Neocortical in vivo focal and spreading potassium responses and the influence of astrocytic gap junctional coupling

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ABSTRACT

Raised extracellular potassium ion (K+) concentration is associated with several disorders including migraine, stroke, neurotrauma and epilepsy. K+ spatial buffering is a well-known mechanism for extracellular K+ regulation/distribution. Astrocytic gap junction-mediated buffering is a controversial candidate for K+ spatial buffering. To further investigate the existence of a K+ spatial buffering and to assess the involvement of astrocytic gap junctional coupling in K+ redistribution, we hypothesized that neocortical K+ and concomitant spreading depolarization (SD)-like responses are controlled by powerful local K+ buffering mechanisms and that K+ buffering/redistribution occurs partially through gap junctional coupling. Herein, we show, in vivo, that a threshold amount of focally applied KCl is required to trigger local and/or distal K+ responses, accompanied by a SD-like response. This observation indicates the presence of powerful local K+ buffering which mediates a rapid return of extracellular K+ to the baseline. Application of gap junctional blockers, carbenoxolone and Gap27, partially modulated the amplitude and shape of the K+ response and noticeably decreased the velocity of the spreading K+ and SD-like responses. Opening of gap junctions by trimethylamine, slightly decreased the amplitude of the K+ response and markedly increased the velocity of redistribution of K+ and SD-like events. We conclude that spreading K+ responses reflect powerful local K+ buffering mechanisms which are partially modulated by gap junctional communication. Gap junctional coupling mainly affected the velocity of the K+ and SD-like responses.

1. Introduction

Potassium spatial buffering is known to be one of the mechanisms responsible for K+ regulation and distribution in the brain. It was proposed that astrocytic gap junctional coupling has a role in extracellular potassium ion concentration ([K+]o) buffering (Orkand et al., 1966; Ransom, 2000), although measurements of electrolyte and extracellular space volume changes could be only partly explained by spatial buffering currents (Dietzel et al., 1989). Wallraff et al. (2006) showed that gap junction-mediated currents represented only about 30% of whole-cell currents in wildtype astrocytes in hippocampal slices. Mice lacking coupled astrocytes displayed changes in [K+]o dynamics, but the major capacity of K+ redistribution was maintained, suggesting that gap junction-mediated buffering only partially accounted for hippocampal K+ buffering (Wallraff et al., 2006). Contribution of gap junctions in [K+]o movement might be dependent on the location within hippocampal layers; only in locations 400–500 um away from the stratum pyramidale, were [K+]o increases in mice lacking astrocytic coupling much smaller than those in wild type mice (Wallraff et al., 2006). It has been shown that, in hippocampal slices, acute astroglial gap junctional uncoupling increased the amplitude of local K+, only when the K+ amplitude exceeded 10 mM, and not with transient and smaller K+ increases (Breithausen et al., 2020).

In pathological situations in the brain, gradual extracellular K+ increases can occur. For example, a gradual K+ increase by a few millimolar before the occurrence of Spreading Depolarization (SD) is observed in ischemia due to opening of calcium-activated K+ channels (Erdemli et al., 1998; Müller and Somjen, 2000; Revah et al., 2016).
High $[K^+]_o$ contributes to the occurrence of SD in addition to a seizure (Hablitz and Heinemann, 1989). The depression pattern determines the neurological deficit seen in pathological conditions such as migraine aura, migraine stroke, transient ischemic attack or typical stroke (Hadjikhani et al., 2001; Major et al., 2019; Olesen et al., 1981).

The role of gap junctional coupling in SD propagation has long been studied. While many groups showed that gap junctional blockage by octanol and heptanol prevented or reduced SD propagation (Nedergaard et al., 1995; Martins-Ferreira et al., 2000), others reported that inactivation of specific gap junctions (CX43) accelerates SD (Theis et al., 2003). Theis et al. (2003) observed that mice lacking Cx43 showed defective $K^+$ redistribution in slices leading to a more abrupt local $[K^+]_o$ increase and an increase in the velocity of hippocampal SD. On the other hand, usage of carbenoxolone (CBX) neither prevented SD nor altered SD propagation (Peters et al., 2003). While SD was blocked using kynurenic acid, MK-801 and octanol, CBX had no effect on in vitro neocortical SD propagation (Vilàglí et al., 2001).

Hence after years of studies, the topic of $K^+$ spatial buffering and the extent of the involvement of gap junctional coupling in $K^+$ buffering remains debatable and inconclusive. We still lack a comprehensive representation of local buffering with regards to fast clearance of local increases in $[K^+]_o$ and its relationship to the electrophysiological measure of SD. Also, quantitative and relative contributions of astrocytic gap junctional coupling to the overall $K^+$ dynamics and SD propagation are still unclear. Answering these questions is difficult because simultaneous direct representation of SD along with $K^+$ buffering in gap junctionally coupled astrocytes in vivo is technically challenging (Bellot-Saez et al., 2017). To overcome the technical barriers, we enhanced well-developed electrophysiological techniques by coupling $K^+$-sensitive and Local Field Potential (LFP) recording electrodes together; 2 sets of the coupled electrodes were placed horizontally ~2 mm apart. SD-like events were characterized by transient events with negative slow current shifts recorded by LFP electrodes (Dreier and Reiffurth, 2015).

