Increased Seizure Susceptibility in Mice Lacking Metabotropic Glutamate Receptor 7

Gilles Sansig,[1] Trevor J. Bushell,[2] Vernon R. J. Clarke,[2] Andrei Rozov,[3] Nail Burnashev,[3] Chantal Portet,[1] Fabrizio Gasparini,[1] Markus Schmutz,[1] Klaus Klebs,[1] Ryuichi Shigemoto,[4] Peter J. Flor,[1] Rainer Kuhn,[1] Thomas Knoepfel,[1] Markus Schroeder,[1] David R. Hampson,[5] Valerie J. Collett,[2] Congxiao Zhang,[6] Robert M. Duvoisin,[6] Graham L. Collingridge,[2] and Herman van der Putten[1]

[1]*Nervous System Department, Novartis Pharma AG, CH-4002 Basel, Switzerland,* [2]*Medical Research Council Center for Synaptic Plasticity, Department of Anatomy, The School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, United Kingdom,* [3]*Abteilung Zellphysiologie, Max-Planck-Institut für Medizinische Forschung, D-69120 Heidelberg, Germany,* [4]*Division of Cerebral Structure, National Institute for Physiological Sciences, Myodaiji, Okazaki 444-8585, Japan,* [5]*Faculty of Pharmacy and Department of Pharmacology, University of Toronto, Ontario, Canada M5S 2S2, and* [6]*Margaret M. Dyson Vision Research Institute, Department of Ophthalmology, Cornell University Medical College, New York, New York 10021*

To study the role of mGlu7 receptors (mGluR7), we used homologous recombination to generate mice lacking this metabotropic receptor subtype (mGluR7$^{-/-}$). After the serendipitous discovery of a sensory stimulus-evoked epileptic phenotype, we tested two convulsant drugs, pentylenetetrazole (PTZ) and bicuculline. In animals aged 12 weeks and older, subthreshold doses of these drugs induced seizures in mGluR7$^{-/-}$, but not in mGluR7$^{+/-}$, mice. PTZ-induced seizures were inhibited by three standard anticonvulsant drugs, but not by the group III selective mGluR agonist (*R,S*)-4-phosphonophenylglycine (PPG). Consistent with the lack of signs of epileptic activity in the absence of specific stimuli, mGluR7$^{-/-}$ mice showed no major changes in synaptic properties in two slice preparations. However, slightly increased excitability was evident in hippocampal slices. In addition, there was slower recovery from frequency facilitation in cortical slices, suggesting a role for mGluR7 as a frequency-dependent regulator in presynaptic terminals. Our findings suggest that mGluR7 receptors have a unique role in regulating neuronal excitability and that these receptors may be a novel target for the development of anticonvulsant drugs.

*Key words: epilepsy; mGluR7; knock-out; mice; group III mGluR; (*R,S*)-4-phosphonophenylglycine*

An imbalance in glutamatergic excitatory neurotransmission and GABAergic synaptic inhibition in the vertebrate CNS can cause seizures and may be a major cause of epilepsy. There is, therefore, considerable interest in how these neurotransmitter systems are regulated physiologically. Metabotropic glutamate receptors (mGluRs) couple to G-proteins and can modulate L-glutamate release, GABA release, and neuronal excitability (Conn and Pin, 1997). They are subdivided into groups I (mGluR1, mGluR5), II (mGluR2, mGluR3), and III (mGluR4, mGluR6, mGluR7, mGluR8) on the basis of homology, intracellular messengers, and ligand selectivity (Conn and Pin, 1997). mGluR7 is the most highly conserved member, and its mGluR7a-isoform is distributed widely throughout the CNS (Kinzie et al., 1995; Ohishi et al., 1995; Bradley et al., 1996; Brandstaetter et al., 1996; Flor et al., 1997; Shigemoto et al., 1997). The two isoforms of the receptor are localized presynaptically, close to release sites (Bradley et al., 1996; Brandstaetter et al., 1996; Shigemoto et al., 1996; Kinoshita et al., 1998).

In recombinant expression systems L-2-amino-4-phosphonobutyrate (L-AP4), L-serine-*O*-phosphate (L-SOP), and (*R,S*)-4-phosphonophenylglycine [(*R,S*)PPG] activate mGluR7 and its coupling to adenylate cyclase inhibition (Gasparini et al., 1999). Among the group III mGluRs, mGluR7 has the lowest affinity for these group III mGluR selective ligands and the endogenous ligand L-glutamate (Okamoto et al., 1994; Saugstad et al., 1994; Flor et al., 1997). In a variety of preparations L-AP4 and L-SOP reduce excitatory synaptic transmission (Koerner and Cotman, 1981; Davies and Watkins, 1982; Lanthorn et al., 1984; Anson and Collins, 1987; Bushell et al., 1995; Manzoni and Bockaert, 1995; Vignes et al., 1995; Pisani et al., 1997) via a putative presynaptic mechanism (Baskys and Malenka, 1991; Gereau and Conn, 1995) or via heterosynaptic effects on interneuron terminals (Salt and Eaton, 1995; Wan and Cahusac, 1995; Cartmell and Schoepp, 2000; Semyanov and Kullmann, 2000).

The notion that group III mGluRs are potential targets for

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G.S. and T.J.B. contributed equally to this work.

Correspondence should be addressed to Herman van der Putten, Nervous System Department, Novartis Pharma AG, K125.5.13, CH-4002 Basel, Switzerland. E-mail: p_herman.van_der_putten@pharma.novartis.com.

T. Knoepfel's present address: Laboratory for Neuronal Circuit Dynamics, The Institute of Physical and Chemical Research (RIKEN) Brain Science Institute, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan.

T. Bushell's present address: Imperial College, Department of Biophysics, Prince Consort Road, London SW7 2BW, UK.

C. Zhang's present address: National Eye Institute, National Institutes of Health, 10 Center Drive, Bethesda, MD 20982-1857.

R. Duvoisin's present address: Neurological Sciences Institute, Oregon Health Sciences University, Portland, OR 97201.

novel antiepileptic drugs is supported by results in rodent models of epilepsy in which group III selective agonists showed prolonged anticonvulsant actions [L-AP4, L-SOP (Tizzano et al., 1995; Tang et al., 1997); (*R,S*)-PPG (Chapman et al., 1999; Gasparini et al., 1999); L-SOP (Yip et al., 2001)] and increased seizure threshold (L-AP4; Suzuki et al., 1999) or seizure latency (Thomsen and Dalby, 1998). In addition, in epilepsy the changes have been noted in the agonist sensitivity (Neugebauer et al., 2000), expression (Aronica et al., 1997; Liu et al., 2000; Yip et al., 2001), and receptor responses of group III mGluRs (Holmes et al., 1996; Neugebauer et al., 1997; Dietrich et al., 1999; Klapstein et al., 1999).

The lack of specific ligands to address mGluR7 function prompted us to generate mice lacking these receptors. A previous study that used these animals revealed deficits in taste aversion and fear responses (Masugi et al., 1999). The present study describes a role of mGluR7 in epilepsy.

MATERIALS AND METHODS

Generation of mGluR7$^{-/-}$ mice

A genomic fragment of the mouse mGluR7 gene was isolated from a 129SV/J λFIX phage library (Stratagene, La Jolla, CA) and probed with a human mGluR7 cDNA. A 2.055 kb *Nhe*I–*Nhe*I DNA fragment comprising the first coding exon was sequenced. It contained 405 bp of 5′-untranslated region (UTR; as judged by homology to rat mGluR7 cDNA), followed by codons for the first 164 amino acids of mouse mGluR7. The targeting vector was constructed by inserting a 0.6 kb *Nru*I–*Xho*I DNA fragment (comprising 115 bp of 5′-UTR) 5′ of the pMCNeo cassette into a *Stu*I–*Xho*I cleaved pTV-0 vector that contains the herpes virus thymidine kinase (TK) gene for negative selection. A 7 kb *Nhe*I–*Nhe*I DNA fragment comprising genomic sequences downstream of the 2.055 kb *Nhe*I–*Nhe*I fragment was inserted into a *Nhe*I site located between pMCNeo and pMCTK in pTV-0. Proper targeting resulted in deleting 0.585 kb of the first coding exon and 0.73 kb of the next intron of the mGlu7 gene. Embryonic day 14 (E14) embryonic stem (ES) cells [129/Ola; genotype A^w (agouti), c^{ch} (albino), p (pink-eyed dilution)] were transfected with 30 μg of *Not*I-linearized and dideoxynucleotide-end-filled (using Klenow enzyme) targeting vector by electroporation (250 V and 500 μF; Bio-Rad Gene Pulser, Munich, Germany). G418 (600 μg/ml) and Ganciclovir (Gancv; 2 μM) selection were applied 24 and 48 hr later, respectively. DNA from double-resistant ES colonies was subjected to PCR analysis by using either one of two PCR primers matching sequences in the *Nhe*I–*Nru*I fragment located just 5′ to, but not contained within, the targeting vector (primer-1, 5′-cttctgccagagctgacagtcaaag-3′; primer-2, 5′-gtcagcaccaatatcgcgactcatc-3′) and either one of two primers located in the *neo* gene (primer-3, 5′-gcgtgcaatccatcttgttcaatgg-3′; primer-4, 5′-gcgctgacagccggaacacg-3′). Combinations of primer-1 or primer-2 and either one of two primers matching sequences in the coding region of the first coding exon (primer-5, 5′-gaaagtgagcgactgttcgagcg-3′; primer-6, 5′-gatgttggctaccatgatggagaccg-3′) served to detect the presence of a wild-type mGluR7 allele. Two of 112 $G418^r Gancv^r$ double-resistant ES cell clones carried a correctly targeted mGluR7 allele, as assessed by PCR and confirmed by Southern blot analysis of genomic DNA digested with *Nhe*I and *Nco*I, respectively, and probed with probe A (158 bp *Nhe*I–*Nru*I fragment), probe B (0.6 kb *Nru*I–*Xho*I fragment), and a *neo* gene probe (probe D) (Fig. 1). Southern blot analysis that used a complete mGluR7 cDNA probe (probe D) revealed no additional rearrangements in the locus (data not shown). Wild-type (+) and mutant alleles (−) are indicated by the presence of a 2 kb (+) versus 1.8 kb (−) *Nhe*I and a 2.5 kb (+) versus a 2.3 kb (−) *Nco*I DNA fragment when probed with probe A or B (Fig. 1*a,b*). The diagnostic sizes for a properly targeted mGluR7 allele when probed with *neo* (probe D) are 1.8 kb (*Nhe*I) and 2.3 kb (*Nco*I). Both ES clones were used successfully to produce germ line chimeras (11 for each clone) by aggregation for 2–3 hr with 10^6 ES cells per milliliter.

