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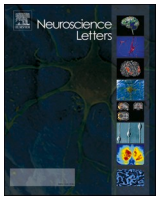

Research article

Galanin diminishes cortical spreading depolarization across rodents – A candidate for treatment?

Fátima Gimeno-Ferrer [a,c], Annett Eitner [b], Hans-Georg Schaible [a], Frank Richter [a,*]

[a] Institute of Physiology 1/Neurophysiology, Jena University Hospital, D-07740 Jena, Germany
[b] Department of Trauma, Hand and Reconstructive Surgery, Experimental Trauma Surgery, Jena University Hospital, D-07740 Jena, Germany
[c] Present address: University of Augsburg, Faculty of Medicine, Institute for Theoretical Medicine, Vascular Biology Lab, D-86159 Augsburg, Germany

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ABSTRACT

Galanin (Gal) is a neuropeptide with the potential to ameliorate cortical spreading depolarization (CSD), an electrophysiological phenomenon occurring after brain injury or in migraine aura. Gal is expressed in all cortical neurons both in rat and in mouse cortices. Here we investigated whether the effect of Gal on CSD previously described in the rat is conserved in the mouse cortex. In rats, the topical application of Gal to the cortex for 1 h did not induce any change in CSD amplitudes, propagation velocity, or threshold of elicitation. Rather, topical application of Gal for 3 h was necessary to obtain a significant decrease in these CSD parameters and to develop a remarkable increase in the KCl threshold to elicit a CSD in rat cortex. In contrast, the topical application of Gal on cortical surface for 1 h in mice was sufficient to significantly attenuate CSD amplitudes and increase threshold. A thinner cortex, a faster diffusion or different affinity/expression of receptors for Gal are possible reasons to explain this difference in the time course between rats and mice. Our data are relevant to postulate Gal as a potential target for inhibition of CSD under pathological situations such as stroke or ischemia.

Significance statement: The neuropeptide Galanin (Gal) is expressed in all neurons throughout the cerebral cortex, both in rats and mice, and is able to reduce or even inhibit Cortical Spreading Depolarization, thus, Gal has the potential to control neuronal excitability that may identify Gal as a target in drug development against CSD.

1. Introduction

Cortical Spreading Depolarization (CSD) is a massive depolarization wave that propagates in the cortical grey matter under pathological conditions such as stroke, ischemia, and migraine aura. CSD is a self-regenerating process that leads to a potentially toxic overload of the intraneuronal space with sodium and calcium ions [1]. If prolonged, this leads to cell death. Recovery from a CSD consumes a large amount of energy for the activation of membrane pumps. In the case of severe ischemia, this energy is not available in sufficient quantities, so that the neurons cannot recover from CSD and die. Therefore, it is essential to identify molecules and mediators that modulate CSD in order to reduce the damage that these waves can generate in the cortex.

The neuropeptide Galanin (Gal) is involved in a wide range of functions and shows inhibitory effects in epilepsy and CSDs. The role of Gal in epilepsy has been widely described, including the development of seizure activity in knockout mice for Gal or Gal receptors (GalRs). A restoration of the normal activity was achieved with exogenous application of Gal [2–5]. A link between Gal and CSD was first shown by upregulation of Gal and GalR1 mRNA that has been detected 7 to 28 days after CSD [6]. Recently, our group described that Gal decreased amplitudes and propagation velocity of CSD and increased the threshold for triggering CSD by KCl in rat cortices [7]. However, the magnitude of the effect of Gal on CSD amplitude and threshold in rats was variable: some rats responded to Gal with a complete abolition of CSD or increase in threshold, and other animals showed no change in CSD parameters [7]. Although only few studies have unraveled the role of Gal in cortex, its function seems to be essential to control excitability as all cortical neurons in rats express this neuropeptide, and the main action is the modulation of cortical excitability [7]. These findings support the neuroprotective role of Gal in cortical functions.

The present study assessed the effect of Gal on CSDs in mouse cortex in order to test whether the Gal effect on CSD is conserved in rodents. The effect of Gal on CSD in mouse and rat cortex was compared for the main CSD parameters.

