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Astrocytic gap junction blockade markedly increases extracellular potassium without causing seizures in the mouse neocortex

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ABSTRACT

Extracellular potassium concentration, $[K^+]_o$, is a major determinant of neuronal excitability. In the healthy brain, $[K^+]_o$ levels are tightly controlled. During seizures, $[K^+]_o$ increases up to 15 mM and is thought to cause seizures due to its depolarizing effect. Although astrocytes have been suggested to play a key role in the redistribution (or spatial buffering) of excess K^+ through Connexin-43 (Cx43)-based Gap Junctions (GJs), the relation between this dynamic regulatory process and seizure generation remains unknown. Here we contrasted the role of astrocytic GJs and hemichannels by studying the effect of GJ and hemichannel blockers on $[K^+]_o$ regulation *in vivo*. $[K^+]_o$ was measured by K^+-sensitive microelectrodes. Neuronal excitability was estimated by local field potential (LFP) responses to forepaw stimulation and changes in the power of resting state activity. Starting at the baseline $[K^+]_o$ level of 1.61 ± 0.3 mM, cortical microinjection of CBX, a broad spectrum connexin channel blocker, increased $[K^+]_o$ to 11 ± 3 mM, Cx43 GJ/hemichannel blocker Gap27 increased it from 1.9 ± 0.7 to 9 ± 1 mM. At these $[K^+]_o$ levels, no seizures were observed. Cx43 hemichannel blockade with TAT-Gap19 increased $[K^+]_o$ by only ~1 mM. Microinjection of 4-aminopyridine, a known convulsant, increased $[K^+]_o$ to ~10 mM and induced spontaneously recurring seizures, whereas direct application of K^+ did not trigger seizure activity. These findings are the first *in vivo* demonstration that astrocytic GJs are major determinants for the spatial buffering of $[K^+]_o$ and that an increase in $[K^+]_o$ alone does not trigger seizures in the neocortex.

1. Introduction

A long standing question in epilepsy research is the mechanism of neuronal (hyper)excitability underlying seizure generation. The neuronal excitability is dictated by extracellular ionic concentrations and synaptic activity (Florence et al., 2009). Non-synaptic mechanisms of seizure generation have been observed (Jefferys and Haas, 1982; Taylor and Dudek, 1982; Haas and Jefferys, 1984) and consist preponderantly of ionic fluctuations in the extracellular space. Experimental observation and computer models suggest neuronal excitability is primarily dictated by the intra- *vs.* extra-cellular potassium gradient (for review see, Florence et al., 2009). In particular, the observation that extracellular potassium ($[K^+]_o$) rises during seizures (Moody et al., 1974; Pedley et al., 1974; Heinemann et al., 1977, Raimondo et al., 2015) led to the "potassium accumulation hypothesis", which posits that an initial supra-threshold increase in $[K^+]_o$ without any other experimental manipulations (such as low calcium or the presence of a convulsant) triggers seizures *via* a positive feedback mechanism (Traynelis and Dingledine, 1988; Frohlich et al., 2008). Although it is widely recognized that understanding the underlying relationship between $[K^+]_o$ and seizure generation is paramount, this hypothesis has hitherto not been tested *in vivo* in the neocortex. It is known that astrocytes are involved in the regulation of $[K^+]_o$ *via* the uptake of K^+ through ion transporters and its dissipation *via* the so-called K^+ spatial buffering (Orkand et al., 1966; Somjen, 2001; Kofuji and Newman, 2004). This process relies on inter-astrocytic communication *via* Gap Junctions (GJs), which are prevalently formed by Connexin43 (Cx43, for review (Scemes and Spray, 2012)) and Cx30 (Kunzelmann et al., 1999). While potassium spatial buffering is well established for the Müller cells of the retina (Newman, 1984, 1993), the importance of Cx43 GJs on K^+ buffering *via* astrocytes in the Central Nervous System has been confirmed only *in vitro* (Wallraff et al., 2006). This mechanism has been presumed to regulate $[K^+]_o$ globally, determine neuronal excitability, and, furthermore, play a key role in seizure generation. To recapitulate the link between $[K^+]_o$ dynamics and seizures, 4-Amino Pyridine (4-AP), a known K_v channel blocker and a convulsive agent (Reddy and Kuruba, 2013) without a direct action on GJs, is widely employed in preclinical