Genotyping

F1 mice carrying a targeted mGluR7 allele were identified by Southern blot analysis (Fig. 1*b*). F2 mice, derived from matings of pairs of heterozygous parents, were screened by PCR and used pairs of one of three different forward primers (5′-cttctgccagagctgacagtcaaag-3′ or 5′-gtcagcaccaatatcgcgactcatc-3′ or 5′-acagtcaaagatcagactcaggggc-3′ or 5′-ctccccataagtcagcaccaatatc-3′) and one of two Neo-specific primers (primer-3 or primer-4) (Fig. 1*a*) to detect the targeted allele. A combination of primer-7 (5′-gagagatggatagcaagcaagggag-3′) and primer-8 (5′-gtgtccctggaacaagtgtccag-3′) served to detect the endogenous mGluR7 allele in mGluR7$^{+/+}$ and mGluR7$^{+/-}$ mice and to confirm its absence in

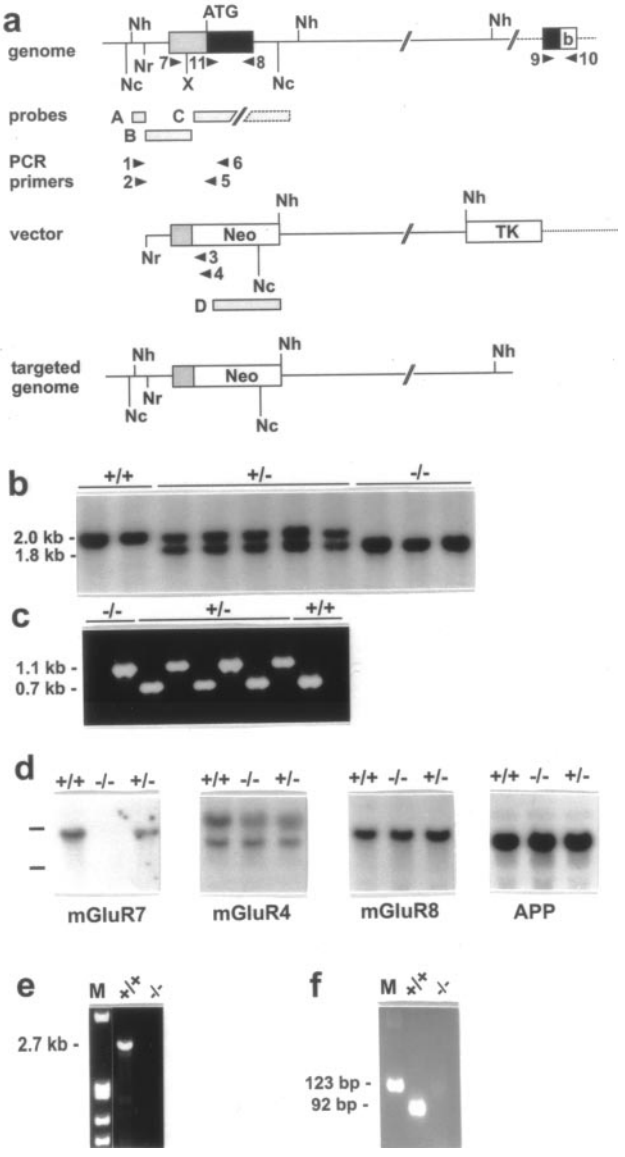

Figure 1. Targeted disruption of the mouse mGluR7 gene and its molecular analysis. *a*, Scheme of mGluR7 genomic DNA, targeting vector, disrupted gene, probes (*stippled bars*), and PCR primers (*arrows*). *Neo*, Neomycin resistance gene; *TK*, herpes virus thymidine kinase gene; *Nh*, *Nhe*I; *Nc*, *Nco*I; *Nr*, *Nru*I; *X*, *Xho*I. *b*, Southern blot analysis. Shown is the result of a representative litter of F2 mice obtained by crossing a pair of mGluR7$^{+/-}$ F1 mice. DNA was *Nhe*I digested. Probe A was used (as shown in *a*). Wild-type and mutant alleles are represented by DNA fragments of 2.055 and 1.885 kb, respectively. *c*, PCR genotyping. Example of a typical PCR result with the use of tail DNA of mGluR7$^{+/+}$, mGluR7$^{+/-}$, and mGluR7$^{-/-}$ mice. Primer pairs 1 + 3 yield a 1.1 kb product (mutant allele). Primers 7 + 8 yield a 0.7 kb DNA fragment (wild-type allele). *d*, Northern blot analysis. Total RNA was isolated from mGluR7$^{+/+}$, mGluR7$^{+/-}$, and mGluR7$^{-/-}$ brains. cDNA probes were APP, mGluR7, mGluR4, and mGluR8. *e, f*, RT-PCR. mGluR7b-specific RT-PCR products of expected sizes 2.7 kb (primers 11 + 10) and 0.092 kb (primers 9 + 10) were detected readily with mGluR7$^{+/+}$, but not with mGluR7$^{-/-}$, brain RNA as a template.

mGluR7$^{-/-}$ mice. mGluR4 mutant mice were genotyped as described previously (Pekhletski et al., 1996). mGluR8 (Duvoisin et al., 1995) mutant mice (R. Duvoisin and C. Zhang, unpublished results) were genotyped by PCR (hot-start PCR, TaqStart antibody; Promega, Madison, WI) according to the manufacturer's instructions. Annealing was 45 sec at 68°C; primer extension was at 74°C for 45 sec for 34 cycles. One combination of two primers (taactaccaggtggacgaactctc; cacaaaggtggtggcaatgattcc) was used to diagnose the endogenous mGluR8 allele in mGluR8$^{+/+}$ and mGluR8$^{+/-}$ mice and to confirm its absence in mGluR8$^{-/-}$ mice. Another primer (taatatgcgaagtggacctgggac) combined with the first one shown above served to detect the targeted allele.

DNA and RNA analysis

Southern and Northern blot analyses, sequencing, PCR, and RT-PCR were performed according to standard protocols. For Northern blot analysis the following probes were used: a 3 kb *Eco*RI fragment of mouse amyloid precursor protein (APP) cDNA, a 3 kb *Hin*dIII fragment of human mGluR7b cDNA, a 0.57 kb *Pst*I human mGluR4 cDNA fragment (encoding amino acids 520–710), and a 1.150 kb rat mGluR8 cDNA probe (encoding amino acids 1–350 of mGluR8). Hybridization with APP cDNA served as a control for loading equivalent amounts of total brain RNA in each lane. RT-PCR of mGluR7$^{+/+}$ and mGluR7$^{-/-}$ brain RNA was performed with several pairs of oligonucleotide primers, including primer-1 and primer-4, primer-1 and primer-10, primer-11 and primer-10, and primer-9 and primer-10. Primer-9 and primer-10 were designed specifically to detect sequences comprising exon b (92 bp) encoding one of two (a and b) C-terminal splice variants of mGluR7 (Flor et al., 1997).

Western blot and immunocytochemical analysis

Immunoblot procedures and immunocytochemistry were as described previously (Shigemoto et al., 1996, 1997; Kinoshita et al., 1998). Briefly, for Western blot analysis the crude membrane preparations from mouse cerebellum, hippocampus, and combined brain regions other than the cerebellum and hippocampus were separated by 7% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were reacted with an affinity-purified antibody for mGluR7a (Shigemoto et al., 1996). For detection, an alkaline phosphatase-labeled anti-rabbit secondary antibody (Chemicon, Temecula, CA) was used. For immunocytochemistry, wild-type and mGluR7$^{-/-}$ mice were anesthetized deeply and perfused transcardially with 3.5% paraformaldehyde, 1% picric acid, and 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were removed, cryoprotected (30% sucrose in 0.1 M PB overnight at 4°C), and cut on a freezing microtome. The 40-μm-thick sections were incubated with antibodies for mGluR7a, mGluR7b, mGluR4a, or mGluR8a (Shigemoto et al., 1997; Kinoshita et al., 1998) in PBS containing 2% normal goat serum and 0.1% Triton X-100 overnight at 15°C. After washes in PBS the sections were incubated with biotinylated goat anti-rabbit or goat anti-guinea pig IgG (Vector Laboratories, Burlingame, CA). Then the sections were washed again, reacted with the ABC kit (Vector Laboratories), and finally incubated with 0.05% diaminobenzidine and 0.0006% hydrogen peroxide.

Chromosomal mapping of the mGluR7 gene

A 129 mouse bacterial artificial chromosome (BAC) was identified and isolated by the PCR screening of a genomic 129SV DNA bank in pBeloBAC11 (Research Genetics, Huntsville, AL). The PCR primers were 7 and 8 (Fig. 1). These amplified specifically the first coding exon of the mGluR7 gene, as confirmed by sequencing. Southern blot analysis was performed with a mGluR7 cDNA probe to confirm the presence of unrearranged and diagnostic mGluR7 genomic DNA fragments. For fluorescent *in situ* hybridization (FISH), BAC DNA was labeled with digoxigenin-dUTP by nick translation and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts. These analyses were performed at Genome Systems (St. Louis, MO).