* Corresponding author at: Institute of Physiology 1/Neurophysiology, Jena University Hospital, Teichgraben 8, 07743 Jena, Germany.
E-mail address: Frank.Richter@med.uni-jena.de (F. Richter).

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2. Materials and methods

This study was approved by the Government of Thuringia (UKJ-17-037; UKJ-19-023) and conducted in accordance with the Animal Welfare Act of the Federal Republic of Germany. Animals were treated in accordance with the Declaration of Helsinki and the guidelines for the care and use of animals. Data collection, analysis and presentation were performed according to the ARRIVE guidelines.

2.1. Histology

Naïve rats (n = 3) and mice (n = 3) were perfused with PBS buffer under deep anesthesia with sodium thiopental (Trapanal®, Inresa, Freiburg, Germany) until euthanasia, followed by perfusion with 4 % ice-cold phosphate-buffered paraformaldehyde (PFA; Sigma-Aldrich, Saint Louis, USA, MI). Brains were removed, postfixed in 4 % PFA for at least 24 h, equilibrated in 30 % sucrose and frozen at −80 °C. Coronal slices of 10 μm thickness were cut using a Leica CM3050S cryostat (Leica Biosystems, Nussloch, Germany). Immunolabeling protocol was performed according to Gimeno-Ferrer et al. [7]. The antibody rabbit anti-Gal (1:100; Enzo BML-GA1161-0100, Enzo Life Sciences GmbH, Lörrach, Germany) as primary antibody and Alexa Fluor 488 goat anti-rabbit (1:200; #A11008 Thermo Fisher Scientific, San Diego CA, USA) as secondary antibody were used. For staining of GalRs in mice the antibodies rabbit anti-GalR1 (1:50 Alomone #AGR-011, Alomone Lab Ltd., Jerusalem, Israel), rabbit anti-GalR2 (1:100 Alomone #AGR-012), and rabbit anti-GalR3 (1:50 Novus Biologicals NLS204, Novus Biologicals LLC, Centennial, CO) were used as primary antibodies and Alexa Fluor 488 goat anti-rabbit as secondary antibody for green color. Negative control experiments were performed without primary antibodies. Images were captured using a TCS SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). Contrast and brightness of the micrographs were adjusted using Image J.

2.2. Surgery and electrophysiology

Adult male Wistar rats (n = 7 for Gal, n = 6 for controls; 350–450 g, aged 10 to 15 weeks, housed in the Animal Facility of the University Hospital Jena) and male C57BL/6 mice (n = 11 for Gal, n = 6 for controls; 20–30 g, aged 15 to 25 weeks) were deeply anaesthetized with sodium thiopental (initially 100–125 mg/kg intraperitoneally [i.p.]). During surgery, the depth of anesthesia was regularly monitored, and additional doses of sodium thiopental i.p. (maximum 20 mg/kg) were administered if necessary. The trachea was cannulated for spontaneous breathing. Electrocardiogram was monitored. Body temperature was maintained at 37 °C. Surgical preparation of the skull with trephination was performed according to the previously described protocol [8]. In rats, two trephinations were made over the left hemisphere of the skull to expose the brain using a minidrill and cooling during the procedure with artificial cerebrospinal fluid (ACSF). The composition of ACSF was (mmol/L): NaCl 138.4, KCl 3.0, $CaCl_2$ 1.3, $MgCl_2$ 0.5, NaH_2PO_4 0.5, urea 2.2 and glucose 3.4, pH 7.4, warmed to 37 °C and equilibrated with 5 % CO_2 in O_2. In mice, the head was fixed, and a single trephination was performed over the left hemisphere of the skull, exposing the brain. The underlying dura and arachnoid were removed, and the exposed cortex was kept moist with ACSF. A barrier of dental acrylic was placed on the skull only around the frontal trephination in rats and the trephination in mice, forming a pool with a capacity of 100 μL for topical application of Gal to a restricted cortical area.