So here, using an enhanced well-developed technique and sets of gap junctional modulating drugs (CBX, Gap27 and TMA), we first investigated the existence of a buffering mechanism contributing to $K^+$ clearance. We studied properties of KCl-evoked local and distal spreading $K^+$ responses and their preceding SD-like responses. We analyzed $K^+$ and LFP dynamics leading to SD-like events and provided further details with regard to involvement of the gap junctionally coupled astrocytes in $K^+$ buffering and SD propagation. In the present work, we hypothesized that $K^+$ and SD-like responses are constrained by local $K^+$ buffering mechanisms, and that astrocytic gap junctional coupling is partially responsible for the observed $K^+$ buffering over the neocortex.

We observed that a threshold effect existed for detecting a local and/or distal spreading response; this response was only partially dependent on astrocytic gap junctional coupling. Gap junctional communication had a more pronounced role in speeding up the spread of $K^+$ and SD-like responses, with partial effect on the amplitude and shape of the $K^+$ responses but no noticeable effect on the amplitude or shape of the SD-like events. The present findings are the first in vivo results recapitulating the regenerative and threshold-based nature of spreading KCl-evoked $K^+$ and SD-like responses. Furthermore, this study provides a quantitative assessment of contributions of astrocytic gap junctional coupling in $K^+$ redistribution using an enhanced experimental platform.

2. Methods

2.1. Animal preparation and craniotomy

Experiments were conducted on one to two month old, 18 to 30 g CD-1 mice. Animals were housed in a 12/12 h light cycle with ad lib access to water and food. All experiments were performed in vivo and all experimental procedures are approved by the Krembil Research Institute. The mice were anesthetized with 5% isoflurane with oxygen flowing at 1 mL/min for inducing anesthesia. During the surgery and during the experiment, inhaled isoflurane was turned down to 1.5–2% and the oxygen flow was reduced to 0.4 mL/min. Craniotomy was performed with a precision drill, removing a circular region of the skull 5 mm in diameter over the right somatosensory cortex. Phosphate Buffered Saline (PBS, pH 7.4, Sigma) was applied over the exposed cortex, filling the cavity of the skull, to prevent tissue damage and dehydration. After craniotomy, the mouse was transferred to the recording chamber. 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.

2.2. K-LFP electrode preparation, implantation for measuring $[K^+]_o$ and LFP

In order to maintain an accurate measurement of $K^+$ in the $K^+$-sensitive electrodes, it is necessary to account for the component arising from an electrical field. To this end, a local reference electrode was used to mitigate distortion of $K^+$-sensitive electrode readings. We have coupled a $K^+$-sensitive electrode (K) with a double-barreled local field potential recording electrode (LFP), creating a K-LFP recording electrode (Fig. 1a & d). The double-barreled electrode was filled with saline and cemented to the K$^+$-sensitive electrode such that the distance between the tips of the electrodes was approximately 50 μm apart (Fig. 1).

First, the K$^+$-sensitive electrode was mounted to a head-stage for use with an Axopatch 200B amplifier sampled at 10 kHz. A differential reference electrode for the head-stage was inserted into one chamber of the double-barreled LFP electrode; the other chamber was used to record the extracellular LFP. This latter signal was differentially recorded from a common ground wire, attached to the scalp. This arrangement was duplicated to have a K-LFP recording available at each recording site (Fig. 1). All four amplifiers were then processed by Digidata 1440, Axon digitizers. LFP and extracellular $[K^+]_o$ signals were low pass filtered at 5 kHz. This preparation allowed for simultaneous and effective recording of LFP and $[K^+]_o$ responses (K-LFP response) from the same location in the cortex. Readings from the LFP electrodes denoted negative DC shifts representing SD-like events.

In the case of the K$^+$-sensitive electrode, the interior wall of the capillary was silanized with dimethyl dichlorosilane vapor and dried at 120 °C for 2 h (Bazzigaluppi et al., 2017b). K$^+$-sensitive electrodes were filled with K$^+$ ionophore I-cocktail B (Sigma-Aldrich Canada Ltd., Oakville) at the tip and back filled with 0.2 M KCl solution (Bazzigaluppi et al., 2017b). Electrodes were lowered into the cortex in steps of 0.1 mm. Under an Olympus BX-61 W1 microscope with 4× PlanN objectives, two sets of K-LFP electrodes (Fig. 1a & d), were inserted at 0.3 mm depth into the right

![Fig. 2. Local and regenerative K-LFP responses constrained by K$^+$ buffering mechanism.](image-url)
somatosensory cortex (layer II-III), such that their tips were about 2 mm apart in a horizontal plane. An injection micropipette (Fig. 1e) filled with 50 mM KCl, was placed ~10 μm from one of the K-LFP recording electrodes. The recording site immediately adjacent to the KCl injection site was called the peri-injection site (peri-IS) whereas the other recording site was called the remote-injection site (remote-IS) (Fig. 4A).

Following 10 min of baseline recording, 50 mM KCl was focally injected before topical application of a drug, and again 10 min after the drug application. The K⁺-sensitive recording electrodes were then calibrated using different concentrations (0, 2.5, 4.5, 6.5 and 22.5 mM) of KCl solutions. The relationship between the measured voltage and the K⁺ concentration of the respective solution was derived using the Nicolsky-Eisenmann equation (Miller, 1995) which is a commonly used method by experts in the field of K⁺ recording. The latter calibration lines which were semi-logarithmic and close to linear were used to determine [K⁺]o in the brain (Walz et al., 1984).

2.3. KCl injection, experimental plan and drug selection

Focal application of 50 mM KCl was performed by 1 to 7 repetitions (every 0.3 s) of microinjections lasting 3 ms to 8 ms (PicoSpritzer III, Parker), resulting in a total injection volume of 0.07 μl to 3 μl. In order to account for variability in the injection micropipette tip diameter, each trial is compared to a baseline KCl puff response in each experiment.

