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Brain Localization and Behavioral Impact of the G-Protein-Gated K^+ Channel Subunit GIRK4

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Neuronal G-protein-gated potassium (K_G) channels are activated by several neurotransmitters and constitute an important mode of synaptic inhibition in the mammalian nervous system. K_G channels are composed of combinations of four subunits termed G protein-gated inwardly rectifying K^+ channels (GIRK). All four GIRK subunits are expressed in the brain, and there is a general consensus concerning the expression patterns of GIRK1, GIRK2, and GIRK3. The localization pattern of GIRK4, however, remains controversial. In this study, we exploit the negative background of mice lacking a functional GIRK4 gene to identify neuronal populations that contain GIRK4 mRNA. GIRK4 mRNA was detected in only a few regions of the mouse brain, including the deep cortical pyramidal neurons, the endopiriform nucleus and claustrum of the insular cortex, the globus pallidus, the ventromedial hypothalamic nucleus, parafascicular and paraventricular thalamic nuclei, and a few brainstem nuclei (e.g., the inferior olive and vestibular nuclei). Mice lacking GIRK4 were viable and appeared normal and did not display gross deficiencies in locomotor activity, visual tasks, and pain perception. Furthermore, GIRK4-deficient mice performed similarly to wild-type controls in the passive avoidance paradigm, a test of aversive learning. GIRK4 knock-out mice did, however, exhibit impaired performance in the Morris water maze, a test of spatial learning and memory.

Key words: potassium channel; G-protein; GIRK; Kir3.0; in situ *hybridization; Morris water maze; passive avoidance; locomotor activity; mice*

G-protein-gated K^+ (K_G) channels are found throughout the CNS and are activated by a large number of neurotransmitters including acetylcholine, adenosine, ATP, dopamine, GABA, opioids, cannabinoids, serotonin, norepinephrine, and somatostatin (North, 1989; Brown, 1990; Nicoll et al., 1990; Hille, 1994). K_G channels are heterotetrameric complexes formed by the assembly of members of the GIRK (Kir3.0) subfamily of inwardly rectifying K^+ channel subunits (Duprat et al., 1995; Ferrer et al., 1995; Inanobe et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995a; Lesage et al., 1995; Liao et al., 1996; Silverman et al., 1996; Velimirovic et al., 1996; Isomoto et al., 1997; Wischmeyer et al., 1997; Corey et al., 1998; Yoshimoto et al., 1999). Four mammalian GIRK (GIRK1–4) subunits have been identified (Dascal et al., 1993; Kubo et al., 1993; Ferrer et al., 1995; Kobayashi et al., 1995; Krapivinsky et al., 1995a,b; Lesage et al., 1995), each containing a hydrophobic sequence common to all potassium-selective channels, two membrane-spanning domains, and cytoplasmic N and C terminals.

The muscarinic-gated atrial potassium channel I_{KACh} is the prototypical member of the K_G channel family. I_{KACh} is formed by stoichiometric assembly of GIRK1 and GIRK4 subunits and is a component of the parasympathetic-signaling pathway that culminates in heart rate slowing (Krapivinsky et al., 1995a; Wickman and Clapham, 1995; Corey et al., 1998). Acetylcholine released after vagal stimulation binds muscarinic receptors on the surface of sinoatrial and atrioventricular nodal cells and atrial myocytes, triggering a G-protein-signaling cascade that stimulates I_{KACh} and modulates the activity of several other ion channels and enzymes. Previously, we generated mice lacking a functional GIRK4 gene (Wickman et al., 1998). Although the mice were viable and appeared normal, cardiac myocytes obtained from the mice lacked the I_{KACh} conductance. Furthermore, the mice exhibited a blunted heart rate response to vagal stimulation and adenosine administration and were resistant to pacing-induced arrhythmias (Wickman et al., 1998) (P. Kovoor, K. Wickman, C. Maguire, W. Pu, J. Gehrmann, C. Berul, and D. Clapham, unpublished observations).

Although the physiological significance of cardiac I_{KACh} is well understood, the role of K_G channels in brain is relatively unclear. Definitive maps of the temporal and spatial expression patterns of each GIRK subunit are required to understand the role(s) of K_G channels in neuronal functions. Indeed, expression patterns for the four GIRK subunits in the developing and adult rodent CNS have been estimated using *in situ* hybridization and immunohistochemical techniques (Karschin et al., 1994, 1996; Spauschus et al., 1996; Chen et al., 1997; Iizuka et al., 1997; Karschin and Karschin, 1997; Murer et al., 1997). Results from these studies suggest that all four GIRK subunits are expressed in the rodent brain and exhibit overlapping yet distinct expression patterns. Of the four subunits, it has been most difficult to reach a consensus concerning the expression pattern of GIRK4. Previous cloning and localization studies suggested that GIRK4 was expressed throughout the brain and, in particular, in the hippocampus, cerebellum, olfactory bulb, and basal ganglia (Spauschus et al., 1996; Iizuka et al., 1997). Because their activity is tightly controlled by many neurotransmitters, K_G channels present in these neuronal populations could influence important behaviors such as learning and memory, locomotion, and sensory perception. In this study, we used mice lacking a functional GIRK4 gene to establish definitively the expression pattern of GIRK4 in the mouse brain. In addition, we tested whether locomotor function, sensory perception, and learning and memory processes were intact in GIRK4 knock-out mice.

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MATERIALS AND METHODS

In situ hybridization

Three 11-d-old wild-type and two GIRK4 knock-out mice were killed by CO_2 asphyxiation. Their brains and hearts were removed and placed on powdered dry ice, stored at −80°C, and subsequently processed in a blind manner. Sixteen micrometer sections were cut by cryostat, thaw-mounted onto glass slides, air-dried for 2–5 min, and fixed for 5–10 min in freshly prepared 4% paraformaldehyde in 1× PBS, pH 7.4. Slides were then washed twice in 1× PBS for 10 min each, dehydrated via a graded ethanol series (50, 70, 90, and 100%), and stored under ethanol (95%) at 4°C until further use.

