Glial cells have long been considered to have only supporting role in the central nervous system. Substantial advances in the past two decades, however, shed light on the various physiological functions they perform and led to the current view that they are active participants of the tripartite synapse. Accordingly, astrocytes are increasingly recognized as important players in the modulation of physiological neuronal function and various pathophysiological conditions and diseases. In addition to modulation by the supplement or lack of metabolic support, they also directly control neuronal activity. In particular, growing evidence demonstrate the involvement of astroglia in epileptiform activity by various mechanisms. In addition to the glia-neuron communication pathways, we have previously revealed, in the current project we aimed to investigate several novel glial mechanisms through which astrocytes can contribute to the generation, propagation and termination of seizure-like events and validate them as potential targets in anti-epileptic drug development in in vitro and in vivo epilepsy models. The work summarized in this report will both deepens our understanding how astrocytes contribute to the emergent neuronal synchronization and overexcitation observed under epileptic conditions and reveals novel potential AED targets, opening up new possibilities for the treatments of epilepsy.

WORK PACKAGE 1

1.1 UNDERSTANDING ASTROCYTIC CONTRIBUTION TO SEIZURE-LIKE EVENTS IN VITRO

In the first work package of the project, we investigated the correlation between neuronal and astrocytic activity in vitro by simultaneous Ca^{2+} imaging in both cell types using a fast, two-photon microscope combined with standard electrophysiological observation of seizure-like events (SLEs) in the low-[Mg^{2+}] epilepsy model. We prepared 400 μm thick hippocampal-entorhinal cortex slices from P11-15 male Wistar rats. The specific astrocyte marker sulforhodamine-101 (SR101, 1 μM) was bulk loaded into astrocytes followed by bulk loading of the Ca^{2+}-sensitive dye Oregon Green BAPTA-AM (OGB, 10 μM) that loads into both neurons and astrocytes.

In order to explore the role of long-range astrocytic communication in SLE propagation, a large field of view (approximately 1x1 mm) was observed. Importantly, despite astrocytic Ca2+ signals are generally considered to be slow (operating on the second- and minute-scale), we previously observed that astrocytic Ca2+ signals during epilepsy can be as fast as neuronal ones. Unfortunately, however, the speed of galvo scanners does not enable to acquire full frame images at this scale if sampling frequency, sufficiently high to follow neuronal and astrocytic Ca2+ transients, needs to be achieved. Therefore, we developed a MATLAB-based custom acquisition script to automatically identify cells before the acquisition process and perform high-speed line scan imaging on these cells. The fully automated acquisition method 1) split the whole field of view into 10x10 overlapping sections; 2) image each section by non-linear zooming to the given region; 3) identify cells with defined size and shape on the zoomed image; 4) classify cells as neurons or astrocytes based on the ratio of intensities of the astrocyte-specific SR101 and the unspecific OGB fluorescence intensities; 5) save the coordinates of the cell's major axis as a line segment; 6) perform line scan measurements on the identified line segments in the whole 1x1 mm field of view. Using this approach, we could identify 189±52 cells (77-326) in the field of view and monitor their Ca^{2+} dynamics with 12 (77 cells) to 29 (326 cells) ms sampling interval. Considering that neuronal and glial Ca^{2+} signals typically lasts >100 ms, this sampling frequency enables high-precision determination of their activation sequence.

The fluorescent signals from neurons and astrocytes were subjected to cross-correlation analysis to determine the correlation and the time lag between the activation of the two cell types. In these data we did not find any lag between CA3 and CA1 cell-level activity or any lag between neuronal and astrocytic Ca2+ transients.