Three experimental groups were used to check for the absence or presence of a K-LFP response following application of different volumes of KCl. In these groups, existence of a threshold effect for the K⁺ response (n = 3) and for the dual K-LFP responses (n = 5) were examined. Next, KCl volume titration and the existence of a ceiling level was tested (n = 5).

Four experimental groups were used to study the role of gap junctional communication in K⁺ redistribution. In these groups 0.1 ml of a general gap junction blocker (Carbenoxolone (CBX) (Bazzigaluppi et al., 2017a, 2017b; Medina-Ceja et al., 2008); 500 μM, n = 5) or a relatively specific astrocytic connexin43 gap junction blocker (Gap27 peptide (Bazzigaluppi et al., 2017b; Evans and Boitano, 2001); 500 μM, n = 6), or a specific connexin43 hemichannel inhibitor peptide (TAT-Gap19 (Gap19) (Bazzigaluppi et al., 2017h; Wang et al., 2013); 500 μM, n = 10), or a general gap junction opener (Trimethylamine (TMA) (Medina-Ceja et al., 2008; Medina-Ceja and Ventura-Mejia, 2010); 100 mM, n = 6) was applied topically onto the neocortex over the recording electrode sites.

2.4. Statistical analysis

Data analysis was performed using MATLAB 2016. In light of the low number of data samples (CBX: n = 5, CBX-2: n = 3, Gap27: n = 6, TMA: n = 6, TAT-Gap19: n = 10), non-parametric statistical analysis was performed. A two-tailed hypothesis was tested and the p-value was calculated using Wilcoxon-Mann-Whitney test. A p-value below 0.05 was considered significant. Statistical analysis was performed to evaluate changes in amplitude, area under the curve (AUC), rise time constant (RTC), decay time constant (DTC), lag time (time difference between the onset of the peri & remote responses) and velocity (distance between the peri & remote electrode divided by lag time) for each animal before and after drug application in both recording sites. Results were reported in three different ways: a) average of individual responses before or after drug application in each group ± SEM, b) average of individual percent variations due to drug application in each group ± SEM, c) percent variation of group averages due to drug application in each group ± SEM.

3. Results

3.1. Local K⁺ buffering restraints local and spreading K-LFP responses; increased focal KCl application triggers regenerative propagating K-LFP responses

In 3 animals we first demonstrated that a threshold amount of KCl was needed to trigger a measurable K⁺ response locally and/or distally, suggesting powerful local K⁺ buffering (Bazzigaluppi et al., 2017a, 2017b). Very small amounts (Fig. 2A, 1p(3) ~ 0.07 µl) of focally injected KCl did not generate a local K⁺ response. The initially obtained K⁺ response was small in amplitude (~4 mM) and the injected amount (Fig. 2A, 2p(3) ~ 0.14 µl) could not trigger a K⁺ response remotely (about 2 mm away from the injection site). By doubling the amount (Fig. 2A, 4p(3) ~ 0.3 µl) of injected KCl, a K⁺ response could be observed remotely (~9 mM) which was preceded by a local K⁺ response. Next we demonstrated that KCl-evoked K⁺ measurements were stable over time; the same amount of KCl application in the same animal led to K⁺ responses with the same properties (Fig. 2B).

Next, in 5 animals, we examined KCl evoked responses using our dual K-LFP experimental platform which allowed for evaluating coupled K-LFP responses in 2 different sites, located about 2 mm apart. Consistent with the previous finding, we observed that a threshold amount of KCl was needed to trigger a measurable K⁺ response locally (Fig. 2C, 1p(3) ~ 0.1 µl), a slightly higher amount (Fig. 2C, 3p(3) ~ 0.3 µl) of KCl application led to peri and remote K⁺ responses and a LFP response was seen locally, but not remotely. Finally a large enough volume of injected KCl (Fig. 2C, 4p(3) ~ 0.4 µl & Fig. 2D, 5p(3) ~ 0.5 µl) resulted in a K-LFP response both locally and distally. In each site, the K⁺ response preceded the LFP by about 1.0 ± 0.5 s which is in line with the observation by Müller and Somjen (2000) where they reported that [K⁺]o began to rise before SD onset (Müller and Somjen, 2000).
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**Responses at peri-IS**

1. **K⁺ sensitive electrode**
2. **Local reference electrode**
3. **LFP electrode**
4. **KCl injection micropipette**

**CBX: n=5 mice**

**Responses at remote-IS**

**D**

| K⁺-amp (mM) | K⁺-AUC (mM*min) |
|-------------|-----------------|
| 15          | 17              |
| 13          | 15              |
| 11          | 13              |
| 9           | 11              |

**E**

| K⁺-amp (mM) | K⁺-AUC (mM*min) |
|-------------|-----------------|
| 13          | 11              |
| 11          | 10              |
| 9           | 8               |

**F**

- **Lag time (min):**
  - Control K⁺: 0.7
  - CBX K⁺: 0.3

- **Velocity (mm/min):**
  - Control LFP: 9
  - CBX LFP: 3

*(caption on next page)*
Fig. 4. Effects of CBX on K-LFP responses locally and remotely (n = 5). (A) a schematic of electrodes configuration in peri & remote Injection Site (IS). (B) & (C) Blue upward curve represents K⁺ response before CBX, red upward curve represents K⁺ response after CBX; green downward curve represents LFP response before CBX and yellow downward curve represents LFP after CBX. (D) & (E) Box and whisker plots comparing amplitude, AUC, RTC, DTC of K⁺ response before and after CBX application; (B) & (D) are peri-IS and C & (E) are remote-IS. (F) Box and whisker plots comparing lag-time and velocity of the response spreading from peri to remote-IS, before and after CBX. In Box and whisker plots: blue is before and red is after drug application, the smallest and largest data points are shown as lines at the tip of the whiskers, the remaining of the data points are represented as empty circles within the box (in some cases data points were equal to quartiles or were overlapping), X represents the mean and horizontal line in the box represents median, * indicates significance (p < 0.05). In (B) & (C): black arrows indicate the injection time, #p sign represents the number of puffs focally applied and (#) represents duration of injection in ms (3p(7)–1 µl). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Ceiling level