For radioactive hybridizations, synthetic oligonucleotides were chosen from the 5′ portion of the mouse GIRK4-coding sequence exhibiting the least homology to other K_{ir} sequences. Antisense oligonucleotides designed with the least predicted tendency for forming hairpins and self-dimers were as follows: (1) 5′-GTG ACT CCT ATC TCC ATG TCT TGA TTC ATA GCA TTC CTA GAA TCA CC-3′ and (2) 5′-GCC TTC TGT CAG CAG GCG GGT GCG GTC TGT GGC AAT GGG GAT GTA-3′. Oligonucleotides were 3′ end-labeled with ^{35}S-dATP or ^{33}P-dATP (New England Nuclear, Boston, MA; 1200 and 1000 Ci/mmol) by terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). Probes were used for hybridization at concentrations of 2–10 pg/μl (400,000 cpm per slide) in hybridization solution (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 0.03 M Tris-HCl, pH 7.4, 0.004 M EDTA, 1× Denhardt's solution, 0.5 mg/ml polyA DNA, 0.5 mg/ml salmon sperm DNA, 50 mM DDT, and DEPC-treated water). Brain sections were processed for radioactive hybridization and exposed to x-ray film as described previously (Karschin et al., 1996). For analysis with bright- and dark-field optics, slides were Nissl-stained with cresyl violet, put into xylene, and coverslipped. Structures in mouse brains were identified and confirmed according to Paxinos and Watson (1986).

For nonradioactive hybridizations, digoxygenin-labeled sense and antisense cRNA probes were transcribed with T3 and T7 polymerase from a rapid amplification of cDNA ends fragment of the mouse GIRK4 gene subcloned into pBluescript (Stratagene, La Jolla, CA) according to the manufacturer's protocol (RNA Labeling and Detection Kit; Roche Diagnostics). Transcripts were used at a concentration of ~800 pg/μl of hybridization buffer and label-detected by alkaline phosphatase-coupled antibodies to digoxygenin (Bartsch et al., 1992).

Although comparison of expression patterns between sections from wild-type and GIRK4 knock-out mice effectively revealed the extent of nonspecific and/or cross-specific interactions between the probes and tissue, wild-type sections were subjected to additional controls: (1) adjacent sections were pretreated with RNase, (2) adjacent sections were hybridized with labeled sense probes, and (3) adjacent sections were hybridized with labeled probe in the presence of a 20-fold excess of unlabeled probe. All control treatments resulted in a complete loss of hybridization signals.

Behavioral studies

Locomotor activity and Morris water maze were performed in a temperature-controlled, dimly lit, and well ventilated room. In total, 11 pairs (six male and five female) of wild-type and GIRK4 knock-out littermates were tested for differences with respect to spontaneous horizontal locomotor activity. These 11 pairs, plus an additional 10 pairs (five male and five female) were tested in the passive avoidance and Morris water maze paradigms. Pairs were obtained from the litters of three breeding pairs consisting of adults heterozygous for the GIRK4 null mutation. Heterozygous parents were obtained from the fifth generation of backcrossing onto the C57BL6/J mouse strain. Ages of the mice tested ranged from 3 to 9 months. No statistically significant differences in performance were observed between genders in any of the tests. Thus, all data were collapsed into single groups of wild-type and GIRK4 knock-out mice.

Locomotor activity

Horizontal locomotor activity of the wild-type and knock-out mouse pairs was measured using an automated photobeam activity system. Mice were introduced into a novel environment (standard rat cage), and the number of infrared photobeam interruptions was totaled for each 5 min interval during a 1 hr session. Between sessions, the rat cage was wiped clean with 70% ethanol. All mice were tested between 1 and 4 P.M.

Morris water maze

The Morris water maze consisted of three phases.

Hidden platform test. A circular opaque plastic tub 1 m in diameter was filled with water (20.5–22°C). A 10-cm-square transparent Plexiglas platform was submerged 0.5 cm beneath the water surface and positioned consistently throughout the course of the hidden platform tests. On day 0, mice were allowed to swim until they encountered the platform or until 60 sec had elapsed, at which point they were placed on the platform for 15 sec. On days 1–12, each mouse was given four trials (mice were placed into a different quadrant of the tub for each trial), and the time to find the hidden platform was recorded and averaged daily. The maximum allowable swim time was 60 sec. Swim time and all other measurements for each experimental trial were made using the Poly-Track Video Tracking System (San Diego Instruments). After each trial, mice were placed or allowed to remain on the platform for 15 sec. The intertrial interval was 3.5 min. Experiments were performed during the afternoon. Performance did not improve significantly between the tenth and twelfth days of testing.

Transfer test. On day 13, the platform was removed, and each mouse was placed in the center of the tub facing the same direction and allowed to swim for 60 sec. The number of entries into the target area (where the platform had been positioned on days 1–12), time spent in each quadrant, path length, and first latency to entry into the target area were recorded.

Visible platform test. On days 1–3 of the visible portion of the Morris water maze, each mouse was given four trials (mice were placed in a different quadrant of the tub for each trial), and the time to find a flagged platform was recorded and averaged for each session. The maximum allowable swim time was 60 sec. After each trial, mice were allowed to sit or were placed on the platform for 15 sec. The intertrial interval was 3.5 min. Two four-trial sessions were performed each day, and performance did not improve significantly between sessions 3 and 5. Five pairs of mice were removed because of excessive floating throughout the course of the testing.