Electrophysiology

Hippocampus. The 400-μm-thick slices were prepared from 5- to 29-week-old mutant mice and littermate wild types via standard procedures, as described previously (Conquet et al., 1994). Slices were submerged in a medium that comprised (in mM) 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, and 10 D-glucose (bubbled with 95% O_2/5% CO_2, pH 7.4); the medium was perfused at a rate of ~4 ml/min (29–31°C). Extracellular recordings were obtained from stratum radiatum or stratum pyramidale of area CA1 in response to low-frequency (0.033 Hz) stimulation of the Schaffer collateral–commissural pathway. For each protocol one slice was used per animal; hence *n* values give the number of slices per mice used. Results were analyzed via Student's *t* tests or ANOVA, with $p < 0.05$ taken to indicate statistical significance. Animals were genotyped by PCR and presented to the experimenter in a randomized and blind manner.

Neocortex. Brain slice preparation and visualization of neurons in the living slice are described previously (Stuart et al., 1993; Markram et al., 1997). During recordings the slices were maintained at room temperature (20–24°C) in extracellular solution consisting of (in mM) 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$, pH 7.2. Whole-cell voltage recordings were performed simultaneously from two neurons with pipettes filled with (in mM) 115 K-gluconate, 20 KCl, 4 ATP-Mg, 10 phosphocreatine, 0.3 GTP, and 10 HEPES, pH 7.3 (310 mOsm). In synaptically connected neurons a suprathreshold intracellular stimulation of presynaptic pyramidal cells evoked depolarizing EPSPs. Presynaptic pyramidal cells were stimulated with a 10 Hz train of two to three suprathreshold current pulses. Typically, the trains were delivered at intervals of 5–7 sec for mGluR7$^{+/+}$ mice and at 30 sec for mGluR7$^{-/-}$ mice so that recovery from short-term modification was complete, as evidenced by the lack of systematic changes in the amplitude of the first EPSP of a train during successive trains of stimuli. For recovery from facilitation measurements, two action potentials delayed at variable time intervals (Δt) were delivered every 30 sec in both mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice. Voltage traces that are shown are averages of 50–100 sweeps. The amplitude of the first EPSP of the train is defined as the difference between the peak of the EPSP and baseline. For the second (or third) EPSP the amplitude is the difference between the peak of the EPSP and the baseline measured just before the onset of the EPSP. Stimulus delivery, data acquisition, and analyses were performed with macros in IGOR (Wavemetrics, Lake Oswego, OR).

Drug administration

Pentylenetetrazole (PTZ; Metrazol, Knoll AG, Liestal, Switzerland) was given intraperitoneally at a subthreshold dose of 40 mg/kg or at a suprathreshold dose of either 60 (in mGluR4 wild types and mutants) or 70 mg/kg (in mGluR7 and mGluR8 wild types and mutants). A dose of 40 mg/kg PTZ induced clonic or clonic-tonic seizures in mGluR7$^{-/-}$ mice only, whereas it failed to induce seizures that were visible behaviorally in mGluR4$^{+/+}$, mGluR4$^{+/-}$, mGluR4$^{-/-}$, mGluR7$^{+/+}$, mGluR7$^{+/-}$, mGluR8$^{+/+}$, mGluR8$^{+/-}$, or mGluR8$^{-/-}$ mice. A PTZ dose of 70 mg/kg induced clonic seizures of ~5 sec duration in at least 90% of all of the mGluR mutant and wild-type mice. Anticonvulsant drugs were given 1 hr before PTZ. Doses used in mGluR7$^{+/+}$, mGluR7$^{+/-}$, and mGluR7$^{-/-}$ mice treated with 40 mg/kg PTZ were as follows: valproate (VPA; Depakine, Sanofi, Paris, France) (500 mg/kg, p.o.), ethosuximide (ESM; Galenica, Berne, Switzerland) (500 mg/kg, p.o.), clonazepam (CZP; Rivotril, Roche, Gipf-Oberfrick, Switzerland) (0.1 mg/kg, p.o.). In the experiment in which mGluR7 mutant mice were given 70 mg/kg PTZ (see Fig. 4, *black bars*), at 1 hr before PTZ the mice received placebo (water, p.o.), 500 mg/kg VPA, 750 mg/kg ESM, or 1 mg/kg CZP. In this experiment the dosing of ESM and CZP was increased to assure maximum chances of success for counteracting the seizures in mGluR7$^{-/-}$ mice. Note that a dose of 70 mg/kg PTZ is far above threshold in mGluR7$^{-/-}$ mice. Bicuculline (Sigma, St. Louis, MO) was given subcutaneously at 2.5 mg/kg (mGluR7$^{+/-}$ and mGluR7$^{-/-}$ mice) or 3.5 mg/kg [Maus Auszucht Geigy (MAG) mice]. (*R,S*)-4-phosphonophenylglycine (PPG; Tocris, Bristol, UK) was dissolved in 0.9% NaCl, pH-adjusted to 6–7, and injected intracerebroventricularly into the mice under light Fluothane anesthesia. Injection volume was 2.5 μl/mouse. Intracerebroventricular administration of PPG in 0.9% NaCl or placebo (0.9% NaCl) occurred 15 min before PTZ was given. Doses required to evoke seizures in ≥80% of anesthetized (and 0.9% NaCl placebo injected intracerebroventricularly) wild-type (OF-1, MAG, 129Ola×C57BL/6) or heterozygous mGluR4, mGluR7, or mGluR8 mutant mice with different and mixed genetic backgrounds including 129Sv/J×CD-1 (mGluR4), 129Ola×C57BL/6 or 129Ola×BALB/c (mGluR7), and 129Sv/J×C57BL/6 (mGluR8) were 60 mg/kg (for 129×CD-1) and 70 mg/kg (for all others). Concentrations and application modes are indicated in the text and legends. All whole animal experiments were approved and conducted according to the Swiss legislation and guidelines on animal experimentation.

Seizure scoring

Mice were considered protected from seizures and scored as such when neither clonic nor clonic-tonic seizures were observed within the first 10 min after PTZ treatment and within 30 min after bicuculline treatment. After PTZ or bicuculline treatment, clonic convulsions (myoclonic jerks, forelimb clonus) of ~5 sec duration and clonic-tonic (hindlimb extension) convulsions were scored by using behavioral monitoring, as described previously (Schmutz et al., 1990).

EEG recordings

Stainless steel screw electrodes were implanted bilaterally over the frontal and parietal cortex under isoflurane/O_2/N_2O (0.5 l/min) anesthesia. An indifferent electrode served as ground electrode and was positioned at bregma F1.6/l2.5. All screw electrodes were connected by insulated stainless steel wiring to a four-pole socket embedded in dental cement. The electroencephalogram (EEG) that was analyzed was the differential between the frontal (bregma F − 1.0/l3.2) and parietal (F − 3.0/l3.2) electrodes of the same hemisphere compared with the combined reference electrodes. Bipolar leads from the mice were recorded via cables connected to a slipstring system, at the earliest 21 d after the operation. The behavior of the animals, which were housed singly in wooden observation cages measuring 32 × 32 × 40 cm, was observed over a closed circuit TV system. The EEGs were amplified by an isolated four-channel bipolar EEG amplifier (Spectralab EEG-2104, Kilchberg, Switzerland), recorded on a thermorecorder (MTK95, Astromed), and collected on a personal computer.

RESULTS

Generation of mGluR7$^{-/-}$ mice

mGluR7$^{-/-}$ mice were generated by homologous recombination (Fig. 1*a*). They completely lacked mGluR7 mRNA (Fig. 1*d–f*) in agreement with previously shown *in situ* hybridization results (Masugi et al., 1999). mGluR7a (Fig. 2*a–c*) and mGluR7b proteins (data not shown) were absent in mGluR7$^{-/-}$ mice. Brain mRNA expression levels for other group III mGluRs (mGluR4 and mGluR8) were unchanged (Fig. 1*d*). Gross histological abnormalities in brains of mice aged 12 weeks were not apparent, neither in standard hematoxylin and eosin-stained sections nor after immunohistology. The latter analysis included antibodies specific for the group III mGluRs mGluR4a and mGluR8a, respectively, as well as antibodies directed against other mGluR subtypes (Shigemoto et al., 1997) (data not shown).

Previous observations in 6- to 8-week-old animals from our colony also had not revealed any histological abnormalities (Masugi et al., 1999). Aging and health status of mGluR7$^{-/-}$ mice did not differ from mGluR7$^{+/-}$ littermates, except for a slight reduction in body weight (mGluR7$^{-/-}$, 25 ± 4 gm, n = 10 vs mGluR7$^{+/-}$, 31 ± 3 gm, n = 9; age 4 months) and poor fecundity. There was no major morbidity except for seizures in mice aged from 10 to 12 weeks and abnormal fear and conditioned taste aversion responses in mice aged 6–8 weeks (Masugi et al., 1999).

An epilepsy-prone phenotype of mGluR7$^{-/-}$ mice

Spontaneous seizures were precipitated repeatedly in standard pathogen-free (SPF) mGluR7$^{-/-}$ mice. The seizures were observed in mice aged from 10 weeks to 9 months (oldest age that was examined), but not in 6- to 8-week-old mGluR7$^{-/-}$ mice. They were never observed in mGluR7$^{+/+}$ or mGluR7$^{+/-}$ littermates, but an observer (unaware of the genotype) was able to detect the phenotype in 17 of 20 mGluR7$^{-/-}$ mice.

