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Functional outcome of pannexin-deficient mice after cerebral ischemia

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Pannexin (Px, Panx) channels have been implicated in several physiological and pathological processes. We recently studied the potential contribution of pannexins in ischemic brain damage using $Px1^{-/-}$ $Px2^{-/-}$ mice and provided evidence that (1) the release of IL-1β and hemichannel function in astrocytes are, in contrast to published data, not affected by the absence of Px1 and Px2, (2) channel function in neurons lacking Px1 and Px2 is impaired and (3) $Px1^{-/-}$ $Px2^{-/-}$ mice had a better functional outcome and smaller infarcts than wild-type mice when subjected to ischemic stroke. Here, we further investigate the neurological outcome of wild-type and pannexin double-knockout mice 48 h after permanent occlusion of the distal middle cerebral artery (MCAO). Pannexin double-knockout mice ($Px1^{-/-}$ $Px2^{-/-}$) were less impaired in parameters such as exploration, anxiety, sensorimotor function and behavioral symmetry.

Introduction

Pannexins are a recently discovered family of channel-forming proteins. Despite a similar three-dimensional structure, they have no sequence homology with connexins, the subunit proteins of gap junctions. In fact, the three members of the family, pannexin 1 (Px1), pannexin 2 (Px2) and pannexin 3, only exceptionally form gap junctions but rather unapposed channels in the membrane that link the cytosol of cells with the extracellular space.[1] Several studies have provided evidence for a physiological role of pannexin channels in diverse cellular functions, including release of ATP into the extracellular space, production of IL-1β, apoptosis and conductance of large electrical currents in neurons.[2] The first round of pannexin research relied on fairly unspecific pharmacological tools and siRNAs to silence pannexins. Recently, targeted deletions of mouse pannexin genes have made it possible to reevaluate the role of pannexins and to explore their role in vivo. We have generated $Px1^{-/-}$, $Px2^{-/-}$ and $Px1^{-/-}Px2^{-/-}$ mice and investigated these animals in the context of cerebral ischemia.[3] The results show that Px1 and Px2 play a detrimental role after focal cerebral ischemia. The double deletion of both pannexins led to a reduced infarct size and a better neurological outcome 24 h after the ischemic stroke.

Infarct size is an outcome parameter that is often used in experimental studies, but what really counts for stroke patients is the degree of functional disability after the event. To re-assess whether pannexins determine the functional outcome, including anxiety, exploration, sensorimotor function, behavioral symmetry and locomotion, 48 h after cerebral ischemia, we used a battery of behavioral tests like the corner test, the latency to move and the open-field test. Our data confirm that Px1 and Px2 contribute to ischemic brain damage and neurological impairment after stroke.

Results

Latency-to-move test. The latency to move may be affected by increased levels of anxiety after MCAO but probably also reflects locomotor activity of mice that is altered after MCAO.[4,5] It has previously been shown that the latency to move is prolonged up to several days after cerebral ischemia.[6] In our study, the latency to move one body length was, as expected, increased after the stroke in wild-type mice, but the time needed was significantly less in double-knockout than in wild-type mice 48 h after permanent distal MCAO (**Fig. 1**).

Corner test. The corner test evaluates sensorimotor function and behavioral symmetry. It is thought that stimulation of the vibrissae prompts animals to rise on the hindlimbs, thus allowing to test both cortical and subcortical functions.[7] It has previously been shown that following MCAO animals showed a preference to turn to the contralateral side of the lesion.[6] In the current study, pannexin double-knockout mice showed a trend to turn less often to the right side 48 h after left-sided MCAO than wild-type mice, although the difference did not reach statistical significance (**Fig. 2**).

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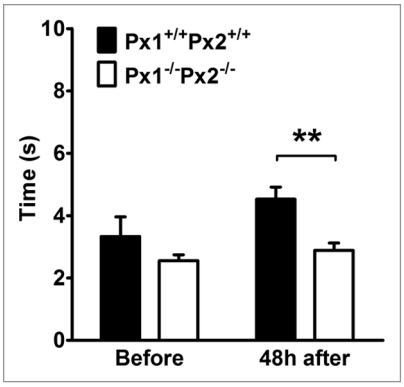

Figure 1. The latency to move was increased after MCAO in $Px1^{+/+}Px2^{+/+}$ wild-type mice. However, in the double-knockout $Px1^{-/-}Px2^{-/-}$ group, the latencies were significantly shorter than in the wild-type group 48 h after MCAO repeated measures ANOVA, $F(1/12) = 11.59$, $p < 0.01$. **$p < 0.01$ (Bonferroni post-hoc test). Values are means ± SEM (n = 5–9).