For recording, an Ag/AgCl reference electrode (containing 2 mol/L KCl) was placed on the nasal bone. Electrodes for direct current (DC) electrocorticogram (ECoG) recordings had a tip diameter of approximately 5 μm, resistance of < 5 MΩ. A microinjector (PLI-100; Harvard Apparatus, Holliston, MA) was used to trigger CSD waves by injections of 0.5 μL 1 mol/L KCl solution with a pressure of 100 kPa. Injection times ranged from 0.1 to 1 s. If a KCl-microinjection of 0.1 s did not trigger a CSD, the injection time was increased in steps of 0.25 s after waiting an appropriate time interval. In both rodents, the KCl pressure-evoked electrode was connected to DC_{rear}. In rats, DC_{rear} was located at a depth of 1200 μm from the cortical surface. Electrode DC_{front} was placed at a depth of 400 μm from the cortical surface. In the mouse, electrodes DC_{rear} and DC_{front} were placed at a depth of 200–250 μm. The depth of 400 μm in rat and 200 μm in mouse cortex corresponds to layer III (Fig. 1).

In rat, two CSDs were induced in each animal by KCl microinjection 20 min apart prior to Gal application to confirm the ability to produce CSDs at all (ACSF in both trephinations) and to establish them as controls. Gal (TOCRIS, Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany) diluted in PBS was then applied topically at the frontal trephination at a concentration of 10^{-7} mol/L. The posterior trephination was kept moist with ACSF throughout the experiment. After Gal application, CSDs were elicited every 30 min for 3 h. In mice, 2 control KCl injections were performed at 20 min intervals before Gal (ACSF) and then Gal 10^{-7} mol/L was applied for 1 h. CSDs were elicited every 30 min after Gal. Six control mice and six control rats with the same surgery underwent the same KCl injection protocol. In these controls only ACSF was applied onto the cortical surface for the whole observation time.

CSDs were evaluated for occurrence in the treated area, maximal amplitudes relative to baseline before the depolarization, duration at half-maximal amplitude, propagation time from elicitation site to frontal area and changes in KCl application time.

To analyze the depression patterns of ECoG activity associated with CSD, DC signals were resampled offline to a rate of 205 Hz and first detrended by appropriate adaptive filtering, followed by bandpass filtering (0.01–45 Hz). To reveal alternating current (AC) ECoG activity, the signals were high-pass filtered with a lower frequency cut-off of 0.5 Hz.

2.3. Data statistics

Bar graphs are presented as mean ± standard error. Line graphs show time course and mean ± standard error at each time point. Scatter plots represent the distribution of individual data-points. Statistics were performed using one-sample *t*-test (against reference) for amplitude and threshold, unpaired *t*-test (Student) for comparisons between control and Gal groups, or Wilcoxon test comparing CSD velocity before and after Gal. Calculations were performed using InStat (Graph Pad, San Diego CA, USA). Significance was accepted at $p < 0.05$.

3. Results

3.1. Expression of Gal and of GalRs in mouse cerebral cortex

It was previously reported that Gal is expressed by all cortical neurons in rats [7]. Here we confirm that Gal expression is conserved in rodents, as we found the same expression in mouse cortex. Therefore, neuronal Gal should play an important role in the rodent cortex (Fig. 2). Commercial antibodies for mouse GalRs stained GalR1 and GalR3 in some neurons, but did not label for GalR2, suggesting that GalR2 is not expressed in mouse cortex (Suppl. Fig. 1). Therefore, the neuronal location of all three GalRs described for rat [7] could not definitely be confirmed in mice.

3.2. Effect of Gal on CSD amplitudes

Topical application of Gal to the cortex decreased CSD amplitudes and velocity and increased the KCl threshold. Fig. 3 shows a representative example of the effect of Gal on rat and mouse CSD.

