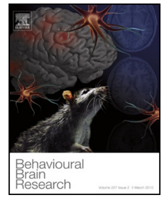
Behavioural Brain Research 318 (2017) 24–27

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Short communication

Sleep-wakefulness cycle and behavior in pannexin1 knockout mice

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HIGHLIGHTS

- Waking is increased and slow wave sleep is decreased in the dark period in mice deficient for Panx1.
- Movement activity is increased throughout the light/dark cycle in mice deficient for Panx1.
- The lack of pannexin modifies animal behavior in vertical and horizontal pole tests.

ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 4 October 2016
Accepted 7 October 2016
Available online 18 October 2016

Keywords:
Pannexin
Sleep
Purinergic signaling
ATP
Adenosine
Hemichannels
Gene knockout models

ABSTRACT

Pannexins are membrane channel proteins that play a role in a number of critical biological processes (Panchin et al., 2000; Shestopalov, Panchin, 2008). Among other cellular functions, pannexin hemichannels serve as purine nucleoside conduits providing ATP efflux into the extracellular space (Dahl, 2015), where it is rapidly degraded to adenosine. Pannexin1 (Panx1) is abundantly expressed in the brain and has been shown to contribute to adenosine signaling in nervous system tissues (Prochnow et al., 2012). We hypothesized that pannexin1 may contribute to sleep-wake cycle regulation through extracellular adenosine, a well-established paracrine factor in slow wave sleep. To investigate this link, EEG and movement activity throughout the light/dark cycle were compared in Panx1$^{-/-}$ and Panx1$^{+/+}$ mice. We found a significant increase in waking and a correspondent decrease in slow wave sleep percentages in the Panx1$^{-/-}$ animals. These changes were especially pronounced during the dark period. Furthermore, we found a significant increase in movement activity of Panx1$^{-/-}$ mice. These findings are consistent with the hypothesis that extracellular adenosine is relatively depleted in Panx1$^{-/-}$ animals due to the absence of the ATP-permeable hemichannels. At the same time, sleep rebound after a 6-h sleep deprivation remained unchanged in Panx1$^{-/-}$ mice as compared to the control animals. Behavioral tests revealed that Panx1$^{-/-}$ mice were significantly faster during their descent along the vertical pole but more sluggish during their run through the horizontal pole as compared to the control mice.

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In the mammalian purinergic signaling system [1], intracellular ATP molecules can reach the extracellular medium via vesicular release (Ves) [2] and/or by direct release through Pannexin1 hemichannels (Panx1) [3–5], as schematically shown in Fig. 1. Adenosine can also diffuse directly via equilibrative nucleoside transporters (ENT) [6]. Extracellular ATP is rapidly degraded into adenosine by a series of ectonucleotidases (ecto) [7]. The receptors in the purinergic signaling system include ligand-gated P2X ion channels and G protein-coupled P2Y receptors that are activated by ATP and G protein-coupled adenosine (A) receptors [1]. The purinergic signaling is essential for the control of sleep and wakefulness [8–10]. Adenosine is known as a powerful somnogenic substance [8]. Fluctuations of adenosine concentration in the anterior hypothalamus are associated with the sleep cycle [11,12]. It has been shown that Pannexin-1 is expressed in the brain [13,14] and was suggested to play an important role in regulating paracrine levels of adenosine [15]. Here, we hypothesize that the channels formed by pannexins may be an essential source of adenosine in the hypothalamus. If our hypothesis is correct, the absence of pannexin

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http://dx.doi.org/10.1016/j.bbr.2016.10.015
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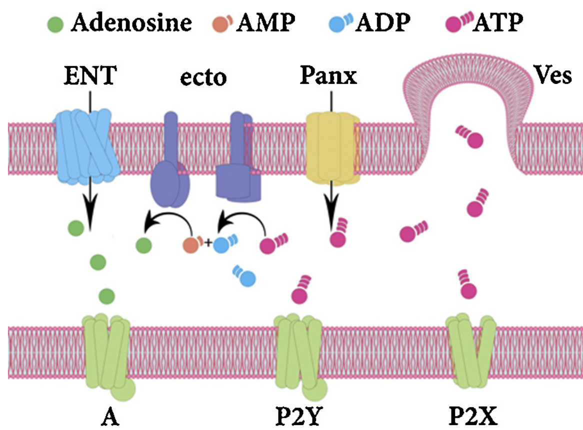