To titrate the injection volumes, we tested whether there is a ceiling level for the K⁺ responses responding to the local application of 50 mM KCl. With n = 5 animals we observed that there was a ceiling level for this response, such that after application of a certain amount of 50 mM KCl solution, the amplitude of the K⁺ response did not exceed 15.5 ± 1.1 mM, even with increasing the volume of 50 mM KCl injection (Fig. 5A, 5p(6) & B, 7p(8)). The maximum injected KCl volume was about 3 µl (n = 5).

3.3. Gap junctional coupling had a pronounced effect on the velocity of the spreading K-LFP response and a partial effect on the shape and amplitude of the K⁺ responses

3.3.1. Carbamoxalone partially modulated local and distal K⁺ responses

Using the newly configured experimental platform (Fig. 4A), we observed that topical application of CBX (n = 5) increased the amplitude (amp), area under the curve (AUC) and decay time constant (DTC) of the K⁺ responses by 21.5 ± 7.8% (p = 0.0214), 38.6 ± 10.7% (p = 0.0214) and 28.3 ± 6.7% (p = 0.012) in the peri-injection site (Table 1 & Fig. 4B, D). Simultaneously, in the remote injection-site, CBX application decreased the K⁺ response amplitude by 19.2 ± 1.5% (p = 0.028) and increased the RTC and DTC by 42.8 ± 11.0% (p = 0.021) and 34.7 ± 6.2% (p = 0.012) respectively in comparison with before drug application responses (Table 1 & Fig. 4C, E). To assess the role of gap junctional communication in the kinetics of the spread of the K-LFP responses, we measured the lag-time and velocity of these responses. We observed that the K-LFP response, spreading from the peri to remote site increased in lag-time by 72.8 ± 16.3% (p = 0.036) and decreased in velocity by 39.5 ± 7.0% (p = 0.012) after CBX application in comparison to before CBX application (Table 1 & Fig. 4F).

Although the decreased amplitude caused by CBX was statistically significant, it did not appear to be the predominant effect as the K⁺ amplitude changed by about 20%. All the LFP responses corresponded with their respective K⁺ responses, but changes in their amplitude and shape caused by CBX were minimal and statistically insignificant (data not shown). CBX had a more pronounced effect on slowing the K-LFP events. These findings led us to examine the effect of CBX on K-LFP responses at different distances from the KCl injection site. We conducted experiments (n = 3) where the injection micropipette was placed between the recording electrodes; 1/3rd (~0.7 mm) away from one of the K-LFP recording electrodes and 2/3rd (~1.3 mm) away from the other recording electrode (Fig. 5A). In comparison to before CBX application, upon CBX administration, K⁺ response decreased in amplitude by 20.1 ± 3.1%, increased in RTC by 64.1 ± 27.9% and in DTC by 49.1 ± 32.7% at 1/3rd away from the injection site (Table 2 & Fig. 5B, D). At 2/3rd away from the injection site, K⁺ response decreased in amplitude by 16.5 ± 0.5%, RTC and DTC increased by 21.2 ± 16.2% and 66.0 ± 32.3% (Table 2 & Fig. 5C, E). Because n = 3, p-value was not calculated although in all experiments changes were consistent.

3.3.2. Gap27 partly altered local and distal K⁺ responses

Because CBX is a non-specific gap junction blocker, we examined the effect of Gap27, which is known to specifically block astrocytic connexin 43 gap junctional communication (Evans and Boitano, 2001; Wang et al., 2013; Evans and Leybaert, 2007). Repeating the same experiments as done for CBX using Gap27 (n = 6), we demonstrated similar but less prominent actions (Table 3 & Fig. 6). The KCl injection micropipette was placed next to one of the K-LFP recording electrodes (Fig. 6A). Upon topical application of Gap27, as compared with before drug application responses, in the peri injection site, the K⁺ response increased in amplitude, AUC and DTC by 13.1 ± 2.8% (p = 0.03), 20.0 ± 7.0% (p = 0.02) and 12.9 ± 7.9% (p = 0.037) respectively (Table 3 & Fig. 6B, D). Simultaneously, at the remote injection site, in compare to before drug administration, Gap27 application fractionally decreased the K⁺ response amplitude by 12.1 ± 2.5% (p = 0.037) and increased DTC by 13.4 ± 1.6% (p = 0.037) (Table 3 & Fig. 6C, E). We observed that the lag-time between peri and distal K-LFP response markedly increased by 60.3 ± 13.5% (p = 0.045) and velocity of K-LFP response propagation decreased by 35.5 ± 5.1% (p = 0.02) after Gap27 application (Table 3 & Fig. 6F). All the LFP responses corresponded to their respective K⁺ responses but we could not detect any noticeable effect on their amplitude caused by Gap27 (data not shown).