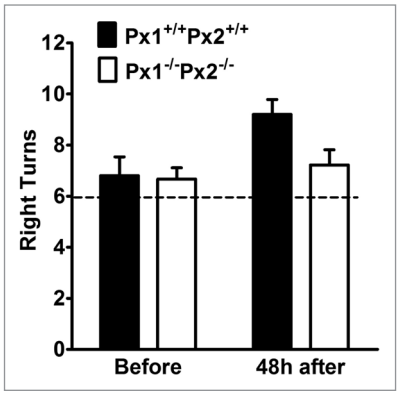

Figure 2. In the corner test, mice turned more often to the right side after left-sided MCAO. Pannexin double-knockout $Px1^{-/-}Px2^{-/-}$ mice tended to turn less often to the right side than wild-type $Px1^{+/+}Px2^{+/+}$ littermates 48 h after MCAO, although the difference did not reach statistical significance. Repeated-measures ANOVA, $F(1/12) = 3.52$, $p = 0.08$. Values are means ± SEM (n = 5–9).

Open-field test. The open-field test provides simultaneous measures of locomotion, exploration and anxiety.[8] It is well-known that healthy animals prefer the periphery rather than the center of an open field, a behavior which is related to the level of anxiety. In our experiment, wild-type animals spent significantly less time in the center of the arena, reflecting higher anxiety levels than pannexin double-knockout animals when tested 48 h after MCAO (**Fig. 3A**). Another parameter that we determined in the open-field test was how often mice reared, an activity which reflects mainly exploration but also locomotion. Double-knockout mice reared significantly more often than wild-type animals 48 h after MCAO (**Fig. 3B**). In contrast, the number of lines crossed in the maze did not differ between the two groups before and 48 h after MCAO showing that locomotor activity was equally reduced in wild-type and double-knockout mice (**Fig. 3C**).

Discussion

Deletion of both Px1 and Px2 reduced dye release through membrane channels of primary neurons and was neuroprotective in vivo.[3] Using a battery of behavioral tests, we show in this study that the functional outcome, evaluated by several parameters such as exploration, sensorimotor symmetry and anxiety, is better in pannexin double-knockout mice than in wild-type littermates even 48 h after MCAO. Interestingly, in the open-field test, it is the fear- and the interest-related behavior that is less affected in mice lacking pannexin channels and not that much locomotion, which was equally decreased in the two genotypes after cerebral ischemia. These findings may be useful for further investigation of the functional role of pannexins after ischemia and for the development of specific pannexin inhibitors.

So far, we have only investigated neurological function in the acute phase after stroke because, in our mouse model, the functional deficit disappears within days, even without treatment.[6] Future work will have to evaluate whether the functional benefit of pannexin inhibition or deletion persists during the chronic phase of recovery. In order to investigate the effects of pannexins in the chronic phase of recovery after cerebral ischemia, further experiments using different models of cerebral ischemia and probably an extended spectrum of functional tests are needed. This would provide information as to whether pannexins influence mechanisms that control the delayed phase of brain damage after cerebral ischemia or recovery.[9] Before a translation to the clinic will be possible, further important questions have to be solved. Do pannexins promote ischemic brain damage also in old individuals and in the presence of comorbidities? Is there a sex difference? Finally, a second species will have to be investigated.[10]

To answer these questions, a specific pharmacological pannexin inhibitor would be helpful. Which channel subunits should be targeted by such an inhibitor? Interestingly, a recent study reported that neuronal deletion of only Px1 was sufficient to protect retinal ganglion cells against ischemia.[11] In contrast, our data suggest that for a robust neuroprotection, both Px1 and Px2 would have to be inhibited. In single $Px1^{-/-}$ or $Px2^{-/-}$ mice, there was a trend toward smaller cortical infarcts that did not reach statistical significance.[3] This discrepancy might reflect a different contribution of Px1 and Px2 to tissue vulnerability during ischemia dependent on the anatomical site. However, the expression of Px1 and Px2 has a rather similar distribution in the brain.[12] Therefore, it seems more likely that Px1 and Px2 partially compensate for each other, thus requiring inhibition of both subunits for a robust neuroprotection.

