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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ABSTRACT

The search for antiepileptic drugs that 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 type 2 (GalR2) to inhibit kindling epileptogenesis and studied signaling cascades that mediate their effects. Wistar rats received 24-h-long intrahippocampal infusion of a GalR1/2 agonist galanin(1–29), GalR1 agonist M617 [galanin(1–13)-Gln14-bradykinin(2–9)-amide], or GalR2 agonist galanin(2–11). The peptides were administered alone or combined with an inhibitor of Gi protein pertussis toxin (PTX), Gi-protein activated K⁺/H11001 channels (GIRK) inhibitor tertiapin Q (TPQ), Gq/11 protein inhibitor [D-Arg1,D-Trp5,7,9,Leu11]-substance P (dSP), or an inhibitor of intracellular Ca²⁺/H11001 release dantrolene. Sixteen hours into drug delivery, the animals were subjected to rapid kindling—60 electrical trains administered to ventral hippocampus every 5 min. M617 delayed epileptogenesis, whereas 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 inverted 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 the proconvulsant effect of PTX/galanin(1–29) and PTX/galanin(2–11) combinations. We conclude that hippocampal GalR1 exert their disease-modifying effect through the Gi-GIRK pathway. GalR2 is antiepileptogenic through the Gi mechanism independent of GIRK. A secondary proconvulsant pathway coupled to GalR2 involves Gq/11 and intracellular Ca²⁺. The data are important for understanding endogenous mechanisms regulating epileptogenesis and for the development of novel antiepileptogenic drugs.

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; Mazarati et al., 2004a; Lundström et al., 2005a). Second, chronic overexpression of galanin in the brain mitigated seizure activity (Mazarati et al., 2000; Kokaia et al., 2001; Haberman et al., 2003; Lin et al., 2003). Third, deletion of galanin or galanin receptors through either genetic mutations or antisense technique, resulted in a proconvulsant phenotype (Mazarati et al., 2000, 2004a,b; Jacoby et al., 2002). 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, to be effective in temporal lobe epilepsy, an antiepileptic drug should target hippocampal circuitry.

Of the three galanin receptor types known do date, type 1 (GalR1) and type 2 (GalR2) are expressed in the hippocam-
Galanin Receptors and Kindling Epileptogenesis

Materials and Methods

Animals. The experiments were performed on 10- to 12-week-old male Wistar rats (HARLAN, Indianapolis IN). The animals were individually housed with a 12-h 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 stereotoxic instrument (model 902; David Kopf Instruments, Tujunga, CA). A bipolar stimulating electrode (PlasticsOne, Roanoke, VA) was implanted into the left ventral hippocampus (4.8 mm posterior and 5.3 mm lateral from bregma and 6.5 mm ventral from the brain surface) (Paxinos and Watson, 1986). A tripolar recording skull electrode (PlasticsOne) was placed on the left side 2 mm anterior from bregma with the ground connected to the screw in the nasal bone. A 5-μl microsyringe model 7105KH (HARLAN, 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 the stereotoxic 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 the 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/h, total infusion duration 24 h, and infusion volume 0.2 ml; DURECT Corporation, Cupertino, CA), which had been prefilled with the solution (see below) connected to the infusion cannula of Brain Infusion Kit II (DURECT Corporation) and primed for 2 h 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 GalR1/GalR2 agonist rat galanin(1–29) (5 μM); a GalR1/GalR2 antagonist chimeric peptide M35 (galanin1–13)-bradykinin(2–9) amide) (10 μM, 5 μl) (Kask et al., 1995). The following compounds were infused through the ALZET osmotic pump: a nonspecific GalR1/GalR2 agonist rat galanin(1–29) (5 μM; Bachem California); a preferential GalR1 agonist chimeric peptide M617 (galanin1–13)-Gln14-b Bradykinin(2–9)-amid e) (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); or 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 the Hamilton microsyringe and from the ALZET osmotic pumps.

Kindling Procedure. We used the rapid kindling protocol, originally described by Lothman et al. (1985). In contrast with conventional kindling, in which electrical stimuli are delivered hours apart and which requires weeks 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 grad-

pus (O’Donnell et al., 1999; Mennicken et al., 2004). 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 the 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 (Mazarati et al., 2000; Counts et al., 2002; Lundström et al., 2005a). The main pathway downstream from GalR2 is through coupling to Gq/11 protein and includes the increase of inositol triphosphate accumulation and the increase of intracellular Ca2+. The main pathway downstream from GalR2 is through coupling to Gq/11 protein and includes the increase of inositol triphosphate accumulation and the increase of intracellular Ca2+ (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 Gq 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 the kindling process.