Passive avoidance

Experiments were performed using a passive avoidance apparatus (Ugo Basile, Verase, Italy), which consisted of a unit with one black, unlit chamber and one white, lit chamber separated by a controllable door. The floor consisted of a metal grid wired to deliver shocks of controlled intensities and durations. The sensitivities of the two groups of mice to increasing foot shock intensities were assessed before testing. Mice were scored for jumping and vocalization responses to shocks ranging from 0.2 to 2 mA. Shock thresholds for vocalization [0.5 ± 0.11 vs 0.61 ± 0.11 mA, wild-type vs knock-out mice (± SEM)] and jumping [0.96 ± 0.11 vs 1.02 ± 0.11 mA, wild-type vs knock-out mice (± SEM)] were not significantly different between the two groups. On day 1 of the test, each mouse was placed into the white compartment and allowed to explore both chambers of the apparatus for 5 min. On day 2, each mouse was put into the white chamber of the apparatus. After the mouse entered the dark chamber, the door closed, and the mouse received a 2 mA shock of 1 sec duration. The latency to enter the dark compartment was recorded. After the shock, mice were removed and returned to their cage. On day 3, each mouse was placed into the white chamber, and the latency to enter the black chamber was measured. No shock was delivered. Mice were removed if they failed to enter the dark compartment after 5 min and were assigned a latency of 300 sec. The apparatus was wiped down with 70% ethanol between trials. Trials were performed between 6 and 7 P.M.

Statistical considerations

Data from the locomotor activity test, passive avoidance, and Morris maze (path length, target entries, and first latencies) were analyzed by a two-tailed *t* test assuming equal variances (variances were assessed to be equal using the *F* test). The Morris maze performance curves were analyzed by repeated measures ANOVA with one within-subject variable (each genotype) and one between-subject variable (comparing genotypes) using the Statistical Program for the Social Sciences (SPSS). All values are given ± SEM.

RESULTS

GIRK4 brain localization

In situ hybridizations with both radioactive and nonradioactive probes were performed in blind format on sections of brain and heart obtained from wild-type and GIRK4 knock-out mice. Sections of heart tissue from wild-type mice served as positive controls for GIRK4 expression (Fig. 1). In mice and other mammals, atrial tissue hosts both GIRK1 and GIRK4 subunits that combine to generate I_{KACh} (Krapivinsky et al., 1995a; Wickman et al., 1998). In mouse, highest GIRK4 expression levels were observed in the heart atria (Fig. 1*B*). GIRK4 mRNA was also detected in the heart ventricle, although at lower levels. In contrast, no expression of GIRK4 was detected in heart sections obtained from GIRK4 knock-out mice (Fig. 1*D*). Similarly GIRK4 mRNA signals were completely absent from the brain sections obtained from GIRK4 knock-out mice (Fig. 2*F*). Lack of signal in brain sections from GIRK4 knock-out mice was not caused by poor tissue quality or inappropriate experimental conditions, because hybridization with probes specific for a different potassium channel subunit (K_{ir}2.3) yielded strong signals consistent with previously established expression patterns (data not shown) (Karschin et al., 1996). Nissl-stained sections did not reveal any gross morphological or cytoarchitectural differences between the sections of different genotypes.

GIRK4 brain expression patterns revealed by both labeled oligonucleotide and cRNA probes were consistent for the three wild-type mice tested. GIRK4 mRNA was restricted to only a few

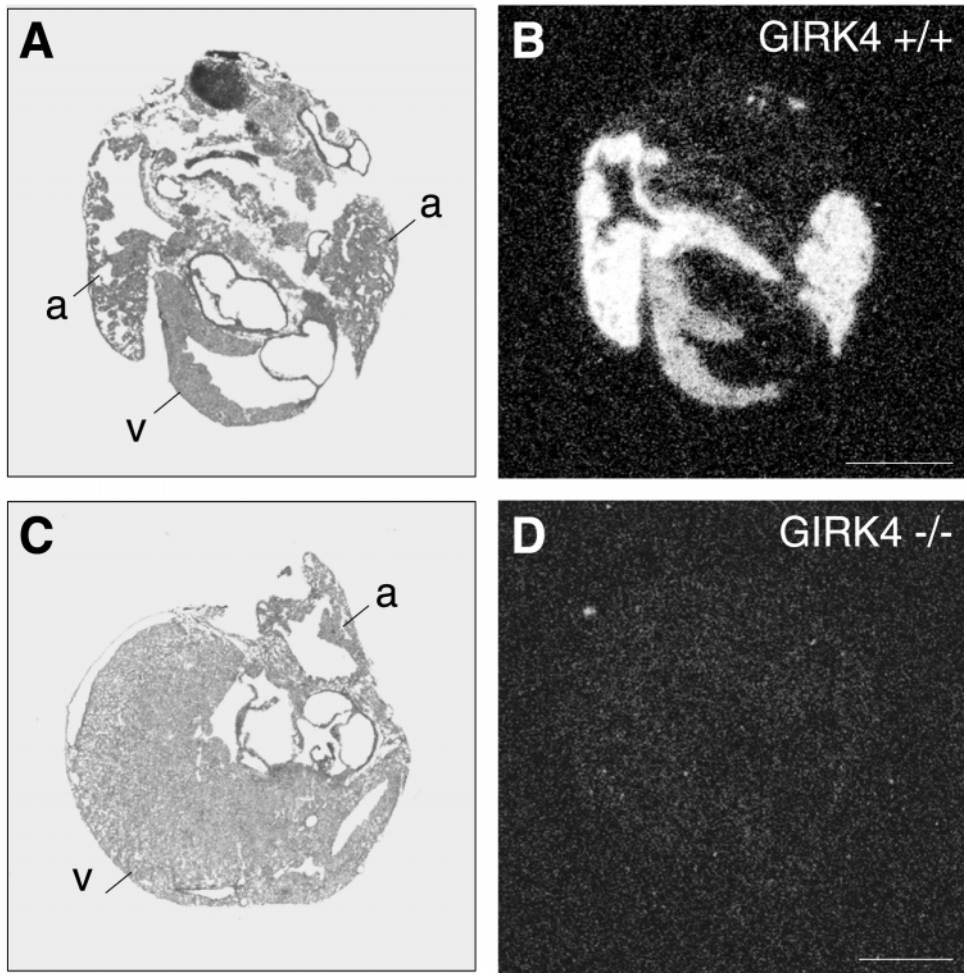

Figure 1. Localization of GIRK4 mRNA in mouse heart as revealed by *in situ* hybridization. X-ray film autoradiographs of horizontal sections through the heart of wild-type mice (*A, B*) and GIRK4 knock-out mice (*C, D*) hybridized with GIRK4-specific ^{35}S-labeled oligonucleotide probes. Sections shown in *A* and *C* were Nissl-counterstained with cresyl violet. Exposure time was 38 d. Scale bars: *A, B*, 1 mm; *C, D*, 1 mm. *a*, atria; *v*, ventricle.