Next we investigated the role of gap junctional connexin43 hemichannels, not the gap junction itself, by using TAT Gap19 (Wang et al.,

Table 1

| CBX | K⁺ responses at peri injection site | K⁺ responses at remote injection site | Spreading from peri to remote IS |
|-----|----------------------------------|------------------------------------|-----------------------------------|
|     | Amp (mM) | Amp (mM) | Amp (mM) | Amp (mM) | Amp (mM) |
|     | AUC (min. mM) | AUC (min. mM) | AUC (min. mM) | AUC (min. mM) | AUC (min. mM) |
|     | RTC (min) | RTC (min) | RTC (min) | RTC (min) | RTC (min) |
|     | DTC (min) | DTC (min) | DTC (min) | DTC (min) | DTC (min) |
|     | Lag time (min) | Velocity (mm/min) | Lag time (min) | Velocity (mm/min) |
| Before CBX Ave | 11.2±0.6 | 10.2±0.5 | 0.29±0.01 | 0.99±0.03 | 10.3±0.4 | 9.0±0.4 | 0.29±0.01 | 0.96±0.06 | 0.27±0.05 | 7.5±0.9 |
| After CBX Ave ± SEM | 13.5±0.4 | 14.0±0.7 | 0.33±0.02 | 1.26±0.06 | 8.4±0.5 | 10.0±0.6 | 0.41±0.03 | 1.28±0.04 | 0.43±0.05 | 4.3±0.3 |
| Ave of individual %variations ± SEM (1/1) | 121.5±7.8* | 38.6±10.7* | 113.6±5.5 | 28.3±6.7* | 19.2±1.5* | 11.6±9.7 | 142.8±11.0* | 134.7±6.2* | 172.8±16.3* | 139.5±7.0* |
| % of group ave variation (1/1) | 120.2* | 137.1* | 113.8 | 28.0* | 19.0* | 110.6 | 142.1* | 133.5* | 161.9* | 142.3* |

Table 1 Effect of CBX on amplitude (Amp), Area Under the Curve (AUC), Rise Time Constant (RTC) and Decay Time Constant (DTC) at peri & remote injection site plus lag time and velocity of the spreading response. (1/1) indicates increase or decrease of the response in the presence of CBX. * represents significance of variations.
Fig. 5. Effects of CBX on K-LFP responses at 1/3rd and 2/3rd away from the injection site (n = 3). (A) a schematic of electrodes configuration in 1/3rd (~0.7 mm) & 2/3rd (~1.3 mm) away from the injection site (IS). (B) & (C) Blue upward curve represents K\(^+\) response before CBX; red upward curve represents K\(^+\) response after CBX; green downward curve represents LFP response before CBX and yellow downward curve represents LFP after CBX. (D) & (E) Box and whisker plots comparing amplitude, AUC, RTC, DTC of K\(^+\) response before and after CBX application. (B) & (D) are 1/3rd (~0.7 mm) away from the IS, (C) & (E) are 2/3rd (~1.3 mm) away from the IS. In Box and whisker plots: blue is before and red is after drug application, the smallest and largest data points are shown as lines at the top and bottom of the box, third data point is represented as an empty circles in the box, X represents the mean and the horizontal line in the box represents median. In (B) & (C): black arrows indicate the injection time, #p sign represents the number of puffs focally applied and (#) represents duration of injection in ms (3p(7) ~ 1 μl). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

| CBX                        | K\(^+\) responses 1/3rd away from injection site | K\(^+\) responses 2/3rd away from injection site |
|---------------------------|-----------------------------------------------|-----------------------------------------------|
|                           | Amp (mM)  | AUC (mM*min) | RTC (min) | DTC (min) | Amp (mM)  | AUC (mM*min) | RTC (min) | DTC (min) |
| Before CBX Ave ± SEM      | 11.6±0.1 | 10.2±0.4     | 0.24±0.03 | 0.80±0.05 | 11.0±0.1 | 9.1±0.6      | 0.32±0.01 | 0.75±0.06 |
| After CBX Ave ± SEM       | 9.2±0.3  | 10.6±1.3    | 0.39±0.03 | 1.16±0.17 | 9.2±0.1  | 9.6±0.5      | 0.38±0.04 | 1.20±0.13 |
| Ave of individual %variation± SEM (1/1) | 120.1±3.1  | 3.8±1.2      | 164.1±27.9 | 149.1±32.7 | 121.2±16.2 | 166.0±32.3 |
| % of group ave variation (1/1) | 120.2  | 3.8      | 158.9   | 144.8   | 120.0  | 5.7       | 160.7   |
| P- 2tail                  | Because n = 3, P value was not calculated | Because n = 3, P value was not calculated |

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had a limited role in modulating K buffering. We demonstrated that neocortical gap junctional coupling away from the application site (Kraio and Nicholson, 1978; Dietzel et al., 2013) (n = 10) which is a connexin43 hemichannel inhibitor. We observed that following TATGap19 application, the amplitude, AUC, RTC and DTC remained unchanged (changes below 1%) as compared to before drug application.

3.3.3. Trimethylamine markedly increased the speed of K-LFP responses and slightly decreased the amplitude of K+ responses

We then investigated how opening (Medina-Ceja et al., 2008; Medina-Ceja and Ventura-Mejia, 2010) instead of closing gap junctions affects the K-LFP propagation dynamics. With the same experimental configuration (Fig. 7A), application of TMA (n = 6), a general gap junctional opener, at the peri injection site, partly decreased the amplitude and AUC by 13.4 ± 3.3% (p = 0.02) and 20.6 ± 2.1% (p = 0.03) (Table 4 & Fig. 7B, D) in comparison to before drug application. In the remote injection site, amplitude and AUC decreased by 16.6 ± 2.3% (p = 0.037) and 21.6 ± 3.2% (p = 0.045) respectively (Table 4 & Fig. 7C, E). The lag-time between the peri and remote response decreased significantly by 45.9 ± 9.3% (p = 0.03) and velocity of the response increased markedly and significantly by 103.7 ± 23.4% after TMA application as compared with before drug administration (Table 4 & Fig. 7F). We could not detect any obvious effect of TMA on the amplitude and shape of the recorded LFPs (data not shown).