1.2 GENERATION AND VALIDATION OF EPILEPTIC WAG/RIJ RATS EXPRESSING FLUORESCENT CA^{2+} SENSITIVE PROTEIN IN NEURONS AND ASTROCYTES

In order to follow astrocyte activity during epileptic seizures in vivo, our transgenic rat line expressing the Ca^{2+}-sensitive fluorescent protein GCaMP2 was crossbred with WAG/Rij rats. All pups were viable and survived. Normal behavior (e.g., grooming, social interactions etc.) and body weight were observed

in 100% of crossbred rats. Unfortunately, however, only 25 % of crossbred animals displayed epileptic seizures and only to a very limited extent (0-2 SWDs/hour). Next, we crossbred WAG/Rij rats with another transgenic rat line expressing GCaMP6. This attempt also led to viable offspring, but they also did not develop significant amount of seizures even after 8 months. Therefore, as contingency plans, we developed two different methods that allowed us to measure astrocytic Ca2+ activity in naïve WAG/Rij rats. We bulk-loaded astrocytes with OGB-1 AM and imaged the astrocytic activity in the cortex using conventional two-photon imaging and also by the Miniscope approach. Although the Miniscope method offered some advantages, especially the opportunity to measure in awake, unperturbed animals, we also identified some serious disadvantages, that prevented us from routinely applying it. First, it requires a rather long surgical protocol, taking approximately 3 weeks from the first surgery to imaging. Moreover, the camera – and therefore the imaged field – should be fixed days before the actual imaging experiment. Consequently, we obtained rather low success rate for imaging in well labeled areas. Therefore, we opted to fine-tune the surgical and imaging protocols for the conventional two-photon imaging. We successfully developed a method that allowed us to label very high ratio (nearly 100 %) of astrocytes with OGB-1 AM. Since rats are much more difficult to immobilize than mice, motion artefacts had to be handled. To reduce the movements of animals, rats were sedated with a mixture of fentanyl (0.1 mg/kg) + haloperidol (3.0 mg/kg). Head was fixed using a flexible mount on the 2P microscope stage. Body temperature was maintained by a heating pad. A mixture of dyes (0.02 mM SR101 and 0.05 mM OGB) was slowly injected into the subarachnoid space to stain the target brain area.

Later we further improved this experimental method. By proper training of the animals to tolerate immobilization during wake-up (when seizures most frequently occur), we could eliminate the use of the sedation drug haloperidol and still be able to continue imaging during sleep-wake transitions.

WORK PACKAGE 2

2.1. VALIDATION OF ANTI-EPILEPTIC POTENTIAL OF GLIAL GLU/GABA EXCHANGE IN VITRO

We have previously shown that astrocytes are able to significantly contribute to the tonic inhibition of neurons by releasing GABA. The glial Glu/GABA exchange (Héja et al., 2012, 2009) mechanism has been shown to be triggered by glial uptake of synaptically released Glu. Since the Glu transporters gain their driving force from the downhill transport of Na^+ (Héja et al., 2019), their activity increases the intracellular sodium concentration, which, in turn, reverses the GABA transporters that operate close to their equilibrium potential. The released glial GABA (formed from putrescine) activates extrasynaptic $GABA_A$ receptors, therefore provides tonic inhibition to the overexcited neurons. Moreover, the negative feedback provided by the astrocytes is proportional to the network activity, making this mechanism an attractive target for AED development. In the current project, we investigated two pathways, by which the Glu/GABA exchange mechanism can be intensified in order to increase inhibitory feedback during seizures.

In the current project, we investigated what conditions are required to reverse the glial GAT-3 transporter. To determine GAT-3 operation direction, we measured the tonic inhibition changes following application of the GAT-3 specific blocker SNAP-5114 in the in vitro low-[Mg2+] epilepsy model. Removal of Mg2+ from the extracellular medium induced recurrent SLEs in 73 % of the slices. In these slices, application of 100 μM SNAP-5114 decreased tonic current on CA1 pyramidal cells by 20.2 ± 7.4 pA, demonstrating that GAT-3 transporters operate in the reversed direction, releasing GABA to the extracellular space. In the remaining 27 % of slices, however, 100 μM SNAP-5114 increased tonic current by 6.2 ± 2.8 pA, despite the enhanced neuronal activity due to Mg2+ removal. Therefore, GAT-3 reversal cannot be triggered solely by enhanced network activity, it requires actual generation of SLEs. Next, we investigated what are the relative contributions of neuronal vs. glial mechanism to the generated tonic current. We applied 100 μM NNC-711, a specific inhibitor of the neuronal GAT-1 transporter and 20 μM (R)-baclofen, a specific GABAB agonist. Both drugs increased the tonic current of CA1 pyramidal cells by 7.0 ± 3.7 and 4.1 ± 1.9 pA, respectively. Comparing these data to the 20.2 pA tonic current generated by GAT-3 reversal suggests that GAT-3 may play the most prominent role in tonic current generation during seizures.