neuronal populations, and the overall expression pattern was primarily consistent with that described for the developing rat (Karschin and Karschin, 1997). In the neocortex, a conspicuous cell band adjacent to the white matter was detected, composed of strongly labeled neurons, alternatively termed layer VIb, layer VII, or subplate neurons (Figs. 2*D*, 3*A*). In neurogenesis, these deep cortical neurons are among the first generated and differentiated. In the cat visual and auditory cortex, layer VII neurons are required for cortical target selection and ingrowth by thalamic axons (Ghosh and Shatz, 1993). As described for the rat (Karschin and Karschin, 1997), GIRK4 expression in layer VII neurons also declines as the animal progresses to adulthood (data not shown), suggesting a developmental role for channels composed of GIRK4. Strong GIRK4 expression was also observed in the claustrum and adjacent endopiriform nucleus, components of the insular cortex and the central autonomic system. Other central autonomic system centers, the thalamic parafascicular and paraventricular nuclei, were among the most prominently labeled structures. Lower levels of GIRK4 mRNA were detected in the laterodorsal and lateral posterior thalamic nuclei (Fig. 2*D*). In the hypothalamus, only the ventromedial hypothalamic nucleus displayed a signal (Fig. 2*D*).

In several nuclei, only small subsets of neurons contained GIRK4 mRNA. This is true, for instance, in the globus pallidus, the nuclei of the diagonal band of the forebrain, and the superior colliculus of the midbrain and for distinct brainstem nuclei such as the medial vestibular and dorsal tegmental nuclei (Fig. 3*B,C*). Similarly GIRK4 mRNA was not detected in the principal cell layers in the hippocampus but was observed in a few cells in the dentate gyrus molecular layer, often in close proximity to the hippocampal fissure (Fig. 3*D*). It is possible that the GIRK4 cDNA cloned from a human hippocampal library derived from these cells (Spauschus et al., 1996). Furthermore, although the main olfactory bulb was not labeled, a few cells of unknown identity and function in the anterior olfactory nucleus displayed strong probe reactivity (Fig. 3*F*). Neurons expressing GIRK4 (and GIRK1) in a similar location were detected in the developing rat brain (Chen and Yu, 1994; Karschin and Karschin, 1997).

Although Purkinje neurons in the rat cerebellum were reported to express GIRK4 mRNA and protein (Iizuka et al., 1997; Murer et al., 1997) (see Discussion), we did not detect GIRK4 mRNA in the molecular, granule cell, or Purkinje cell layers of the mouse cerebellum. Neurons in the lateral cerebellar nuclei, however, were labeled (Figs. 2*B*, 3*E*). In addition and consistent with previous observations in the developing rat brain, strong GIRK4 signals were detected in the lower brainstem of 11-d-old mice within inferior olive neurons, whose climbing fibers project to cerebellar Purkinje neurons (Fig. 2*B*).

Behavioral characterization of GIRK4 knock-out mice

Mice lacking GIRK4 are viable, appear normal, and display a normal life expectancy (Wickman et al., 1998). The phenotypes detected in GIRK4 knock-out mice to date relate to cardiac function and include a blunted heart rate decrease in response to vagal stimulation and a resistance to pacing-induced arrhythmias (Wickman et al., 1998) (Kovoor, Wickman, Maguire, Pu, Gehrmann, Berul, and Clapham, unpublished observations). Despite evidence of expression of GIRK4 in pancreatic β-cells (Ferrer et al., 1995), GIRK4 knock-out mice have resting blood glucose levels and weights similar to those of their wild-type littermates (data not shown). Initial mRNA and protein localization experiments suggested that GIRK4 was expressed in several brain regions, including the hippocampus, basal ganglia, and cerebellum (Karschin et al., 1996; Iizuka et al., 1997). Because these brain structures influence several important behaviors such as learning and memory,

