Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K⁺ channel GIRK2
(GIRK1/embryonic stem cells/genetics/weaver/cerebellum)

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Contributed by Lily Y. Jan, November 21, 1996

ABSTRACT  G protein-gated, inwardly rectifying K⁺ channels (GIRK) are effectors of G protein-coupled receptors for neurotransmitters and hormones and may play an important role in the regulation of neuronal excitability. GIRK channels may be important in neurodevelopment, as suggested by the recent finding that a point mutation in the pore region of GIRK2 (G156S) is responsible for the weaver (wv) phenotype. The GIRK2 G156S gene gives rise to channels that exhibit a loss of K⁺ selectivity and may also exert dominant-negative effects on GIRK1 function. To investigate the physiological role of GIRK2, we generated mutant mice lacking GIRK2. Unlike wv/wv mutant mice, GIRK2 −/− mice are morphologically indistinguishable from wild-type mice, suggesting that the wv phenotype is likely due to abnormal GIRK2 function. Like wv/wv mice, GIRK2 −/− mice exhibit much milder cerebellar abnormalities than wv/wv mice, indicating a dosage effect of the GIRK2 G156S mutation. Our results indicate that the weaver phenotypes arise from a gain-of-function mutation of GIRK2 and that GIRK1 and GIRK2 are important mediators of neuronal excitability in vivo.

G protein-gated, inwardly rectifying K⁺ channels (GIRK) are regulated by neurotransmitters and hormones through G protein-coupled receptors (1–3). GIRK channels are believed to determine neuronal membrane excitability by selectively permitting the flux of K⁺ ions near the resting membrane potential (4–7). The weaver mouse, a neurological mutant characterized by extensive cerebellar granule cell death during development (8–10), age-dependent dopaminergic neuronal loss in the substantia nigra (11, 12), male infertility (13), and spontaneous seizures (14), carries a G156S point mutation in the pore-forming region H5 of GIRK2 (15). This mutation leads to a loss of K⁺ selectivity of homomeric GIRK2 channels and strongly reduces heteromeric GIRK1/GIRK2 channel function (16–18). Electrophysiological recordings from weaver and wild-type cerebellar granular cells have yielded conflicting reports, supporting either a loss of K⁺ selectivity (16) or a loss of channel function (19). To study the physiological effects of GIRK2 in vivo and to address the question whether the phenotypic defects in the weaver mouse are due to gain-of-function effects such as the loss of K⁺ selectivity or due to loss-of-function or dominant-negative effects on GIRK1/
Rabbit antibodies were affinity-purified, and sections were incubated in 1–2 μg/ml primary antibody overnight (23, 24). Monoclonal antibodies against tyrosine hydroxylase (TH; Pel-Freeze Biologicals) were used at 1:1000. Biotinylated donkey anti-rabbit or anti-mouse IgG Fab (The Jackson Laboratory) were used at 1:200, and sections were developed with the ABC kit (Vector Laboratories) and diaminobenzidine.

**Induction of Seizures Using Pentylentetrazole (PTZ).** PTZ (Sigma) was dissolved in PBS and injected i.p. at a dose of 50 mg/kg in ~0.1 ml. Animals were housed in a room with controlled light/dark cycle (12 hr light/12 hr dark) and temperature (23°C). All experiments were performed between 11 a.m. and 1 p.m. Animals were injected and observed without prior knowledge of their genotype. Each mouse was placed in a transparent cage and observed for 30 min after injection. All mice were littersmates between 10 and 14 weeks of age and weighed ~20 g.

**RESULTS AND DISCUSSION**

**Generation of GIRK2 Null Mice.** The GIRK2 gene was disrupted in ES cells by homologous recombination using a targeting vector in which exon 2 was disrupted and partially deleted by a pgk-neomycin resistance cassette (Fig. 1A). One ES cell clone that carried the targeted allele was used to generate chimeric male animals that passed the mutant allele to their offspring. GIRK2 +/– mice were indistinguishable from wild-type mice and were inbred to produce GIRK2 –/– mice (Fig. 1B). No normal GIRK2 mRNA could be detected in brains of adult GIRK2 –/– mice by RT-PCR analysis, but a truncated GIRK2 mRNA was present (Fig. 1C). No GIRK2 immunoreactivity was detectable using antibodies against either the N terminus or C terminus of GIRK2 (Figs. 2A and 3 A and B; data not shown). We conclude, therefore, that we have generated GIRK2 null mice.

