Regulation of kindling epileptogenesis by hippocampal galanin type 1 and type 2 receptors: the effects of subtype selective agonists and the role of G-protein mediated signaling.

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[D-Arg¹,D-Trp⁵⁷⁹,Leu¹¹]-substance P; GalR1- galanin receptor type 1; GalR2: galanin receptor type 2; GIRK- G-protein coupled inwardly rectifying K⁺ channels; PTX- pertussis toxin; TPQ-
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Abstract

Search for antiepileptic drugs which are capable of blocking the progression of epilepsy (epileptogenesis) is an important problem of translational epilepsy research. The neuropeptide galanin effectively suppresses acute seizures. We examined the ability of hippocampal galanin receptor type 1 (GalR1) and 2 (GalR2) to inhibit kindling epileptogenesis, and studied signaling cascades that mediate their effects. Wistar rats received 24 hour long intrahippocampal infusion of a GalR1/2 agonist galanin(1-29), GalR1 agonist M617, or GalR2 agonist galanin(2-11). The peptides were administered alone, or combined with an inhibitor of G\textsubscript{i} protein pertussis toxin (PTX), G\textsubscript{i}-protein activated K\textsuperscript{+} channels (GIRK) inhibitor tertiapin Q (TPQ), G\textsubscript{q/11} protein inhibitor [D-Arg\textsuperscript{1},D-Trp\textsuperscript{5,7,9},Leu\textsuperscript{11}]-substance P (dSP), or an inhibitor of intracellular Ca\textsuperscript{2+} release dantrolene. Sixteen hours into drug delivery, the animals were subjected to rapid kindling - sixty electrical trains administered to ventral hippocampus every 5 minutes. M617 delayed epileptogenesis, while galanin(1-29) and galanin(2-11) completely prevented the occurrence of full kindled seizures. TPQ abolished anticonvulsant effect of M617, but not of galanin(2-11). PTX blocked anticonvulsant effects of M617 and inversed the action of galanin(1-29) and galanin(2-11) to proconvulsant. dSP and dantrolene did not modify seizure suppression through GalR1 and GalR2, but eliminated proconvulsant effect of PTX+galanin(1-29) and PTX+galanin(2-11) combinations. We conclude that hippocampal GalR1 exert disease - modifying effect through G\textsubscript{i}-GIRK pathway. GalR2 is antiepileptogenic through G\textsubscript{i} mechanism independent of GIRK. Secondary proconvulsant pathway coupled to GalR2, involves G\textsubscript{q/11} and intracellular Ca\textsuperscript{2+}. The data are important for understanding endogenous mechanisms regulating epileptogenesis, and for the development of novel antiepileptogenic drugs.
**Introduction**

Three lines of evidence suggest that the neuropeptide galanin is a powerful inhibitor of seizure activity. First, acute administration of galanin receptor agonists inhibited seizures (Bartfai et al., 2004; Lundström et al., 2005a; Mazarati et al., 2004a). Second, chronic overexpression of galanin in the brain mitigated seizure activity (Haberman et al., 2003; Kokaia et al., 2001; Lin et al., 2003, Mazarati et al., 2000). Third, deletion of galanin or galanin receptors through either genetic mutations or antisense technique, resulted in a proconvulsant phenotype (Jacoby et al., 2002; Mazarati et al., 2000, 2004a,b).

Anticonvulsant effects of galanin were shown, by and large, under conditions of acute seizures. However, a desirable property in an antiepileptic drug is the potential ability to prevent epileptogenesis, that is the *development* of chronic epilepsy (Stables et al., 2003). One of the common forms of drug-resistant epilepsy is temporal lobe epilepsy, in which the primary epileptic focus is located in the hippocampus. Hence, in order to be effective in temporal lobe epilepsy, an antiepileptic drug should target hippocampal circuitry.

Of the three galanin receptor types known to date, type 1 (GalR1) and type 2 (GalR2) are expressed in the hippocampus (Mennicken et al., 2004; O'Donnel et al., 1999). Activation of both hippocampal GalR1 and GalR2 is anticonvulsant (Mazarati et al., 2004a,b); however, the two types of galanin receptors are coupled to distinct signaling cascades. Similar to other members of G-protein coupled receptor family, the signaling mediated by galanin receptors is multifaceted, with apparently only certain mechanisms relevant to their anticonvulsant effects. Thus, coupling of GalR1 to Gi protein opens G-protein mediated inward rectifier K⁺ channels (GIRK), or ATP-sensitive K⁺ channels, which in turn results in presynaptic inhibition of glutamatergic transmission (Counts et al., 2002; Lundström et al., 2005a; Mazarati et al., 2000). The main
pathway downstream from GalR2 is through coupling to G_{q/11} protein, and includes the increase of inositol triphosphate accumulation, and the increase of intracellular Ca^{2+} (Wang et al. 1998, Lundström et al., 2005a); this would presumably stimulate neuronal activity and neurotransmitter release. Such an effect was indeed reported for GalR2 - serotonin interaction in the dorsal raphe nucleus (Mazarati et al. 2005). In addition, GalR2 activates mitogen activated protein kinase through coupling to G_o protein (Wang et al., 1998). Furthermore, both GalR1 and GalR2 inhibit cyclic AMP-responsive element binding protein (CREB), an effect possibly pertaining to the inhibition of long term potentiation (Badie-Mahdavi et al., 2005).