It is possible that redundancy and compensation are not limited to the pannexin family, because connexins also form large-pore membrane channels. Compensation by a connexin or another protein may explain why a knockout of Px1 and Px2 genes had no effect on inflammasome activation and the production of IL-1β by macrophages, although the short-term inhibition of pannexin function or expression was effective.[3,13-15] Concerning the composition of channels in astrocytes, no consensus has been reached. The notion that Cx43 is the essential

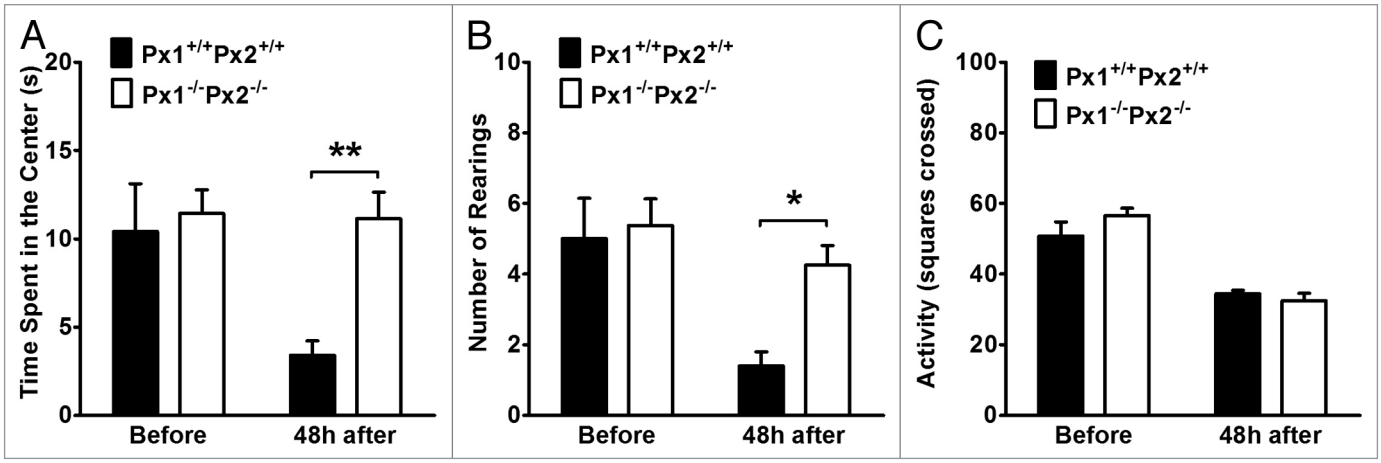

Figure 3. Exploration, anxiety and locomotion were assessed using the open-field test. In the open-field test, $Px1^{-/-}Px2^{-/-}$ mice spent significant more time in the center of the arena [(**A**), Repeated-measures ANOVA, $F(1/10) = 6.63$, $p < 0.05$; Bonferroni post-hoc test, $^{**}p < 0.01$] and reared more often [(**B**), Repeated-measures ANOVA, $F(1/11) = 3.55$, $p = 0.086$; Bonferroni post-hoc test, $^{*}p < 0.05$] than their $Px1^{+/+}Px2^{+/+}$ littermates 48 h after MCAO. The number of lines crossed did not differ between the two groups (**C**). Values are mean ± SEM (n = 5–7).

subunit of astrocyte channels and both Px1 and Px2 play no role has been challenged by a recent study claiming that Px1 is the key channel subunit of astrocytes.[3,16-18] Possibly, there is a partial compensation between pannexins and Cx43 in astrocytes. Irrespective of channel function in astrocytes, there is good evidence that pannexin channels are a useful target for stroke therapy, as interference with pannexin function robustly improves the functional outcome after stroke.

Materials and Methods

Mice and model of cerebral ischemia. Generation of the $Px1^{-/-}Px2^{-/-}$ mouse line and the model of permanent occlusion of the distal middle cerebral artery (MCAO) have been described before.[3] Here, we report additional data from the experiment initially described in Figure 4 of our previous publication.[3]

Corner test. We used the corner test to assess sensorimotor function and behavioral symmetry.[7,19] Mice were investigated twice, before MCAO and 2 d after MCAO. The test device consists of two vertical boards (each 30 cm × 20 cm × 1 cm) connected on one side at an angle of 30°. The animals were left in the test device for 2 min to explore the environment. Subsequently, they were placed halfway in the corner. A food pellet in a small opening between the two boards stimulated mice to enter the corner. Thereafter, animals performed the task spontaneously. After reaching the corner, vibrissae were stimulated bilaterally, mice reared and turned to the right or left side. Only complete turns were counted if mice fully reared. Mice were left to perform the test 12 times.

Latency-to-move test. Mice were placed on a plate and the time to move one body length (about 7 cm) was recorded. The test was performed three times for each mouse and the mean value was calculated.

Open-field test. Mice were placed in the center of an arena (72 cm × 72 cm) and allowed to explore the apparatus for 60 sec. Subsequently, mice were returned to their home cages. Thereafter, the arena was cleaned with 60% alcohol and permitted to dry between two consecutive tests. The time spent in the central area of the arena, the number of rearings and the total number of lines crossed with all four paws were measured.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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