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Vlgr1 knockout mice show audiogenic seizure susceptibility

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Abstract

Susceptibility to audiogenic seizures, which are reflex seizures provoked by loud noise, can be induced in rodents by acoustic priming (exposing animals to strong auditory stimuli at an early developmental stage). Some strains of mice and rats are susceptible to audiogenic seizures without priming and these have been used as good experimental models with which to study epilepsies. Here we identified *Vlgr1d* and *Vlgr1e*, novel alternatively-spliced variants of *Vlgr1b*/*MGR1*, which, upon sequence analysis, were shown to be transcripts from a locus previously characterized as mass1. *Vlgr1* (*Vlgr1b, Vlgr1d* and *Vlgr1e*) mRNA is expressed predominantly in the neuroepithelium of the developing mouse brain. Our protein-tagged experiment suggested that Vlgr1d and Vlgr1e are secretory molecules, while Vlgr1b is a receptor. Knockout mice lacking exons 2–4 of *Vlgr1* were susceptible to audiogenic seizures without priming, although there were no apparent histological abnormalities in their brains. Ninety-five percent of these knockout mice exhibited wild running, a feature typical of the preconvulsive phase of audiogenic seizures triggered by loud noise (11 kHz, 105 dB), and 68% exhibited tonic convulsions at 3 weeks after birth. Our monogenic mice, which have a unique genetic background, serve as a useful tool for further studies on seizures.

Keywords: Calx-β, epilepsy, gene targeting, Mass1, neuroepithelium, reflex seizure.

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Epileptic seizures and syndromes, which are estimated to affect 1–3% of the population (Hauser *et al*. 1996), are defined as the occurence of recurrent seizures. Although most seizures are unprovokable and unpredictable, some stimuli induce seizures even when no obvious structural, metabolic or neurological abnormalities exist. One typical model for this type of seizure in rodents is audiogenic seizures, generalized self-sustained convulsive seizures in which acoustic stimulations evoke wild running and tonic flexion and extension (Ross and Coleman 2000). They are induced by exposing animals to a loud noise during the early stages of their development (acoustic priming; Henry 1967; Miyakawa *et al*. 1995). While acoustic priming is crucial for the induction of audiogenic seizures in resistant mouse strains such as C57BL/6J, in some strains, such as DBA/2 and *Frings*, seizures may be provoked by auditory stimuli

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The nucleotide sequences of the gene reported in this paper (*Neurepin1/Vlgr1d* and *Neurepin2/Vlgr1e*) have been submitted to the DDBJ (DNA Databank of Japan) database with accession numbers AB086166 and AB086167.

Abbreviations used: BrdU, bromodeoxyuridine; DAB, diaminobenzidine; DBH, dopamine beta-hydroxylase; E, embryonic day; EGF, epidermal growth factor; ES, embryonic stem; EYFP, enhanced yellow fluorescent protein; FGF2, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5-HT_{2C}, 5-hydroxytryptamine type 2C receptor; neo, neomycin-resistant gene; P, postnatal day; PBS, phosphate-buffered saline; PGK, phosphoglycerol kinase; tk, thymidine kinase.

without priming (Ross and Coleman 2000). Interestingly, susceptibility to such seizures is regulated developmentally. In many strains, including DBA/2, susceptibility peaks at 3 weeks after birth and declines subsequently, adults being almost resistant (Seyfried 1979; Misawa *et al*. 2002).

The fact that susceptibility to audiogenic seizures is hereditary indicates that it is attributable to the mutation of specific genes. To date, most idiopathic epileptic seizures and syndromes in humans are caused by mutations in ion channels. For example, mutations in voltage-gated or ligand-gated channels result in idiopathic epilepsies such as febrile seizures, benign neonatal convulsions and autosomal dominant nocturnal frontal-lobe epilepsy (Steinlein 2002). It is now recognized that channelopathies are relevant to these diseases in humans. However, other types of molecules have been added to the list of seizure-responsible molecules in model animals. For example, a mouse with mutated 5-hydroxytryptamine type 2C receptor (5-HT_{2C}) exhibits susceptibility to audiogenic seizures (Brennan *et al*. 1997). Following a recent detailed genetic and physical mapping of the audiogenic seizure-related locus in the *Frings* mouse, it was reported that Mass1, which is not a component of either an ion channel or a receptor, is responsible for audiogenic seizures (Skradski *et al*. 2001).