Modification of epileptogenesis by galanin has been described in a single report, in which galanin – overexpressing mice exhibited a delay in the progression of kindled seizures (Kokaia et al., 2001). However, the study did not address the role of galanin receptor types and downstream signaling pathways that mediated the inhibition of kindling process.

The present study had two goals. First, we examined whether GalR1 and GalR2 in the hippocampus exerted antiepileptogenic effect. A common approach for evaluating such an effect in antiepileptic drugs is to study their influence on the development of chronic epilepsy resulting from neuronal injury and synaptic reorganization caused by status epilepticus (Löscher 2002; Morimoto et al., 2004). For our study, however, we used the kindling model of epileptogenesis (Löscher 2002; Morimoto et al., 2004). While being different from post-status epilepticus - induced epileptogenesis, kindling represents a useful tool for proving the principle. On the one hand, spontaneous seizures following status epilepticus have random temporal distribution and variable frequency, which requires large number of experimental subjects and prolonged period of observation in order to obtain reliable results. On the other hand, kindling offers a controlled situation, in which seizures evolve through the “silent” period to the occurrence and progression.
of overt limbic seizures in response to repetitive subthreshold electrical stimulation of certain brain areas. More importantly, despite obvious differences in pathophysiological substrate, the two models showed significant overlap in terms of sensitivity to several antiepileptic drugs (Löscher, 2002).

Furthermore, we attempted to outline the mechanisms through which hippocampal GalR1 and GalR2 regulate kindling epileptogenesis. Based on the pathways which might mediate anticonvulsant effects of galanin, we examined how inhibition of G\textsubscript{i/o} protein, GIRK, G\textsubscript{q/11} protein, and intracellular Ca\textsuperscript{2+} mobilization affected kindling progression and anticonvulsant effects of GalR1 and GalR2 agonists.
Methods

Animals. The experiments were performed on 10-12 week old male Wistar rats (Harlan, Indianapolis, IN). The animals were individually housed with 12 hour dark-light cycle and free access to food and water. The experiments were done in accordance with National Institutes of Health policy and were approved by the UCLA Office for Protection of Research Subjects.

Surgery. Animals were anesthetized with Isoflurane and placed in the stereotaxic instrument model 902 (David Kopf Instruments, Tujunga, CA). Bipolar stimulating electrode (PlasticsOne, Roanoke, VA) was implanted into the left ventral hippocampus (4.8 mm posterior and 5.3 mm lateral from Bregma, 6.5 mm ventral from the brain surface, Paxinos and Watson, 1986). Tripolar recording skull electrode (Plastics1) was placed on the left side 2 mm anterior from Bregma with the ground connected to the screw in the nasal bone. A five microliter microsyringe model 7105KH (Hamilton, Reno, NV) was placed into the injector of the infusion pump model sp310i (World Precision Instruments, Sarasota, FL), which had been mounted on the arm of stereotaxic instrument. A hole was drilled on the left, 4.16 mm posterior and 2.5 mm lateral from Bregma. The needle of the syringe was lowered 2.5 mm ventral from brain surface into the CA1 of the hippocampus (Paxinos and Watson, 1986). Five microliters of solution (see below) were infused at a rate of 5 µl/min.

A subcutaneous pocket was made between the rat’s shoulders. An ALZET osmotic pump model 2001D (infusion rate 8.3 µl/hour, total infusion duration 24 hour, infusion volume 0.2 ml, DURECT Corporation, Cupertino, CA) which had been pre-filled with the solution (see below) connected to the infusion cannula of Brain Infusion Kit II (DURECT Corporation), and primed for 2 hours in saline at 37°C, was placed into the subcutaneous pocket. The infusion cannula was lowered into the same place in the hippocampus, where the solution had been injected through
the microsyringe. The electrodes and the cannula were cemented to the skull using Cerebond adhesive (MyNeurolab.com, St. Louis, MO).

**Drug injections.** One of the following substances was injected through the Hamilton microsyringe. A G_{i/o} protein inhibitor Pertussis toxin (PTX, 0.5 µg in 5 µl, List Biological Laboratories, Campbell, CA) (Bokoch et al., 1983); a blocker of GIRK 1-4 subunits tertiapin Q (TPQ, 10 nM, 5 µl, Tocris Coockson, Bristol, UK) (Jin and Lu, 1998); a G_\text{q/11} protein inhibitor ([D-Arg^1,D-Trp^{5,7,9},Leu^{11}]-substance P) (dSP, 10 µM, 5 µl, Bachem, Torrance, CA) (Mitchell et al., 1995; Sinnett-Smith et al., 2000); an inhibitor of Ca^{++} release from endoplasmic reticulum dantrolene sodium salt (10 µM, Tocris Coockson) (Lauckner et al., 2005); a GalR1/GalR2 antagonist - chimeric peptide M35 [galanin(1-13)-bradykinin(2-9) amide] (10 µM, 5 µl, Kask et al., 1995). The following compounds were infused through the ALZET osmotic pump: a non selective GalR1/GalR2 agonist rat galanin (1-29) (5 µM, Bachem, Torrance, CA); a preferential GalR1 agonist chimeric peptide M617 (Galanin(1-13)-Gln^{14}-bradykinin(2-9)-amide) (5 µM, synthesized by Lundström et al., 2005b); a preferential GalR2 agonist galanin (2-11) (5 µM, Sigma) (Liu et al., 2001; Lundström et al., 2005a). The peptides were administered alone, or in combination with one of the following agents: PTX (0.5 µg); TPQ (5 µM); dSP (5 µM); dantrolene (10 µM); M35 (10 µM). Selected concentrations for each compound had been optimized in pilot experiments. All substances were dissolved in saline, except dantrolene, which was dissolved in polyethylene glycol 300 (Sigma). Control treatments consisted of the administration of respective vehicles both through Hamilton microsyringe and from ALZET osmotic pumps.