Fig. 1. Schematic depicting mechanisms of purinergic and adenosine signaling in the brain. Pannexin1 hemichannels (Panx) release ATP. Equilibrative nucleoside transporters (ENT) transport nucleoside substrates like adenosine across the membranes. ATP molecules can reach the extracellular medium via vesicular release (Ves). Extracellular ATP is degraded into ADP and adenosine by ectonucleotidases (ecto). Ionotropic P2X receptors are gated by ATP. G protein-coupled P2Y receptors are activated by ATP and ADP. G protein-coupled adenosine receptors (A) respond to adenosine.

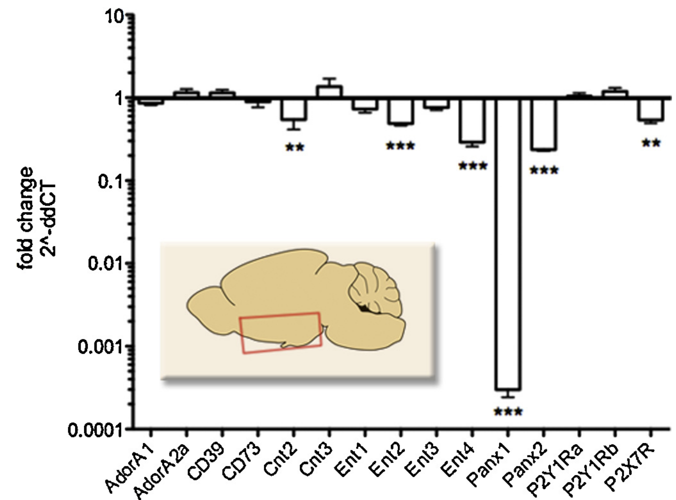

Fig. 2. Changes in the expression levels of 15 genes reportedly implicated in ATP signaling, ATP and adenosine transport in the brain of the Panx1$^{-/-}$ vs. C57Bl6 mice, used as a reference. Graphs depict the fold change of the corresponding value in the control WT brain sample ± SEM after normalizing the transcript abundance value of each gene to that of P-actin. Global deficiency in Panxl transcript is validated by a ~800-fold drop of correspondent reading. See Supplement for gene abbreviations. Data are presented as mean± SD; **P < 0.005, ***P < 0.0005, n = 3. Inset shows mouse anterior hypothalamus (preoptic area) brain region, dissected for RT-PCR analysis of gene expression. (AdorA1 and AdorA2a – adenosine receptors; CD39 and CD73-ectonucleotidases; Cnt2 and Cnt3 – concentrative nucleoside transporters; ENT 1–-4 – equilibrative nucleoside transporters; Panx1 and Panx2 -pannexins; P2Y1Ra, P2Y1Rb and P2 × 7R – purinergic receptors).

channels in knockout (KO) animals may lead to downregulation of adenosine signaling, and resemble the action of adenosine receptors antagonists, such as caffeine and theophylline, when dosed chronically. Furthermore, adenosine receptors are also strongly expressed in some brain structures involved in the basic behavioral functions (e.g. basal ganglia) [16]. This would suggest that wake-sleep cycling and general behavioral patterns will be altered in mice with a genetically disrupted pathway of Panx1-mediated ATP release.