The present study had two goals. First, we examined whether GalR1 and GalR2 in the hippocampus exerted anti-epileptogenic effects. 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 (Löschler, 2002; Morimoto et al., 2004). For our study, however, we used the kindling model of epileptogenesis (Löschler, 2002; Morimoto et al., 2004). Although being different from post-status epilepticus-induced epileptogenesis, kindling represents a useful tool for proving the principle. On the one hand, spontaneous seizures after status epilepticus have random temporal distribution and variable frequency, which requires large number of experimental subjects and prolonged periods of observation 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 substrates, the two models showed significant overlap in terms of sensitivity to several antiepileptic drugs (Löschler, 2002).

Furthermore, we attempted to outline the mechanisms through which hippocampal GalR1 and GalR2 regulate kindling epileptogenesis. Based on the pathways that might mediate the anticonvulsant effects of galanin, we examined how inhibition of Gq/11 protein, GiRK, Gq/11 protein, and intracellular Ca2+ mobilization affected kindling progression and the anticonvulsant effects of GalR1 and GalR2 agonists.
ual progression of the severity of limbic seizures, and enhanced seizure susceptibility after kindling is complete. Sixteen to 17 h after the implantation of ALZET osmotic pumps (6–7 h before the completion of the infusion), the animals were connected to the DS8000 electrical stimulator via DS100 stimulus isolators (World Precision Instruments) and to the MP100/EEG100B acquisition system (BIOPAC, Santa Barbara, CA). An electroencephalogram was acquired, AcqKnowledge 3.7 software (BIOPAC) was used. Simultaneously, animals’ behavior was recorded by digital video camera. Both an electroencephalogram 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, starting with 0.1 mA increments, delivered every 10 min. Ten minutes after the detection of afterdischarge threshold, evident as a high-frequency response of least 2 s duration following the end of the train, animals underwent a rapid kindling procedure. Kindling consisted of 60 trains delivered every 5 min using the parameters described above and the current of 50 μA above the afterdischarge threshold (total procedure duration was 5 h). 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; and 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., if the animal transitioned from stage 1 to stage 2 seizures without exhibiting stage 2 convulsions, the number of stimulations needed to reach stage 3 was also assigned to stage 2). The number of stimulations required to reach each consecutive seizure score (1 through 5) and the number of full motor seizures (stages 4 and 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 to 200 μl was delivered (90–100% of the originally placed volume). Tissue uptake and distribution of peptides were exemplified by studying the distribution of fluorescein-tagged galanin(1–29). Two animals were injected with human fluorescein-galanin(1–29) (empirical formula C141H211N43O41, molecular weight 3164) and subjected (GraphPad Software Inc., San Diego, CA), and we used a one-way ANOVA followed by the post hoc Dunn’s test. P < 0.05 was accepted for statistical significance. Each group included six animals, unless indicated otherwise. As our stimulation/recording system allowed simultaneous processing of four subjects, each set of animals generally included one of three different treatments and one control.

**Results**

**Distribution of Fluorescein-Galanin(1–29).** A 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 that corresponded approximately to 3.5 and 5.3 mm caudal from bregma, compared with the images of the brain atlas (Paxinos and Watson, 1986). In the coronal plane the signal spanned across 2 to 3 mm, that is, was present in almost the 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 naive rats (n = 10), afterdischarge threshold was 1.1 ± 0.1 mA, and afterdischarge duration was 30.5 ± 2.4 s (Fig. 2A). The 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 to reach stage 2 seizures, and 15.2 ± 0.7 stimuli to develop the first stage 3 seizure.

![Fig. 1. Experimental design. A, experimental protocol. Time on the top: hours from the time of ALZET pump and electrode implantation. Vertical arrows point to 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. C to F, fluorescent images of coronal sections of the hippocampus from the rat that received human fluorescein-galanin(1–29) infusion into the hippocampus and was euthanized 24 h later. Numbers in parentheses indicate approximate distance of sections posterior from bregma (in millimeters), 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 punctate staining away from the infusion site. Scale bar, 500 μm for B and C; 250 μm for D and E; 125 μm for F. G, a figure from The Rat Brain in Stereotaxic Coordinates, G Paxinos and C Watson. Copyright © 1986, with permission from Elsevier, Oxford, UK.](https://www.jpet.aspetjournals.org/content/110/5/702/F1.large.jpg)
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 a decrease of afterdischarge threshold to 0.43 ± 0.1 mA and the increase of afterdischarge duration to 52 ± 2.9 s (p < 0.05 versus 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 seizures (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 to 75% increase in afterdischarge threshold, compared with 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 the control group (46.0 ± 6.9 s) (Fig. 2A).