**Kindling procedure.** We used the rapid kindling protocol, originally described by Lothman et al. (1985). In contrast to conventional kindling, in which electrical stimuli are delivered hours apart,
and which requires weeks in order for overt limbic seizures to develop, in rapid kindling the 
epileptogenesis is compressed to several hours, while still bearing key hallmarks of kindling: 
appearance and gradual progression of the severity of limbic seizures, and enhanced seizure 
susceptibility after kindling is complete. Sixteen-seventeen hours after the implantation of 
ALZET osmotic pumps (6-7 hours prior to the completion of the infusion) the animals were 
connected to the DS8000 electrical stimulator via DSI100 stimulus isolators (World Precision 
Instruments) and to the MP100/EEG100B acquisition system (BIOPAC, Santa Barbara, CA). 
EEG was acquired and using AcqKnowledge 3.7 software (BIOPAC). Simultaneously, animals’ 
behavior was recorded by digital video camera. Both EEG and behavioral responses were 
analyzed off-line.

At the beginning of the experiment, afterdischarge threshold and duration were detected by 
applying trains of electrical stimuli, - 10 s train duration, 20 Hz, 1 ms pulse duration, square wave 
monophasic stimuli, stating with 0.1 mA, with 0.1 mA increments, delivered every 10 minutes. 
Ten minutes after the detection of afterdischarge threshold, evident as a high-frequency response, 
of at least 2 s duration, following the end of the train, animals underwent rapid kindling procedure. 
Kindling consisted of 60 trains delivered every 5 minutes using the parameters described above 
and the current of 50 µA above the afterdischarge threshold (total procedure duration was 5 
hours). Behavioral seizures were scored using the following scale: 1- Motor arrest and whisker 
twitching; 2 – chewing, head bobbing; 3- forelimb clonus; 4- forelimb clonus and rearing; 5-
rearing and falling. If the animals failed to develop seizures of certain score, 60 (number of 
stimulations) was assigned. If the animal skipped a certain phase in seizure progression, the 
number of stimulations required to reach the subsequent phase was also assigned to the skipped 
phase (e.g. in case, when the animal transited from stage 1 to stage 3 seizure without exhibiting
stage 2 convulsion, the number of stimulations needed to reach stage 3 was also assigned to the stage 2. Number of stimulation required to reach each consecutive seizure score (one through five), and the number of full motor seizures (stage 4-5) were calculated.

Twenty four hours after the end of kindling procedure, animals were reconnected to the stimulating/recording system and afterdischarge threshold and afterdischarge duration were detected again. The experimental protocol is summarized in Fig.1A

Verification of infusion. After the second test of afterdischarge properties, animals were euthanized, ALZET pumps were removed, and the content of the pump was withdrawn to verify the injected volume. In all subjects 180-200 µl was delivered (90-100% of the originally placed volume). Tissue uptake and distribution of peptides was exemplified by studying the distribution of fluorescein-tagged galanin (1-29). Two animals were injected with human fluorescein-galanin(1-29) (empirical formula C_{139}H_{210}N_{42}O_{43}, molecular weight 3157) (Anaspec, San Jose, CA) instead of rat galanin(1-29) (empirical formula: C_{141}H_{211}N_{43}O_{41}, molecular weight 3164), and subjected to kindling protocol as described above. Immediately after kindling, the animals were euthanized, and perfused transcardially with 0.9% NaCl followed by 0.4% paraformaldehyde. The brains were postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose, and cut in coronal plane at 40 micron on Leica sliding microtome (Leica, Microsystems, Nussloch, Germany). Sections were coverslipped and examined under green fluorescence using Leica microscope equipped with Micropublisher 5 digital camera (QImaging, Barnaby, BC, Canada).

Statistical analysis. Data were analyzed using Prizm 4 software (GraphPad, San Diego, CA), and employed One way ANOVA followed by the post hoc Dunn’s test. P<0.05 was accepted for statistical significance. Each group included 6 animals, unless indicated otherwise. As our
stimulation/recording system allowed simultaneous processing of 4 subjects, each set of animals generally included one of three different treatments and one control.
Results

*Distribution of fluorescein-galanin (1-29).* Fluorescent signal indicative of the retention of fluorescein-galanin (1-29) in the brain was diffusely distributed adjacent to the site of injection. Along with the presence of fluorescence in the white matter, a selective uptake of the peptide was found in the CA1 area of the hippocampus (Fig.1B). In the sagittal plane the peptide was detected between sections which corresponded approximately to 3.5 mm and 5.3 mm caudal from Bregma, as compared to the images of the brain atlas (Paxinos and Watson, 1986). In the coronal plane the signal spanned across 2-3 mm, that is was present in almost entire CA1, although the strength of the signal substantially decreased in the portions of CA1 more distant from the injection site. In sections adjacent to the site of injection, an uptake of fluorescein-galanin(1-29) was also observed in the middle part of the upper blade of the dentate gyros (Fig.1B).