Methodological details for this study are summarized in Supplement materials. In brief, we used the Panx1$^{-/-}$ mouse strain developed by Dvoriantchikova and co-authors [17]. The Panx1$^{-/-}$ mice are fertile and phenotypically indistinguishable from WT littermates [18,19]. Epidural electrodes (four per each animal) were implanted under general (avertine) anesthesia to the frontal and parietal neocortex. EEG and animal movements were recorded without animal mobility restrictions. The analysis of the EEG and actogram (mechanogram) was performed using specialized software designed using an open source EDF browser package (see: Suppl. Ref. 3). The standard rodent criteria for waking, slow wave (non-REM, NREM) and paradoxical (REM) sleep in 20-s epochs were used. Behavioral studies were performed using vertical pole and horizontal pole ("beam walking") tests (see: Suppl. Refs. 4, 5) which allow the estimation of sensorimotor coordination, moving activity and general emotionality of the animals. Details on real-time PCR analysis and primers used are also provided in Supplement materials.

Panx1 is a membrane channel that may allow ATP release in the brain and other organs [13,14]. Then released ATP could be converted extracellularly into adenosine, the sleep-promoting factor in mammals [8]. To test whether PANX1 ablation affects the major transporters of adenosine, and the enzymes controlling adenosine metabolism and transport in the brain, we analyzed changes in expression levels of genes encoding these enzymes in Panx1$^{-/-}$ forebrains relative to WT control tissues by quantitative RT-PCR (See supplement for primers and gene list). Our analysis (Fig. 2) revealed that the majority of genes encoding alternative ATP release pathways did not change significantly in Panx1$^{-/-}$ brains, apart from down-regulation of Cnt2, Ent2, Ent4 (2.5-, 3.0- and 6.0-fold change, respectively). Also downregulated were genes encoding Panx2 and the P2 × 7 receptor (7-fold and 2.5-fold, respectively). These results indicate that changes in ATP and adenosine signaling, caused by the PANX1 protein deletion are not compensated by alternative pathways, such as Ent. Instead, PANX1 ablation causes coordinated suppression of the ATP signaling pathway, involving the P2 × 7 receptor and the PANX2 protein that was suggested to synergistically interact with PANX1 channel. This is in agreement with earlier report on high transcriptional correlation between Panx1 and Panx2 genes in the brain [19,20]. Similar to our previous results [21], expression of Panx3 transcript in brain samples was not detected, and this gene was omitted from the analysis.

As depicted in Fig. 3A, Panx1$^{-/-}$ mice demonstrated a significantly higher percentage of wakefulness during the light (+12%; $p < 0.05$) and, especially during the dark period of the nychthemeron (+32%; $p < 0.01$), as compared to controls. Corresponding decrease in percentage of NREM sleep was observed in both light (−10%; $0.05 < p < 0.06$) and dark (−40%, $p < 0.01$) periods. Importantly, we detected an inversion of the waking/NREM ratio during daylight, which is not typical for nocturnal rodents [22]. REM sleep decreased by 30% during the dark time and increased by 10% during the light time in Panx1$^{-/-}$ mice relative to controls. EEG spectra did not differ significantly between knockout and control mice.

Next, we performed a 6-h sleep deprivation study during the daylight using the "soft" behavioral arousing method [22] (rocking the cages, knocking on the cage walls, introduce air puffs using a rubber bag, etc.) and were monitored using polysomnography. The comparison between knockout and control mice did not reveal significant differences in 6-h rebound sleep duration and structure as compared to the controls (data not shown).

Several research groups have exploited a similar approach to identify other players in the purinergic signaling network in the brain. The type 1 equilibrative nucleoside transporter (ENT1) has been previously implicated in regulating levels of extracellular adenosine. Similar to our observations in Panx1$^{-/-}$ mice, the baseline NREMS amount is decreased in ENT1 knockout mice [6]. The authors demonstrated that adenosine perfusion directly into the basal forebrain increased NREMS in knockout animals. Taken

