Galanin expressed in the excitatory fibers attenuates synaptic strength and generalized seizures in the piriform cortex of mice

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Abstract

The neuropeptide galanin is considered to be an endogeneous antiepileptic agent, presumably acting via inhibition of glutamate release. Previously, we have demonstrated that in mice ectopically overexpressing galanin in cortical and hippocampal neurons, particularly in granule cells and their axons, the mossy fibers, hippocampal kindling epileptogenesis is suppressed, and is associated with attenuated frequency facilitation in mossy fiber-CA3 cell synapses. We hypothesized that changes in synaptic transmission might occur also in other excitatory synapses of the galanin overexpressing (GalOE) mouse, contributing to seizure suppression. Lateral olfactory tract (LOT) synapses, formed by axons of olfactory bulb (OB) mitral cells and targeting piriform cortex (PC) pyramidal cells, ectopically express galanin in GalOE mice. Using whole-cell patch-clamp recordings, we found that excitatory synaptic responses recorded in PC pyramidal cells during high frequency stimulation of the LOT were attenuated in GalOE mice as compared to wild-type controls. This effect was mimicked by bath application of galanin or its agonist galnon to wild-type slices, supporting the notion of ectopic galanin action. Since the high frequency activation induced *in vitro* resembles epileptic seizures *in vivo*, we asked whether the observed synaptic inhibition would result in altered epileptogenesis when animals were kindled via the same synapses. In male GalOE mice we found that the latency to convulsions was prolonged, and once animals had experienced the first stage 5 seizure, generalized seizures were less sustainable. These data indicate that the PC is a possible target for epilepsy treatment by ectopically overexpressing galanin to modulate seizure activity.

Keywords: epilepsy, kindling, olfactory bulb, neuropeptides, patch-clamp, brain slices

Introduction

Galanin is a 29 amino acid neuropeptide (Tatemoto, K., Rokaeus, A., et al. 1983) which is widely distributed throughout the peripheral and central nervous system, and is involved in a broad range of brain functions such as food intake (Kalra, S.P. and Horvath, T.L. 1998; Leibowitz, S.F. 2005), cognition (Crawley, J.N. 1996; McDonald, M.P., Willard, L.B., et al. 1998; Ogren, S.O., Schott, P.A., et al. 1998), mood (Barrera, G., Echevarria, D.J., et al. 2005; Fuxe, K., Jansson, A., et al. 1998; Weiss, J.M., Bonsall, R.W., et al. 1998), and pain (Liu, H.X. and Hokfelt, T. 2002; Wiesenfeld-Hallin, Z. and Xu, X.J. 2001; Xu, X.J., Hokfelt, T., et al. 2000) . In the brain, galanin co-exists with other neurotransmitters like catecholamines, GABA and 5-hydroxytryptamine (Melander, T., Hokfelt, T., et al. 1986), and is thought to act by modifying the release of these neurotransmitters. Three receptors for galanin have been cloned, GalR1-3 (Branchek, T., Smith, K.E., et al. 1998; Branchek, T.A., Smith, K.E., et al. 2000; Iismaa, T.P. and Shine, J. 1999), and they are found in the locus coeruleus, hypothalamus, dorsal raphe nucleus, amygdala, and at more moderate levels in the prefrontal cortex and hippocampus (Jacobowitz, D.M., Kresse, A., et al. 2004; O'Donnell, D., Mennicken, F., et al. 2003), correlating well with the distribution of galanin-immunoreactive (IR) nerve terminals. In the olfactory system, including the olfactory tubercle and piriform cortex (PC), a high density of galanin binding sites is detected alongside a sparse number of galanin containing fibers (Jacobowitz, D.M., Kresse, A., et al. 2004; Melander, T., Kohler, C., et al. 1992).

In the rat hippocampus, a common structure of seizure generation in human temporal lobe epilepsy, galanin is found mainly in noradrenergic fibers stemming from the locus coeruleus (Gabriel, S.M., Knott, P.J., et al. 1995; Melander, T., Staines, W.A., et al. 1986; Xu, Z.Q., Shi, T.J., et al. 1998), but there is also evidence

for cholinergic and serotonergic inputs (Gabriel, S.M., Knott, P.J., et al. 1995; Melander, T., Hokfelt, T., et al. 1986; Melander, T., Staines, W.A., et al. 1985). Galanin-like immunoreactivity (LI) in these fibers is dramatically decreased 24 hours after chemically or electrically induced seizures (Mazarati, A.M., Liu, H., et al. 1998). At the same time, galanin mRNA is upregulated in the hippocampal formation, as detected by quantitative real-time polymerase chain reaction (PCR) (Wilson, D.N., Chung, H., et al. 2005), and galanin-LI is increased in neurons of the dentate gyrus (Fetissov, S.O., Jacoby, A.S., et al. 2003; Mazarati, A.M., Liu, H., et al. 1998; Wilson, D.N., Chung, H., et al. 2005). Similarly to other neuropeptides such as NPY (Vezzani, A. and Sperk, G. 2004), these seizure-induced changes in galanin expression have been taken to suggest that galanin is involved in seizure regulation, presumably exerting a protective action. Indeed, accumulating evidence indicates that galanin can modulate seizure activity in the brain, as shown in different animal models of epilepsy (Kokaia, M., Holmberg, K., et al. 2001; Mazarati, A.M. 2004; Mazarati, A.M., Hohmann, J.G., et al. 2000; Mazarati, A.M., Liu, H., et al. 1998; Mazarati, A.M., Lu, X., et al. 2004b; Mazarati, A.M. and Wasterlain, C.G. 2002; McColl, C.D., Jacoby, A.S., et al. 2006). For example, infusion of exogeneous galanin into the rat dentate gyrus before or during the self-sustained phase of status epilepticus (SE) shortened or completely abolished seizure activity (Mazarati, A.M., Liu, H., et al. 1998; Mazarati, A.M. and Wasterlain, C.G. 2002). In line with these observations, galanin gene knockout mice were shown to be more prone to develop SE seizures, while transgenic mice overexpressing the galanin gene in noradrenergic neurons under the dopamine β-hydroxylase promoter were more resistant to the induction of SE (Mazarati, A.M., Hohmann, J.G., et al. 2000). Earlier, we have demonstrated that even ectopic galanin overexpression under the PDGF-B promotor suppressed hippocampal kindling

epileptogenesis in mice, and was associated with attenuated frequency facilitation in the mossy fiber - CA3 cell synapses (Kokaia, M., Holmberg, K., et al. 2001).