*Kindling in control animals.* In naïve rats (*n=10*), afterdischarge threshold was 1.1±0.1 mA and afterdischarge duration was 30.5±2.4 s (Fig.2A). Kindling procedure led to the occurrence and the progression of behavioral convulsions; it took 5.5±0.5 stimulations to reach stage 1 seizures, 10.7±0.6 stimulations – for stage 2 seizures, and 15.2±0.7 stimuli to develop the first stage 3 seizure. The first full motor seizure (stage 4) occurred after 23.6±0.5 stimuli, and the first stage 5 seizure was observed after 25.8±0.4 stimulations (Fig.2B). After the first full motor seizure, animals responded with either stage 4 or 5 convulsions to 17.4±1.8 of stimulations. Twenty four hours after the last kindling train, all animals showed decrease of afterdischarge threshold to 0.43±0.1 mA and the increase of afterdischarge duration to 52±2.9 s (*p*<0.05 vs. the values before kindling, Fig.2C). All animals developed behavioral convulsions in response to the threshold stimulation, although none of the rats developed full motor seizure (average seizure...
score was 2.9±0.2, Fig.2D). Rats injected with fluorescein-galanin(1-29) followed the pattern of kindling progression observed in animals treated with rat galanin(1-29), but the data were not included in the statistical analysis.

*Effects of galanin receptor agonists.* Animals treated with galanin(1-29) and M617 exhibited a 60-75% increase in afterdischarge threshold, as compared to controls (p<0.05, Fig.2A). Galanin(2-11), in contrast, produced a statistically significant decrease in afterdischarge threshold (0.5±0.1 mA), and afterdischarge duration was significantly longer than in control group (46.0±6.9 s) (Fig.2A).

During kindling, galanin (1-29) significantly delayed the occurrence of behavioral seizures of all stages as compared with control (Fig.2B). Furthermore, 2 out of 6 animals, failed to exhibit stage 5 seizures. Treatment with M617 also produced a significant delay in the development of behavioral seizures, however the peptide did not block the occurrence of stage 5 convulsions. Galanin(2-11) treated rats were not different from control in the development of stage 1 and 2 convulsions, but it took more stimulations, than in controls to develop stage 3-5 seizures; in addition, 4 out of 6 animals failed to exhibit stage 5 convulsions, and the rest developed stage 5 seizure only in response to the last stimulation (Fig.2B). In the animals which were treated with a combination of M617 and galanin(2-11) kindling followed the pattern similar to that in galanin(1-29) - treated rats (Fig.2B). The number of full motor seizures was significantly lower in rats which received the injections of galanin(1-29) (0.7±0.2, p<0.05 vs. Control), galanin (2-11) (0.6±0.3, p<0.05 vs. Control), or a combination of M617 and galanin (2-11) (0.9±0.4, p<0.05 vs. Control) with no differences across the three groups. M617 treated animals developed 7.7±1.2 full motor seizures (p<0.05 vs. Control, galanin(1-29) and galanin (2-11)). Testing afterdischarge properties 24 hours after kindling revealed that the animals treated with galanin
(1-29), galanin (2-11), or with a M617/galanin(2-11) combination had significantly higher afterdischarge threshold and shorter afterdischarge duration than control animals, and those who received M617 (Fig. 2C). Furthermore, the animals of the first three groups either failed to develop behavioral seizures in response to the threshold stimulation, or only exhibited stage 1 convulsions (three animals in galanin(1-29) and two animals in M617+galanin(2-11) treated groups.

Effects of M35. Intrahippocampal administration of M35 (n=5) decreased afterdischarge threshold (0.56±0.05 mA, p<0.05 vs. control), but did not modify afterdischarge duration. Progression of kindled seizures was not different between M35 – treated and control groups (data not shown, n=5 per group). M35 prevented the increase of afterdischarge threshold induced by M617, but did not affect changes in afterdischarge properties observed after administration of galanin(2-11) (Fig. 3). Co-administration of M35 abolished inhibition of kindling observed after treatment by each of the peptides alone (data not shown).

Effects of PTX. Intrahippocampal infusion of PTX alone significantly increased afterdischarge threshold (Fig. 4A). Animals treated with the combination of PTX and galanin(1-29), or with PTX and galanin (2-11) exhibited a significantly longer afterdischarge, as compared with both controls and with PTX alone injected rats. Furthermore, combination of either of the two peptides with PTX led to a significant reduction of afterdischarge threshold as compared to PTX alone. There were no differences between PTX and PTX+M617 treated animals (Fig.4A).

PTX treatment significantly delayed the progression of kindled convulsions, and decreased the number of stage 4-5 seizures (11±1, p<0.05 vs. Control) although it did not prevent the development of the latter (Fig.4B). Surprisingly, a combined administration of PTX with either galanin (1-29), or galanin (2-11) facilitated kindling progression, as the animals reached full
motor seizures significantly faster than controls (13.1±0.7 and 15.5±1.0 respectively, p<0.05 vs. both controls and PTX treatment, Fig.4). The number of full motor seizures also significantly increased, (29.7±2.1 for PTX+Galanin(1-29) and 31.1±2.4 for PTX+Galanin(2-11), p<0.05 vs. both Control and PTX alone). Animals treated with a combination of PTX and M617 showed very similar pattern of kindling progression (Fig.4B) and the reproducibility of stage 4-5 seizures (10.5±1.1), to that of PTX-treated rats.