In rats it was possible to record CSD with two sets of electrodes in two different cortical areas, one treated with Gal 10^{-7} mol/L and the other untreated (ACSF) (see Fig. 1A). An “all or none” response to Gal 10^{-7} mol/L was observed, with some animals responding to the neuropeptide

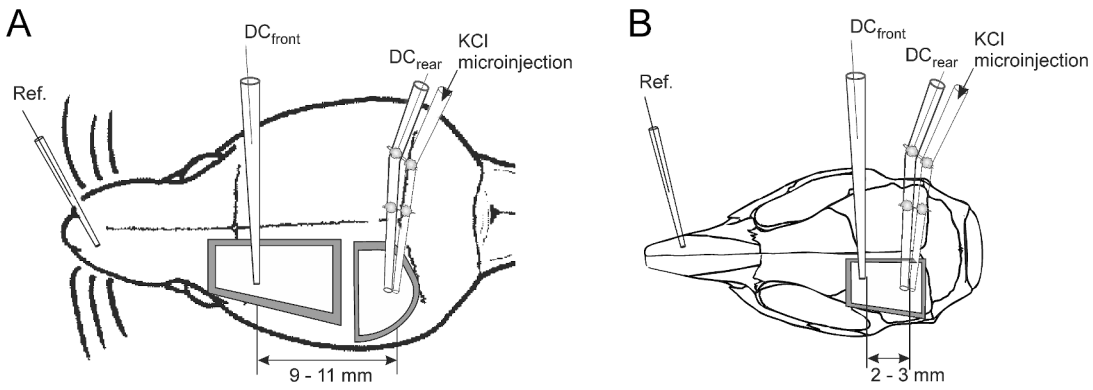

Fig. 1. Scheme of rat and mouse skull with electrodes for CSD recording. **A)** Rat skull with two trepanations and electrodes (KCl microinjection for CSD elicitation and DC_{rear} in the untreated posterior area; and DC_{front} in the treated frontal area). **B)** Mouse skull with only one trepanation and the same electrode distribution.

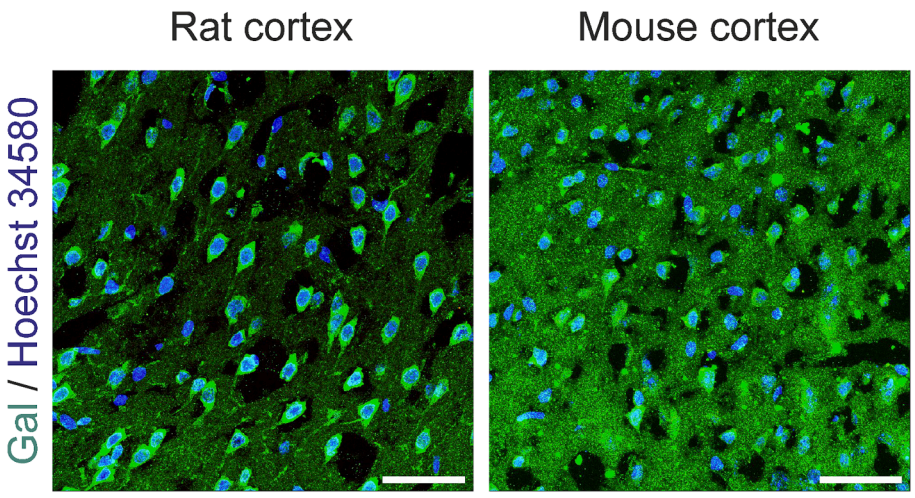

Fig. 2. Cortical localization of Gal was conserved in rodents. By immunolabelling with anti-Gal (green), the expression of the neuropeptide was found in all cortical neurons in rat (left) and mouse cortex (right). Nuclei labelled with Hoechst 34580 (blue). Scale bars 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

