hrs post-CCI, Fluoro-Jade B staining also revealed substantial neuronal death in the striatum (Fig.1a-c and 2a,b) and low-to-modest neuronal death in a number of other forebrain regions, including the hippocampus, thalamus and some cortical areas (not shown). This neuronal death was considered secondary to CCI as the dying neurons were located outside the cortical region that was directly subjected to the CCI impact. The neuronal death in the striatum was analyzed and showed significant reduction in WT mice that received a single intraperitoneal injection of mefloquine, a relatively selective blocker of Cx36-containing gap junctions (Fig. 1d and 2a,b), or LY341495, an antagonist of group II mGluRs (Fig.1e and 2a,b). The level of striatal neuronal death also was lower in saline-treated Cx36 knockout than in saline-treated WT mice (Fig.1f and 2a,b). However, there was no statistical difference in the amount of striatal neuronal death between saline-, mefloquine- and LY341495-treated Cx36 knockout animals (Fig.2c,d).

4. Discussion

In a previous study we showed [18] that NMDAR-mediated neuronal death induced in adult WT mice by intraperitoneal administration of NMDA is prevented by co-administration of mefloquine and does occur in Cx36 knockout mice. In the same study, we also demonstrated that mefloquine administration in WT mice or Cx36 knockout dramatically reduced neuronal death caused by photothrombotic focal cortical ischemia, an *in vivo* model of ischemic stroke. We concluded [18] that neuronal (Cx36-containing) gap junctions play a critical role in NMDAR-mediated excitotoxicity and ischemic neuronal death. In the present study, we used CCI in adult mice, an *in vivo* model of TBI. Here, we observed the neuroprotective effects that were virtually similar to those in the previous study: that mefloquine administration or Cx36 knockout significantly reduced the secondary neuronal death that occurs in the mouse striatum 24 hrs following CCI. This suggests that neuronal gap junctions play critical role in the CCI-dependent secondary neuronal death.

In another previous study [19] we used focal cerebral ischemia in adult mice and four models of neuronal injury *in vitro* employing mouse mature neuronal cortical cultures. These included: hypoosmotic shock as a model of cytotoxic and osmotic edemas that occur during TBI and ischemic stroke; hydrostatic pressure injury that represents mechanical aspects of TBI; oxygen-glucose deprivation as a model of stroke; and administration of 4-aminopyridine as a model of epileptic seizures. Using these five injury models, we showed that neuronal gap junction coupling and the expression of Cx36 rapidly increase (within 2 hrs) following neuronal injury and that group II mGluRs control this increase. Specifically, we demonstrated that activation of group II mGluRs elevated background levels of coupling and Cx36 expression and inactivation of group II mGluRs prevented the injury-mediated increases in the coupling and Cx36. Moreover, inactivation of group II mGluRs dramatically reduced injury-mediated neuronal death, as demonstrated in the same study with the use of five *in vivo* and *in vitro* injury models. We concluded that not only do group II mGluRs control the injury-mediated increase in the coupling, but via this increase they also contribute significantly to injury-mediated neuronal death [19]. The results obtained in the present study, demonstrating that blockade of group II mGluRs significantly reduces the CCI-mediated secondary neuronal death, agree with those previous observations described above [19]. In combination with the mefloquine and Cx36 knockout data (shown in Fig. 1 and 2), these results also indirectly support the previous conclusion that group II mGluRs play a role in the injury-mediated neuronal death, most likely via up-regulation of neuronal gap junction coupling.

In a previous study with the use of NMDAR-mediated excitotoxicity and focal cerebral ischemia in adult mice [18], we found that administration of mefloquine did not have any additional neuroprotective effects in Cx36 knockout animals. Based on this, we concluded that the neuroprotective effect of mefloquine very likely was based upon blockade of Cx36-containing gap junctions. Similar results were obtained in the present study with injections of mefloquine and LY341495 in Cx36 knockout mice following CCI (Fig. 2c,d), i.e., no additional neuroprotection was observed. This suggests that the neuroprotective effects of mefloquine and LY341495 likely are via blockade of Cx36 gap junctions (mefloquine) or inactivation of the mechanisms for their increase following injury (LY341495).

Altogether, the results from our previous [7,15,18,19] and present study suggest that neuronal gap junctions are critical for neuronal death that occurs following neuronal injury, including ischemia, TBI and presumably epilepsy. The results also identify neuronal gap junction coupling as a critically important therapeutic target for the development of new neuroprotective agents.

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Research Highlights

- > Controlled cortical impact in mice induces secondary neuronal death in the striatum
- > This neuronal death is reduced by blockade of neuronal gap junctions or Cx36 knockout
- > It is also reduced by inactivation of group II metabotropic glutamate receptors
- > Results suggest critical role for neuronal gap junctions in secondary neuronal death

Fig. 1.

CCI induces the secondary neuronal death in the striatum of adult mice that is reduced by pharmacological and genetic blockade of neuronal gap junctions and by inactivation of group II mGluRs. Representative images of Fluoro-Jade B staining in mouse brain sections from saline-treated WT (**a-c**), mefloquine-treated WT (**d**), LY341495-treated WT (**e**) and saline-treated Cx36 knockout (Cx36 KO; **f**) mice are shown. All images are taken at bregma 0.15-0.20 mm. Images in **a-c** are taken from the same brain section and the regions that are boxed in **a** and **b** are shown at a higher magnification as, respectively, figures **b** and **c**. All injections were done 10 min post-CCI and the staining was conducted 24 hrs post-CCI. CPu, caudate putamen (striatum); MS, medial septum; SSp, primary somatosensory cortex. In **a**, **b** and **d-f**, lesion cavity is indicated by asterisk.

Fig. 2.

Statistical analysis of Fluoro-Jade B staining in the striatum of adult mice following CCI. The number of stained pixels (**a**, **c**) and size of area of the secondary neuronal death (**b**, **d**) are analyzed 24 hrs post-CCI. (**a**, **b**) Blockade of neuronal gap junctions (with mefloquine) and inactivation of group II mGluRs (with LY341495) in WT mice as well as Cx36 knockout all reduce the CCI-dependent secondary neuronal death. Analysis: Student's *t*-test; statistical difference is shown relative to the saline-treated WT mice; *n* = 4-5 per group; mean $\pm$ S.E.M. (**c**, **d**) In Cx36 knockout mice, injections of mefloquine or LY341495 did not produce neuroprotective effects; the analysis did not reveal statistical difference between the three groups of Cx36 knockout mice: *P* > 0.05, Student's *t*-test; *n* = 3-5 per group; mean $\pm$ S.E.M.