Twenty four hours after kindling, PTX and PTX+M617 treated animals still showed lower excitability than controls (Fig.4C). The animals failed to respond, or responded with minimal behavioral seizures to the afterdischarge stimulation. At the same time, kindled animals which had been treated with PTX+galanin(1-29), and PTX+galanin(2-11) were not different from control group in terms of AD properties. However afterdischarge threshold was significantly lower (0.9±0.1 and 0.6±0.1 mA respectively), than in PTX treated rats (3.2±0.3 mA, Fig.4D). Furthermore, in PTX+galanin(1-29) and PTX+galanin (2-11) treated animals the threshold current elicited behavioral seizures (2.4±0.2 and 3.6±0.2 respectively) with the severity similar to controls, and more severe than in PTX animals (Fig.4C).

**Effects of TPQ.** Intrahippocampal infusion of TPQ led to the decrease of afterdischarge threshold (0.15±0.015 mA). Animals treated with a combination of TPQ with galanin(1-29), M617, or galanin(2-11) showed similar decrease in afterdischarge properties (Fig. 5A). TPQ - treated rats progressed through kindling stages 2 through 5 faster (Fig.5B), and showed higher number of full motor seizures (29.3±1.8), as compared to controls. TPQ+M617 administration also accelerated kindling rate, and increased the number of full motor seizures to 27.5±1.3 (p<0.05 as compared to controls), but the indices did not differ from those in TPQ-only injected rats.
Animals which received TPQ+galanin(1-29), or TPQ+galanin(2-11) combination exhibited kindling rate similar to control group; kindling progression through stages 2-5 was significantly slower as compared to TPQ-only treated rats (Fig.5B). The number of stage 4-5 seizures in these rats was not different from controls, but was significantly lower than in TPQ-treated animals (14.5±1.4 and 18.5±1.1 respectively). Afterdischarge properties and seizure response to the test stimulation twenty four hours after kindling were similar among all TPQ – treated groups (not shown).

Effects of dSP. Intrahippocampal delivery of d-SP altered neither afterdischarge properties, nor the rate of kindling epileptogenesis, nor it affected anticonvulsant profile of M617 (Fig.6A,B). However, dSP abolished the galanin(2-11) - induced increase of hippocampal excitability before kindling (Fig.6A). At the same time, dSP did not affect galanin(2-11) – induced delay of kindling rate and the decrease of the incidence of full motor seizures (0.7±0.3, p<0.05 vs. galanin(2-11 alone). However, adding dSP to PTX+galanin(2-11) combination inversed the effect of such a treatment from kindling - facilitating (Fig.4B) to anticonvulsant (Fig.6B), and abolished the increase of the number of full motor seizures (16.2±0.9 p<0.05 vs. PTX+galanin(2-11).

Combined administration of PTX and dSP without galanin receptor ligands affected all examined parameters in the same way as PTX treatment alone (p>0.05, data not shown).

Effects of dantrolene. Infusion of dantrolene into the hippocampus affected neither afterdischarge threshold, nor afterdischarge duration in non-kindled animals (Fig.6C). Dantrolene did not modify the increase of afterdischarge threshold induced by M617, however, it abolished the increase of hippocampal excitability due to galanin (2-11) (Fig 6C). Animals which were treated with intrahippocampal dantrolene failed to develop kindling; the maximal severity of seizures were of stage 1; such seizures developed in all rats, but only in response to 10-19 out of 60
stimulations. Animals injected with dantrolene+M617, dantrolene+galanin(2-11),
PTX+dantrolene, or PTX+dantrolene+galanin(2-11) showed pattern of kindling progression
similar to that of dantrolene-only treated rats (data not shown).
Discussion

Preferential activation of GalR1 and GalR2 exerted differential effects upon kindling epileptogenesis through certain G-protein coupled pathways. Since the modulators of the signal transduction by themselves modified kindling epileptogenesis, there effects are discussed first.

Effects of PTX, TPQ, dSP and dantrolene. Intrahippocampal administration of PTX decreased hippocampal excitability and interfered with the progression of kindling. PTX uncouples G_{i/o} proteins by catalyzing ADP-ribosylation of α subunits (Bokoch et al., 1983). ADP-ribosylation has been implicated in kindling epileptogenesis (Suzuki et al., 1997). Furthermore, inhibitory and anticonvulsant effects of galanin, somatostatin, neuropeptide Y, GABA acting at GABAB receptor, glutamate acting at groups II and III metabotropic glutamate receptors, serotonin acting at 5HT1A receptor, adenosine acting at A1 receptor are coupled to G_{i} protein (Counts et al., 2002; Moldrich et al., 2003; Wickenden, 2002). Hence, PTX should facilitate, rather than inhibit seizures. Paradoxically, PTX inhibited kindled seizures not only in our experiments, but in earlier studies as well (Watanabe et al., 1991). Inhibition of long term potentiation (Goh and Pennefather, 1989) in the hippocampus further implicates PTX in inhibiting synaptic activity. Conceivably, signal transduction downstream of PTX affects seizures in different ways depending on mechanisms predominantly involved in seizure regulation in particular experimental paradigms. PTX inhibition of kindling epileptogenesis is galanin receptor – independent, and may recruit other mechanisms relevant to the evolvement of kindled seizures (e.g. cholinergic transmission, Burchfiel et al., 1979).