Next, we explored whether astrocytic GABA production can be increased by direct application of the GABA precursor putrescine (PUT) (Kovács et al., 2022a). Exogenous PUT application was observed to

dose-dependently shorten seizure-like events (SLEs) in the low-[Mg2+] in vitro model of temporal lobe epilepsy. SNAP-5114 reversed the anti-epileptic effect of PUT, further confirming that PUT reduces seizure duration by triggering glial GABA release. In accordance, we observed that PUT specifically reduces the frequency of excitatory synaptic potentials, suggesting that it specifically acts at excitatory synapses. We also identified that PUT specifically eliminated the tonic depolarization-induced desynchronization of SLEs.

2.2. UNDERSTANDING ASTROCYTIC CONTRIBUTION TO SEIZURE-LIKE EVENTS IN VIVO

Applying the improved protocol described in 1.2, we successfully explored cell- and subcellular-level astrocytic Ca2+ activity in high temporal and spatial resolution in WAG/Rij rats in vivo during seizures and control periods. During acquisition, astrocytes and neurons were identified by MATLAB routines online using the method developed in 1.1. Ca2+ signals from SR101 + astrocytes were monitored in line scan mode and analyzed offline. To reveal any plausible concurrent neuron-astrocyte interaction in transgenic WAG/Rij rats, the fluorescent data were correlated to the time-matched electrophysiological LFP recording. The onset and termination of spontaneous SWD on the LFP signal were automatically identified by a custom MATLAB routine. The green fluorescent signal was normalized and fluorescent activity during interictal periods were compared to those in identified SWD periods. These results (Péter et al., publication in preparation) show that during SWDs, astrocytes also display oscillatory Ca2+ activity with a peak of approximately 7 Hz, identical to that of neuronal SWD characteristics. To our knowledge, this is the first recording of seizure-associated astrocytic activity in the literature. Even more importantly, however, wavelet analysis revealed that astrocytes show this oscillatory activity not only during seizures, but also in the control periods. Moreover, the power of 5-10 Hz frequency components was 34 ± 9 % higher in the 10 s periods preceding SWDs than during the SWDs themselves. Similarly, the power of 10-20 Hz frequency components was 72 ± 17 % higher in the 10 s periods preceding SWDs than during SWDs. Astrocytic Ca2+ oscillations with 7 Hz peak frequency appeared in astrocytes up to 20 minutes before the first detectable SWD.

By analyzing the spatial distribution of astrocytic Ca2+ oscillations with 7 Hz peak frequency, we revealed that they first appear in astrocytic processes before getting prevalent in the soma of astrocytes. How such Ca2+ oscillations emerge in astrocytic processes is completely unknown to date. They likely originate from very thin (width < 100 nm) processes that cover the synapse, but Ca2+ activity in these microdomains cannot be resolved by experimental methods. Therefore, to better understand their basic properties, their ability to generate large-scale neuronal synchronization and also to identify potential drug targets by which these pathological neuronal synchronizations could be prevented, we continued our ongoing efforts to simulate Ca2+ dynamics in astrocytic leaflets. First we showed that activity of Na+/Ca2+ exchanger (NCX) alone has been able to generate markedly stable Ca2+ oscillation in an astrocytic leaflet model (Héja and Kardos, 2020). Continuing this approach, we used experimentally determined genuine 3D geometries of 208 excitatory synapses and simulated [Ca2+] in the surrounding astrocytic processes under control conditions and following synaptic Glu release (Héja et al., 2021). Applying this model, we demonstrated that the surface/volume ratio of peri-synaptic astrocytic processes is the main parameter that defines whether or not spontaneous [Ca2+] oscillations shall arise due to NCX activity. These results further support the principal role of the dynamically changing astrocyte shape in the generation of intrinsic [Ca2+] oscillations and their spreading over larger astrocytic compartments and potentially to neuronal ensembles.