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research to generate seizures with a concomitant increase in $[K^+]_o$ (Avoli et al., 1996a, 1996b, for review Avoli and de Curtis, 2011) In the present work, we examined the role of astrocytic Cx43-Gjs and hemichannels in $[K^+]_o$ regulation and seizure generation in the mouse neocortex *in vivo*, contrasting the effects on $[K^+]_o$ of the broad-spectrum connexin channel blocker Carbenoxolone (CBX), of the Gap27 peptide (blocking Cx43 hemichannels and gap junctions, Evans and Boitano, 2001; Evans and Leybaert, 2007; Wang et al., 2012; Wang et al., 2013a), and of the TAT-GAP19 peptide (specifically blocking Cx43 hemichannels without inhibiting gap junctions (Wang et al., 2013b)). We observed a marked rise in $[K^+]_o$ following blockade of GJs and hemichannels with CBX and Gap27. However, specific block of Cx43 hemichannels with TAT-Gap19 did not influence $[K^+]_o$, indicating that mainly inter-astrocytic GJs were responsible for $[K^+]_o$ alterations, in line with the primary role of GJs in spatial K^+ buffering. Although the ensuing increase in $[K^+]_o$ from blocking interastrocytic GJs was comparable to that observed after the 4-AP application, and local field potentials evoked by peripheral stimulation were depressed, no seizures were observed. These data suggest that 4-AP convulsive action is probably due to its blocking effect on K_v channels. To rule out definitively the hypothesis that high $[K^+]_o$ alone causes cortical seizures *in vivo*, we progressively increased $[K^+]_o$ by applying solutions with increasing $[K^+]$ onto the exposed cortex. We observed a bimodal effect of exogenous K^+ solutions on neuronal excitability, in line with the endogenous K^+ increases: increased somatosensory evoked responses and neuronal power for concentrations between 4 and 10 mM followed by depressed neuronal transmission for concentrations higher than 12 mM. We did not observe seizures at any of the concentrations tested. The present findings are the first *in vivo* data on the effects of local astrocytic GJ blockade on $[K^+]_o$ homeostasis, neuronal excitability, and responsivity to peripheral stimulation.

2. Material and methods

2.1. Animal preparation

Experiments were conducted on young adult CD-1 mice (4–5 weeks of age) in accordance with the guidelines of the animal welfare committee of the University Health Network. Mice were anesthetized by intraperitoneally injected Ketamine-Xylazine (respectively 95 and 5 mg/kg b.w.) and placed into a stereotaxic frame. The animal body temperature was maintained at 37.5 °C using a heating pad (Physitemp, TCAT-2DF). Hind limb withdrawal reflexes and breathing rates were observed at regular intervals throughout the experiment to ensure that the animal remained at a surgical plane of anesthesia. Forepaw stimulation (train of 3 pulses of 100 μs at 10 Hz, 0.5–0.8 mA) was delivered *via* needles implanted into the left forepaw. A local anesthetic (Sensoricaine, AstraZeneca Canada Inc.) was injected subcutaneously into the scalp region to be incised. A small craniotomy (diameter of ~2 mm) was performed over the right somatosensory cortex of the mouse, leaving the dura mater intact and a well was built around the opening to preserve constant level of CSF over the brain. Phosphate Buffered Saline (PBS, pH 7.4, Sigma) was applied over the exposed cortex to prevent tissue damage and dehydration. Five groups were studied, each undergoing a different cortical microinjection (see below): Control (PBS, $n = 4$), TAT-GAP19 ($n = 3$), Carbenoxolone (CBX, $n = 7$), GAP-27 ($n = 7$), and 4-AP ($n = 5$). In the exogenous K^+ experiments we sequentially applied onto the cortex buffered saline (pH 7.4, 0.4–0.5 ml) solutions with increasing $[K^+]$ (4, 6, 8, 12 and 20 mM).