Audiogenic seizures vary considerably with regard to the age of maximum risk, and their severity and duration. So far, the molecular mechanisms underlying the susceptibility to audiogenic seizures have not been fully elucidated. Genetically-engineered mice with a single genetic background and in which the responsible gene has been modified are invaluable if the etiology of idiopathic epilepsies is to be elucidated. Moreover, such mice may serve as good models with which to study the development of brain function, especially as it appears that developmental regulation is pivotal to susceptibility to audiogenic seizures.

During a search for genes that are expressed predominantly in the neuroepithelium, we identified novel alternatively-spliced variants of Vlgr1b that are expressed predominantly in the neuroepithelium. Vlgr1b (MGR1) is a very large membrane protein that is related to Mass1 (McMillan *et al*. 2002; Ptácek and Fu 2003). Here, we provide data that show that disruption of the *Vlgr1* gene causes audiogenic seizure susceptibility. This observation confirms and extends work that has shown that disruption of Vlgr1b or Mass1 in mice increases audiogenic seizure susceptibility (Skradski *et al*. 2001; Yagi *et al*. 2003; McMillan and White 2004).

Materials and methods

Animals

Animals were obtained from local vendors: Wistar rats were from Keari (Osaka, Japan), DBA/2J mice were from Charles River Japan (Yokohama, Japan), and C57BL/6J mice and ICR mice were from SLC (Hamamatsu, Japan). The day that the presence of a vaginal plug was confirmed was defined as embryonic day 0 (E0). For pups, the day of birth was designated postnatal day 0 (P0). Before experiments, all animals were deeply anesthetized by hypothermia (for P0–P5), or by intraperitoneal injection of sodium pentobarbital (40 mg/kg). For embryos, their dams were deeply anesthetized with sodium pentobarbital (40 mg/kg). All experiments were conducted in accordance with the guidelines for animal experiments of Osaka City University and University of Fukui.

DNA database inquiries

A homology search was carried out using the BLAST program at http://www.ncbi.nlm.nih.gov/BLAST/ through the internet. Enquiries into the human genomic DNA database were carried out at http://www.ncbi.nlm.nih.gov/genome/guide/human/, and motifs and specific domains were searched at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. Mouse genome resources were searched at http://www.ncbi.nlm.nih.gov/genome/guide/mouse/. Polyadenylation site prediction was carried out at http://125.itba.mi.cnr.it/~webgene/wwwHC_polya.html. NCBI Locuslink is at http://www.ncbi.nlm.nih.gov/LocusLink/. According to the NCBI LocusLink database nomenclature, the locus from where *Vlgr1* and *Mass1.1* are transcribed is called mass1.

Isolation of a rat cDNA fragment by differential display and *in situ* hybridization

We used mRNA differential display and *in situ* hybridization to identify genes that are expressed predominantly in the neuroepithelium (ventricular zone) during the early stage of corticogenesis. Details of the procedures have been described previously (Takamura *et al*. 2001; Nagano *et al*. 2002). In brief, polyadenylated [poly(A)+] RNAs were extracted from the telencephalons of E11 and E18 rat embryos, reverse-transcribed (RT), then subjected to polymerase chain reaction (PCR) using sets of two primers to display mRNAs differentially and isolate DNA fragments. Two hundred DNA fragments that were expressed predominantly in the telencephalon of E11 embryos, but not significantly in that of E18 embryos, were obtained as candidates. By determining their nucleotide sequences and making enquiries into the DNA databank, 80 independent clones were identified. We then performed a further screen using *in situ* hybridization to identify genes that are expressed in the ventricular zone, but not in the cortical plate, of the developing telencephalon. The conventional protocol was used for *in situ* hybridization (Nagano *et al*. 2000). Finally, among the clones that we obtained, we identified a novel 384-base pair (bp) DNA fragment that was temporarily designated rat-*Neurepin*.

Screening of a rat cDNA library

A rat E12 forebrain cDNA library constructed in lambda ZAP II was screened using a rat-*Neurepin* DNA fragment labeled with [α-^{32}P] dCTP using the BcaBest labeling kit (Takara Bio, Ohtsu, Japan). We then identified a longer cDNA fragment of rat-*Neurpein* that was 4.2 kb in size.