Keeping in mind the ubiquity of PTX-mediated intracellular signaling, we narrowed it down to a candidate that might mediate anticonvulsant effects of galanin – GIRK. All of neuropeptides and neurotransmitters mentioned above, exert their effects through GIRK (Wickenden, 2002).
Activation of GIRK inhibits glutamatergic transmission both pre- and postsynaptically through membrane hyperpolarization (Wickenden, 2002). Indeed, injection of TPQ in our experiments yielded a more predictable outcome, as it facilitated kindling epileptogenesis.

Inhibition of Gq/11 by dSP did not affect kindling progression. Gq/11 is coupled to inositol triphosphate production and intracellular Ca2+ release, which should enhance neuronal excitability. Receptors of proconvulsant agents, such as substance P (Liu et al., 1999) and metabotropic glutamate receptors 1 and 5 (Moldrich et al., 2003) are coupled to Gq/11. The absence of the effects of dSP itself is congruent with the previously shown “silent” behavior of this compound: dSP inhibited bombesin mediated activation of phospholipase C, but itself did not affect the enzyme activity (Mitchell et al., 1995). However, direct inhibition of Ca2+ release from endoplasmic reticulum by dantrolene did exert a rather predictable anticonvulsant effect.

Thus, the diversity of signal transduction pathways regulated by G proteins complicates a clear-cut prediction of the net effects of their inhibitors on seizures. However, reducing G-protein coupled signaling cascades to likely candidates of seizure modulation produces more predictable effects. Importantly, such an approach allowed us to correlate regulation of seizures by GalR1 and GalR2 to certain downstream mechanisms.

Role of GalR1. Presumable activation of GalR1 decreased ambient excitability of the hippocampus, and delayed, although did not prevent kindling epileptogenesis (Table 1). The experiments with PTX and TPQ suggested the involvement of G1 protein and downstream GIRK in the anticonvulsant action of M617 and galanin (1-29), while studies with dSP and dantrolene excluded Gq/11 and intracellular Ca2+ as targets for GalR1; such conclusions are in line with previously delineated properties of GalR1 (Counts et al., 2002; Lundström et al., 2005a; Wang et
al., 1998). Hence, hippocampal GalR1 exerts disease-modifying, but not antiepileptogenic effects in kindling epileptogenesis.

**Role of GalR2.** Galanin(2-11) exhibited strong anti-kindling effect (Table 1). In fact, the action of galanin(2-11) could be identified as antiepileptogenic, as judged by the complete prevention of both full motor seizures and of a post-kindling increase of hippocampal excitability. It should be noted, that although galanin(2-11) has equal affinity towards GalR2 and galanin receptor type 3 (Lu et al., 2005), the latter is absent from the hippocampus (Menniken et al., 2004).

Inhibition of kindling by galanin(2-11) depended on G_i/o, as it was PTX sensitive, and indeed coupling of GalR2 to G_i/o has been well established (Lundström et al., 2005a; Wang et al., 1998). However, the failure of TPQ to eliminate anticonvulsant action of galanin(2-11) and galanin(2-19) argued against the involvement of GIRK. Thus the mechanisms by which galanin(2-11) inhibited kindling require further studies. CREB is one possible candidate: CREB activity was enhanced by kindling in a temporally specific manner (Kashihara et al., 2000), while galanin(2-11) inhibited CREB activity (Badie-Mahdavi et al., 2005). At this point it is reasonable to conclude that the anticonvulsant effects of GalR2 G_i/o protein coupled, but are GIRK independent.