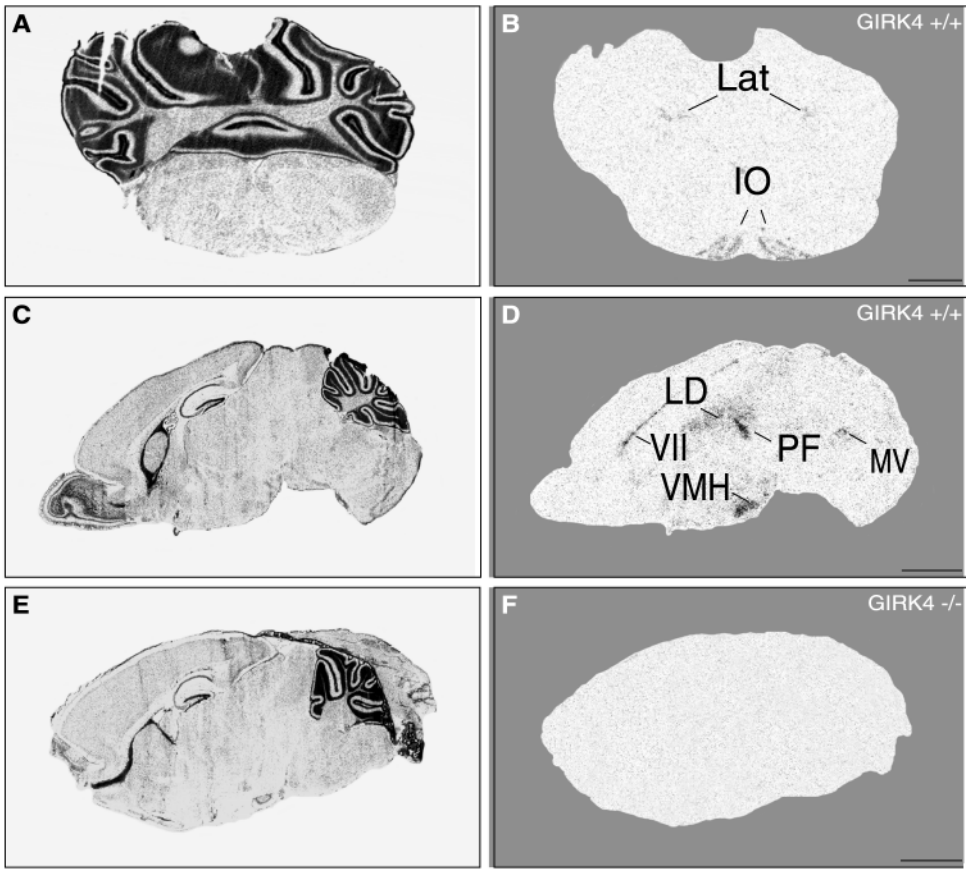

Figure 2. Coronal and sagittal brain sections of wild-type mice (*A–D*) and GIRK4 knock-out mice (*E, F*) hybridized with ^{35}S- and ^{33}P-labeled GIRK4-specific oligonucleotide probes. Nissl-counterstained sections are shown on the *left* for anatomical reference (*A, C, E*). Exposure time was 32 d. *IO*, Inferior olive; *Lat*, lateral cerebellar nuclei; *LD*, laterodorsal thalamic nucleus; *MV*, medial vestibular nucleus; *PF*, parafascicular thalamic nucleus; *VMH*, ventromedial hypothalamic nucleus; *VII*, cortex layer VII. Scale bars: *A*, *B*, 1 mm; *C*, *D,* 2 mm; *E, F*, 2 mm.

locomotion, and coordination, we became interested in testing for phenotypes related to these functions in mice lacking GIRK4.

As a simple test of spontaneous horizontal locomotor activity, same-sex sibling pairs of wild-type and GIRK4 knock-out mice were introduced into a novel environment, and their activity levels were monitored for 1 hr. This test has been used to reveal gross differences related to activity levels and coordination (Crawley and Paylor, 1997; Picciotto and Wickman, 1998). Furthermore, because most well established behavioral paradigms rely on locomotion, it is important to ascertain whether the two groups of subjects to be tested exhibit comparable levels of activity. We observed no significant difference in locomotor activity between genotypes or genders (Fig. 4). In addition, mice from both groups exhibited comparable levels of habituation (decrease in locomotor activity over time) to the environment.

We next tested for differences between the two genotypes in the Morris water maze, a test of spatial learning and memory. Genetic and lesion studies have confirmed the importance of hippocampal function for successful performance of this task (Sutherland et al., 1983; Schenk and Morris, 1985; Morris et al., 1986; Sakimura et al., 1995; Cho et al., 1998; Giese et al., 1998). The water maze is a three-phase test. In the visible platform test, the speed with which a mouse locates a visible platform in a pool of water is measured over a series of trials. Mice with sufficient visual acuity, swimming ability, and motivation to escape the water perform well in this phase of testing. Indeed, both GIRK4 knock-out mice and wild-type controls performed equally well in the visible platform test (Fig. 5*A*).

In the hidden platform test, mice use visual cues from the environment to locate a transparent platform submerged beneath the water surface. The time taken to find the hidden platform is measured over the course of several sessions. A trend toward poorer performance by the GIRK4 knock-out mice was observed over the 12 d of testing in this phase of the water maze (Fig. 5*B*). Indeed, the GIRK4 knock-out mice as a group performed worse than did wild-type mice on 10 of the 12 d of trials, the only exceptions being equivalent performances on days 2 and 3. By day 12, wild-type mice found the platform in 17 ± 3 sec (n = 19), whereas GIRK4 knock-out mice found the platform in 22 ± 3 sec (n = 16). This trend did not reach statistical significance (p = 0.11), however, and both performance curves reflect improvements over time.

Improvement in the hidden platform test related to spatial learning is assessed using the transfer test, during which the platform is removed and the exploratory pattern of the mouse is recorded. Wild-type mice entered the target area (location of platform in hidden platform test) 4.1 ± 0.4 times during a 60 sec swim, whereas GIRK4 knock-out mice entered the target area only 2.2 ± 0.5 times (Fig. 5*C;* $^{**}p$ = 0.009). Four GIRK4 knock-out mice failed to enter the target area, whereas all of the tested wild-type mice entered the target area at least once. This difference was not caused by different swimming abilities, because there was no significant difference in the distance traveled by the two groups (1420 ± 40 vs 1320 ± 50 cm; p = 0.088). First latencies to enter the target area during the transfer test reflected the trend observed in the hidden platform phase of testing, with wild-type mice entering the target area more quickly than GIRK4 knock-out mice (16 ± 3 vs 25 ± 6 sec; p = 0.11). An assessment of quadrant occupancy during the transfer test demonstrated that wild-type mice spent most of their time in the quadrant where the platform had been located, whereas GIRK4 knock-out mice showed preference for an adjacent quadrant (Fig. 5*D*). Despite the overall differences observed between the performance of the two groups, it should be noted that some of the GIRK4 knock-out mice performed well in all phases of the test.