4. Discussion

To modulate the pathophysiological levels of K+, it is crucial to identify the various contributing mechanisms involved in K+ homeostasis and redistribution (Bellot-Saez et al., 2017). In this study, using a novel modification of established electrophysiology techniques, we investigated the involvement/role of gap junction blockers and activators in the K+ dynamics preceding SD-like events. When KCl is applied in vivo in the neocortex. KCl application leads to an increase in [K+]o that can be recorded remotely from the KCl application site with sufficient amount of applied KCl, as buffering mechanisms rapidly distribute K+ away from the application site (Kraio and Nicholson, 1978; Dietzel et al., 1989; Enger et al., 2015). In brief, we demonstrated a powerful local K+ buffering mechanism which prevented the local [K+]o from going higher than the normal baseline level. Upon passing a threshold level, with higher amounts of KCl injection, K+ responses and their concomitant SD-like events became apparent. Next, we investigated the degree of the contribution of astrocytic gap junctional coupling in the observed K+ buffering. We demonstrated that neocortical gap junctional coupling had a limited role in modulating K+ amplitude responses and its role was more pronounced in the kinetics and velocity of the K+ and SD-like responses, with no significant effect on the amplitude of SD-like events.

We found that it was necessary to focally inject a sufficiently high volume (>0.1 μl) of 50 mM KCl to generate a measurable local increase in [K+]o, with even more required to measure a peri-injection site LFP. Higher volumes (>1 μl) were needed to observe a spreading distal K-LFP response. The amplitude of the K+ response was dependent on the volume of the applied KCl, until a ceiling level was reached, which is in line with studies that reported a smooth decrease of [K+]o from the surface to 3 mm deep into the brain after topical application of KCl solution (Fisher et al., 1976; Cordingley & Somjen, 1978), Cordingley and Somjen (1978) reported that with direct electrical stimulation of the cortical surface, the dissipation rate of accumulated K+ during stimulation was correlated with the response magnitude. The observed rapid dissipation of a local K+ response and the absence of a K+ response remotely with lower volumes of KCl denote a powerful local extracellular K+ buffering system which is designed to maintain the local [K+]o at the baseline level and restore the local K+ homeostasis (Brown, 2017).

We then assessed the debated role of neocortical astrocytic gap junctional coupling, in vivo, as one of the possible contributors in K+ buffering. We proposed that due to blockade of gap junctional coupling (using CBX/Gap27) in the affected area, K+ responses were partially “trapped” locally, hence leading to a partially increased local K+ response. When gap junctional coupling was impaired, the amplitude of the distal K+ response decreased to some extent and the decay time constant increased in both sites, indicating a longer time for K+ washout. Remotely, area under the curve did not change significantly, reflecting the combination of decreased amplitude and increased DTC. Although we demonstrated some changes in amplitude, AUC, RTC and DTC due to gap junctional blockade, these changes were not predominant as they were always less than 22%. Gap junctions seemed to have a more important role in speeding up the K+ and SD-like responses rather than shaping them, as percent variations corresponding to lag time and velocity were above 36%. On the other hand, global opening of the gap junctions (using TMA), permitted slightly enhanced K+ diffusion leading to a fractional decrease in the K+ amplitude in both recording sites by less than 17%, whereas velocity of the K+ and SD-like responses was affected markedly and it was doubled (104% increase). These findings reinforce the concept that gap junctional coupling is more critical for increasing the speed of the spreading responses rather than their amplitudes and shapes as gap junctional modulation did not prevent the K+ response from happening and a large capacity of K+ buffering was conserved in the presence of gap junctional blockers or an opener.

The above findings indicate that astrocytic gap junctional communication has only a partial role in K+ redistribution/buffering. We have demonstrated these findings in vivo in the neocortex, whereas other groups have confirmed our findings in vitro in hippocampal slices. Wallraff et al. (2006) showed that a total interruption of hippocampal astrocytic gap junctional coupling (Cx30/Cx43 knockout), which is known to be essential for K+ redistribution, resulted just in a slight delay in K+ clearance, Breithaupt et al. (2020) also suggested a limited involvement of hippocampal astrocytic gap junctional coupling in extracellular K+ buffering. Moreover, as explained by MacAulay and

| Table 3 | Effect of Gap27 on amplitude, AUC, RTC and DTC at peri & remote injection site plus lag time and velocity of the spreading response. (1/1) indicates increase or decrease of the response in the presence of Gap27. * represents variations of significances. |
|---------|---------------------------------------------------|
|         | n = 6                                                                 |
|         | K+ responses at Peri injection site                 |
|         | K+ responses at remote injection site               |
|         | Spreading from Peri to remote IS                    |
|         | Amp (mM) | AUC (min. mM) | RTC (min) | DTC (min) | Amp (mM) | AUC (min. mM) | RTC (min) | DTC (min) | Lag time (min) | Velocity (mm/min) |
| Gap27   |          |             |          |          |          |             |          |          |              |                   |
| Before Gap27 Ave ± SEM | 10.7±0.3 | 10.5±0.4 | 0.15±0.01 | 1.13±0.05 | 10.2±0.2 | 9.5±0.2 | 0.21±0.02 | 1.05±0.03 | 0.21±0.03 | 9.4±0.9 |
| After Gap27 Ave ± SEM | 12.1±1.0 | 12.5±1.2 | 0.17±0.03 | 1.27±0.10 | 8.9±0.9 | 9.9±0.7 | 0.28±0.08 | 1.19±0.12 | 0.32±0.04 | 5.9±0.4 |
| Ave of individual % variations ± SEM (1/1) | 113.2±2.8* | 120.0±7.0* | 112.9±7.9 | 112.9±5.0* | 112.1±2.5* | 3.6±3.8 | 130.9±11.8 | 113.4±1.6* | 160.3±13.5* | 35.5±5.1* |
| % of group ave variation (1/1) | 129.4* | 119.1* | 111.1 | 112.2* | 112.0* | 3.3 | 131.2 | 113.5* | 152.8* | 37.2* |
| P- 2tail | 0.03 | 0.02 | 0.42 | 0.037 | 0.037 | 0.9 | 0.037 | 0.37 | 0.17 | 0.037 |
|         | 0.045 | 0.02 |
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**Responses at peri-IS**