These sensory stimulus-evoked seizure episodes occurred after cage transfer. The seizures were clonic (myoclonic jerks, forelimb clonus) and sometimes tonic in nature. A lag phase of ≥3 d generally was required before mGluR7$^{-/-}$ mice showed renewed susceptibility to the same type of stimulus. Whatever its chemical nature (so far unresolved), it derived from the bedding material and, most likely, was olfactory in nature (data not shown). Its further characterization proved difficult because of the variability that was seen in seizure frequency with different batches of bedding material. Interestingly, a series of other visual, vestibular, and olfactory sensory stimuli that were tested failed to identify another stimulus that was evoking seizures (data not shown). In contrast, two chemical convulsants reproducibly evoked seizures in mGluR7$^{-/-}$ mice at doses that were significantly below threshold for mGluR7$^{+/-}$ and mGluR7$^{+/+}$ mice (see below).

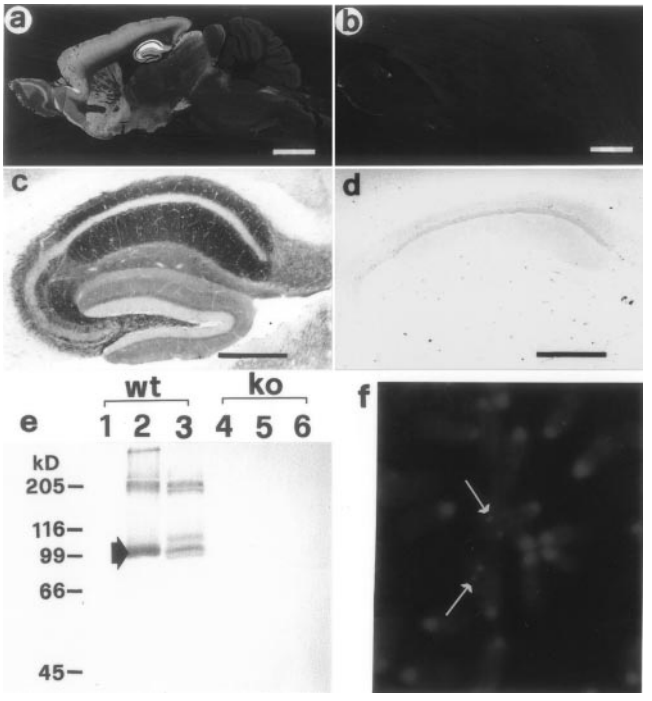

Figure 2. Lack of mGluR7 protein in mGluR7$^{-/-}$ mice. *a–d*, Immunocytochemical analysis comparing reactivity of the mGluR7a-specific antibody in a brain section of a mGluR7$^{+/+}$ mouse (*a* and hippocampus shown enlarged in *c*) and lack of reactivity in mGluR7$^{-/-}$ mouse brain (*b* and hippocampus shown enlarged in *d*). Scale bars: 2 mm (*white horizontal line* in *a, b*) and 400 μm (*black horizontal line* in *c, d*), respectively. *e*, Immunoblot that uses a polyclonal rabbit mGluR7a-specific antibody (Shigemoto et al., 1996, 1997; Kinoshita et al., 1998) of homogenates of mGluR7$^{+/+}$ (*wt*) and mGluR7$^{-/-}$ (*ko*) brain regions, including cerebellum [*lanes 1, 4*; serving also as a negative control because this brain region contains undetectable levels of mGluR7a (Kinoshita et al., 1998)], hippocampus (*lanes 2, 5*), and the other combined brain regions without hippocampus and cerebellum (*lanes 3, 6*). The *arrow* indicates the position in the gel of the bulk of mGluR7a protein. *f*, Localization of the mouse mGluR7 gene to chromosome 6E1 by fluorescent *in situ* hybridization (FISH). *White arrows* indicate the position of the fluorescent signal on chromosome 6.

The epilepsy-prone phenotypes appeared in mGluR7$^{-/-}$ mice derived from two independently targeted ES cell clones and in mutants with different genetic backgrounds [in 129Ola×C57BL/6 hybrid mice and in mice back-crossed for several generations on either C57BL/6 (F3–F14) or BALB/c (F6)].

The mouse mGluR7 gene was localized to chromosome 6E1 (Fig. 2*f*), and 70 kb of 129Ola mouse genomic DNA was sequenced around the disrupted mGluR7 exon (S. D. McDonald, S. Goff, H. van der Putten, unpublished results). Neither procedure provided links to genes (other than the mGluR7 gene) known for predisposing to epilepsy (Allen and Walsh, 1996; McNamara and Puranam, 1998; Bate and Gardiner, 1999; Frankel, 1999), strongly

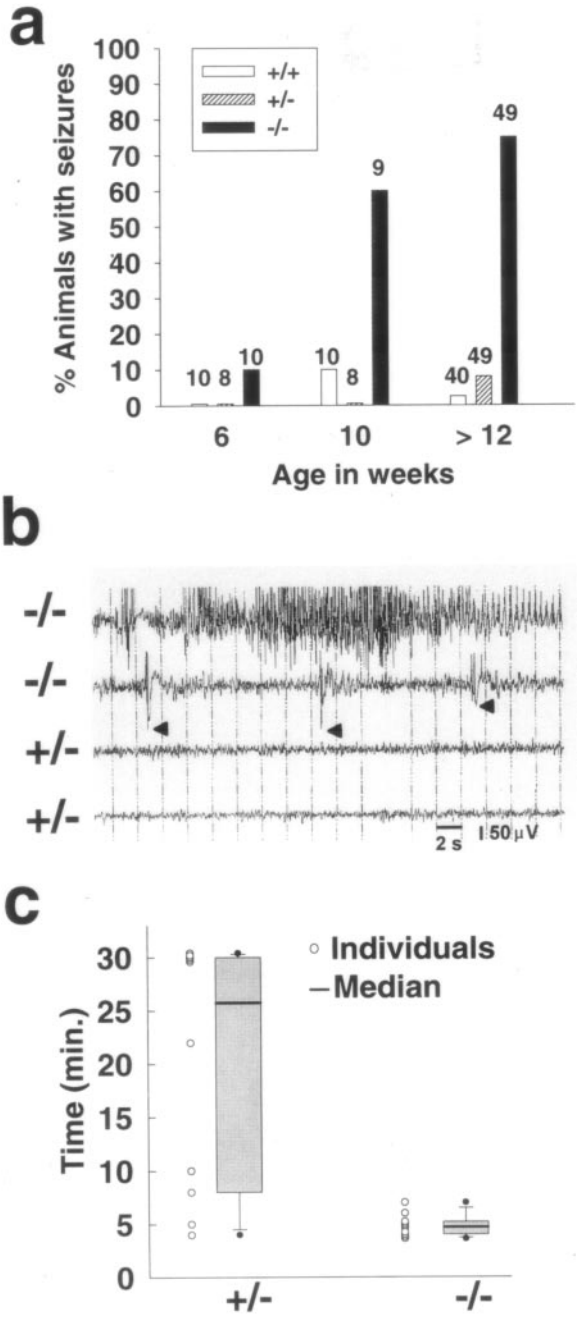

Figure 3. Increased PTZ susceptibility of mGluR7$^{-/-}$ mice to convulsant drugs. *a*, Age-dependent development of a seizure-prone phenotype in mGluR7$^{-/-}$ mice (129Ola×C57BL/6 and 129Ola×BALB/c mixed strain backgrounds). Groups of 8–10 mGluR7$^{+/+}$, mGluR7$^{+/-}$, and mGluR7$^{-/-}$ mice aged 6, 10, and older than 12 weeks (this last group included a group of nine mGluR7$^{+/-}$ and nine mGluR7$^{-/-}$ mice aged 36 weeks) were given PTZ (40 mg/kg, i.p.). Seizures (tonic in mGluR7$^{+/+}$ and mGluR7$^{+/-}$ mice, and either tonic or tonic-clonic in mGluR7$^{-/-}$ mice) were scored behaviorally. Animals were considered protected from the convulsant effect of PTZ when neither clonic nor clonic-tonic convulsions were observed within the first 10 min after PTZ (Schmutz et al., 1990). The *numbers* above the *bars* correspond to the number of mice tested in an age group. The group aged >12 weeks included groups of mGluR7$^{+/+}$, mGluR7$^{+/-}$, and mGluR7$^{-/-}$ mice aged 12–14, 14–18, and 22–24 weeks, respectively. It also included one group aged 36 weeks, of nine mGluR7$^{+/-}$ and nine mGluR7$^{-/-}$ mice, but no mGluR7$^{+/+}$ mice. Of these mice, one of nine mGluR7$^{+/-}$ and eight of nine mGluR7$^{-/-}$ showed seizures. Confidence limits in the groups of >12 weeks were ±10% for the mGluR7$^{+/-}$ groups and ±15% for the mGluR7$^{-/-}$ groups. *b*, Electrographic seizures in mGluR7$^{-/-}$ mice. EEGs recorded for 1 week and for 24 hr continuously in freely moving mGluR7$^{-/-}$ and mGluR7$^{+/-}$ mice (each group $n = 4$, aged 16–20 weeks) revealed no spontaneous epileptiform activity (data not shown). After the 1 week of recording the same mice were given 40 mg/kg PTZ, and the seizures were recorded by behavior as well as by electroencephalography. For each genotype group two representative EEG recordings from two different individuals are shown, starting at 210 sec after PTZ injection. The *top two traces* are from two different mGluR7$^{-/-}$ mice; the *bottom two traces* are from two different mGluR7$^{+/-}$ mice. The first mGluR7$^{-/-}$ mouse (*top trace*) had tonic-clonic seizures; the second mGluR7$^{-/-}$ mouse (*second trace* from *top*) had clonic seizures manifested behaviorally as body jerks (*arrowheads* in EEG). None of the mGluR7$^{+/-}$ mice (*bottom two traces*) showed epileptiform activity. Time scale is shown in seconds, and amplitude is in microvolts. *c*, Increased susceptibility to seizures for the $GABA_A$ receptor antagonist bicuculline, as shown by comparing two groups of 10 mGluR7$^{+/-}$ and mGluR7$^{-/-}$ mice (aged 14–16 weeks) that were given 2.5 mg/kg bicuculline. Seizures were scored behaviorally. Plotted is the time in minutes for individual animals to develop clonic seizures. Observation time was 30 min after drug application, and mice plotted at this value were without seizures.

suggesting that it is the homozygous mutant mGluR7 genotype that determines the epilepsy-prone phenotype in these mice.

mGluR7$^{-/-}$ mice have a lower seizure threshold for convulsants

The initial discovery of sensory stimulus-evoked seizures was not suitable as an experimental paradigm for further studies, first because of the unresolved chemical nature of the stimulus and second because of the great degree of variability in seizure incidence of mGluR7$^{-/-}$ mice housed in different environments. Nevertheless, our initial findings in SPF-housed mGluR7$^{-/-}$ mice suggested a significantly reduced threshold for seizures in these mice. Our failure to identify another defined sensory stimulus that could provoke seizures reproducibly in mGluR7$^{-/-}$ mice prompted us to test subconvulsant doses of two drugs, pentylenetetrazole (PTZ) and bicuculline.