We next tested for phenotypes related to learning and memory subserved by different brain structures. Passive avoidance is a test of aversive memory that involves pairing of a mild foot shock with a specific environment and then measuring the extent of aversion displayed toward that environment after subsequent exposure (Brioni and McGaugh, 1988; Mathis et al., 1994a,b; Picciotto and Wickman, 1998). Lesion and pharmacological studies have demonstrated that the amygdala and striatum are crucial for learning in

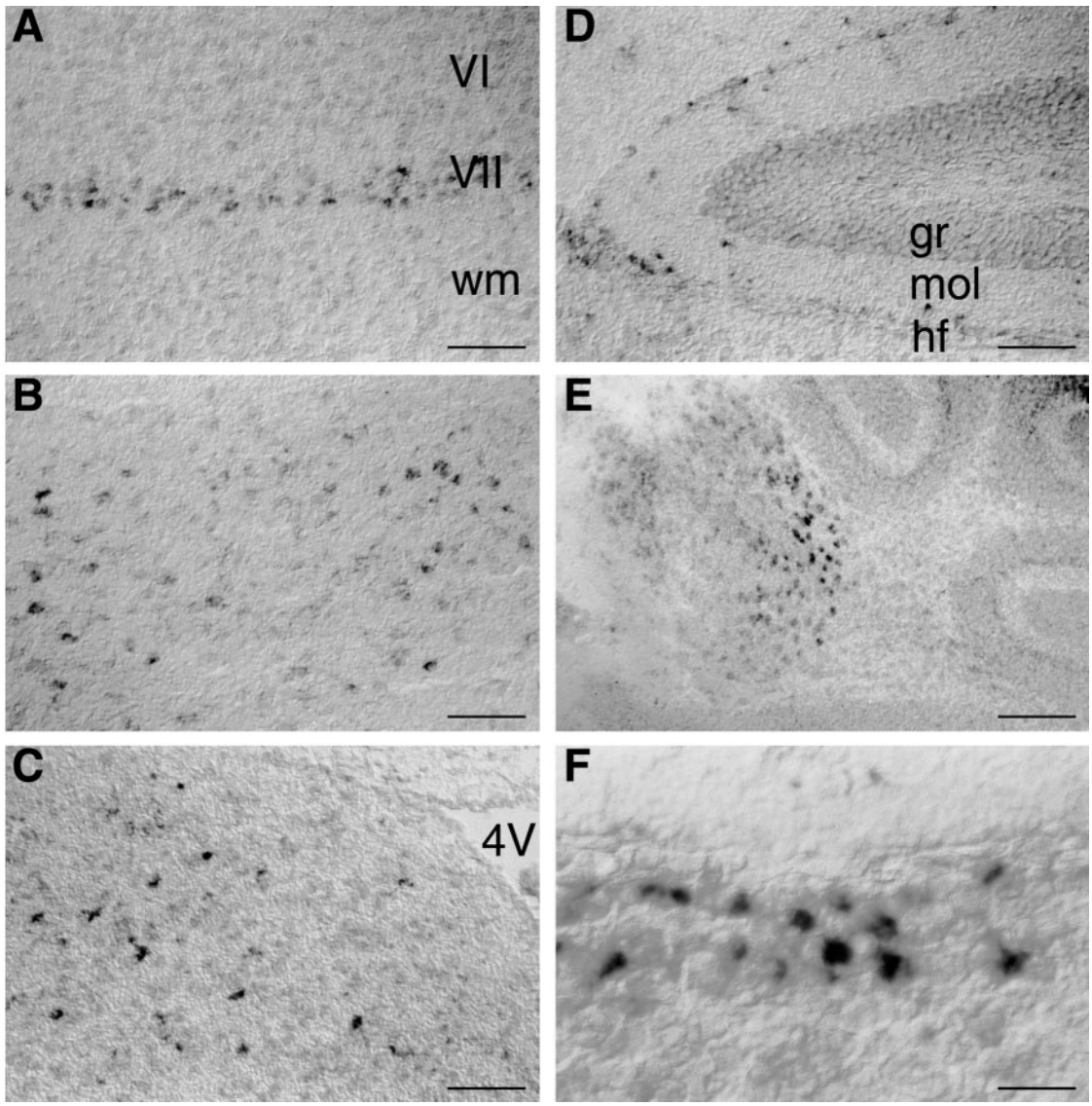

Figure 3. *A—C*, Nomarski optics bright-field photomicrographs of wild-type mouse brain sections hybridized with digoxygenin-labeled GIRK4 mRNA probes show positive signals in neurons of cortical layer VII (*A*), the globus pallidus (*B*), and the medial vestibular nucleus (*C*). Note that only a few neurons are strongly positive in each region. *D,* Small cells in the dentate gyrus molecular layer close to the hippocampal fissure strongly express GIRK4 mRNA. *E,* Neurons in the lateral cerebellar nucleus are positive, but not in the granule cell or Purkinje cell layer. *F,* High-power micrograph of GIRK4-positive cells in the dorsal anterior olfactory nucleus is shown. *gr*, Dentate gyrus granule cell layer; *hf*, hippocampal fissure; *mol*, dentate gyrus molecular layer; *VI, VII*, cortical layers VI and VII; *wm*, white matter; *4V*, fourth ventricle. Scale bars: *A—D*, 100 μm; *E*, 200 μm; *F*, 30 μm.

this paradigm (Dubrovina and Ilyutchenok, 1996; Stewart et al., 1996). Because the negative stimulus in this test is a foot shock, it is important first to confirm that both groups of mice respond similarly to the stimulus. Indeed, both groups responded similarly, by vocalization and jumping, over a range of foot shock intensities. On the first day of testing, wild-type and GIRK4 knock-out mice entered the dark compartment with identical latencies (Fig. 6; 14 ± 3 vs 14 ± 2 sec), at which point they received a 2 mA foot shock of 1 sec duration. Twenty-four hours later, both groups of mice displayed marked delays before entering the dark environment (140 ± 24 vs 182 ± 23 sec, wild-type vs knock-out mice; $p = 0.21$); several mice from each group avoided the dark environment altogether. Thus, there was no significant difference in performance between the two groups in this behavioral test.

DISCUSSION

One goal of this study was to address the discrepancies between previous studies of GIRK4 localization in the rodent CNS. Because most previous studies have been performed in the rat, a related goal was to establish the GIRK4 expression pattern in the mouse brain. All previous studies have agreed that GIRK4 is expressed in the rodent brain, although at lower levels than the other three GIRK family members (Karschin et al., 1994, 1996; Spauschus et al., 1996; Chen et al., 1997; Iizuka et al., 1997; Karschin and Karschin, 1997; Murer et al., 1997). Differences have centered mainly on the level and pattern of GIRK4 expression. In this study, we exploited the negative background of a GIRK4 knock-out mouse to reveal the GIRK4 expression pattern in wild-type mice.