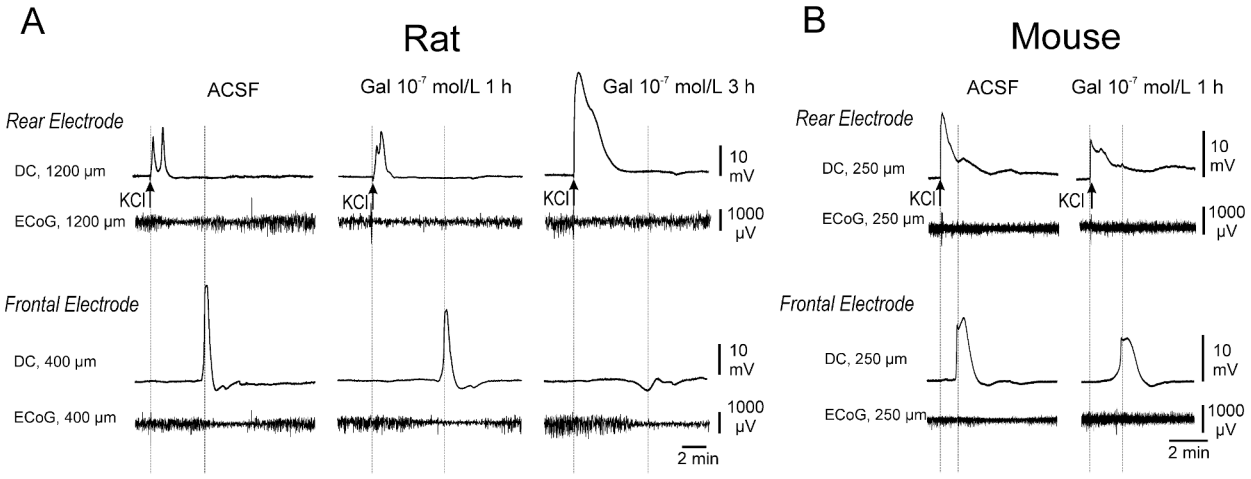

Fig. 3. Effect of Gal on CSD parameters in rodents. **A)** Representative CSDs in rat cortex from rear and frontal areas of the cortex before (ACSF) and after 1 h and 3 h of Gal 10^{-7} mol/L application. **B)** CSD example from mouse cortex before (ACSF) and after topical application of Gal 10^{-7} mol/L for 1 h. In A) and B), arrows mark KCl microinjection to induce CSD. The Direct Current electrocorticogram (DC-ECoG) and the corresponding high-pass filtered ECoG data (0.5–45 Hz) are shown to demonstrate the depression of ECoG activity. Note that in A, after 3 h Gal, the DC shift at DC_{rear} is greater because a greater amount of KCl was required to induce a CSD (increased threshold).

and others with CSD not affected by Gal. It was necessary to apply Gal for 3 h to visualize a consistent effect of reduced mean amplitude (Fig. 3A). A similar effect was seen for CSD propagation velocity and threshold. Note that in Fig. 3A, at 3 h Gal, a larger CSD was recorded in DC1 due to the increase in KCl threshold.

In mice, CSDs were recorded with two sets of electrodes placed only in one area in the cortex due to the animal size (see Fig. 1B). After establishing CSD control parameters (amplitude, velocity, and threshold), Gal was applied at a concentration of 10^{-7} mol/L for 1 h and induced comparable effects in all animals tested. This application time was sufficient for Gal to induce in mouse cortex the effects on CSD previously reported in rat cortex (Fig. 3B). However, in rats, this short application time was not sufficient to induce any effect on CSD and the 3 h application was necessary to show inhibitory signs on CSD.

When analyzing each parameter in detail, the Gal-induced reduction of CSD amplitudes (normalized to percentages, where control amplitude is 100 %) was conserved in both rodents. In rats, CSD amplitudes showed a slight decrease to 84 % within the first 1 h of Gal application. A significant decrease (p-value = 0.0275) to 53 % was observed only after 3 h of Gal treatment, with almost complete abolition in 2 animals. However, several rats showed no change in amplitudes even after 3 h of Gal. In control rats, an insignificant decline in CSD amplitudes in the