The mechanisms of antiepileptic action of galanin are not well understood. However, it has been shown that galanin, via interaction with its receptor subtypes GalR1 and -3 (Branchek, T., Smith, K.E., et al. 1998; Branchek, T.A., Smith, K.E., et al. 2000; Iismaa, T.P. and Shine, J. 1999), can cause the opening of ATP-dependent K^+ channels (Kask, K., Berthold, M., et al. 1997; Zini, S., Roisin, M.P., et al. 1993) and/or blockade of voltage gated Ca^{++} channels (Palazzi, E., Felinska, S., et al. 1991). These effects of galanin can presynaptically lead to decreased glutamate release, and thus inhibition of seizure activity. The role of the galanin receptor 2 (GalR2) is less explicit. GalR2 has been shown to increase Ca^{++} mobilization in different cell types including neurons *in vitro* (Kerekes, N., Mennicken, F., et al. 2003; Smith, K.E., Forray, C., et al. 1997), and has also been suggested to partly mediate the anti-epileptic effect of galanin *in vivo* (Mazarati, A.M. and Lu, X. 2005; Mazarati, A.M., Lu, X., et al. 2004a).

To date, evidence for the involvement of galanin in epileptic activity is limited to its action in the hippocampus, whereas its role in other pathways crucial for the progression of seizures remains unexplored. One such area is the PC, which has been shown to play an important role in the seizure generalization process (Haberly, L.B. and Sutula, T.P. 1992; McIntyre, D.C. and Plant, J.R. 1989). The PC, also called primary olfactory cortex due to its major input from the olfactory bulb (OB), is directly and reciprocally connected to the limbic system, i.e. the amygdala and the hippocampus, via the entorhinal cortex (Carlsen, J., De Olmos, J., et al. 1982; Krettek, J.E. and Price, J.L. 1977; Ottersen, O.P. 1982; Russchen, F.T. 1982). In animal studies, the PC has been shown to be most susceptible to electrical or chemical

induction of limbic seizures (Loscher, W. and Ebert, U. 1996), and bilateral lesion of the PC blocked generalization of seizures induced by kindling from the hippocampus or the OB (Loscher, W. and Ebert, U. 1996). It is thought that the extensive network of associative fibers found within the deep layers of the PC underlies a multiplication of the excitatory input from limbic structures, thus triggering the generalization of seizure activity (Haberly, L.B. and Sutula, T.P. 1992; Hoffman, W.H. and Haberly, L.B. 1991; McIntyre, D.C. and Plant, J.R. 1989; Racine, R.J., Mosher, M., et al. 1988).

In the present study we aimed to explore the effect of ectopically overexpressed galanin on synaptic plasticity and kindling epileptogenesis in excitatory circuits of the PC. We particularly chose the synapses formed by OB mitral cell axons onto the pyramidal cells of the PC, because (1) galanin is ectopically expressed in the OB mitral cell bodies and axons of galanin overexpressing (GalOE) mice (Kokaia, M., Holmberg, K., et al. 2001; Kuteeva, E., Calza, L., et al. 2004), and (2) these synapses can play an important role in the spread of epileptic seizures from the OB.

Materials and Methods:

Animals

Adult (3 to 5 months old) male and female GalOE mice and WT littermates were housed with a 12-h light/dark cycle and *ad libitum* access to food and water. GalOE mice were generated as described earlier (Kokaia, M., Holmberg, K., et al. 2001), and the transgenic status of animals was confirmed by PCR (Kokaia, M., Holmberg, K., et al. 2001). All experiments were performed according to National Institutes of Health guidelines and approved by the local Ethical Committee.

Electrophysiology

Mice were anaesthetized and decapitated, and brains were removed and placed in ice-cold artificial cerebrospinal fluid (aCSF), which was gassed by a mixture of 95 % O_2 and 5 % CO_2. The composition of aCSF was as follows (in mM): NaCl (119), KCl (2.5), $MgSO_4$ (1.3), $CaCl_2$ (2.5), $NaHCO_3$ (26.2), NaH_2PO_4 (1) and glucose (11). Coronal slices of 300 µm thickness were cut through the anterior PC (1.7 to 0.0 mm anterior to bregma) (Franklin, K.B.J. and Paxinos, G. 1997) on a Vibratome (TPI, Redding, CA), and were stored submerged in a chamber with gassed aCSF. For electrophysiolgical recordings, the slices were transferred to the recording chamber, which was permanently perfused with gassed aCSF (4 mL/min) at room temperature. Paired-pulse (with interstimulus intervals, ISIs, of 25, 50, 100 and 200 ms, frequency 0.067 Hz) or high frequency stimulations (HFS, 10 pulses at 40 Hz) were delivered via a stainless steel bipolar electrode placed in the lateral olfactory tract (LOT) at the PC level. Field excitatory postsynaptic potentials (fEPSPs) were recorded using a pipette filled with 3M NaCl (resistance 0.5–1 MOhm) placed in the outer plexiform layer of the PC. The potentials were amplified and filtered at 1 kHz, and sampled at 10 kHz on EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Whole-cell patch-clamp recordings with visual approach enabled by IR-DC videomicroscopy were performed with glass pipettes filled with (in mM): Cs-gluconate (97.5), CsCl (17.5), HEPES (10), BAPTA (10), NaCl (8), MgATP (2), GTP (0.3), and QX-314 (5), with osmolarity of 295 mOsm, and resistance of 4-6 MOhm. EPSCs were recorded at a holding potential of -70 mV. The EPSCs were filtered at 2.9 kHz and sampled at 10 kHz with the same amplifier. Voltage-step commands at the end of each recorded trace were applied to constantly monitor the series resistance, and recordings were discarded when it varied more than 20 %. All data were stored on a G4 Macintosh

computer for off-line analysis. The measurements of the initial slope of the fEPSPs were made over 1-2 ms, and paired-pulse facilitation (PPF) was calculated as percentage change of the second fEPSP slope as compared to the first one. The average synaptic response during HFS was calculated by dividing the total integral of all ten EPSCs in the train (total response current, 0-400 ms) by the integral of the first EPSC (0-25 ms). Galanin (Bachem, Bubendorf, Switzerland) and galnon (a kind gift from Dr. T. Bartfai, La Jolla, CA, USA) were bath-infused into the recording chamber for 10 min at a concentration of 0.5 µM and 1 µM, respectively.