When administered intraperitoneally at a dose of 40 mg/kg, PTZ is generally subthreshold for inducing clonic seizures in control animals. In mice 12 weeks and older, it induced seizures in 1 of 40 mGluR7$^{+/+}$ and in 4 of 49 mGluR7$^{+/-}$ mice. In contrast, it evoked seizures in the majority of mGluR7$^{-/-}$ littermate mice (43 of 58; 74%) (Fig. 3*a*). Note that all of these mice were aged 10 weeks or older before testing to assure development of the epilepsy-prone phenotype in mGluR7$^{-/-}$ mice. The PTZ-evoked seizures in mGluR7$^{-/-}$ mice were frequently both clonic and tonic in nature and were followed by death (>50% of the mice). For comparison, in mGluR7$^{+/+}$ and mGluR7$^{+/-}$ mice, 70 mg/kg PTZ was required to produce seizures that consistently were generally only clonic in nature.

In mice aged 6 weeks the PTZ (40 mg/kg) failed to evoke seizures in a statistically significant manner (Kruskal–Wallis one-way ANOVA on ranks, $p = 0.407$). In only 1 of 10 mGluR7$^{-/-}$ mice were the seizures observed. When this mouse was eliminated from the group and the others were retested (40 mg/kg PTZ) at age 10 weeks, six of nine mGluR7$^{-/-}$ mice showed clonic seizures. In parallel and at both ages only one mouse of two groups of $n = 10$ mGluR7$^{+/+}$ mice (MAG; parental strains, NIH and Maus Inzucht Geigy) and no mGluR7$^{+/-}$ mice showed seizures ($p = 0.003$, Kruskal–Wallis one-way ANOVA on ranks revealed a significant difference among the genotypes; $p = 0.023$, Mann–Whitney rank–sum test for +/− vs −/−).

Five different groups ($n = 9$–10 per group and genotype) of

mGluR7$^{-/-}$, mGluR7$^{+/+}$, and mGluR7$^{+/-}$ mice were tested at ages 12–36 weeks, and the overall results are shown (Fig. 3*a*). One of 40 (2.5%) mGluR7$^{+/+}$ and 4 of 49 (8%) mGluR7$^{+/-}$ mice showed seizures (clonic only). In contrast, 37 of 49 (75%) mGluR7$^{-/-}$ mice showed clonic seizures that often (in >60% of these mice) progressed to tonic seizures. Often, mGluR7$^{-/-}$ mice with tonic seizures died. Statistical significance for the results in the groups aged >12 weeks is given by pairwise comparison of the genotype groups with the Mann–Whitney rank–sum test ($p = < 0.001$ for the +/+ vs −/− and the +/− vs −/− groups; $p > 0.05$ and no statistical significance when comparing +/+ and +/− groups). At age 36 weeks, differences in PTZ sensitivity remained statistically significant ($p = 0.002$, Mann–Whitney rank–sum test; comparison of a single group of nine mGluR7$^{+/-}$ and nine mGluR7$^{-/-}$ mice).

Spontaneous epileptiform activity was not detected during 1 week of continuous (24 hr/d) EEG recordings by using bilaterally implanted frontal and parietal electrodes in mGluR7$^{+/-}$ ($n = 4$) and mGluR7$^{-/-}$ ($n = 4$) mice. A subsequent injection of PTZ (40 mg/kg, i.p.) rapidly triggered epileptiform discharges and seizure manifestations that were specific to the mGluR7$^{-/-}$ mice. The same dose of PTZ injected into the mGluR7$^{+/-}$ mice triggered no detectable discharges (Fig. 3*b*), indicating a significantly reduced threshold for PTZ-induced discharges and seizures in the mGluR7$^{-/-}$ mice.

Susceptibility to seizures also was increased for the competitive $GABA_A$ receptor antagonist bicuculline. Two groups of 10 mGluR7$^{+/-}$ and mGluR7$^{-/-}$ mice (aged 14–16 weeks) were given 2.5 mg/kg bicuculline subcutaneously and were observed for 30 min. The latency to first seizure was plotted (Fig. 3*c*) for individual animals showing clonic seizures. Mice plotted at the 30 min value were without seizures. The median (*horizontal bar* in *bold*) for each group was 4 min (mGluR7$^{-/-}$, 25% at 4 min and 75% at 5 min) versus 26 min (mGluR7$^{+/-}$, 25% at 8 min and 75% at 30 min), respectively (Mann–Whitney rank–sum test, $p = 0.003$). For reference, nine of nine mGluR7$^{+/+}$ MAG mice treated with the same lot, and for MAG mice a standard suprathreshold dose of 3.5 mg/kg bicuculline, showed clonic seizures with a mean onset time of 3.2 min (data not shown).

Effects of standard anticonvulsant drugs

Three widely used mechanistically different antiepileptic drugs (White, 1997), ethosuximide (ESM), clonazepam (CZP), and valproate (VPA), were tested in mGluR7$^{-/-}$ mice for protection from PTZ-evoked seizures (Fig. 4).

First (Fig. 4*a,b*, *gray bars*), three groups of 10 mGluR7$^{+/-}$ and 10 mGluR7$^{-/-}$ mice (all aged 14–20 weeks) received pretreatment (1 hr before 40 mg/kg PTZ, i.p.) with an antiepileptic drug (CZP, 0.1 mg/kg, p.o.; ESM, 500 mg/kg, p.o.; VPA, 500 mg/kg, p.o.). In parallel, another group of 10 mGluR7$^{+/-}$ and 10 mGluR7$^{-/-}$ mice and, in addition, 10 mGluR7$^{+/+}$ wild-type (MAG) mice received a placebo (Methocel as reference for ESM and CZP; water compared with VPA). At 1 hr after placebo or anticonvulsant drug treatment all of the mice were given PTZ (40 mg/kg, i.p.). PTZ induced seizures in 60–90% of each of the placebo-treated mGluR7$^{-/-}$ groups ($n = 3 \times 10$), but not in any of the placebo-treated mGluR7$^{+/-}$ ($n = 3 \times 10$) or mGluR7$^{+/+}$ (MAG; $n = 3 \times 10$) mice. ESM, CZP, and VPA protected 100% of the mGluR7$^{-/-}$ mice ($n = 10$ per group and compound) from PTZ-induced seizures (statistical significance for each drug-treated versus placebo group is given by $p < 0.05$, Mann–Whitney rank–sum test).

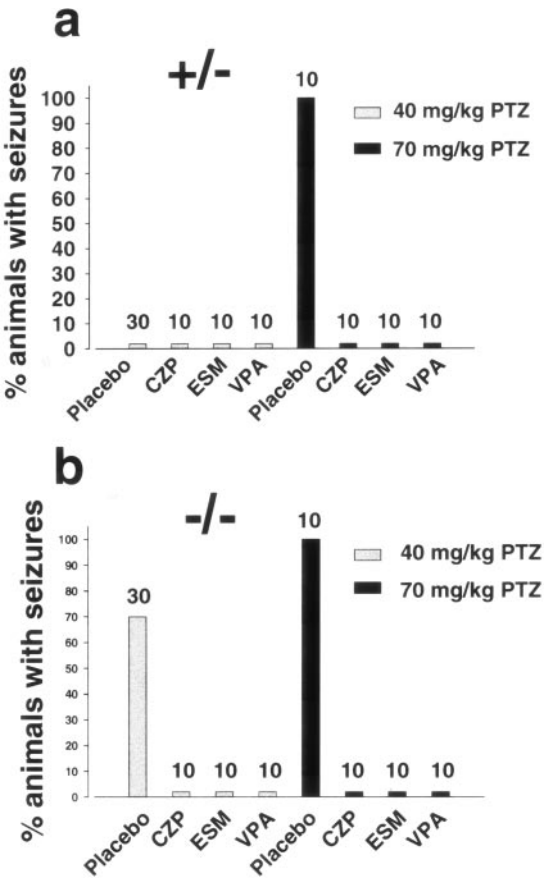

Figure 4. The actions of three standard anticonvulsants are unimpaired in mGluR7$^{-/-}$ mice. *a, b*, Plotted is the percentage of mice with seizures, scored behaviorally, for a period of 10 min in response to PTZ (40 or 70 mg/kg) given intraperitoneally. The *number* above each *bar* indicates the group size. The *gray bars* represent groups of mice given 40 mg/kg PTZ that were pretreated 1 hr before PTZ with either placebo (Methocel in two groups of $n = 10$ per genotype and as reference for ESM and CZP; water was the placebo in one group of $n = 10$ per genotype and compared with VPA) or anticonvulsant (0.1 mg/kg CZP, p.o.; 500 mg/kg ESM, p.o.; or 500 mg/kg VPA, p.o.). The *black bars* in *a* and *b* represent groups of 10 mice per genotype that had received PTZ (70 mg/kg, i.p). At 1 hr before PTZ all of the mice in a group had received the placebo (water, p.o.), CZP (1 mg/kg, p.o.), ESM (750 mg/kg, p.o.), or ESM (500 mg/kg). Unlike in mGluR7$^{-/-}$ mice (*b*), the 40 mg/kg PTZ dose is below threshold for inducing seizures in mGluR7$^{+/-}$ (*a*) and wild types (mGluR7$^{+/+}$ mice; data not shown). Therefore, mGluR7$^{+/-}$ mice displayed no seizures, regardless of pretreatment with placebo or anticonvulsant. In contrast, 70% of all mGluR7$^{-/-}$ mice showed clonic or clonic-tonic seizures when given placebo (*b*). The injection of 70 mg/kg PTZ evoked seizures (clonic in mGluR7$^{+/-}$ and mGluR7$^{+/+}$ mice; clonic or clonic-tonic in mGluR7$^{-/-}$ mice) in at least 90% of all placebo-treated mice, regardless of their mGluR7 genotype. CZP, ESM, and VPA fully protected from PTZ-induced clonic seizures in mGluR7$^{+/-}$ (*black bars* in *a*) and mGluR7$^{+/+}$ mice (data not shown) as well as from PTZ-induced clonic or clonic-tonic seizures in mGluR7$^{-/-}$ mice.