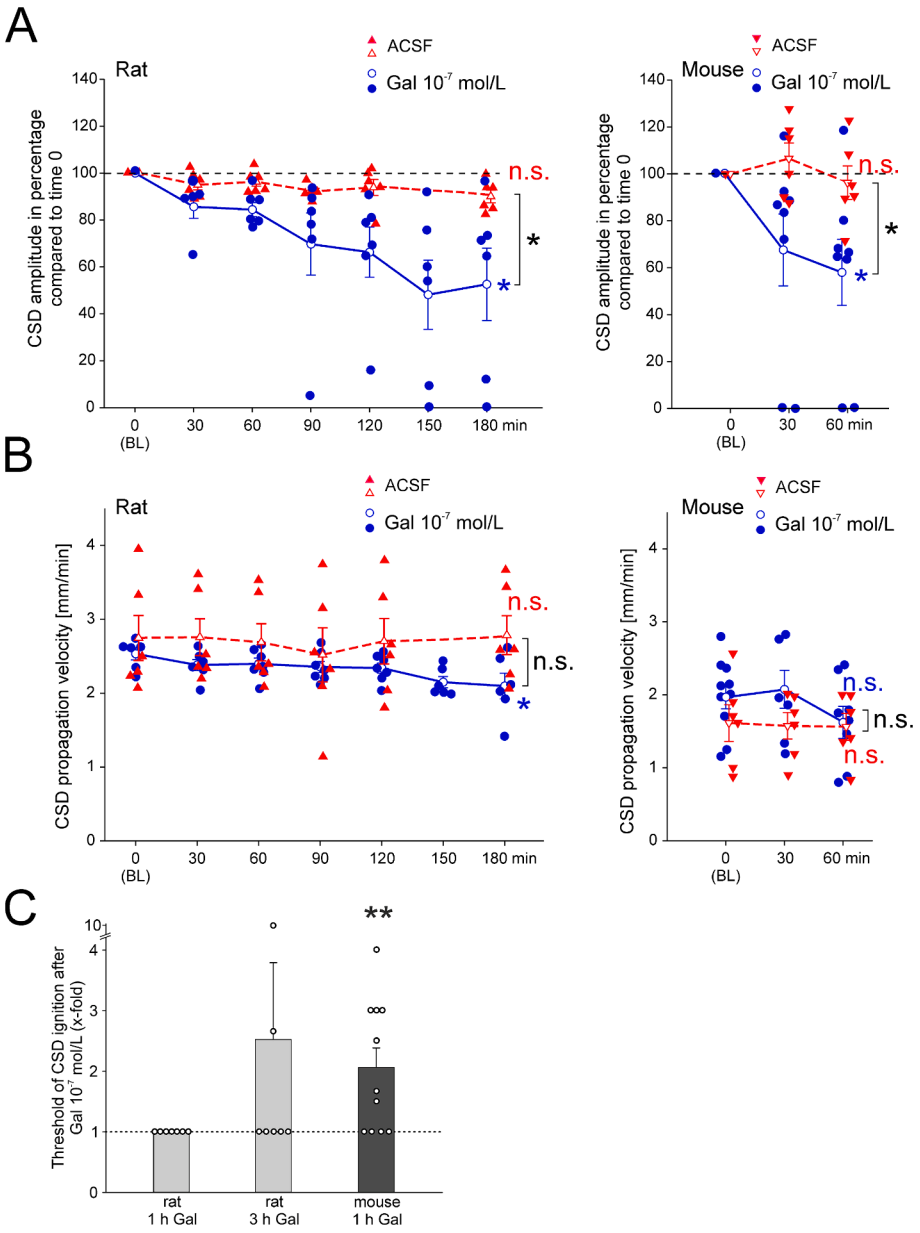

Fig. 4. Effect of Gal on CSD parameters in rodents. **A)** Effect of ACSF (dashed red lines) or of Gal 10^{-7} mol/L (solid blue lines) on CSD amplitudes. Values are normalized as percentages of the control amplitude (set at 100 %, dashed black line). Gal induced a decrease in CSD amplitudes in both rodents. **B)** Effect of ACSF (dashed red lines) or of Gal 10^{-7} mol/L (solid blue lines) on CSD propagation velocity. In A) and B), empty dots and triangles represent mean ± standard error of amplitude and velocity respectively, filled dots and triangles represent single data points. **C)** Effect of Gal on CSD initiation threshold (initial threshold during ACSF is fixed as 1, dashed line). Gal at 10^{-7} mol/L increased the application time of KCl required to elicit a CSD in rodents. Bars represent mean increase ± standard error (x-fold compared to control set as 1), dots represent individual data points. Discrepancies between number of animals and data points amongst A) and B) are due electrode failure. Non-propagating CSD have no velocity data. In A) and C) one-sample *t*-test was performed between the actual value and the reference (set at 100 % in A and set at 1 in C). In A) significance in colored symbols. In A) and B) the unpaired *t*-test was performed between data at 180 min Gal vs. ACSF (rat) or at 60 min (mouse), significance in black symbols. In B), Wilcoxon test between control velocity and velocity after Gal was performed, colored symbols. *p < 0.05; **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

same time range reached 86 % of the baseline values. This decline differed significantly from the amplitude decrease in Gal treated rats (*t*-test Gal vs control, p-value = 0.038).