Kindling

Twenty-two mice (12 GalOE and 10 WT) were used in total. Animals were placed in a Kopf stereotaxic frame under halothane anaesthesia. Bipolar stainless-steel stimulating/recording electrodes were implanted into the right OB (1.0 mm anterior to the rostral confluens of sinuses, 1.0 mm lateral to midline, 1.2 mm ventral to dura; tooth bar at 0) (Ferland, R.J. and Applegate, C.D. 1998), and reference electrodes were placed between the skull and the right temporal muscle. Electrodes were connected to a plastic pedestal and fixed to the skull bone using dental cement. Animals were allowed to recover for one week after surgery before kindling was initiated. Kindling stimulations (1 ms square wave pulses of 100 Hz for 1s) were given once a day at threshold intensity determined on the first days of stimulation. The thresholds were defined by inducing stimulations at increasing current intensity (by 10 µA steps), starting with 10 µA, until focal epileptiform activity (afterdischarge, AD) of at least 5 sec duration could be elicited. Behavioural seizures were scored according to the scale of Racine et.a. (1988) (stage 1: facial twitches; stage 2: chewing and nodding; stage 3: forelimb clonus; stage 4: rearing, body jerks, tail rising; stage 5:

imbalance). The group identity of individual animals was unknown to the experimenter conducting the scoring of seizure stages. Electroencephalographic (EEG) activity was recorded on a MacLab system before, during, and at least 1 min after the end of each AD. Animals were considered kindled after having exhibited 5 stage 5 seizures. Four weeks after termination of kindling, the AD threshold was re-determined and mice were re-kindled using the same stimulation parameters as during initial kindling. Mice were considered re-kindled after exhibiting 3 stage 5 seizures. Animals were killed 24 h after the last re-kindling stimulation for immunohistochemistry. Unstimulated GalOE and WT mice were used to determine baseline galanin levels by immunohistochemistry.

Immunohistochemistry

Mice were deeply anesthetized with pentobarbital and perfused through the ascending aorta with 0.9 % NaCl followed by 4 % paraformaldehyde (PFA). After post-fixation in PFA (overnight, 4° C), brains were transferred to 20 % sucrose and stored overnight at 4° C. Slices (30 µm thick) were cut on a microtome and collected in antifreeze solution. Free-floating sections were rinsed in PBS and processed according to the protocol for the tyramide signal amplification system (TSA) (Adams, J.C. 1992) using a commercial kit (NEN Life Science Products, Boston, MA, USA). Briefly, quenched (0.03 % H_2O_2 for 30 min) sections were incubated with rabbit antiserum to rat galanin (Theodorsson, E. and Rugarn, O. 2000) for 3 days at 4° C, rinsed, and incubated with secondary biotinilated goat-anti rabbit antibody. Further incubation with Streptavidine-HRP, biotinyl tyramide reagent and Alexa-488 fluorophore finalized the reaction. The specificity of antibody binding was confirmed by omission

of the primary antibody on a subset of sections (Kokaia, M., Holmberg, K., et al. 2001; Kuteeva, E., Calza, L., et al. 2004).

Statistical Analysis

Statistical analysis of differences between groups was performed using Student's unpaired *t*-test. Differences were considered significant at $p < 0.05$. Data are presented as average ± SEM.

Results

Galanin expression in GalOE mice

In GalOE mice, galanin-LI was found in the mitral cells of the olfactory bulb (Fig. 1 A, insert) and the outer plexiform layer of the PC, where the LOT and its presynaptic endings are located (Fig.1 B). In WT animals, on the other hand, galanin-LI was in these areas not higher than background staining, suggesting absence of galanin (Fig. 1 C, D). These data are supported by *in situ* hybridization studies showing galanin mRNA expression in mitral cells as well as in the deep layers of the PC in GalOE mice but not in WT animals (Kokaia, M., Holmberg, K., et al. 2001; Kuteeva, E., Calza, L., et al. 2004). However, no galanin-IR cell bodies could be detected in the PC. This pattern of galanin-LI in the OB and PC of GalOE and WT mice was not changed substantially 24 h after the last re-kindling stimulation.

Basal synaptic transmission

We first explored whether basal synaptic transmission in LOT synapses was affected by galanin overexpression. We stimulated the LOT with increasing intensity every 20 sec and recorded fEPSPs in the outer molecular layer of the PC. Resulting fEPSP

slopes were plotted against the amplitude of the presynaptic fiber volley (PSFV), as shown for averaged data in Fig. 2 A. There was no difference between these curves in slices from GalOE and WT mice, indicating that the basal synaptic transmission was not changed in the LOT synapses of GalOE mice.

Short-term synaptic plasticity

Next we investigated PPF, a form of short-term synaptic plasticity determined predominantly by presynatic mechanisms (Zucker, R.S. and Regehr, W.G. 2002; Zucker, R.S. and Stockbridge, N. 1983), in LOT synapses. The LOT was stimulated with paired-pulses with ISIs of 25, 50, 100 and 200 ms. Analysis of induced fEPSPs showed no significant alterations in PPF in LOT synapses of GalOE mice at any ISI studied as compared to WT animals (Fig. 2 B). These data indicate that galanin overexpression had no effect on PPF in GalOE mice.

High Frequency Stimulation

We hypothesized that during the low frequency paired-pulse or single stimulations of LOT, overexpressed galanin was not released in sufficient amounts from the presynaptic terminals to affect excitatory glutamatergic transmission in these synapses. To facilitate galanin release, we therefore increased the number and frequency of stimulations applied to the LOT to 10 stimulations at 40 Hz. In these experiments, postsynaptic responses (i.e. EPSCs) evoked by LOT stimulation were recorded from individual pyramidal cells of the PC using the whole-cell patch-clamp method. We observed that the total synaptic response was considerably smaller in GalOE (Fig. 3 B) as compared to WT mice (Fig. 3 A). Quantitative analysis from several cells showed a significant reduction of the synaptic responses in GalOE mice

as compared to the WT controls during HFS (Fig. 3 C, D). These results were taken to suggest that galanin was released from LOT synapses during HFS and could inhibit glutamatergic transmission in these synapses. To confirm that this effect was indeed caused by extracellular galanin, we next applied galanin through the perfusion medium onto the slices from WT animals during HFS, and compared resulting EPSC responses to those before galanin application. The results clearly demonstrated that galanin (Fig. 4 A), as compared to aCSF (Fig. 4B), caused a significant reduction of the total synaptic responses induced by HFS of the LOT. Averaged data are presented in Fig. 4 **C**. To further substantiate our results, we used the non-peptide analog of galanin, galnon (Saar, K., Mazarati, A.M., et al. 2002; Sollenberg, U., Bartfai, T., et al. 2005), in the same experimental paradigm as galanin. Similar to galanin, galnon (Fig. 4 D), as compared to aCSF (Fig. 4E), led to decreased EPSC responses after HFS of the LOT. Moreover, we were able to observe a partial wash-out of the galnon effect, probably due to the fact that it is a non-peptide analog of galanin and is more readily washed out from the slice tissue (Fig. 4 F).