2.2. Electrophysiological recordings

The procedure used to manufacture the K^+-sensitive electrodes was similar to the one described in previous studies (Dufour et al., 2011; Bazzigaluppi et al., 2015; Wang et al., 2016). Briefly, K^+-sensitive electrodes were made from pulled borosilicate capillaries (tip diameter ~1 μm, World Precision Instruments, Sarasota, FL). The interior wall of the capillary was silanized with dimethyldichlorosilane vapor and dried at 120 °C for 2 h. The tip was then filled with the potassium Ionophore I-cocktail B (Sigma-Aldrich Canada Ltd., Oakville). The rest of the barrel was backfilled with a 0.2 M KCl. The signal at the reference barrel was subtracted from the signal at the K^+-selective barrel to obtain a signal proportional to $[K^+]_o$. Local field potentials (LFP) were recorded with a pulled borosilicate capillary filled with PBS (in the control experiments), or a mixture of PBS and Carbenoxolone (CBX, 1 mM, Sigma), or the GAP27 peptide (500 μM, (Samoilova et al., 2008), Severn Biotech), or the TAT-GAP19 peptide (500 μM, (Wang et al., 2013b)) or 4-AP (5 mM, Sigma). The focal application of the pharmacological agents used (or vehicle) was performed by ten repetitions of 3 to 5-ms microinjections from the LFP pipette (PicoSpritzer III, Parker), applied over a time period of 5 min; this resulted in a total injection volume of ~1 μl. The signal recorded with the LFP electrode was subtracted from that recorded from the K^+-sensitive electrode. Data was acquired with Axopatch 200B amplifiers and sampled at 10 kHz. Under an Olympus BX-61W1 microscope with 4X PlanN objectives, both electrodes were placed into the cortical layers 2–3 of the forelimb region of the mouse somatosensory cortex (−0.3 to −0.5 mm from bregma, 1 to 1.3 mm from midline, 150 to 250 μm depth), so that their tips were within 10 μm of each other. LFP and extracellular K^+ signals were low pass filtered (5 kHz) and digitized (Digidata 1440, Axon instruments). K^+-sensitive electrodes were calibrated using solutions containing 2.5, 4.5, 6.5 and 22.5 mM KCl. The relationship between the measured voltage and the K^+ concentration of the respective solution was derived using the Nicolsky-Eisenmann equation (Ammann, 1986).

2.3. Data analysis and statistics

Given the relatively slow dynamics of $[K^+]_o$ increase following drug application, resting state $[K^+]_o$ was estimated at: before injection (*i.e.* baseline) and at 10 min, 30 min, 60 min and 90 min after injection, and expressed as a 1 min average preceding each somatosensory stimulation. In the same epochs, the Fast Fourier Transform of the cortical EEG was computed to estimate the power of the neuronal activity. In the experiments where exogenous K^+ was applied onto the cortex, we waited up to 10 min for $[K^+]_o$ to stabilize before recording spontaneous and evoked activity. Raw power at different time points was divided into the following frequency bands: Theta (4–8 Hz), Alpha (9–14 Hz), Beta (15–30 Hz). The raw power in each band was averaged across animals and then normalized to the baseline value (*i.e.* before any somatosensory stimulation or pharmacological manipulation) in that band. Twenty trains of somatosensory stimuli were delivered at each time point (or at different $[K^+]_o$s) and the evoked field (LFP) responses were estimated as the ten-repetitions-average. We assessed normality of data distribution within groups with normal probability plots and Kolmogorov-Smirnov test, then tested for significance with one way-ANOVA (for normally distributed data) or with Kruskal-Wallis ANOVA (for non-normally distributed data). Within each group, statistical difference between the different time points was assessed *via* one-way-ANOVA or Kruskal-Wallis one-way-ANOVA (see Statistic Table – Supplementary Table 1) followed by Least Significant Difference post-hoc test for multiple comparisons. The p-values represent the result of one-way-ANOVA. Analysis was performed in Matlab. All the values represent mean ± SEM.

3. Results

3.1. Connexin channel blockade increases $[K^+]_o$

We performed measurements of intracortical (parenchymal) $[K^+]_o$ and LFPs making use of two electrodes placed in the forelimb region of mouse somatosensory cortex positioned at the level of cortical layers 2–3. Given the slow changes in the $[K^+]_o$ (see examples in Fig. 1A), we