The results that seemed paradoxical was that treatment with PTX inversed the effects of both galanin (2-11) and galanin (1-29) from anti- to proconvulsant. Furthermore, the mentioned effect of PTX+galanin(2-11) combination was sensitive to both dSP and dantrolene. We speculate, that along with a G_i dependent pathway, which mediates inhibition of kindling, GalR2 in the hippocampus activate G_q/11 and downstream intracellular Ca^{2+}, which ultimately increases neuronal activity and promotes epileptogenesis. When both G_i and G_q/11 are intact, the first pathway dominates, while the secondary, excitatory component can be unmasked through G_i.
inhibition. Such a suggestion was confirmed in the experiments with galanin (1-29), an endogenous neuropeptide which equally acts at GalR1 and GalR2 (GalR1 and GalR2 are equally expressed in the rat hippocampus, Mennicken et al., 2004; O'Donnel et al., 1999). In the intact hippocampus the effects of galanin (1-29) were congruent with the effects of both GalR1 (decreased ambient excitability) and GalR2 (antiepileptogenic action). Blocking of G_i dependent cascades changed the effects of galanin(1-29) in such a way that the peptide acted similar to galanin(2-11). Coupling of GalR2 to G_{q/11} has been known (Lundström et al., 2005a; Wang et al., 1998). Furthermore, the activation of G_{q/11} inactivates GIRK (Lei et al., 2003), which could further promote seizures. However, the fact that in our experiments both dSP treatment and galanin(2-11) administration did not facilitate kindled seizures proves that the role of G_{q/11} in promoting epileptogenesis is secondary to antiepileptic effects of G_i – coupled pathways. Blocking of galanin receptors by M35 decreased afterdischarge threshold, and negated inhibitory effects of M617. Both M617 and M35 are chimeric peptides which contain C-terminus of bradykinin (Kask et al., 1995; Lundström et al., 2005a). The antagonism between the two peptides argues against the involvement of bradykinin receptors in anticonvulsant effects of M617. Despite the increase of hippocampal excitability by M35, M35+galanin(2-11) combination did not show an additive effect, thus suggesting that facilitation of afterdischarge by M35 and galanin(2-11) occurs through different mechanisms. Abolishing inhibitory effects of M617 and galanin(2-11) on kindling progression by M35 proves galanin receptor-specific effects of both galanin receptor agonists. M35 itself did not affect the evolution of rapid kindling. Our previous studies indicated that M35 acted as proconvulsant in pentylenetetrazole seizures, and facilitated self-sustaining status epilepticus (Mazarati et al., 1998). The mentioned discrepancies likely reflect differences in pathophysiology of seizures depending on experimental model.
Our previous studies suggested that the two galanin receptor subtypes differently regulated the progression of limbic status epilepticus: while GalR1 inhibited status epilepticus during the initiation phase, the major anticonvulsant effect of GalR2 was observed during seizure maintenance (Mazarati and Lu, 2005). The present data extend the principle of receptor subtype specificity to kindling model, when antiepileptogenic and disease modifying effects of galanin occur predominantly through GalR2 and GalR1 respectively.

Clearly, our studies did not provide unambiguous conclusions on the mechanisms of seizure modulation by hippocampal galanin receptors. Three major problems can be identified. First, the complexity and the diversity of the mechanisms of \textit{in vivo} epileptogenesis. Second, currently available pharmacological tools for studying galanin receptors are still not perfect. M617 and galanin(2-11) are the only available agonists of GalR1 and GalR2 respectively, but they do not exhibit absolute preference over galanin receptor subtypes (Lundström et al., 2005a). Third, the diversity of signal transduction pathways affected by \textit{in vivo} administration of G protein inhibitors, that might translate in either anti- or proepileptogenic effects. Despite these, and possibly other limitations, the reported results hopefully expand our understanding of how galanin suppresses seizures, bring up the complexity of the effects of the peptides in the hippocampus, and might be ultimately useful for the development of new antiepileptic drugs.

In conclusion, GalR1 and GalR2 in the hippocampus modified kindling epileptogenesis through different downstream signaling cascades, in either facilitatory or inhibitory fashion. The net effect of GalR1 and GalR2 activation, appears to be antiepileptic; however, hippocampal GalR2 might appear proconvulsant under certain conditions. On this note, our previous studies showed that activation of GalR1 in dorsal raphe nucleus facilitated, rather than inhibited seizures (Mazarati et al., 2005). The established variety of the effects of galanin receptor subtypes,
particularly, different patterns of anticonvulsant activity (e.g. disease modification versus inhibition of epileptogenesis in kindling model, or regulation of the initiation versus the maintenance of status epilepticus), and furthermore seizure – facilitating effects which occur under certain conditions, should be kept in mind while developing antiepileptic drugs acting at galanin receptors (Bartfai et al., 2004).
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References


Burchfiel JL, Duchowny MS, and Duffy FH (1979) Neuronal supersensitivity to acetylcholine induced by kindling in the rat hippocampus *Science*, **204**: 1096-1098.


Footnotes

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Legends for Figures

Figure 1. Experimental design. A: Experimental protocol. Time on the top - hours from the time of ALZET pump and electrode implantation. Vertical arrows point at the time points when animals underwent afterdischarge test and kindling protocol. The period of drug infusion is outlined by the shaded area. AD- afterdischarge B: Cresyl violet - stained image and C-F: Fluorescent images of coronal sections of the hippocampus from the rat which received human fluorescein-galanin(1-29) infusion into the hippocampus and was euthanized 24 hours later. Numbers in parentheses indicate approximate distance of sections posterior from Bregma (in mm), with the reference to the rat brain atlas (Paxinos and Watson, 1986). Arrowheads indicate the site of and adjacent to the cannula track. DG- dentate gyrus. Fluorescent staining is visible throughout the CA1 area of the hippocampus with the maximal continuous signal adjacent to, and a weaker punctuate staining away from the infusion site. Scale bar: 500 micron for B and C; 250 micron for D and E; 125 micron for F. G - a figure from the rat brain atlas (Paxinos and Watson, 1986), corresponding to the coronal section of rat brain 4.8 mm caudal from Bregma. Arrow points to the site of the placement of stimulating electrode (S); arrowhead points to the infusion site (I). (Inset: © Academic Press, San Diego, 1986)

Figure 2. Effects of galanin receptor agonists on afterdischarge properties and kindling progression. A: Afterdischarge threshold (ADT, left Y-axis) and duration (ADD, right Y-axis) before kindling. B: kindling progression, presented as a function of number of stimulations required for reaching each behavioral seizure score; C: Afterdischarge properties 24 hours after the last kindling stimulation; bar coding is identical to that in A. On C the outlined area of the graph indicates behavioral seizure score in response to threshold stimulation and is on the right Y-axis. Abbreviations: Gal- galanin, ADT- afterdischarge threshold, ADD- afterdischarge.
duration. Data are presented as Mean±SEM. *-p<0.05 vs. Control (One way Anova + Dunns test). Statistical difference symbol pertains to all points outlined by the oval.