During kindling, galanin(1–29) significantly delayed the occurrence of behavioral seizures of all stages compared with controls (Fig. 2B). Furthermore, two of six 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 controls in the development of stage 1 and 2 convulsions, but it took more stimulations, than in controls to develop stage 3 to 5 seizures; in addition, four of six 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 that 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 that received the injections of galanin(1–29) (0.7 ± 0.2, p < 0.05 versus control), galanin(2–11) (0.6 ± 0.3, p < 0.05 versus control), or a combination of M617 and galanin(2–11) (0.9 ± 0.4, p < 0.05 versus control) with no differences across the three groups. M617-treated animals developed 7.7 ± 1.2 full motor seizures [p < 0.05 versus control, galanin(1–29) and galanin(2–11)]. Testing afterdischarge properties 24 h after kindling revealed that the animals treated with galanin(1–29), with 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 the afterdischarge threshold (0.56 ± 0.05 mA, p < 0.05 versus 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). Coadministration 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, compared with both controls and with PTX alone-injected rats. Furthermore, a combination of either of the two peptides with PTX led to a significant reduction of afterdischarge threshold compared with 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 to 5 seizures (11 ± 1, p < 0.05 versus 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 versus 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 versus both control and PTX alone]. Animals treated with a combination of PTX and M617 showed a pattern of kindling progression (Fig. 4B) and the reproducibility of stage 4 to 5 seizures (10.5 ± 1.1) very similar 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 that had been treated with PTX + galanin(1–29) and PTX + galanin(2–11) were not different from control group in terms of afterdischarge 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 severity similar to those in controls and more severe than those in PTX-treated 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 decreases 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) compared with controls. TPQ +

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**Fig. 4.** Effects of 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 h 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. Gal, galanin. Data are presented as means ± S.E.M. *, p < 0.05 versus control; †, p < 0.05 versus PTX (one-way ANOVA + Dunn’s test). Statistical difference symbols pertain to all points outlined by the oval.

**Fig. 5.** Effects of 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. Gal, galanin. Data are presented as means ± S.E.M. *, p < 0.05 versus control; †, p < 0.05 versus TPQ (one-way ANOVA + Dunn’s test). Statistical difference symbols pertain to all points outlined by the oval.
M617 administration also accelerated kindling rate and increased the number of full motor seizures to 27.5 ± 1.3 (p < 0.05 compared with controls), but the indices did not differ from those in TPQ-only injected rats.

Animals that received the TPQ + galanin(1–29) or TPQ + galanin(2–11) combination exhibited kindling rate similar to control group; kindling progression through stages 2 to 5 was significantly slower compared with TPQ-only treated rats (Fig. 5B). The number of stage 4 to 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 24 h after kindling were similar among all TPQ-treated groups (not shown).

**Effects of dSP.** Intrahippocampal delivery of dSP altered neither afterdischarge properties nor the rate of kindling epileptogenesis, nor did it affect anticonvulsant profile of M617 (Fig. 6, A and 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 versus galanin(2–11) alone). However, adding dSP to the PTX + galanin(2–11) combination inverted 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 versus 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 nonkindled 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 that were treated with intrahippocampal dantrolene failed to develop kindling. The maximal severity of seizures was stage 1; such seizures developed in all rats, but only in response to 10 to 19 of 60 stimulations. Animals injected with dantrolene + M617, dantrolene + galanin(2–11), PTX + dantrolene, or PTX + dantrolene + galanin(2–11) showed patterns of kindling progression similar to those 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. Because 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 receptors, glutamate acting at groups II and III metabotropic glutamate receptors, serotonin acting at 5-hydroxytryptamine1A receptors, and adenosine acting at A1 receptors are coupled to Gi-protein (Counts et al., 2002; Wickenden, 2002; Moldrich et al., 2003). 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 evolution 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 THE 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.
Inhibition of $G_{q/11}$ by dSP did not affect kindling progression. $G_{q/11}$ is coupled to inositol triphosphate production and intracellular $Ca^{2+}$ 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 $G_{q/11}$. 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 $Ca^{2+}$ 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. It is noteworthy that such an approach allowed us to correlate regulation of seizures by GalR1 and GalR2 to certain downstream mechanisms.