In mice, Gal is able to reduce the amplitude down to 67 % of control amplitude after only 30 min of treatment and significantly to 57 % after 1 h of treatment (p-value = 0.0198). In 2 animals, complete abolition of CSD was observed within 30 min of application. In control mice treated with ACSF only, CSD amplitudes went down to 91 % of baseline. As in the rats, the decline in CSD amplitudes in Gal-treated mouse cortex was significantly larger (*t*-test Gal vs. control, p-value = 0.0484). Longer treatment with Gal in mice was discarded because the effect of Gal after 1 h was comparable to the effect in rats after 3 h of Gal. Overall, the amplitude effect was more consistent and faster in mouse cortex (Fig. 4A).

3.3. Effect of Gal on CSD propagation velocity

The propagation velocity of the CSD spread (mm/min) was determined for ACSF during the control phase. Gal at 10^{-7} mol/L induced an increase in CSD propagation time from the elicitation site (rear) to the recording site (frontal). In rats, 1 h of Gal-application induced a decrease in CSD propagation only to 95 % of the initial velocity (from 2.53 to 2.38 mm/min), and a statistically significant decrease (p-value = 0.0313) to 83 % of the CSD propagation velocity was observed after 3 h (2.09 mm/min). In control rats, in the same time interval CSD propagation did not change (from 2.77 to 2.81 mm/min). However, in mice, a decrease in CSD velocity to 82 % of the baseline was observed after 1 h of Gal, with a non-statistically significant slowing of velocity (from 1.97 to 1.62 mm/min). Interestingly, slight decline in CSD propagation velocity to 96 % of the baseline (from 1.61 to 1.55 mm/min) was also seen in control mice, Therefore, we cannot exclude that i) the surgical preparation of the mouse brain itself, and ii) the insertion of two sets of glass microelectrodes at a short distance had partially contributed to the slowed CSD propagation (Fig. 4B).

3.4. Effect of Gal on KCl threshold to elicit CSD

The last CSD parameter studied was the elicitation threshold (KCl application time to elicit a CSD). The use of a microinjector guaranteed same amounts of KCl when the same injection time was used and higher amounts of KCl with increased injection times. The lower the amount of KCl to ignite a CSD wave, the higher the susceptibility to CSD and the more excitable the brain. The initial threshold (microinjection time of KCl to induce CSD in controls with ACSF) was normalized to 1 (for 1-fold). Gal is able to increase the threshold. In rat cortex, no change was observed after 1 h of Gal. Further application of Gal to rat cortex for 3 h showed an insignificant increase in KCl threshold (2.52 ± 1.27-fold). In contrast, in mice, after 1 h of Gal 10^{-7} mol/L, the threshold for CSD elicitation was significantly increased to 2.06 ± 0.32-fold (p-value = 0.0084), indicating that a double amount of KCl is required for CSD elicitation (Fig. 4C). In contrast, both in control rats and in control mice the elicitation threshold for CSD did not change (maintained at 1, data not shown).

4. Discussion

CSD is critical to the impact and outcome of cortical pathologies such as migraine aura, or stroke, or traumatic brain injury (TBI) [1,9]. Regulation and minimization of CSD is essential to reduce cortical damage under pathological conditions. Gal emerges as a neuropeptide capable of modulating and inhibiting CSD: it is able to reduce susceptibility to the development of CSD (increasing threshold) and to reduce the severity of CSD (decreasing velocity and amplitude) and, in some animals, to inhibit it completely.