The GIRK4 mRNA localization pattern we describe for 11-d-old mice is primarily consistent with results from a previous study in the developing rat, with a few minor differences (Karschin and Karschin, 1997). For example, we detected no GIRK4 in the subthalamic nucleus and little GIRK4 in the anterior pretectal nuclei, regions that tested positive for GIRK4 in the rat brain. In addition, the expression of GIRK4 in subsets of neurons within a given nucleus (e.g., globus pallidus) was not reported previously in rat. This feature of GIRK4 expression was revealed in mice only by using efficiently labeled probes, long exposure times, and comparisons with analogous sections from GIRK4 knock-out mice. The

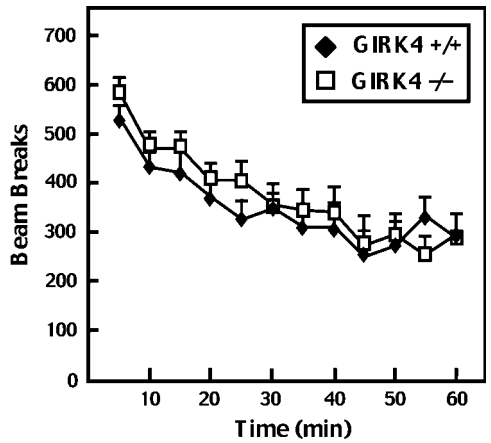

Figure 4. Spontaneous horizontal locomotor activity of GIRK4 knock-out and wild-type mice. Both groups showed habituation over the course of the experiments. There was no statistically significant difference in the activity levels in the different groups of mice.

small differences between our study and the study in the developing rat could reflect genuine species differences, which have been noted for other GIRK subunits. For example, GIRK2 is more evenly distributed throughout the thalamus in the mouse in comparison with the rat (Liao et al., 1996). Alternatively, the small differences could simply reflect a signal-to-noise problem. Indeed, the low levels of GIRK4 mRNA approached the limits of detection of targets for *in situ* hybridization.

Species differences probably do not account for the major differences between our study and two GIRK4 immunohistochemical studies (Iizuka et al., 1997; Murer et al., 1997). Using a combination of *in situ* hybridization and immunohistochemical approaches, Iizuka et al. (1997) reported that GIRK4 was expressed at high levels in the rat hippocampus, cerebral cortex, basal ganglia, and several brainstem nuclei. In contrast, we detected no GIRK4 mRNA in these brain regions. In general, our findings were more consistent with the immunohistochemical study by Murer et al. (1997). We both, for instance, observed a limited GIRK4 expression pattern and found GIRK4 in the deep layers of the cortex and in specific subsets of neurons within several nuclei (e.g., globus pallidus). Murer et al. (1997) reported GIRK4 expression, however, in the habenula. Although we did not detect GIRK4 expression in the habenula, we did detect a strong signal in the paraventricular and parafascicular thalamic nuclei, which surround the habenular nucleus. Because of the agreement between our study and previous *in situ* hybridization studies (Karschin et al., 1996; Chen et al., 1997; Karschin and Karschin, 1997) and the immunohistochemical study of Murer et al. (1997), the weight of evidence argues that problems related to antibody and *in situ* probe specificity led to the overestimation by Iizuka et al. (1997) of GIRK4 expression levels and distribution in rat brain.

One discrepancy between our findings and both immunohistochemical studies relates to the expression of GIRK4 in cerebellar Purkinje neurons. Although both antibody studies detected GIRK4 protein in Purkinje neurons, we failed to detect GIRK4 mRNA in these neurons. Cerebellar Purkinje neurons receive input from the terminal axon fields of climbing fibers originating in the inferior olive, neurons we found to possess high levels of GIRK4 mRNA. It is possible that the detected immunoreactivity reflected GIRK4 protein found in axon terminals of climbing fibers synapsing on Purkinje neurons. Other explanations are also tenable. As mentioned previously, antibody nonspecificity or cross-specificity could explain the immunoreactivity in Purkinje neurons. Alternatively, the level of GIRK4 mRNA in some neurons could be below the limits of detection by *in situ* hybridization, yet sufficient to produce detectable protein by a sensitive antibody.

Before completing our GIRK4 mRNA localization studies, we initiated a series of behavioral tests aimed at detecting phenotypes