Kindling and re-kindling

Next we asked whether the changes observed in LOT synapses would affect spread and generalization of seizures from the OB to the PC via these synapses. To address this question, we used kindling in the OB, from which the LOT represents the main output to the PC (Ojima, H., Mori, K., et al. 1984; Price, J.L. 1973; Schwob, J.E. and Price, J.L. 1984; Shipley, M.T. and Adamek, G.D. 1984). In these experiments both male and female mice were used. When comparing the kindling development between GalOE and WT animals, we did not find significant differences in the average seizure stages (Fig. 5 A) or the number of stimulations required to reach different seizure

stages and the fully kindled stage (Fig. 5 B). The threshold currents to induce ADs, as well as the duration of the ADs and behavioral seizures were also similar in both groups (Table 1).

Since female hormone fluctuations have been reported to influence kindling epileptogenesis (Ebert, U., Rundfeldt, C., et al. 1994; Edwards, H.E., Burnham, W.M., et al. 1999; Teskey, G.C., Hutchinson, J.E., et al. 1999) and galanin levels in the brain (Hilke, S., Theodorsson, A., et al. 2005), we speculated that the use of female mice could introduce high variation in our kindling data. To rule out this possibility, we excluded females from the analysis. The kindling development even in this case was not significantly different between GalOE and WT mice (Fig. 6 A). However, after exhibiting the first stage 5 seizure we observed a significant increase in latency to behavioural convulsions during the consecutive stage 5 seizures (Fig. 6 B), as well as a significantly higher number of stimulations required to induce further stage 5 seizures in GalOE mice as compared to WT animals (Fig. 6 C). When animals were re-kindled four weeks after the last kindling stimulation, no difference between the groups of males was found in the number of stimulations needed to express three stage 5 seizures (7.7 ± 1.5 stimulations for GalOE and 9.0 ± 3.1 for WT, respectively), the latency to occurance of the first stage 5 seizure (1.5 ± 0.3 stimulations for GalOE and 4.5 ± 2.9 for WT, respectively), or any of the other seizure parameters studied (data not shown).

Discussion

Here we demonstrate that galanin overexpressed ectopically in mitral afferents selectively modulates high frequency excitatory synaptic transmission, as well as attenuates some parameters of generalized seizures induced by kindling in the OB.

This finding shows for the first time that galanin overexpressed in the forebrain outside the hippocampal formation has a significant effect on excitatory synaptic transmission, which could contribute to its seizure-inhibiting action in the brain.

The analysis of immunoreactivity as well as our electrophysiological data suggest that galanin overexpressed in the mitral cells of the OB can be transported anterogradely to the PC and presumably released there, most likely during high frequency synaptic activity (Hokfelt, T. 1991; Merighi, A. 2002; Zupanc, G.K. 1996). As neuropeptides in general are released “extrasynaptically”, they may diffuse in the extracellular space to affect galanin receptors at some distance, so called volume transmission (Fuxe, K. and Agnati, L.F. 1991). Therefore it is conceivable to suggest that in addition to LOT, other PC synapses could also be affected by galanin. In fact, a high densitiy of ^{125}I-galanin binding sites, as well as GalR1 mRNA, have been demonstrated throughout the rat PC (O'Donnell, D., Mennicken, F., et al. 2003).

Present data indicate that endogenously overexpressed galanin in the excitatory synapses can modulate synaptic responses. This galanin-induced modulation of LOT synaptic transmission was observed only during HFS, while PPF and the input-output relationship were not altered. This observation suggests that a sufficient amount of galanin is released only during repetitive, harsh synaptic activation, resembling epileptic seizures. Exogenous application of galanin or its non-peptide analog, galnon, had a similar, although less pronounced effect in LOT synapses of WT mice during HFS. This could be due to the lack of activity-dependent release of galanin from LOT synapses during the course of HFS in WT animals (as opposite to what is probably the case in GalOE mice), resulting in less inhibitory effect on glutamate release, and, consequently, less pronounced suppression of successive EPSCs, as compared to GalOE mice. It has to be mentioned, however,

that galnon has recently been shown to act not only at GalR1-3 receptors but also other G-protein-coupled receptors and both at extra- and intracellular sites (Floren, A., Sollenberg, U., et al. 2005). The present results are well in line with our previous observations showing that overexpressed galanin attenuates frequency facilitation of fEPSPs in mossy fiber-CA3 cell synapses of the hippocampus (Kokaia, M., Holmberg, K., et al. 2001). Taken together, these data support the idea that ectopic galanin might act via suppression of glutamate release from the presynaptic sites (Palazzi, E., Felinska, S., et al. 1991; Zini, S., Roisin, M.P., et al. 1993).

Despite the pronounced effect on LOT synapses *in vitro*, the kindling epileptogenesis from the OB was unaltered in GalOE animals as compared to WT mice. Neither threshold nor duration of ADs in the OB was different between the GalOE and WT mice (see Table 1), all speaking against a local effect of galanin in the OB (McIntyre, D.C., Kelly, M.E., et al. 1999). It seems reasonable to conclude that the observed decrease in high frequency synaptic transmission in LOT synapses was not sufficient to inhibit the strength and rate of seizure propagation from the OB to the PC in GalOE mice. However, once male GalOE mice had expressed the first generalized stage 5 seizure, they needed significantly more stimulations to reach the fully kindled state (determined as 5 stage 5 seizures), compared to their WT counterparts. In the same group, the onset of generalized convulsions became significantly delayed, while it remained unchanged in control animals. These changes associated with the later stages of kindling could occur if galanin inhibited the recurrent network in the deeper layers of the PC, which are thought to be responsible for the spread and expression of generalized seizure activity in the brain (Loscher, W. and Ebert, U. 1996). Therefore, one could speculate that galanin in GalOE mice either diffused and reached the deep PC layers after being released from the LOT synapses

during seizure activity, or, alternatively, was released after being translated into the peptide from the mRNA found in the deep PC layers (Kokaia, M., Holmberg, K., et al. 2001; Kuteeva, E., Calza, L., et al. 2004).

When animals were re-kindled, no differences could be found between the genotypes in any of the kindling parameters evaluated. The loss of effect on generalized seizures in the re-kindling period could be due to several reasons. Downregulation of galanin may have occurred, which seems unlikely, since our immunohistochemical and in-situ hybridization analysis show no significant changes of galanin levels in GalOE mice after re-kindling. Alternatively, the expression of galanin receptors, or their downstream signaling, could have changed. Seizure-induced downregulation of GalR2 (but not GalR1) has been reported 20 h after SE (Lu, X., Mazarati, A., et al. 2005). However, this issue needs further, more detailed investigation.