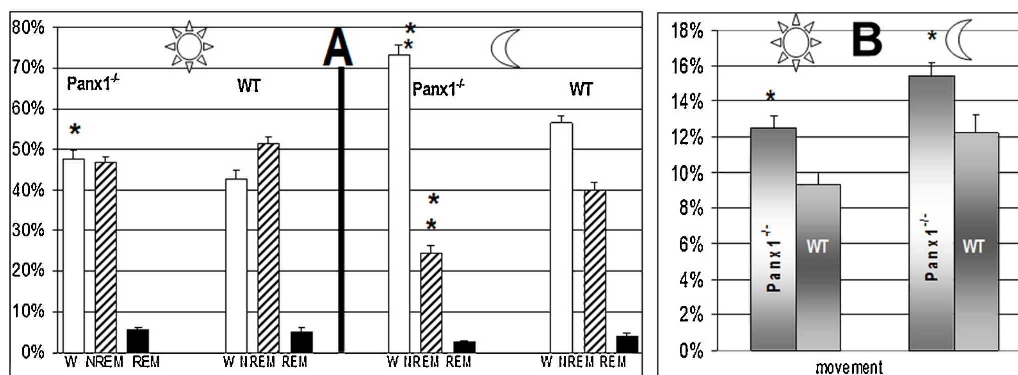

Fig. 3. **A** Sleep-wakefulness cycle in Panx1$^{-/-}$ (N = 13) and control (WT, N = 10) mice during the light (marked by the Sun) and dark (marked by the crescent) periods of the nychthemeron. White columns – wake, cross-hatched – NREM, black – REM sleep. Y-axis – percentages of the recording time (12 h = 100%). Asterisks denote significant changes between knockout and control mice during the corresponding light/dark period: $^{*}p < 0.05$; $^{**}p < 0.01$ (*U* test). B: Movement activity of Panx1$^{-/-}$ (N = 13) and WT (N = 13) mice as revealed by the accelerometer (100% – maximal possible deviation) during light (marked by the Sun) and dark (marked by the crescent) periods of nychthemeron, $^{*}p < 0.01$.

together, our findings suggest that the similar effects observed in PANX1 and ENT1 knockout mice resulted from the decrease in concentration of extracellular adenosine. Interestingly, in the study by Kim et al., the differences in sleep characteristics between knockout and control mice were more distinct during the light period [6], whereas in our case they were more pronounced during the dark period. Our study (Fig. 2) revealed no compensatory elevation in Ent gene expression, but rather some decrease in the Ent mRNA in Panx1$^{-/-}$ brains, which might aggravate the effects of the PANX1 knockout. Rebound sleep duration after a period of experimental sleep deprivation was not affected in ENT1 KO [6], similar to our PANX1 KO mice. It is significant, in this regard, that Kim et al. suggested that pathways other than ENT1 could contribute to the extracellular adenosine during sleep deprivation [6]. These knockout data may imply that the ENT1 and PANX1 pathways of ATP release could compensate each other in the brain, a plausible model to be explored in future studies.

CD73 is another potential player in the adenosine signaling pathway that was studied using a knockout animal model. This 5′- ectonucleotidase enzyme contributes to extracellular adenosine production by conversion from extracellular AMP that is, in turn, produced by CD39 ectonucleoside from extracellular ATP. Zielinski et al. reported a small effect on spontaneous NREMS in CD73 knockout mice compared to C57BL/6 control mice [7]. Rather unexpectedly, NREMS is enhanced in CD73 knockout mice.

Vesicular gliotransmission was previously suggested as an additional source of extracellular adenosine release in the brain (Fig. 1). Thus, Halassa et al. reported that SNARE-dependent gliotransmission was genetically inhibited in conditional astrocyte-specific transgenic mice, due to reduced adenosine release [2]. The attenuation of gliotransmission did not notably impact baseline sleep time or structure, but significantly reduced the increase in NREM duration following sleep deprivation, another behavioral measure of the homeostatic response.