**Down-Regulation of GIRK1 Protein in GIRK2 Null Mice.** GIRK2 –/– mice are born at the expected frequency and are viable. Given that GIRK2 and GIRK1 have partly overlapping temporal and spatial expression patterns and are known to form functional heteromultimers in vitro (16–18, 25–28) and in vivo (24), we examined the expression of GIRK1 and other related inward rectifier channels by using affinity-purified polyclonal antibodies against GIRK1, IRK1, and GIRK4 in Western blot and immunohistochemical studies of GIRK2 +/–, +/–, and –/– mice. Immunoblot analysis showed that GIRK1 levels were reduced in brain membranes of GIRK2 +/– mice and nearly undetectable in –/– mice, whereas IRK1 protein levels remained constant in mice of all three genotypes (Fig. 2A). RT-PCR analysis showed that GIRK1, GIRK4, and IRK1 mRNA were similar in all animals, suggesting that the down-regulation of GIRK1 in GIRK2 –/– mice occurred posttranscriptionally (Fig. 2B). Immunohistochemical analysis showed dramatic reduction of GIRK1 immunoreactivity in many brain regions in GIRK2 –/– mice, whereas IRK1 and GIRK4 immunoreactivities were normal in these GIRK2 mutants (Fig. 3 A and B; data not shown).

The extent of reduction in GIRK1 varied with the brain regions; expression of GIRK1 in the cerebral cortex and hippocampus was virtually undetectable, whereas in the cerebellum, significant amounts of GIRK1 remained in the granule cell layer (Fig. 3 A and B). The reduction of GIRK1 protein levels throughout the brain suggests that a majority of GIRK1 proteins in the brain interact with GIRK2, and in the absence
of GIRK2, there is a concurrent loss of GIRK1 subunits that normally form heteromers with GIRK2.

**Diffences Between the GIRK2 Null Phenotypes and the Weaver Phenotypes.** The GIRK2 +/- and wv/wv mice showed striking differences. Visual inspection and histological examination of the brain and other organs of GIRK2 +/- animals revealed no anomalies. GIRK2 +/- mice exhibited normal cerebellar morphology except for the reduced GIRK1 and GIRK2 protein expression (Fig. 3B and D). Midbrain dopaminergic neurons and their dendrites also appeared normal despite the absence of GIRK1 protein (Fig. 3C). While male wv/wv mice are infertile, male GIRK2 +/- mice are fertile; superovulated CD-1 mice mated with either GIRK2 +/- males or their wild-type littermates produced a comparable number of fertilized eggs. The apparent normal phenotype in GIRK2 +/- /- mice provides strong evidence that loss of homomeric GIRK2 channel and/or heteromeric GIRK1/GIRK2 channel function is not the primary cause of the weaver phenotype.

**The Weaver Gene Dosage Effect.** When GIRK2 +/- mice were compared with +/- mice carrying one or two copies of the wv allele (GIRK2 wv/- and/or wv/wv), we found that both +/- and wv/wv mice exhibit normal locomotive behavior, unlike the wv/wv mice. In +/-, +/-, and wv/wv animals, there was no obvious loss of TH-positive neurons or dendrites in the substantia nigra pars compacta or in the ventral tegmental area, whereas substantial cell loss was evident in the substantia nigra pars compacta of the wv/wv midbrain (Fig. 3C). In the substantia nigra pars compacta and ventral tegmental area of wv/wv mice, GIRK2 immunoreactivity was present but of lower intensity. In contrast to the heterozygous GIRK2 +/- mice, most of the GIRK2 immunoreactivity in the wv/wv mice was found in the cell bodies of the dopaminergic neurons; the GIRK2 immunoreactivity in the dendrites was much reduced (Fig. 3C). The size and gross morphology of the cerebellum of wv/wv animals are not significantly different from that of the wild-type animals. Histologically, the wv/wv cerebellum appeared more similar to that of the wv/+ (8, 9) than the cerebellum of +/- or wv/wv mice. The wv/wv granule cell layer often appeared thinner. The Purkinje cell layer was disorganized in various locations, and some of the Purkinje cells were found deep in the granule cell layer (Fig. 3D). The similarity between wv/wv and wv/+ cerebella and the difference between wv/wv and wv/+ cerebella suggest that cerebellar development is sensitive to the dosage of the GIRK2 G156S mutant gene.