All three anticonvulsant drugs also provided protection in mGluR7$^{-/-}$ mice when challenged with 70 mg/kg PTZ (Fig. 4*a,b*, *black bars*), a dose that evoked clonic seizures in at least 90% all mGluR7$^{+/-}$ (Fig. 4*b*) and mGluR7$^{+/+}$ mice (data not shown). In this experiment we increased the dose of ESM (to 750 instead of 500 mg/kg) and CZP (to 1 mg/kg instead of 0.1 mg/kg). VPA dosing (500 mg/kg) was not increased, because this can cause lethality in mice. Given that all three anticonvulsants protected mGluR7$^{-/-}$ mice from either 40 or 70 mg/kg PTZ-

induced seizures, we conclude that mGluR7 receptors do not contribute significantly to mechanisms underlying the action of these antiepileptics.

Anticonvulsant effects of PPG are diminished greatly in mGluR7$^{-/-}$ mice

The group III mGluR selective agonist PPG has shown potent and sustained anticonvulsant actions in several rodent models of epilepsy (Chapman et al., 1999; Gasparini et al., 1999). Therefore, we compared its anticonvulsant action against PTZ-evoked seizures in mGluR7$^{-/-}$ mice and two other mGluR group III mutant mice, the mGluR4$^{-/-}$ and mGluR8$^{-/-}$ mutants. All of the mice used in these experiments were aged from 12 to 20 weeks before testing to allow for development of the seizure-prone phenotype in the mGluR7$^{-/-}$ mice. Also, we tested different doses of PTZ to determine, in each of the strain backgrounds of the different mutants, a dose that evoked clonic convulsions in 80% or more of the mice under the experimental conditions that were used, i.e., light Fluothane anesthesia, intracerebroventricular injection of placebo (0.9% NaCl), followed 15 min later by intraperitoneal injection of PTZ. Determining these experimental conditions was necessary because PPG is not active when given systemically (Gasparini et al., 1999), and, when given intracerebroventricularly, it requires brief anesthesia that increases PTZ thresholds (data not shown). In addition, PTZ thresholds depend on multiple chromosomal loci that differ between mouse strains (Kosobud et al., 1992; Ferraro et al., 1999), and mGluR4$^{-/-}$, mGluR7$^{-/-}$, and mGluR8$^{-/-}$ mutant mice have mixed and different genetic contributions from a number of strains. Accordingly, the PTZ doses required under our experimental conditions were 60 mg/kg in mGluR4 mutants (129Sv/J×CD1) and 70 mg/kg in mGluR7 [129Ola×C57BL/6 and (129Ola×C57BL/6)×BALB/c] and mGluR8 [(129×C57BL/6)×C57BL/6] mutants.

Next, dose responses for PPG (see Gasparini et al., 1999) (data not shown) in the different heterozygous mGluR mutant mice revealed that 634 nmol intracerebroventricularly (2.2 mg/kg) could protect ≥70% of the different heterozygous mutant mice from seizures induced by PTZ. This protective effect of PPG was reduced dramatically in mGluR7$^{-/-}$, but not in mGluR4$^{-/-}$ and mGluR8$^{-/-}$, mice (Fig. 5). The loss of the anticonvulsant activity of PPG in mGluR7$^{-/-}$ mice treated with 70 mg/kg PTZ was specific to PPG because CZP, ESM, and VPA provided complete protection in both mGluR7$^{+/-}$ and mGluR7$^{-/-}$ mice challenged with this dose of PTZ (Fig. 4*a,b*; 70 mg/kg PTZ dose results are represented by *black bars* in the histograms).

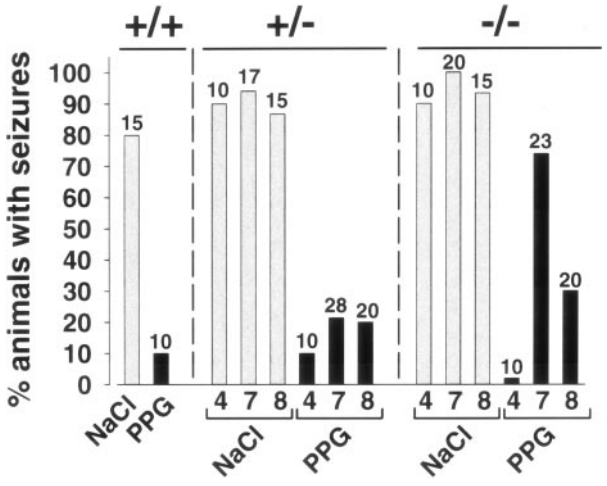

Figure 5. Greatly reduced anticonvulsant action of PPG in mGluR7$^{-/-}$ mice. Shown are the protective effects of 634 nmol of PPG intracerebroventricularly (*black bars*) versus 0.9% NaCl (*gray bars*) in mGluR7$^{+/+}$ mice (+/+) and the different mGluR$^{+/-}$ and mGluR$^{-/-}$ mutant mice as indicated by the numbers *4*, *7*, and *8* on the horizontal axis (all mice aged ≥12 weeks). PPG was given intracerebroventricularly 15 min before the intraperitoneal injection of PTZ [60 mg/kg in mGluR4 mutants; 70 mg/kg in all wild types (+/+) and other mGluR mutants]. Seizures were scored behaviorally for a period of 10 min (Schmutz et al., 1990). The *numbers* above the *bars* indicate the number of mice per group. The protective effects of PPG versus NaCl were 78% (22 of 28) versus 6% (1 of 17) in mice with one mutant mGluR7 allele and 26% (6 of 23) versus 0% (0 of 20) in mice with two mutant mGluR7 alleles. Unlike a comparison of the protective effect of PPG between mGluR7$^{+/-}$ and mGluR7$^{-/-}$ mice, there were no statistically significant differences in the protective action of PPG when mGluR4$^{+/-}$, mGluR4$^{-/-}$, mGluR8$^{+/-}$, and mGluR8$^{-/-}$ groups were compared (all $p > 0.05$). Confidence limits for the groups $n > 10$ were ±10%. For PPG versus NaCl comparisons in all groups, p values were <0.001 (Kruskal–Wallis one-way ANOVA on ranks, Dunn's test). Other values were mGluR7$^{-/-}$ versus mGluR7$^{+/+}$ (wild type), $p = 0.004$; mGluR7$^{-/-}$ versus mGluR4$^{-/-}$, $p < 0.001$; mGluR7$^{-/-}$ versus mGluR8$^{-/-}$, $p = 0.014$ (Mann–Whitney rank–sum tests).

Altered excitability in hippocampal slices

Because of similarities between seizures observed in mGluR7$^{-/-}$ mice and those known to involve limbic systems, we examined electrophysiological responses in hippocampal slices. We focused primarily on synaptic transmission in the CA1 region because CA3-derived Schaffer collateral–commissural terminals are rich in mGluR7a (Shigemoto et al., 1996, 1997) (Fig. 2*c*) and CA3 is a major trigger region for discharge activity that can propagate to CA1 and beyond in different models of epileptic discharge (Wong and Traub, 1983; Barbarosie and Avoli, 1997). Input–output curves relating the initial slope of the field EPSP to either stimulus intensity or presynaptic fiber volley amplitude revealed no significant differences between mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice (Fig. 6*a,b*). Paired pulse facilitation was also similar between groups. For example, with an interpulse interval of 50 msec the facilitation ratios in CA1 were 1.45 ± 0.04 ($n = 9$) and 1.49 ± 0.03 ($n = 9$), respectively.

Next, we determined whether hippocampal slices from mGluR7$^{-/-}$ mice were more excitable when the stimulus intensity was increased to evoke a population spike and activate GABAergic synapses polysynaptically (Fig. 6*c,d*). In the absence of PTZ a very small secondary population spike was apparent in 8 of 11 slices from null mice (0.067 ± 0.020 of the first population spike) but only in 1 of 10 slices from controls (0.009 ± 0.011 of the first population spike; Student's t test, $p < 0.05$). Thus, slices prepared from mGluR7$^{-/-}$ mice were slightly more excitable under control conditions.

Given the lower seizure threshold of mGluR7$^{-/-}$ mice to PTZ, we examined the effects of PTZ on synaptic transmission in CA1. PTZ caused a reduction in synaptic inhibition, manifest as a concentration-dependent appearance of multiple population spikes in both mGluR7$^{-/-}$ and wild-type mice (Fig. 6*c,d*). The effect was such that differences in excitability between mGluR7$^{-/-}$ and wild-type littermates, observed in the absence of PTZ, were no longer evident when higher concentrations of PTZ (1–4 mM) were used.