The pattern of action of overexpressed galanin on seizures seems to be region-specific. This idea is supported by the fact that kindling in the OB and in the hippocampus are differentially affected in the same strain of GalOE mice because, in contrast to the present results, hippocampal kindling was significantly delayed in GalOE mice as compared to the WT controls (Kokaia, M., Holmberg, K., et al. 2001). In line with this hypothesis, recent data also show that viral vector-induced overexpression of galanin confined to specific brain areas is able to suppress different aspects of seizure activity in models of epilepsy (Haberman, R.P., Samulski, R.J., et al. 2003; Lin, E.J., Richichi, C., et al. 2003). Animals transfected with the galanin gene in the hippocampus developed a significantly lower number of seizures and of shorter duration after intrahippocampal kainic acid injection as compared to control animals (Lin, E.J., Richichi, C., et al. 2003). Transfection of the galanin gene into the

inferior colliculus suppressed post-stimulus wild-running when seizures were induced by brief electrical stimulations of the inferior colliculus (Haberman, R.P., Samulski, R.J., et al. 2003).

Our present data also indicate that regions outside the hippocampal formation, such as the PC, could be considered as a possible target for viral vector-based overexpression of galanin as a novel approach to epilepsy treatment. It remains to be explored, however, whether such overexpression would be more effective within the recurrent neuronal networks residing in the deeper layers of the PC, which are responsible for the seizure generalization process, rather than in the superficial layers.

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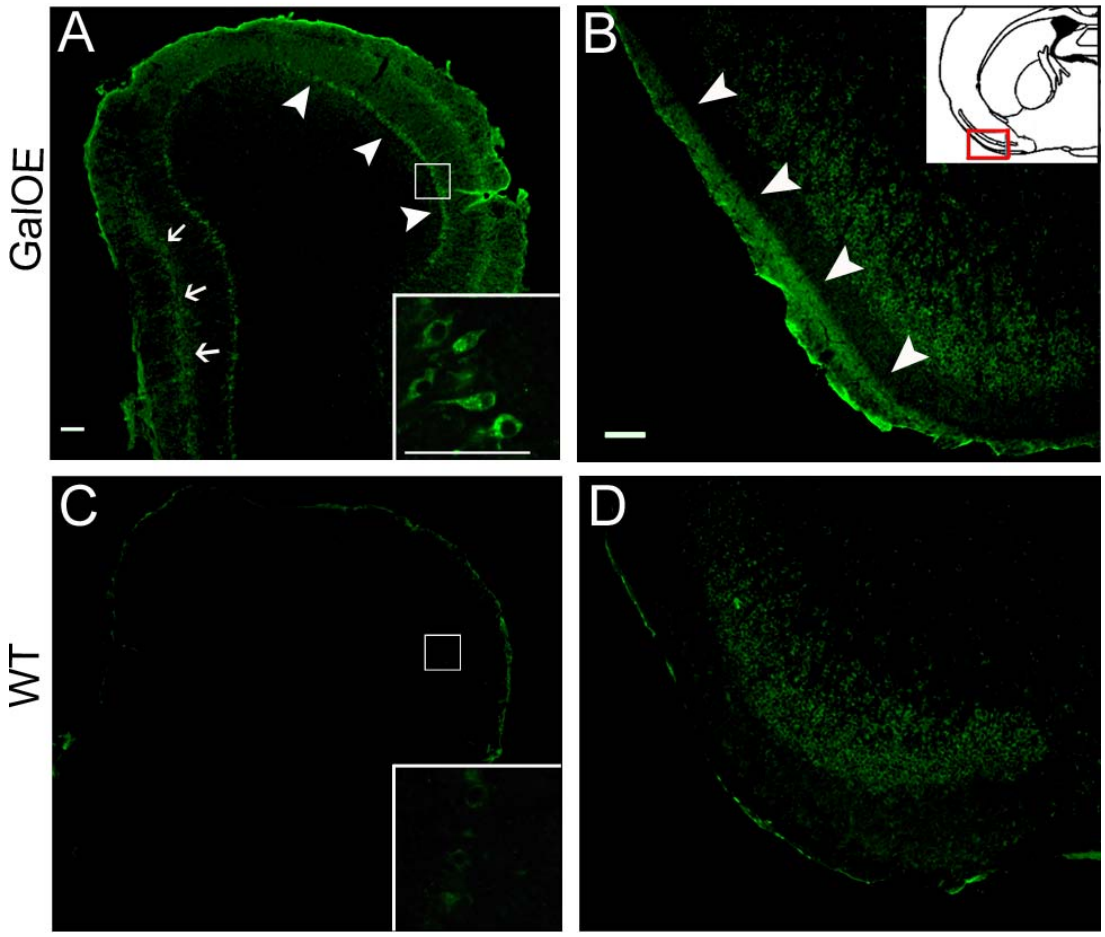

Fig. 1. Expression and distribution of ectopic galanin protein in the OB and PC of GalOE and WT mice. Microphotographs show galanin-LI in the OB **(A, C)** and the PC **(B, D)** of GalOE **(A, B)** and WT **(C, D)** mice. **(A, C)** In the OB, galanin-LI is mainly present in the mitral cells (arrowheads) and in the periglomerular layer (arrows) of the GalOE mouse, whereas no certain specific staining can be seen in the WT mouse. Inserts in A and C show mitral cells at higher magnification**.** In the PC of the GalOE mouse galanin-LI is seen in the outer plexiform layer (arrowheads) of the PC **(B)**. No corresponding staining can be seen in the WT mouse **(D)**. Insert in B shows the approximate location of microphotographs in B and D. Images are representative for the galanin immunoreactivity in WT and GalOE mice. All brain

sections were treated in the same staining session, images were digitally aquired and processed identically. Scale bars are 100 µm (A = C, including insert, B = D).