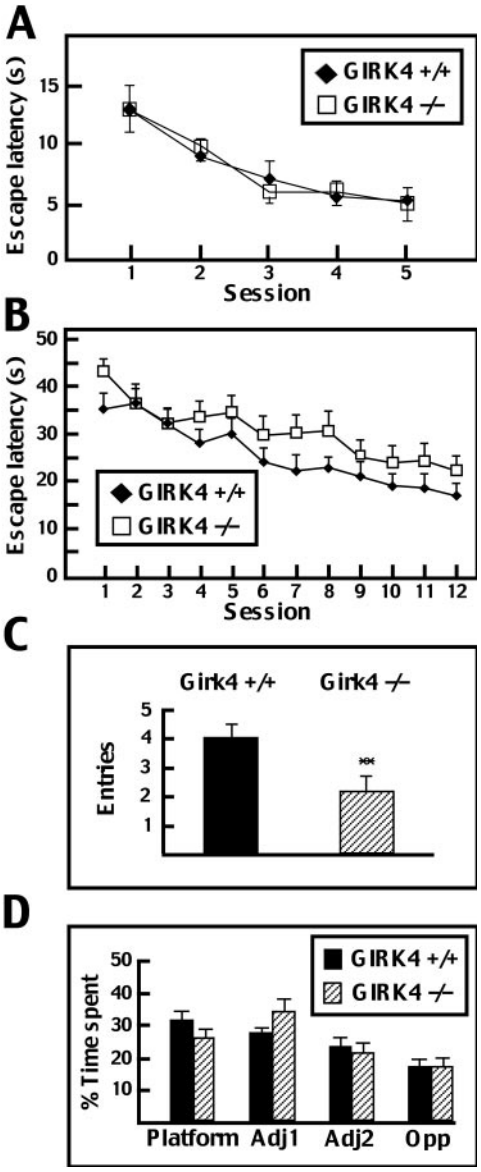

Figure 5. Performance of GIRK4 knock-out and wild-type mice in the Morris water maze. *A,* Learning curves in the visible platform component of the test measured over 5 sessions. Escape latency refers to the time required to find the visible, flagged platform. *B,* Learning curves in the hidden platform component of the test measured over 12 sessions. Escape latency refers to the time required to find the submerged platform. There was a trend toward poorer performance by the GIRK4 knock-out mice over the 12 d of testing, but it did not reach statistical significance. *C,* Number of target area entries during the transfer test. GIRK4 knock-out mice entered the target area approximately one-half as many times as did wild-type control mice. *D,* Percentages of time spent in each quadrant during the transfer test. Note that the wild-type mice spent most of their time in the target area quadrant at the expense of the opposite quadrant. Interestingly, GIRK4 knock-out mice spent most of their time in an adjacent quadrant, the quadrant toward which they were directed when introduced into the water. *Adj*, Adjacent; *Opp*, opposite.

related to locomotor activity and learning and memory. These behaviors were targeted because of reports of GIRK4 expression in the hippocampus, cerebellum, and basal ganglia (Spauschus et al., 1996; Iizuka et al., 1997). We took several steps to minimize the primary sources of variability in behavioral studies, including age, gender, and genetic background. Pairs of same-sex littermates homozygous for either the wild-type or mutant GIRK4 gene were tested. This effectively controls for the effects of gender, age, and environmental factors experienced throughout development between genotype groups. Increased appreciation for the influence of the background strain in behavioral studies has developed over the

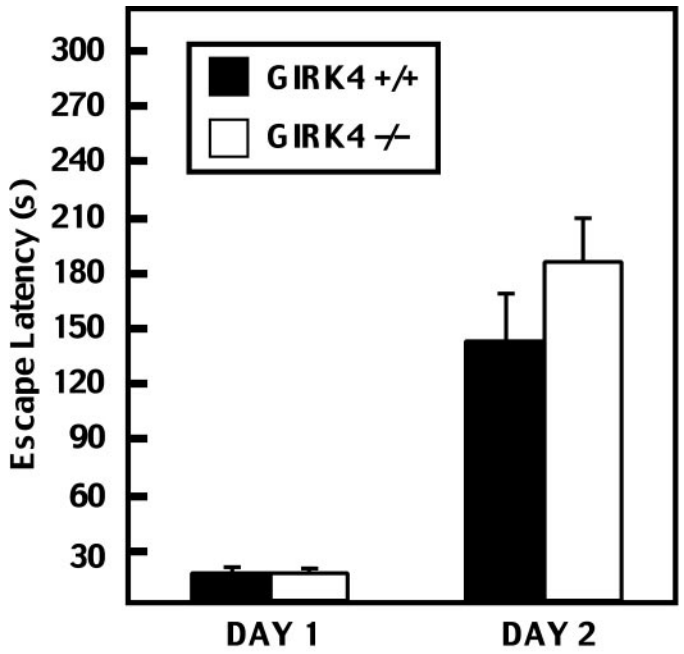

Figure 6. Performance of GIRK4 knock-out and wild-type mice in the passive avoidance test. The latencies to enter the dark chamber for wild-type and GIRK4 knock-out mice were identical on day 1 and not significantly different on day 2, 24 hr after receiving the foot shock.

last several years, emphasizing the importance of controlling for this factor in testing paradigms (Crawley, 1996; Gerlai, 1996; Crawley and Paylor, 1997; Crawley et al., 1997). To minimize the impact of genetic background, we backcrossed the GIRK4 null mutation for five generations onto the C57BL6/J genetic background before establishing the breeding pairs used to generate the mice in this study. Five generations of backcrossing ensures that the majority of gene alleles are identical in the test subjects.

Results from our studies suggest that GIRK4 knock-out mice are not grossly different from wild-type counterparts with respect to locomotor activity, visual acuity, and pain perception (foot shock). Because performance in all phases of the Morris water maze is dependent on swimming and climbing ability, we can infer that coordination is not grossly affected in mice lacking GIRK4. This is not to say that G-protein-gated K^+ channels containing GIRK4 do not play roles in these processes. The presence of other GIRK family members may compensate for the loss of GIRK4. Indeed, the neuronal populations we identified in this study as expressing GIRK4 also have been shown to express one or both of the GIRK4 functional homologs GIRK2 and GIRK3 (Karschin et al., 1996; Chen et al., 1997).

In general, GIRK4 knock-out mice performed poorly relative to wild-type littermates in the hidden platform test and were significantly impaired in the transfer test. Because same-sex sibling pairs of wild-type and GIRK4 knock-out mice were used in these studies and because virtually identical results were obtained in two different testing groups, it seems likely that the effects observed in the Morris water maze were not caused by an uncontrolled genetic or environmental variable.

The Morris water maze is routinely classified as a hippocampal-dependent task that probes spatial learning and memory. We did not, however, detect GIRK4 mRNA in the principal neurons of the hippocampal formation. Furthermore, electrophysiological characterization of hippocampal pyramidal and dentate gyrus neurons in brain slices did not reveal significant differences between wild-type and GIRK4 knock-out mice with respect to K_G (data not shown). Taken together, our results suggest that subtle deficits in the function of brain structures other than the hippocampus are responsible for the poorer performance of GIRK4 knock-out mice in the Morris water maze. Because of the results from our *in situ* hybridization study, we are now in a position to ask questions concerning the function of GIRK4-containing K_G channels in these other neuronal populations. It will be important to ascertain which neurotransmitters couple to K_G channels in these neurons and the impact that K_G activation has on both the host neuron and the linked neuronal circuitry during neurogenesis and in the adult animal. In the absence of specific pharmacological tools for K_G channels, mice lacking GIRK4 and the other GIRK subunits will continue to be valuable negative controls for these studies.

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