It is also to note that we successfully obtained astrocytic calcium imaging with very high sampling rate (> 50 Hz) that helped us to reveal high-frequency astrocytic Ca2+ oscillations that previous studies were completely unaware.

WORK PACKAGE 3

3.1. EXPLORING NEW ASTROCYTIC TARGETS IN VITRO

The synthesis of PUT involves copper amino oxidase (Kardos et al., 2018). Therefore, we explored whether the Cu+/Cu++ homeostasis is involved in astrocytic control on neuronal excitability by regulating polyamine metabolism (Szabó et al., 2021). We observed that the blockade of copper

transporter (CTR1) by AgNO3 (3.6 μM) prevents GABA transporter-mediated tonic inhibitory currents, indicating causal relationship between copper (Cu+/Cu++) uptake and the catabolism of PUT to GABA in astrocytes. In addition, we showed that MnCl2 (20 μM), an inhibitor of the divalent metal transporter DMT1, also prevents the astrocytic Glu-GABA exchange. Furthermore, we found that facilitation of copper uptake by added CuCl2 (2 μM) boosts tonic inhibitory currents. These findings corroborate the hypothesis that modulation of neuron-glia coupling by copper uptake drives PUT to GABA transformation, which leads to subsequent Glu-GABA exchange and tonic inhibition.

3.2. VALIDATION OF ANTI-EPILEPTIC POTENTIAL OF IDENTIFIED ASTROCYTIC TARGETS IN VIVO

The primary objective of this project is to explore and identify potential drug target proteins and mechanisms based on the deeper understanding of astrocytic contribution to seizure generation and maintenance. To this end, we addressed two potential anti-convulsive astrocytic targets, the connexin proteins and the astrocytic GABA transporter subtypes.

By elucidating the role of gap junctions in epilepsy, we have shown (Vincze et al., 2019) that blockade of gap junctions by the non-specific inhibitor carbenoxolone (CBX) significantly suppressed epileptic activity in the low-[Mg^{2+}] epilepsy model. In contrast, opening of the gap junctions by trimethylamine (TMA, 5 mM) increased the ratio of SLE appearance to 100 % of slices. Furthermore, TMA also significantly decreased the onset of the first SLE and the average interictal interval. Interestingly, however, the duration of SLEs were also decreased.

Surprisingly, the same drugs showed opposite effects *in vivo* in WAG/Rij rats. Intraperitoneal administration of CBX (100 mg/kg) increased the SWD number compared to control. In TMA treated (200 mM in 5 μl) rats, the number of SWDs have decreased.

Next, we identified the connexin subtype responsible for in vitro and in vivo seizure formation. We showed that a Cx43-specific antibody and the hemichannel inhibitor La^{3+} could completely eliminate in vitro SLEs, while the Cx36-specific quinine displays only limited anticonvulsive effect both in vitro and in vivo. These results validate the astrocyte-specific Cx43 connexin isoform as a potent mediator of epileptiform activity.

Furthermore, since utilization of Cx43 as an anti-epileptic target is largely hindered by the lack of its specific inhibitors and the deeply limited understanding of the gap junction formation process, we explored the potential binding sites of Cx43 by molecular modelling and molecular dynamics studies (Héja et al., 2022; Simon et al., 2020).

WORK PACKAGE 4

4.1. EXPLORING NEW ASTROCYTIC TARGETS IN VIVO

Since opening and inhibition of gap junctions resulted in differential effects in the in vitro model of a convulsant epilepsy (temporal lobe epilepsy) and in an in vivo model of a non-convulsant epilepsy (absence epilepsy), in the last phase of the project, we focused on utilizing the Glu/GABA exchange mechanism by various ways.

First, we explored whether astrocytic GABA production can be increased by direct application of the GABA precursor PUT (Kovács et al., 2022a). We explored the potential of PUT to inhibit spontaneous SWDs in WAG/Rij rats. Similarly to the anti-convulsant effect demonstrated in the in vitro low-[Mg2+] model described in 2.1, significant shortening of SWDs in response to intraperitoneally applied PUT has been observed. Importantly, this effect could be antagonized by blocking GAT-2/3-mediated astrocytic GABA release with the specific inhibitor SNAP-5114. Direct application of exogenous GABA also reduced SWD duration, suggesting that PUT-triggered astroglial GABA release through GAT-2/3 may be a critical step in limiting seizure duration.