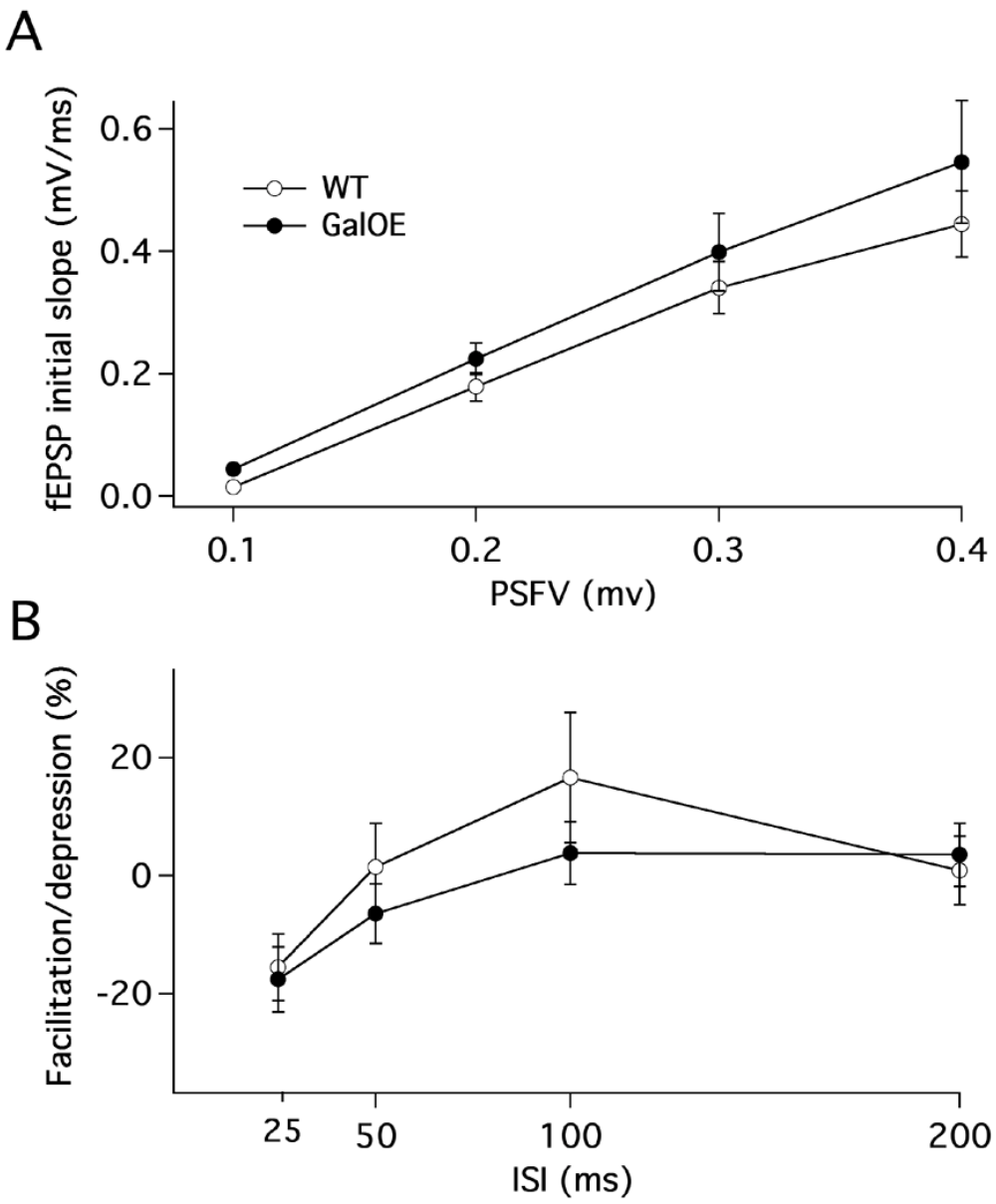

Fig. 2. Basal synaptic properties and short-term plasticity are not altered in the LOT synapses of GalOE mice. (A) The relationship between the average presynaptic fiber volley (PSFV) and the initial slope of field excitatory postsynaptic potential (fEPSP) recorded in the outer molecular layer of PC in response to LOT stimulation. **(B)** Averaged PPF of fEPSPs in LOT synapses, expressed as percent change of the

second fEPSP compared to the first one. GalOE, *n* = 18 slices from 6 animals; WT, *n* = 15 slices from 5 animals.

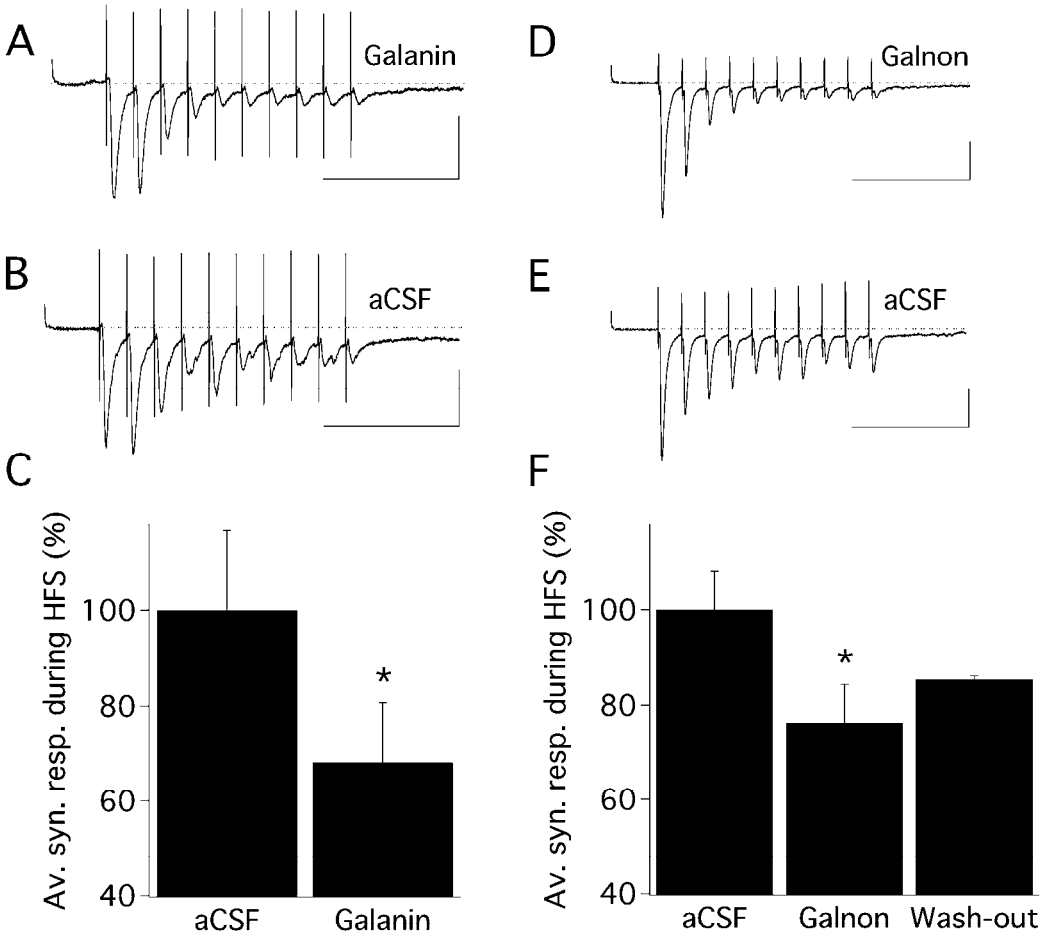

Fig. 3. High frequency stimulation-induced excitatory synaptic responses are attenuated in GalOE mice. Representative traces of EPSCs in WT **(A)** and GalOE **(B)** mice. **(C)** The average ratio of normalized synaptic responses in percent (integral of total EPSC response during 400 ms divided by the integral of the first EPSC during 25 ms) recorded in pyramidal cells of the PC in GalOE and WT mice during HFS of the LOT (*$p < 0.05$). **(D)** Cumulative fraction (probability) curves of the relative synaptic response, defined as integral of the total synaptic response normalized to that of the first EPSC in GalOE and WT mice. (GalOE, *n* = 16 cells from 6 animals; WT, *n* = 17 cells from 5 animals)