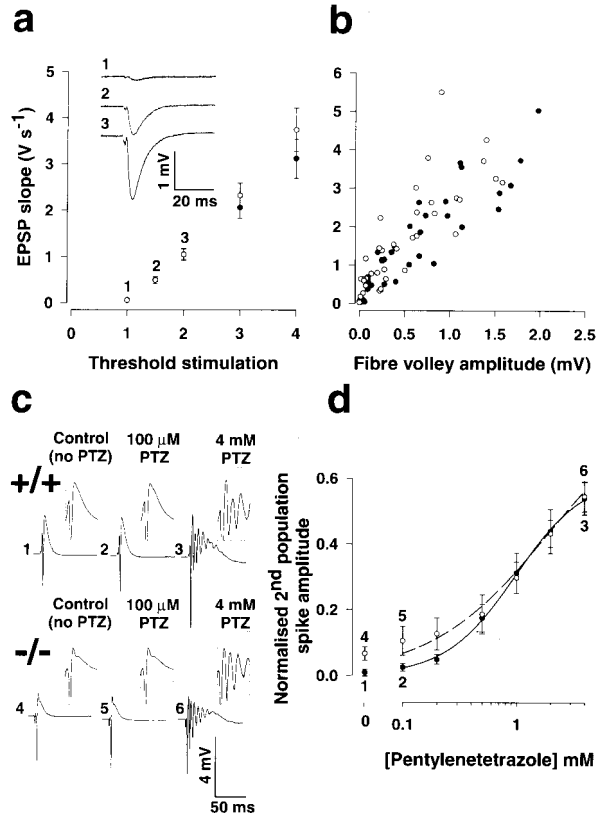

Figure 6. Increased excitability in the CA1 region of hippocampal slices from mGluR7$^{-/-}$ mice. *a*, Plotted is the slope of the field EPSP, recorded in stratum radiatum, versus relative stimulus strength, expressed as a function of the threshold intensity (i.e., the intensity at which a response is just detectable in single records) for mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice. The traces are averages of successive responses from a typical mGluR7$^{-/-}$ mouse at the times indicated (*1–3*); the input–output curves show no significant difference in excitatory synaptic transmission between wild-type ($n = 14$) and null mice ($n = 12$). *b*, Input–output curve, relating the slope of the field EPSP to the presynaptic fiber volley amplitude. *c*, Examples of somatic field recordings from wild-type (*1–3*) and mGluR7$^{-/-}$ mice (*4–6*) to illustrate the effects of increasing concentrations of PTZ. The *insets* are a magnification (2.5×) of the windows shown in *c1* and *c4* to illustrate the generation of multiple population spikes on a faster time base. Field potentials were recorded from stratum pyramidale and input–output curves that were constructed. Then the stimulus intensity was set at that which produced a first population spike of ~30% of the maximum, and PTZ was applied sequentially in increasing concentrations. *d*, Pooled data for 10 wild-type and 11 mGluR7$^{-/-}$ mice (aged between 8 and 29 weeks) of the amplitude of the second population spike, expressed as a function of the primary population spike, versus PTZ concentration. *Open symbols*, mGluR7$^{-/-}$ mice; *filled symbols*, littermate wild types, throughout.

Altered frequency-dependent facilitation in mGluR7$^{-/-}$ mice

To investigate mechanisms that might underlie the changes in excitability, we turned to a different slice preparation and studied transmission between synaptically coupled pyramidal neurons and bitufted interneurons in layer 2/3 of the neocortex. This synapse contains a high density of presynaptic mGluR receptors, including mGluR7 (Shigemoto et al., 1996), and it demonstrates frequency-dependent facilitation (Reyes et al., 1998), which is believed to arise from the elevation of Ca^{2+} at the presynaptic release site (Zucker, 1994; Fisher et al., 1997; Rozov et al., 2001).

As seen in the hippocampus (see paired pulse data in previous section), facilitation in response to brief trains of two or three

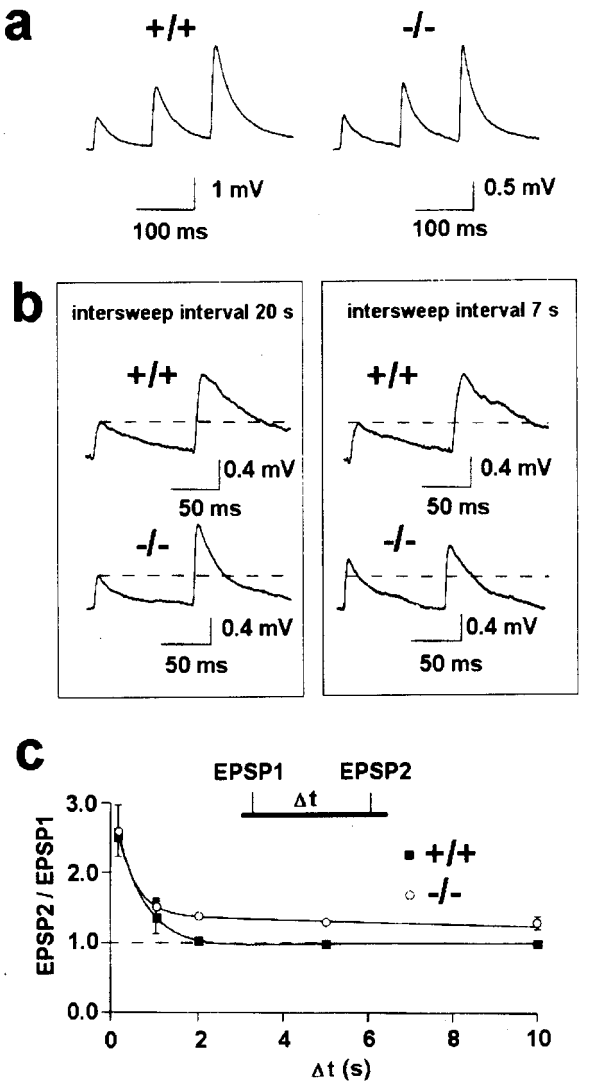

Figure 7. Altered recovery from paired pulse facilitation in mGluR7$^{-/-}$ mice. Simultaneous whole-cell paired recordings from synaptically connected pyramidal and bitufted neurons in layer 2/3 of mGluR7$^{+/+}$ ($n = 9$) and mGluR7$^{-/-}$ ($n = 7$) mice (P14; neocortex). *a*, Averaged EPSPs ($n =$ 50–100) recorded from bitufted neurons of mGluR7$^{+/+}$ and mGluR7$^{-/-}$ mice in response to a train of three action potentials evoked in a projecting pyramidal neuron at 10 Hz. Time intervals between trains (intersweep intervals) were 30 sec in both cases. *b*, Averaged EPSPs of 50–100 sweeps recorded from bitufted neurons in response to two action potentials that were evoked in pyramidal neurons with a 100 msec time interval. The difference between the *left* and *right traces* is the intersweep interval (time between subsequent pairs of action potentials). For the *left traces* it was 20 sec, and for the *right traces* it was 7 sec, as indicated above the traces. The *top traces* in each *box* represent recordings from mGluR7$^{+/+}$ mice, and the *bottom traces* are from mGluR7$^{-/-}$ mice. For each genotype the representative recordings that are shown (i.e., averaged EPSPs of 50–100 sweeps) are from the same bitufted neuron. *c*, Time course of recovery from facilitation measured in bitufted neurons. The ratios of the mean EPSP amplitudes (EPSP2/EPSP1) were plotted against Δt.

action potentials (at 10 Hz in this preparation) was similar in mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice, provided that the intersweep interval time was sufficiently long (Fig. 7*a*; in both cases intersweep interval was 30 sec). However, a marked difference was observed among mice when recovery from facilitation was studied. In one set of experiments paired pulses were delivered with a fixed interpulse interval of 100 msec, but the interval between

paired pulses (intersweep interval) was varied between 5 and 30 sec (Fig. 7*b*). Averaged EPSPs recorded from the same bitufted neuron (see *left trace* in *a*) in a mGluR7$^{+/+}$ mouse in response to a train (10 Hz) of two action potentials delivered at 20 sec (left) and 7 sec (right) intersweep intervals shows that the amplitude of the first EPSP (EPSP1) and the paired pulse facilitation ratio (EPSP2/EPSP1) were similar at 20 and 7 sec intersweep interval times. In this mouse the EPSP1 amplitudes were 0.46 and 0.49 mV, and EPSP2/EPSP1 ratios were 2.24 and 2.12 at the 20 and 7 sec intersweep intervals, respectively. In the mGluR7$^{-/-}$ mouse (*bottom traces*) the EPSP1 amplitude increased from 0.39 mV at the 20 sec intersweep interval to 0.75 mV at the 7 sec intersweep interval, and the EPSP2/EPSP1 ratio decreased from 2.61 to 1.28 at the 20 and 7 sec intersweep intervals, respectively. Overall, in this set of experiments the EPSP1 amplitudes with the 7 sec intersweep interval were 100 ± 12% of those measured with 20 sec interval, and the EPSP2/EPSP1 ratios were 2.51 ± 0.28 and 2.56 ± 0.47 at the 20 and 7 sec intersweep intervals, respectively, in mGluR7$^{+/+}$ mice (n = 4). In the mGluR7$^{-/-}$ group the EPSP1 amplitudes with the 7 sec intersweep interval were 59.7 ± 28.3% larger than those recorded at the 20 sec interval. EPSP2/EPSP1 ratios decreased from 2.87 ± 0.88 to 1.27 ± 0.03 for 20 and 7 sec, respectively. In summary, in mGluR7$^{+/+}$ mice the facilitation recovered within 5–7 sec, as observed previously with rats (Reyes et al., 1998). In contrast, in mGluR7$^{-/-}$ mice full recovery from facilitation required 20–30 sec.