**Seizure Activities of GIRK2 Null Mice.** The GIRK2 +/- mice exhibited sporadic seizures characterized by jerking of head and body, vocalization, and infrequently progression to a tonic-clonic seizure. Typically, the episodes lasted for 30 sec and were followed by complete physical inactivity. All witnessed seizures occurred when some kind of stress was exerted on the animal (changing cages, setting up matings), and the behavior of mice returned to normal after the seizure. Seizures were never observed before weaning and seemed to occur at equal frequencies in young and old mutant mice. Pharmacological challenge with the convulsant agent PTZ (29), a y-aminobutyric acid agonist, revealed that GIRK2 +/- mice were hyperexcitable when challenged with a single injection of PTZ (50 mg/kg). At this dose, 70% of GIRK2 +/- mice but only 25% of heterozygous or wild-type littermates developed severe stage 3 tonic-clonic seizures frequently associated with death (P < 0.004, Mann–Wilcoxon rank sum test). The severity of seizure, in the range from 0 to 3, was shifted toward increased severity in GIRK2 +/- mice as compared with +/- and +/- controls. No statistically significant difference was seen between heterozygous and wild-type mice (Fig. 4A). The time taken to develop seizure activities was significantly shorter in GIRK2 +/- mice compared with +/- and +/- animals (P < 0.002, unpaired t-test; Fig. 4B). Seizure activity has previously been noted in weaver mice and might be due to altered or reduced G protein-activated K+ channel function (14). Our observation that GIRK1/GIRK2-deficient mice are susceptible to spontaneous and pharmacologically induced seizures was consistent with numerous studies demonstrating that agonists of G protein-coupled receptors, such as receptors for opioid peptides, somatostatin, and dopamine, can have significant effects on seizure thresholds in several different experimental seizure model systems (30).

In conclusion, we show that GIRK2-deficient mice have greatly reduced GIRK1 protein levels in the brain, suggesting that the majority of GIRK1 proteins in the brain associate with GIRK2. Phenotypic characteristics of GIRK2 +/-, wv/wv, and wv/wv mice suggest that gain-of-function and gene dosage mechanisms are responsible for the developmental defects in weaver mutants. Moreover, loss of GIRK2 function results in sporadic seizures and increased susceptibility to a convulsant agent, implicating GIRK1 and GIRK2 in the control of neural excitability in vivo.

We thank Paul Slesinger for his suggestions and discussions throughout the course of this work. We also thank Jan L. Breslow, James E. Darnell, Jr., Paolo Mocarelli, Annemarie Walsh, and the Rockefeller transgenic facility for their support. The research was supported by the...
FIG. 3. (A) Coronal sections of brain from GIRK2 +/+ and −/− mouse brains stained with antibodies against the N terminus of GIRK2, the N terminus of GIRK1, and the C terminus of IRK1 show that there is no detectable GIRK2 and dramatically reduced GIRK1 immunoreactivity in the GIRK2 −/− mice. The IRK1 staining patterns are the same for these mice. CTX, cerebral cortex; HP, hippocampus; Th, thalamus. (Bar = 10 mm.) (B) Sagittal views of cerebella from GIRK2 +/+ and −/− mice stained with the antibodies described in A. Although there is no detectable GIRK2 protein in the −/− mice, there is still significant GIRK1 staining, whereas the level of IRK1 expression appears to be the same. g, granule cell layer; m, molecular layer. (Bar = 20 mm.) (C) Coronal sections of ventral midbrain from +/+, −/−, wv/−, and wv/wv mice are stained with antibodies against TH and the N terminus of GIRK2. The TH staining of +/+, −/−, and wv/− midbrain appears similar, whereas there are fewer TH-positive neurons in the substantia nigra pars compacta (SNC) of the wv/wv midbrain. GIRK2 immunoreactivity is absent in the −/− midbrain but can still be found in the wv/− and wv/wv mice, although the dendritic staining is dramatically reduced. SNr, substantia nigra pars reticulata;
Fig. 4. Susceptibility of GIRK2-deficient mice to PTZ-induced seizures. (A) Response of mice receiving one injection of 50 mg/kg PTZ i.p. (0, no response; 1, isolated twitches; 2, tonic-clonic convulsions; 3, tonic extension and/or death). GIRK2−/− mice (n = 16) tend to progress to more severe stages than +/+ or +/− mice (n = 13 and 12, respectively; P < 0.004, Mann–Whitney U–Wilcoxon rank sum test). No statistically significant difference was observed between +/+ and +/− animals. (B) Seizure latency. The PTZ seizure latency was defined as the time elapsed from PTZ injection to the first obvious sign of tonic-clonic convolution or tonic extension. The latency to seizures was shorter for the −/− mice (*, P < 0.002, unpaired test).

National Parkinson Foundation (M.S.), a National Institute of Mental Health grant to the Silvio Conte Neuroscience Center at the University of California at San Francisco, and the University of California at San Francisco Neuroscience Graduate Program. L.Y.J. is a Howard Hughes Medical Institute investigator.