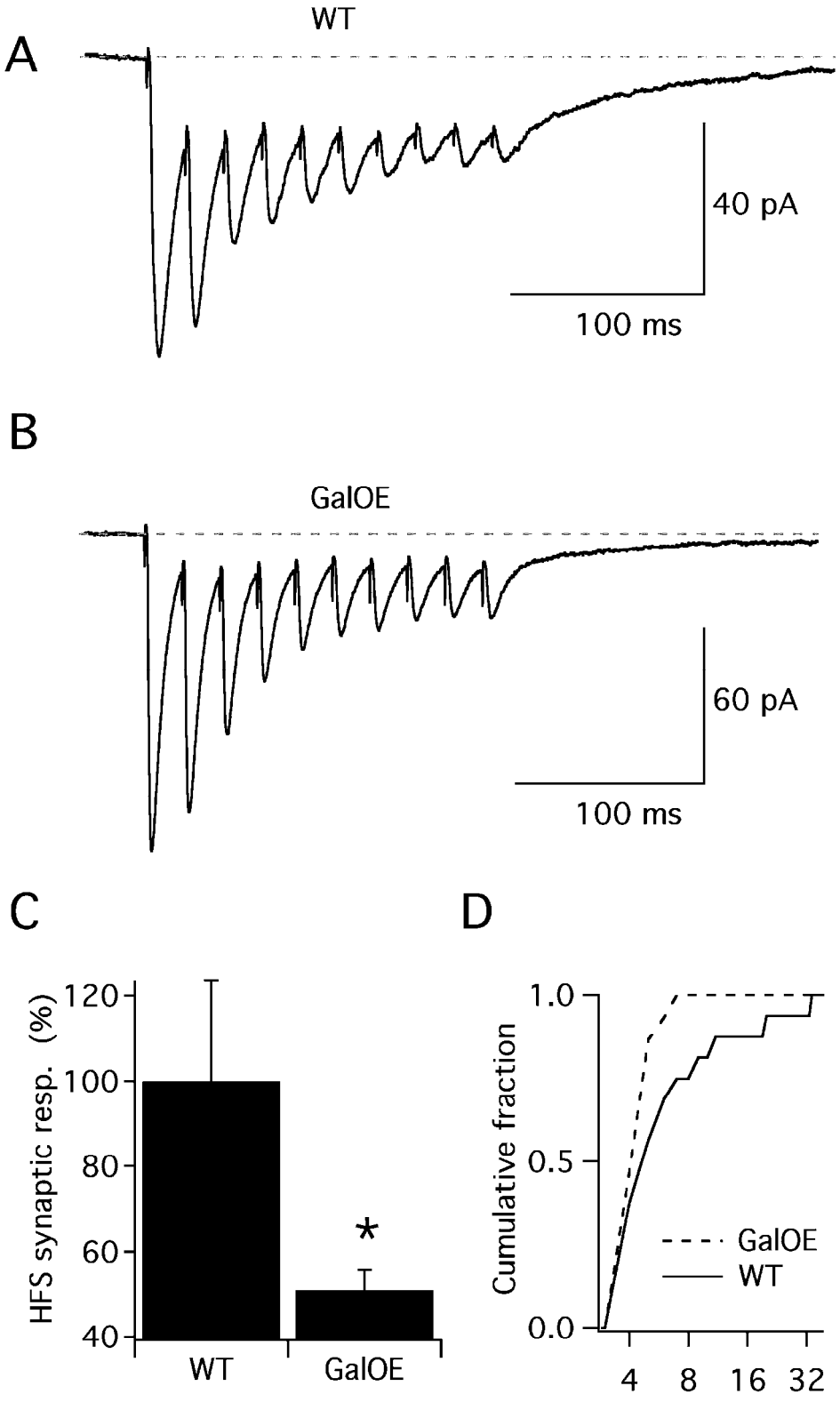

Fig. 4. Galanin and galnon applied to slices of WT mice mimic the changes in the excitatory synaptic responses of GalOE mice. The average ratio of normalized

synaptic responses during HFS of the LOT before and after application of galanin **(C)** (0.5 µM) (*n* = 9 cells from 9 animals, **p < 0.05*) or galnon **(F)** (1.0 µM) (*n* = 5 cells from 5 animals, **p < 0.05*) into the perfusion medium. Representative traces of EPSCs before **(B, E)** and after galanin **(A)** or galnon infusion **(D)**. Scale bars are 100 ms and 50 pA.