**Responses at remote-IS**

**Gap27: n=6 mice**

1. K⁺ sensitive electrode
2. Local reference electrode
3. LFP electrode
4. KCl injection micropipette

**K⁺-amp (mM)**
- Control K⁺: 12 ± 1.5
- Gap27 K⁺: 14 ± 2.0

**K⁺-AUC (mM*min)**
- Control K⁺: 20 ± 3.0
- Gap27 K⁺: 24 ± 4.0

**K⁺-RTC (min)**
- Control K⁺: 0.8 ± 0.2
- Gap27 K⁺: 1.2 ± 0.4

**K⁺-DTC (min)**
- Control K⁺: 1.1 ± 0.3
- Gap27 K⁺: 1.5 ± 0.5

**Lag time (min)**
- Control K⁺: 0.2 ± 0.1
- Gap27 K⁺: 0.5 ± 0.2

**Velocity (mm/min)**
- Control K⁺: 12 ± 2.5
- Gap27 K⁺: 14 ± 3.0

*(caption on next page)*
Zeuthen (2012), the notion of K⁺ spatial buffering through gap junctional coupling can only partially explain other concurrent phenomenology seen with raised [K⁺]o, including intracellular astrocytic K⁺ accumulation and K⁺-dependent astrocytic swelling (MacAulay and Zeuthen, 2012). These studies along with our data imply that gap junctionally mediated K⁺ spatial buffering contributes only to part of the overall K⁺ clearance capacity, and the main contributors to K⁺ spatial buffering remain to be investigated.

Our LFP recordings demonstrated negative DC shifts resembling SD-like events which their amplitude and shape were not noticeably affected by gap junctional blockers or an opener. These findings are in line with the studies done by Peters et al. (2003) and Világi et al. (2001) where they did not see a change in SD following CBX application. On the other hand, others blocked SD, using octanol and heptanol (Nedergaard et al., 1995; Martins-Ferreira et al., 2000), most likely because these drugs cause nonspecific blockade of both neuronal and glial gap junctions. It has been proposed that not seeing an effect on SD following astrocytic gap junctional blockage could be explained by the phenomenon that SD is accompanied by a reduction in intracellular pH (Csíka et al., 1985; Gault et al., 1994) and intracellular acidification closes gap junctional communication (Róig et al., 1996; Spray et al., 1981). Therefore, there is a possibility that SD will be more associated with closure rather than coupling of gap junctions (Világi et al., 2001). However, in our experiments, opening instead of closing gap junctions also did not have a noticeable effect on SD amplitude dynamics. As an alternative explanation for our observation, one could argue that astrocytic gap junctions might attenuate SD propagation by facilitating the uptake of K⁺ or glutamate from the extracellular space but simultaneously CX43 and CX30 will mediate SD propagation through enhancing purinergic propagation of astrocytic calcium waves (Thiels et al., 2003). These cumulative enhancing and depressing effects might explain the lack of significant changes on the amplitude and shape of SD-like events after gap junctional modulation.

Gap junctional communication appears to represent only one of several possible contributors to the total K⁺ redistribution phenomenon. The remote K⁺ response then could be an outcome of a combination of processes such as neuronal depolarization triggered by the excess amount of KCl in the local environment. Among the multiple candidates for K⁺ clearance/redistribution, vasculature and capillary endothelium should also be considered in addition to glia and neurons (Fig. 1). Larsen et al. (2014) suggested that, at least in hippocampal slices, a key contributor to interstitial K⁺ regulation is the neuronal and glial Na+/K⁺-ATPase (Larsen and MacAulay, 2014; Larsen et al., 2014). Na+/K⁺-ATPase is also known to have a crucial role in K⁺ clearance following seizure activity (Bazzigaluppi et al., 2017a). In addition to these mechanisms, large levels of extracellular K⁺ reuptake would result in glutamate release (Larsen et al., 2016). This could also increase the neuronal membrane’s permeability to Na⁺ and increase K⁺ release. This in turn increases glutamate release and results in a positive feedback (Larsen et al., 2016). The Astrocytes-Induced Extracellular Acidification mechanism also takes part in K⁺ redistribution and hinders the interstitial K⁺ concentration from rising above a certain level (Ransom, 2000).

Diffusion is also known to have a role in K⁺ dissipation as explained by Fisher et al. (1976) and Cordingley & Somjen (1978), but our observations could not be explained by passive diffusion. The fast dissipation of local K⁺ increases, existence of a threshold before recording the first event, and the fast velocity of propagation of the responses between the two recording sites cannot be explained by passive diffusion as the K⁺ diffusion coefficient in the cortex is about 1.03 ± 0.16 mm²/h (Fisher et al., 1976), denoting a very slow speed for diffusion. In addition, we are sampling a complex 3-dimensional system with only 2 recording sites, which might bias our observations/results.