Next, we explored whether alteration of the polyamine metabolism in favor of the putrescine (PUT) to GABA conversion may be beneficial in epilepsy (Kovács et al., 2022b). Utilization of the Glu/GABA exchange mechanism in epilepsy is possible by increasing the astrocytic putrescine concentration via the inhibition of putrescine-spermidine conversion. Additionally, intervention into the polyamine metabolism may also offer other ways to combat seizures. The polyamine spermine (SPM), synthesized

form PUT through spermidine (SPD) is known to unblock astrocytic Cx43 gap junction channels and therefore may facilitate astrocytic synchronization. Moreover, SPM released from astrocytes may modulate neuronal NMDA, AMPA and kainate receptors. As a consequence, astrocytic polyamines possess the capability to significantly modulate epileptiform activity. We investigated different steps in polyamine metabolism and coupled GABA release to assess their potential to control seizure generation and maintenance both in the low-[Mg2+] model of temporal lobe epilepsy in vitro and in the WAG/Rij rat model of absence epilepsy in vivo. We found that inhibition of SPD synthesis completely prevented seizure generation in WAG/Rij rats. This anti-epileptic effect is attributed to the subsequent enhancement of PUT to GABA conversion in astrocytes, leading to GABA release through GAT-2/3 transporters. Our findings conclusively suggest that the major pathway through which astrocytic polyamines contribute to epileptiform activity is the production of GABA.

In addition to the polyamine metabolism, we also investigated whether increasing the surface expression of astrocytic GABA transporters (through which GABA is released) is a viable strategy to combat epilepsy. To this end we explored the mechanism of action of the FDA-approved drug levetiracetam. Levetiracetam is effective against absence epilepsy, but the mechanism by which it attenuates seizures is not understood. Importantly, levetiracetam has been shown previously to increase the surface expression of GAT-2/3 transporters. In the current project we showed (Kovács et al., 2022b) that levetiracetam was effective against absence epilepsy in the WAG/Rig rat model in vivo and its anti-epileptic effect could be reversed by application of the specific GAT-2/3 transporter blocker SNAP-5114, demonstrating that the GAT-2/3 expression enhancement is the major route through which levetiracetam exerts its anti-epileptic effect.

In summary, we successfully

1) Identified and validated the glial connexin isoform Cx43 as a potential anti-epileptic target in convulsant epileptiform activity (Vincze et al., 2019). We also explored the potential drug-interaction sites of Cx43 in order to advance drug development (Héja et al., 2022; Simon et al., 2020).
2) Revealed that exogenous application of putrescine can be a viable strategy to enhance the anti-epileptic potential of the astrocytic Glu/GABA exchange (Kovács et al., 2022a).
3) Identified and validated the anti-epileptic potential of intervention into the polyamine metabolism in epilepsy (Kovács et al., 2022b). Importantly, application of the spermidine synthase inhibitor 4-MCHA, which increases putrescine level and correspondingly enhance the Glu/GABA exchange mechanism, completely eliminated seizures in the in vivo absence epilepsy model.
4) Explored the potential of Cu+/Cu++ homeostasis to control on neuronal excitability by regulating polyamine metabolism (Kardos et al., 2018; Szabó et al., 2021).
5) Identified the mechanism of action of the clinically used anti-epileptic drug Levetiracetam. We showed that it mainly exerts its anti-convulsant effect by increasing the surface expression of astrocytic GAT-3 transporter (Kovács et al., 2022b).
6) Imaged astrocytic Ca2+ activity for the first time in in vivo rats during seizures (Péter et al., publication in preparation). We revealed that astrocytes do not only display similar oscillations as neurons, but Ca2+ oscillations with SWD-like frequency appear in astrocytes before neurons. These results suggest that astrocytic activity play a causal role in the generation of in vivo seizures.
7) Explored the role of the Na+/Ca2+ exchanger (NCX) to generate spontaneous astrocytic Ca2+ fluctuations (Héja et al., 2021; Héja and Kardos, 2020).

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