In a separate set of experiments the time course of recovery from facilitation was calculated by using a repeated paired pulse protocol. Every 30 sec a train of two action potentials (indicated as EPSP1 and EPSP2) was evoked. One of several different time intervals was chosen (Δt = 0.1, 1, 2, 5, and 10 sec, respectively) between action potentials 1 and 2, and 50–100 individual sweeps were averaged for each Δt. The intersweep intervals thus were kept constant at 30 sec in all of these experiments to allow the facilitation to recover in mGluR7$^{-/-}$ mice also. The plot shows the summarized results of delivering paired pulses with the different interpulse intervals. In mGluR7$^{+/+}$ mice the recovery from facilitation was evident by 2 sec, whereas even after 10 sec the recovery in mGluR7$^{-/-}$ mice was still incomplete (Fig. 7*c*).

DISCUSSION

Epilepsy-prone phenotype of mGluR7$^{-/-}$ mice, but not other mGluR-deficient mice

In contrast to gene ablations of mGluR 1, 2, 4, 5, 6, and 8, respectively (Aiba et al., 1994; Conquet et al., 1994; Masu et al., 1995; Pekhletski et al., 1996; Yokoi et al., 1996; Lu et al., 1997; Duvoisin and Zhang, unpublished observations), gene disruption of mGluR7 predisposes to epilepsy. This points toward an important role of this particular group III mGluR in regulating the balance between excitatory and inhibitory transmission. The reduced thresholds for PTZ, bicuculline, and sensory stimulus-evoked seizures in mGluR7$^{-/-}$ mice are acute results of the absence in adult tissue of a single or both isoforms of mGluR7 receptors. Alternatively, they are a consequence of absence of the receptor throughout development. Addressing these questions awaits the development of mGluR7-specific antagonists, conditional knock-outs, or mGluR7$^{-/-}$ mice in which receptor expression is reconstituted in the adult.

In either case the mGluR7$^{-/-}$ mouse provides an interesting model of epilepsy, given that (1) the seizure susceptibility phenotype develops gradually, (2) they are uniquely associated with an ablation of this and no other mGluR subtype, and (3) when evoked by PTZ, the seizures are responsive to representatives of three major classes of anticonvulsants [ethosuximide is thought to act via modulation of Na^+ and Ca^{2+} channels (Coulter et al., 1989); clonazepam, a benzodiazepine, increases the frequency of $GABA_A$ receptor chloride channel opening and is especially potent in preventing PTZ-induced seizures (Henriksen, 1998); valproate is a drug with a broad preclinical and clinical profile but is poorly understood mechanistically (McLean and Macdonald, 1986; Kelly et al., 1990; Rogawski and Porter, 1990; Van Erp et al., 1990; Zona and Avoli, 1990)]. Apparently, mGluR7 does not contribute significantly to mechanisms underlying the actions of these antiepileptics.

In contrast, mGluR7 appears to be an important mediator of a mechanistically different (and potentially clinically viable) anticonvulsant principle, namely, the activation of group III mGluRs. This was shown here by using the group III mGluR selective agonist PPG, which proved effective against PTZ-induced seizures in mGluR4$^{-/-}$, mGluR8$^{-/-}$, and mGluR7$^{+/-}$, but not mGluR7$^{-/-}$, mice under experimental conditions in which PTZ evoked primarily clonic seizures. These findings are somewhat surprising, because PPG is least potent on mGluR7 of all of the human and rat recombinant group III mGluRs for the inhibition of forskolin-stimulated cAMP accumulation in mammalian cells (Nakajima et al., 1993; Okamoto et al., 1994; Conn and Pin, 1997; Gasparini et al., 1999). PPG also is anticonvulsant against sound-induced seizures in DBA/2 mice and genetically epilepsy-prone rats (Chapman et al., 1999) in the mouse maximal electroshock model (Gasparini et al., 1999) and in the mouse PTZ model (Fig. 5). Unlike L-AP4 and L-SOP, PPG lacks proconvulsant activity, and its anticonvulsant effects last much longer than those of L-AP4 or L-SOP (Chapman et al., 1999; Gasparini et al., 1999). However, PPG is not active when given systemically, and the compound has sedative effects (Chapman et al., 1999; our unpublished results). Therefore, novel compounds are needed to evaluate the potential of group III and, in particular, mGluR7-selective drugs for treating epilepsy and/or other disorders (Masugi et al., 1999). A recent finding that further strengthens a role for mGluR7 receptors in epilepsy is its selective upregulation in the inferior colliculus of genetically epilepsy-prone (GEP) rats. This was shown to be associated with a prolonged anticonvulsant effect of intracollicular administrated L-SOP against sound-induced seizures in GEP rats (Yip et al., 2001).

One very limited gene polymorphism study in patients (Goodwin et al., 2000) could not provide a link between the mGluR7 gene and epilepsy. This also applies to other group III mGluRs. However, the mGluR7 gene spans 600 kb (http://www.ncbi.nlm.nih.gov/AceView/acegquery.cgi?db=300&ORG=hs&term=GMM7), and more detailed studies need to be performed by using a large number of single nucleotide polymorphisms across the locus before any firm conclusions can be drawn regarding linkage to disease in human.

Interestingly, mGluR4$^{-/-}$ compared with mGluR4$^{+/-}$ mutant mice showed a differential resistance to absence-like seizures induced by 30 mg/kg PTZ (Snead et al., 2000). This finding seems to support a facilitating role of mGluR4 in absence-like seizures. At higher doses PTZ (40–60 mg/kg) evoked clonic convulsions and showed no difference in susceptibility between wild-type and mGluR4$^{-/-}$ mutant mice (Snead et al., 2000). PTZ injections (60 mg/kg) in mGluR4$^{-/-}$ mice under our and different experimental conditions confirmed this. We failed to reveal a role for mGluR4 in mediating the anticonvulsant action of PPG in our PTZ paradigm, but another potent mGluR4 agonist has been

shown to increase the latency of seizure onset in a different PTZ-induced tonic seizure paradigm (Thomsen and Dalby, 1998). Therefore, dependent on the seizure paradigm that is used, the role of particular group III mGluRs may differ.

This hypothesis is tempting, given findings in neuroprotection experiments. When we used different magnitudes of a toxic (NMDA) insult, low doses of PPG substantially reduced toxicity in mGluR4$^{+/-}$ mice, but not in mGluR4$^{-/-}$ mice, whereas higher doses were protective in both genotypes, suggesting that another receptor might play a more important role in protection at higher doses (Bruno et al., 2000).

Potential mechanisms underlying the epileptic phenotype

The very small excitability changes detected in the untreated mGluR7$^{-/-}$ hippocampal slices are consistent and correlate well with the absence of seizures in the mGluR7$^{-/-}$ mice *in vivo* under normal circumstances. The weak epileptogenic effects seen in mGluR7$^{-/-}$ hippocampal slices with a subthreshold concentration of PTZ (for control slices) are consistent with the increased seizure susceptibility for PTZ in the mGluR7$^{-/-}$ mice *in vivo*. The lack of a difference between mGluR7$^{-/-}$ and their wild-type littermates in the excitability in slices exposed to PTZ and, in particular, at higher concentrations of PTZ is suggestive of a common expression mechanism, namely, a net reduction in GABAergic synaptic inhibition, for the two epileptogenic situations (absence of mGluR7 and presence of PTZ).

Recovery from facilitation reflects the restoration of presynaptic Ca^{2+} levels by extrusion while synapses are not active (Zucker, 1994; Fisher et al., 1997). Our results in the paired stimulus paradigms (Fig. 7) are consistent with the hypothesis that deletion of mGluR7 affects a (slow) component involved in presynaptic Ca^{2+} homeostasis. Resolving the underlying molecular mechanism remains a challenge, not only because of the multiple mechanisms implicated in presynaptic Ca^{2+} regulation (Na^{+}–Ca^{2+} exchange, uptake in mitochondria, the plasma membrane Ca^{2+} ATPase) [see Zenisek and Matthews (2000) and references therein] but also because mGluR7 has been linked to multiple effector pathways (Saugstad et al., 1996; Nakajima et al., 1999; O'Connor et al., 1999; Perroy et al., 2000). Regardless of which exact molecular mechanism will prove operational, a delayed recovery from facilitation as observed in mGluR7$^{-/-}$ slices may account, at least in part, for an epilepsy-prone phenotype, given that such alterations share some features that are observed when (presynaptic) K^{+} channels are blocked by convulsant drugs (see, for example, Juhng et al., 1999). The mGluR7-deficient mouse adds to a large and growing list of novel models of epilepsy as a result of gene ablation, recently also including mice that lack the metabotropic receptor for GABA (Prosser et al., 2001; Schuler et al., 2001). Its uniqueness lies in the fact (1) that only few cases have been reported in which the epileptic phenotype is associated and/or caused by a specific presynaptic defect (such as synapsin deficiency; Rosahl et al., 1995) and (2) that no other mGluR gene ablation (mGluR1, 2, 4, 5, and 8) has resulted in an epileptic phenotype despite the fact that two of these receptors (mGluR4 and 8) have a strikingly similar presynaptic location. Given that mGluR4, mGluR7, and mGluR8 modulate different presynaptic parameters and show differential expression patterns (Shigemoto et al., 1997), these receptors might serve as distinct frequency-dependent synaptic transmission filters that accommodate fine tuning and information transfer under normal homeostatic and pathological conditions (for review, see Thomson, 2000). Dissecting the molecular signaling mechanisms underlying mGluR7 and its frequency-dependent regulation of neurotransmission may shed light on why this receptor might have potential as a drug target in epilepsy and/or other indications (Masugi et al., 1999).

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