Aside from dealing with a complex biological mechanism, the experimental design itself had limitations. For instance, gap junctional blockers used in this study are nonspecific and are associated with side effects and probable effects on other processes. CBX blocks pannexin1 and other non-gap junction channels (Ransom, 2006; Larsen et al., 2014), Gap27 is not very potent and TMA is believed to act through elevation of intracellular pH and not specific enough for connexin modulation (Medina-Ceja and Ventura-Mejia, 2010). Despite the concerns, there is already a substantial in vitro literature confirming the efficacy of these drugs on modulating gap junctional coupling (Medina-Ceja et al., 2008; Medina-Ceja and Ventura-Mejia, 2010; Bazzigaluppi et al., 2017b; Evans and Boitano, 2001). Using the FRAP technique, effects of some of these agents on gap junctional uncoupling have been previously demonstrated (Samoilova et al., 2003; Wentlandt et al., 2006b; Samoilova et al., 2008).

One could argue that a caveat to this study is that isoflurane could also act as a potent gap junction channel blocker. However this ability of isoflurane turned to be concentration dependent and have an effect on gap junctional coupling in concentrations higher than what was used in this study. Brain partial pressure will decrease to the maintenance level in a short period of time which is about 3–4 min (Bailey, 1997); therefore, isoflurane during the maintenance phase will not have an effective uncoupling action. Published data in vitro on intravenous versus inhalational anesthetics indicate that the intravenous anesthetics (e.g. thiopental, propofol, ketamine, and dexmedetomidine) also inhibit gap junctional communication at clinically relevant doses for general anesthesia, whereas inhalational anesthetics (e.g. halothane, isoflurane) require doses significantly higher than those required to induce general anesthesia to inhibit gap junctional communication (Wentlandt et al., 2005; Wentlandt et al., 2006a, 2006b; Liu et al., 2016). Miu and Puil (1989) calculated that 1% isoflurane in the gaseous phase is equivalent to 0.2 mM dissolved in ACSF. Manzi et al., 1993 looked directly at the effect of isoflurane on astrocytic gap junctional communication and showed effects at 0.5–2 mM. Peracchia (1991) used 23.6 mM isoflurane to uncouple crayfish axons. In brief, concentrations of isoflurane to obtain strong effects on gap junctional communication are higher than those required to obtain general anesthesia.

It is also important to mention that for such long experiments inhalational anesthetics are the best choice in order to minimize the risks associated with injectable anesthetics. We should reemphasise that isoflurane was used throughout the whole experiment, so if there were any effects of isoflurane, they were present in the baseline and control recordings as well, leading to a reliable comparison between before and after drug application in the presence of isoflurane as an anesthetic. As also demonstrated by other groups studying gap junctional communication, to maintain the anesthetic state of the animal and ensuring the consistency of the isoflurane level throughout the recording, after inducing anesthesia, flowrate was kept constant (1.5–2%) at an optimal
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Responses at peri-IS

Responses at remote-IS

A. Injection Site (IS)
B. K⁺ sensitive electrode
C. Local reference electrode
D. LFP electrode
E. KCl injection micropipette

TMA: n=6 mice

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level throughout the whole experiment (Nilsen et al., 2006; Breithauser et al., 2020; Henneberger et al., 2014; Ander et al., 2014).

In conclusion, we demonstrate that local increases in [K+]o are powerfully regulated, preventing a local or spreading distal K⁺ responses until a threshold amount of K⁺ is locally applied. Further locally applied K⁺ is required to elicit a distal K⁺ response with a concomitant SD-like response. In terms of the many potential factors governing K⁺ buffering, we demonstrate that astrocytic gap junctional coupling only partly modulates K⁺ amplitudes, but has a more pronounced effect on the velocity of the spread of K⁺ and SD-like responses. Much work still needs to be done to unravel many other processes controlling K⁺ buffering and SD propagation in the neocortex, which play important roles in the physiology and pathophysiology of neocortical function.

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References

Anders, S., Minge, D., Griesmann, S., Herde, M.K., Steinhauser, C., Henneberger, C., 2014. Spatial properties of astrocyte gap junction coupling in the rat hippocampus. Philos. Trans. R. Soc. B Biol. Sci. 369 (1654), 20130600.

Bailey, J.M., 1997. Context-sensitive half-times and other decrement times of inhaled LFP responses locally and distally (n = 6). (A) A schematic of electrodes configuration in peri & remote Injection Site (IS). (B) & (C) Blue upward curve represents K⁺ response before TMA, red upward curve represents K⁺ response after TMA; green downward curve represents LFP response before TMA and yellow downward curve represents LFP after TMA. (D) & (E) Box and whisker plots comparing amplitude, AUC, RTC, DTC of K⁺ response before and after TMA application; (B) & (D) is peri-IS and (C) & (E) is remote-IS. (F) Box and whisker plots comparing lag-time and velocity of the K⁺ response spreading from peri to remote-IS before and after TMA. In Box and whisker plots: blue is before and red is after drug application, the smallest and largest data points are shown as lines at the tip of the whiskers, the remaining of the data points are represented as empty circles within the box (in some cases data points were equal to quartiles or were overlapping, X represents the mean and the horizontal line in the box represents median, * indicates significance (p < 0.05). In (B) and (C): black arrows indicate the injection time, #p sign represents the number of puffs focally applied and (#) represents duration of injection in ms (3p(7) ~ 1 μl). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