### Role of GalR1

Presumably, 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 $G_i$ protein and downstream GIRK in the anticonvulsant action of M617 and galanin(1–29), whereas studies with dSP and dantrolene excluded $G_{q/11}$ and intracellular $Ca^{2+}$ as targets for GalR1; such conclusions are in line with previously delineated properties of GalR1 (Wang et al., 1998; Lundström et al., 2005a). Hence, hippocampal GalR1 exerts disease-modifying but not antiepileptogenic effects in kindling epileptogenesis.

### Role of GalR2

Galanin(2–11) exhibited strong antikindling effects (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 postkindling increase of hippocampal excitability. It should be noted that although galanin(2–11) has equal affinity toward GalR2 and galanin receptor type 3 (Lu et al., 2005), the latter is absent from the hippocampus (Mennicken et al., 2004).

Inhibition of kindling by galanin(2–11) depended on $G_{q/11}$ as it was PTX-sensitive, and indeed coupling of GalR2 to $G_{q/11}$ has been well established (Wang et al., 1998; Lundström et al., 2005a). However, the failure of TPQ to eliminate the 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), whereas 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 are $G_{q/11}$ protein-coupled but are GIRK-independent.

The result that seemed paradoxical was that treatment with PTX inverted the effects of both galanin(2–11) and galanin(1–29) from anti- to proconvulsant. Furthermore, the effect of the 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, whereas 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 neuropetide that equally acts at GalR1 and GalR2 (GalR1 and GalR2 are equally expressed in the rat hippocampus) (O’Donnell et al., 1999; Mennicken et al., 2004). 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 reported (Wang et al., 1998; Lundström et al., 2005a). Furthermore, the activation of $G_{q/11}$ inactivates GIRK (Lei et al., 2003), which

### 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 rates of kindling epileptogenesis.

<table>
<thead>
<tr>
<th>Agents Used</th>
<th>Vehicle (Control Values)</th>
<th>Inhibitors of Signal Transduction [Inhibited Signal]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galanin receptor agonists (preferred receptor type)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galanin(1–29) [GalR1 = GalR2]</td>
<td>ADT increased Kindling delayed</td>
<td>ADT decreased Kindling facilitated</td>
</tr>
<tr>
<td>M617 [GalR1 = GalR2]</td>
<td>ADT increased Kindling prevented</td>
<td>ADT not changed Kindling not changed</td>
</tr>
<tr>
<td>Galanin(2–11) [GalR1 &lt; GalR2]</td>
<td>ADT decreased Kindling delayed</td>
<td>ADT not changed Kindling delayed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADT not changed Kindling delayed</td>
</tr>
</tbody>
</table>

ADT, afterdischarge threshold.

* Compared with the inhibitor of signal transduction administered alone. Antiepileptic effects (increase in ADT or delay or prevention of kindling) are emphasized by italics.
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support 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 Gq11 in promoting epileptogenesis is secondary to the antiepileptic effects of Gq-coupled pathways.

Blocking of galanin receptors by M35 decreased the afterdischarge threshold and negated the inhibitory effects of M617. Both M617 and M35 are chimeric peptides that contain the 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 the anticonvulsant effects of M617. Despite the increase of hippocampal excitability by M35, the 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 the inhibitory effects of M617 and galanin(2–11) on kindling progression by M35 proves the 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 a proconvulsant in pentylenetetrazole seizures and facilitated self-sustaining status epilepticus (Mazarati et al., 1998). The discrepancies mentioned probably reflect differences in the pathophysiology of seizures, depending on probably the complexity of the effects of the peptides in the hippocampus, the results reported here hopefully expand our understanding how galanin suppresses seizures, bringing up the complexity of the effects of the peptides in the hippocampus, and might ultimately be 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 a facilitatory or an 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, the different patterns of anticonvulsant activity (e.g., disease modification versus inhibition of epileptogenesis in the kindling model or regulation of the initiation versus the maintenance of status epilepticus), and, furthermore, the seizure-facilitating effects that occur under certain conditions, should be kept in mind in the development of antiepileptic drugs acting at galanin receptors (Bartfai et al., 2004).

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