Gal has been extensively studied in relation to epileptic activity in the hippocampus [2–5], with a manifest epileptic status in knockout mice for Gal and GalRs, suggesting Gal as an endogenous antiepileptic mediator [10]. Moreover, a link between patients with Temporal Lobe Epilepsy (TLE) and mutations in Gal gene was established [11]. In line with this, it was shown that washout of Gal after 3 h of topical application to the rat cortex induced epileptic discharging activity in some animals [7]. Furthermore, we show that Gal is capable to reduce CSD. This present report, together with Gimeno-Ferrer et al. [7], demonstrates the conserved role of Gal on the rodent cortices and its promising therapeutic effect to improve the clinical outcome of CSD-related pathologies. Therefore, it is plausible that the role of Gal is maintained in more rodents, or even in humans.

Several findings indicate that Gal decreased susceptibility to CSD. Gal increased the threshold for triggering CSD by KCl. If CSD were triggered, Gal decreased CSD amplitudes, and slowed their propagation. The interference of Gal with CSD is plausible, since Gal reduced the release of glutamate in hippocampus [12] or suppressed the seizure development in mice [13]. Gal is one of the neuropeptides discussed to be a candidate for the treatment of epilepsy [14].

Gal is similarly widely expressed in rat and mouse cortex. Therefore, we expected a similar distribution of GalRs in the mouse cerebral cortex as previously shown in rats [7]. There is only sparse information on GalRs in mice in the literature: Allen Institute's mouse brain map (based on antisense mRNA) shows only expression of GalR3, but not of GalR1 and GalR2 [15]. Our staining experiments at least agreed with these data when showing GalR3 expressed at neocortical neurons, but we also found a weak staining of GalR1. Gundlach's group showed that there is a general lower [^{125}I]-Gal binding in C57BL/6J than in 129OlaHsd mice and low or even absent [^{125}I]-Gal binding in neocortex [16]. By contrast, mouse cerebellum showed increased levels of GalR1 mRNA expression during the first three weeks in postnatal life [17], suggesting that Gal receptors have a regulatory role in the developing brain. According to our previous rat experiments that confirmed the location of GalRs on rat neurons [7], the action of Gal at the GalR3 could explain the decrease in CSD amplitudes, since GalR3 is linked both to $G_{i/o}$-type G protein to block the adenylate cyclase and to activate GIRK ion channels [18]. The even quicker effect of Gal in mice further underlines the presence of GalRs in murine cortex. It is likely that the quicker effect after topical application of Gal is due to the thinner cortex of mice (in this study records in both rodents were made in layer III) with faster diffusion of Gal through the cortex, but a higher affinity of murine GalRs for Gal cannot be ruled out from our data.

Currently, the COSBID society, experts on the management and treatment of pathologies associated with CSD, recommend the use of ketamine to reduce or minimize the impact of CSD waves on cortex [19]. However, the use of these drugs may be critical, and an exhaustive control is mandatory because of its actions as anesthetic. From this point of view, Gal as endogenic neuropeptide could be under consideration for a better clinical management of CSDs. The upregulation of Gal following CSD [6] suggests that Gal may be an endogenous regulator to restore normal cortical excitability in pathologic situations involving CSD. Notably, this upregulation takes time raising the possibility that this regulatory mechanism develops only slowly.

It is not always the case that reducing cortical excitability improves the patient's outcome by reducing the susceptibility to seizures and/or CSD. Rather a continuum of vulnerability to CSD is discussed that should be based on altered metabolic capacity of the brain tissue [9,20]. There is essential need of development of new drugs to control CSD or the identification of compounds that modulate it in order to reduce the use of narcotics in the clinics for the management of CSD-related pathologies [21]. The development of Gal agonists or compounds based on Gal structure/function may be a target of interest as drug candidates.

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6. Disclosures

All authors have nothing to report.

Author contributions

FGF, AE, H-GS and FR conceived and designed research, performed experiments, analyzed and interpreted results, drafted the manuscript, and approved final version of manuscript.

CRediT authorship contribution statement

Fátima Gimeno-Ferrer: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Annett Eitner:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Hans-Georg Schaible:** Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Frank Richter:** Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2024.137814.

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