We suggest that the PANX1 channel exerts its primary effect on the sleep-wake cycle via adenosine receptors, indirectly activated through the ATP release. The vast literature on genetic ablation of adenosine receptors is reviewed by Wei et al. [23]. In their report, the experiments with knockout models confirmed that adenosine modulates the sleep-wake cycle by acting on A1R and A2AR. In addition to sleep-wake cycle control, adenosine and adenosine receptors participate in a number of brain functions, which dramatically affect behavior patterns [24,25]. We compared these functions in WT control and Panx1$^{-/-}$ mice using basic behavioral tests, including pole tests and activity recordings. We observed significantly shorter descent times in Panx1$^{-/-}$ vs. control mice due to more frequent jumping from the vertical pole directly on the floor ($p = 0.032$; N = 6; Table 1). Mean transit duration along the horizontal round “big” pole (2.3 cm in diameter) was significantly longer in Panx1$^{-/-}$ mice relative to the controls ($p = 0.026$; N = 6; Table 1), due to greater amount of spontaneous stops during the pole transit ($p = 0.015$; N = 6; Table 1). Experiments using 5 other horizontal poles, however, failed to reveal any significant differences, probably due to insufficient numbers of animals in the testing groups. Behavioral observations recorded more frequent stops by Panx1$^{-/-}$ mice during their transiting the horizontal poles. Thus, the animals spent more time orienting in search of direction. In contrast, control animals were faster in overcoming similar obstacles. The activity level in knockout mice was significantly higher during dark and light periods in both duration and intensity as compared to controls (Fig. 3B). Thus, the present study revealed that mice with the Panx1 gene ablation demonstrate increased levels of wakefulness and movement activity and, consequently, decreased levels of NREM sleep (especially pronounced during the dark period), as compared to the control C57Bl/6 mice. A plausible mechanism underlying these differences are a decreased ATP/adenosine release in the brain of the Panx1$^{-/-}$ mice [19,26]. The model of adenosine accumulation during prolonged wakefulness is widely accepted [24,25]. Although our central hypothesis linked the deficiency in Panx1 with a decreased ATP release and deficiency in adenosine signaling, one has to admit that other physiological parameters are known to change due to systemic Panx1 gene ablation. Indeed, recent studies

Table 1
Behavioral study of the Panx1-/- and control mice in vertical and horizontal pole tests.

Group	Number of animals in group	Tests					
		Vertical pole		Horizontal pole			
		Mean descending time in sec (M ± SD)	P-level	Mean pass duration time in sec (M ± SD)	P-level	Mean amount of stops (M ± SD)	P-level
Knockout	N = 6	7.2 ± 8.5	p < 0.05	23 ± 18.1	p < 0.05	5 ± 5.2	p < 0.05
Control	N = 6	34 ± 28.1		6.4 ± 3.5		0.9 ± 1.0	

showed a number of parameters, including blood flow regulation [26], short term memory [15], resistance to ischemia [17,27] were reported to differ between wild type and Panx1$^{-/-}$ mice. Sleep regulation could easily be affected by these systemic alterations. That said, the results of our sleep deprivation experiments were in slight disagreement with adenosine deficiency model, as they did not reveal any changes in sleep rebound after deprivation in Panx1$^{-/-}$ mice. One possible explanation is that, during the 6-h sleep deprivation, the knockout mice could utilize alternative adenosine pathways independent of pannexin-1. Also, accumulation of "sleepiness" during sleep deprivation, which realizes as sleep rebound, might not be due to accumulation of adenosine in basal forebrain either [28].

This study confirms the essential role of Panx1 in behavioral integration. One must also consider the possibility that PANX1 ablation might have effects other than altering adenosine signaling. PANX1 activity is known to act on the P2X or P2Y receptors via ATP efflux [29,30], gap junction function or calcium leak from the endoplasmic reticulum [31]. Although Panxl knockout mice are viable and display no apparent phenotypic abnormalities, studies targeting individual physiological pathways, including this work, clearly show the important role of these proteins in the functioning of the central nervous system.

Acknowledgement

We thank Aleksandr Troshchenko for the design of the recording system. This work was supported by Russian Foundation for Basic Research grant 15-04-06148 (YVP) and NIH NEI grant EY021517 (VIS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.10.015.

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