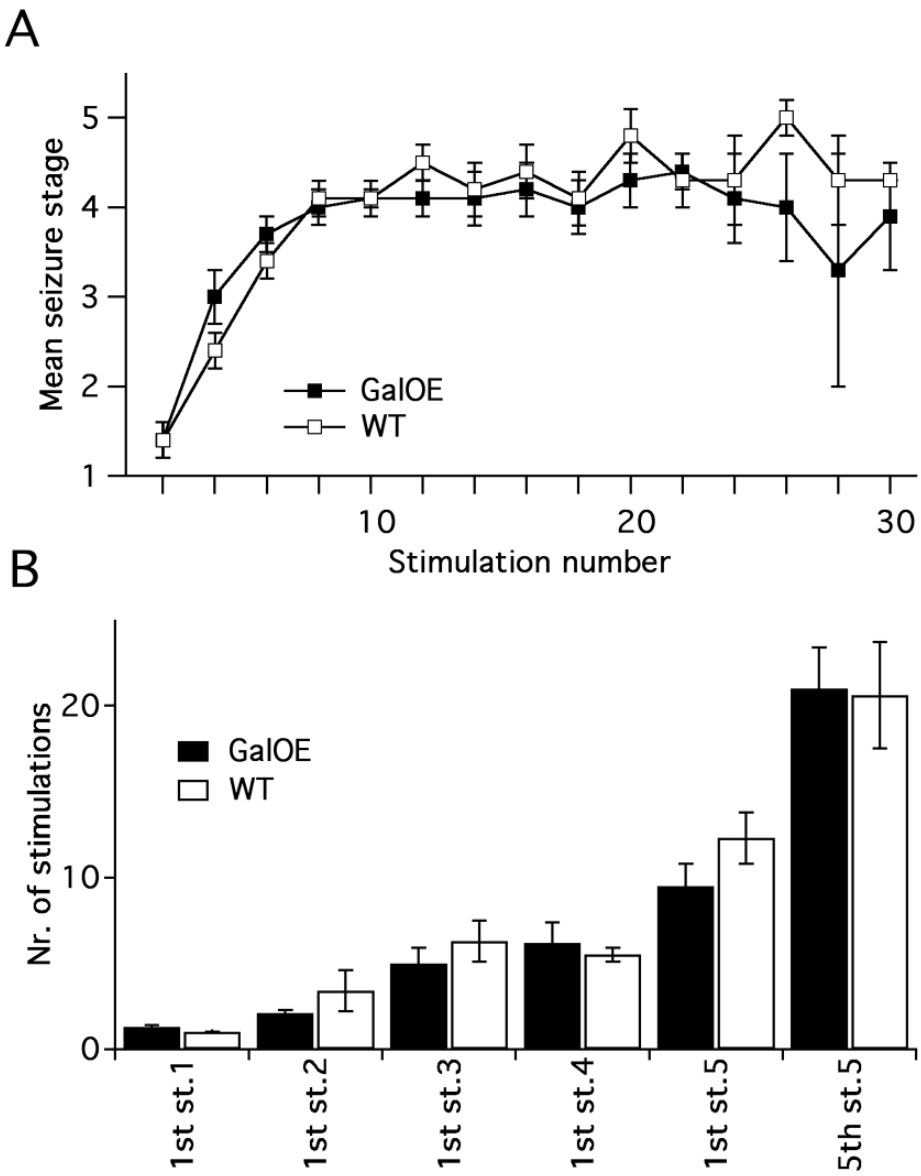

Fig. 5. GalOE and WT mice exhibited similar kindling development. The kindling development, presented as mean seizure stage at each stimulation (day) **(A)** and

average number of stimulations required to reach different seizure stages **(B)** (GalOE, n = 12; WT, n = 10).

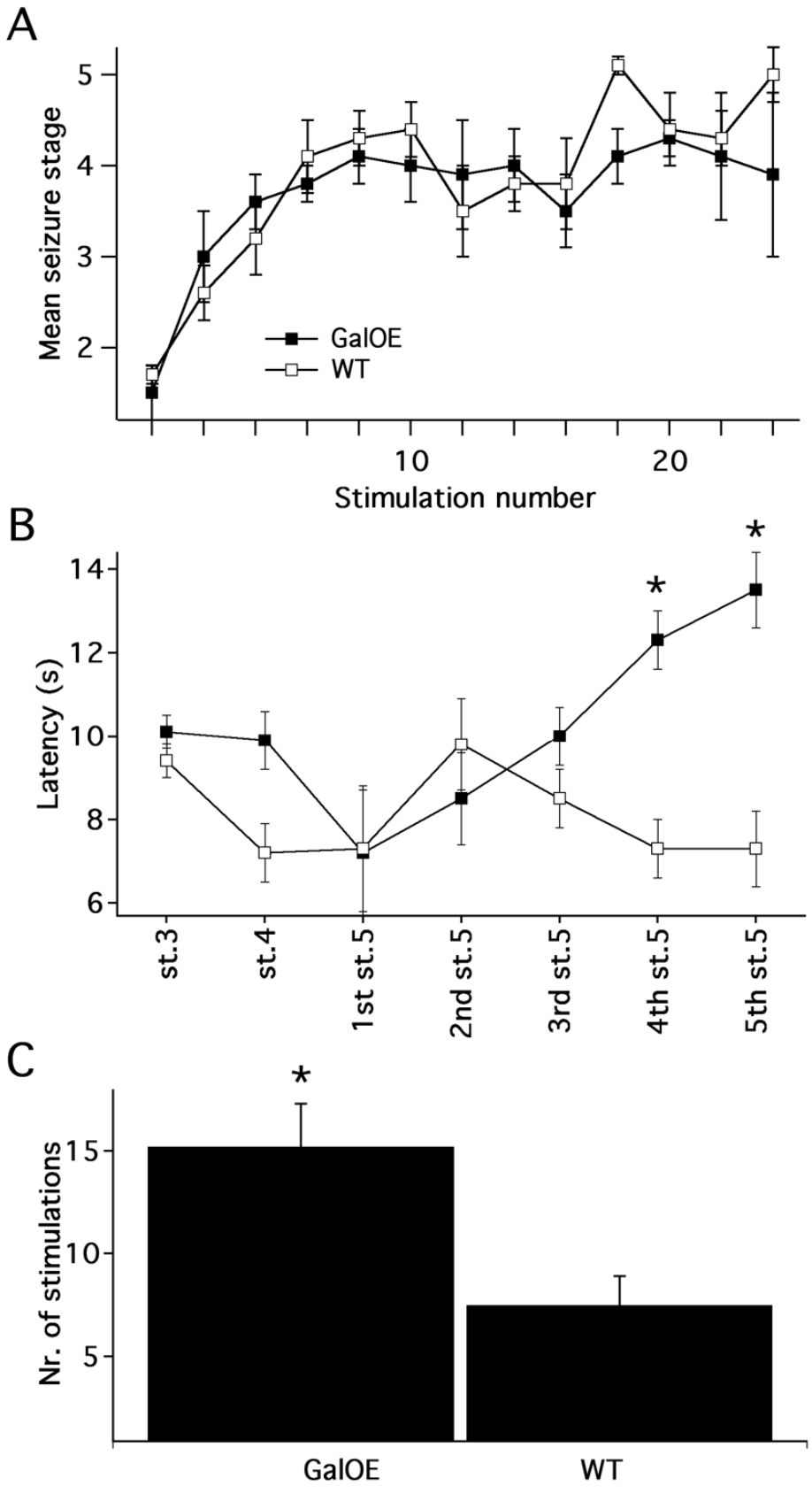

Fig. 6. Male GalOE mice show attenuation of generalized seizures. (A) The kindling development presented as mean seizure stage at each stimulation in GalOE males (n = 6) and WT males (n = 4). **(B)** The average latency time to commencement of behavioural convulsions in GalOE and WT mice during the consecutive stage 5 seizures (*$p < 0.05$). **(C)** The average number of stimulations from the first stage 5 seizure to the fully kindled state in GalOE and WT males (*$p < 0.05$).