**Figure 3. Effects of galanin receptor antagonist M35 on afterdischarge properties before kindling.** Afterdischarge threshold (ADT, left Y-axis) and duration (ADD, right Y-axis) before kindling. *- p<0.04 vs. Control; †- p<0.05 vs. M35.

**Figure 4. Effects of pertussis toxin (PTX) on modulation of afterdischarge and kindling by galanin receptor agonists.** A: Afterdischarge threshold (ADT, left Y-axis) and duration (ADD, right Y-axis) before kindling. B: kindling progression, presented as a function of number of stimulations required for reaching each behavioral seizure score; C: Afterdischarge properties 24 hours after kindling. Bar coding is identical to that in A. On C the outlined area of the graph indicates behavioral seizure score in response to threshold stimulation and is on the right Y-axis. Abbreviations: PTX- pertussis toxin, Gal- galanin, ADT- afterdischarge threshold, ADD- afterdischarge duration. Data are presented as Mean±SEM. *-p<0.05 vs. control, †- p<0.05 vs. PTX. (One way Anova + Dunns test). Statistical difference symbols pertain to all points outlined by the oval.

**Figure 5. Effects of tertiapin Q (TPQ) on modulation of afterdischarge and kindling by galanin receptor agonists.** A: Afterdischarge threshold (ADT, left Y-axis) and duration (ADD, right Y-axis) before kindling. B: kindling progression, presented as a function of number of stimulations required for reaching each behavioral seizure score; Abbreviations: TPQ- tertiapin Q, Gal- galanin, ADT- afterdischarge threshold, ADD- afterdischarge duration. Data are presented as Mean±SEM. *-p<0.05 vs. control, †- p<0.05 vs. TPQ. (One way Anova + Dunns test). Statistical difference symbols pertain to all points outlined by the oval.
Figure 6. Effects of dSP (A,B) and dantrolene (C) on modulation of afterdischarge and kindling by galanin receptor agonists. A, C: Afterdischarge threshold (ADT, left Y-axis) and duration (ADD, right Y-axis) before kindling for dSP and dantrolene respectively. B: kindling progression presented as a function of number of stimulations required for reaching each behavioral seizure score; Abbreviations: Gal- galanin, ADT- afterdischarge threshold, ADD- afterdischarge duration. Data are presented as Mean±SEM. *-p<0.05 vs. control, †- p<0.05 vs. dSP. (One way Anova + Dunns test). Statistical difference symbols pertain to all points outlined by the oval.
Table 1. Summary of the effects of galanin receptor agonists alone or in combination with the inhibitors of signal transduction on afterdischarge properties in naive rats, and on the rats of kindling epileptogenesis.

<table>
<thead>
<tr>
<th>Agents used</th>
<th>Inhibitors of signal transduction [inhibited signal]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTX [G\textsubscript{i/o}]</td>
</tr>
<tr>
<td>Vehicle</td>
<td>ADT increased</td>
</tr>
<tr>
<td>(Control values) Kindling not changed</td>
<td></td>
</tr>
<tr>
<td>Galanin receptor agonists [preferred receptor type]</td>
<td>Gal(1-29) [GalR1=GalR2]</td>
</tr>
<tr>
<td>Kindling prevented</td>
<td></td>
</tr>
<tr>
<td>M617 [GalR1&gt;GalR2]</td>
<td>ADT not changed*</td>
</tr>
<tr>
<td>Kindling delayed</td>
<td></td>
</tr>
<tr>
<td>Kindling delayed</td>
<td></td>
</tr>
<tr>
<td>Kindling delayed</td>
<td></td>
</tr>
<tr>
<td>Kindling prevented</td>
<td></td>
</tr>
<tr>
<td>Kindling prevented</td>
<td></td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>Gal(2-11) [GalR1&lt;GalR2]</th>
<th>ADT decreased</th>
<th>ADT decreased</th>
<th>ADT not changed*</th>
<th>ADT not changed</th>
<th>ADT not changed</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Kindling prevented</td>
<td>Kindling facilitated</td>
<td>Kindling delayed*</td>
<td>Kindling delayed;</td>
<td>Kindling prevented</td>
</tr>
<tr>
<td></td>
<td>+PTX: kindling not changed*</td>
<td></td>
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Molecular target for each of the agents used is indicated in brackets. Abbreviations: GalR1- galanin receptor type 1; GalR2- galanin receptor type 2; PTX- pertussis toxin; TPQ- tertiapin Q; ADT- afterdischarge threshold. Asterisk- as compared to the inhibitor of signal transduction administered alone. Antiepileptic effects (increase in ADT, delay or prevention of kindling) are emphasized by shaded cells.
Figure 1
Figure 2
A. Afterdischarge response before kindling

- Control
- PTX
- PTX+Gal(1-29)
- PTX+M617
- PTX+Gal(2-11)

B. Kindling progression

- Control
- PTX
- PTX+Gal(1-29)
- PTX+M617
- PTX+Gal(2-11)

C. Afterdischarge response after kindling

- ADT, mA

Figure 4
Figure 5
Figure 6