Mouse *Neurepin* cDNA screening

For further genetic manipulation, we screened a mouse cDNA library with this rat-*Neurepin* cDNA fragment; we screened 1×10^6

plaques of the pre-made cDNA library, made from mouse embryonic carcinoma cell line P19 treated with retinoic acid (Stratagene, La Jolla, CA, USA), with the [α-^{32}P] dCTP-labeled rat-*Neurepin* cDNA fragment. Inquiries into the DNA database revealed that the two cDNAs obtained were novel; we named them *Neurepin1* and *Neurepin2* (Yagi *et al*. 2003).

Northern blot analyses

An aliquot of 2–6 μg poly(A)+ RNA from ICR mouse brain was blotted onto a nylon membrane (Hybond N; Amersham Biosciences, Piscataway, NJ, USA) after agarose-formaldehyde gel electrophoresis. Details of the extraction of mRNA and the hybridization procedures are described elsewhere (Yagi *et al*. 1998; Sambrook and Russell 2001). Two probes, approximately 1 kb in size, were used for northern blot analyses: a fragment of the 5′ region of mouse *Neurepin* cDNA (*Neurepin*-probe, nucleotides 153–1340 of *Neurepin1*) and a fragment of the 3′ region of mouse *Vlgr1b* cDNA (*Vlgr1b*-specific probe, nucleotides 17730–19010 of *Vlgr1b*; McMillan *et al.* 2002). Details of *Neurepin* and *Vlgr1b* are described in the Results section. Filters were rehybridized with a [α-^{32}P]-labeled, 0.85 kb fragment of mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). In some cases, expression of each mRNA was analyzed semiquantitatively by measuring their intensities using NIH-image, and optimized based on the intensities of *GAPDH*.

Radiation hybrid mapping

After obtaining partial sequences of human *Neurepin* orthologues, the chromosomal locus of *Neurepin* was elucidated by radiation hybrid mapping using the Stanford G3 panels (Research Genetics, Huntsville, AL, USA), following the manufacturer's protocol. The primers employed were as follows: 5′-AGGAGCTGAGATTAACACCTCT-3′ and 5′-CAACAGAAGGGCTTATTAGCAC-3′. The PCR result was as follows: 00000000000000001000000000000010000010000001001R0000000000000010000100000001001010101, where 1 is positive, 0 is negative and R is 'not determined'. This result was sent to the Stanford Human Genome Center RHserver (http://www-shgc.stanford.edu/index.html) to obtain the chromosomal mapping information. The result of the mapping showed that *Neurepin* was mapped onto chromosome 5, close to SHGC-1762, SHGC-82576 and SHGC-111867. These are close to or within mass1 locus.

Construction of targeting vectors

We screened the 129/Sv mouse genomic library in the Lambda Fix II phage vector (Stratagene) using a 1.2 kb mouse *Neurepin* cDNA fragment as a probe. This probe was obtained by RT-PCR against total RNA extracted from ICR mice with the primers 5′-GATGTCGGTGACCTCAGAGCC-3′ and 5′-CGCTGTATCTTCATCAA-3′, the designs of which are based on the rat-*Neurepin* cDNA sequence. We obtained a mouse genomic fragment encompassing exons 2–6 of the *Neurepin* gene that is identical to those of *Vlgr1b*. In this section, we hereafter designate *Neurepin1*, *Neurepin2* and *Vlgr1b* as *Vlgr1* (see details in the Results section).

To generate *Vlgr1* (*Neurepins* and *Vlgr1b*) knockout mice, we disrupted the region including exons 2–4 by replacing it with a neomycin-resistant gene (*neo*) driven by a phosphoglycerol kinase *(PGK)* promoter (Yagi *et al*. 1998). We inserted a 3.0 kb *Bam*HI-*Hin*dIII fragment of intron 1 and a 1.6 kb *Eco*RI-*Bgl*II fragment of the *PGK-neo* cassette into a *Bam*HI-*Hin*dIII site and a *Cla*I site of the pBluescript SK (Stratagene), respectively, in which the *Ssp*I fragment was replaced with the *PGK-thymidine kinase (tk)* cassette (Yagi *et al*. 1998). To obtain the final targeting vector, we inserted a 5.0 kb *Sal*I-*Not*I fragment, consisting of intron 4, exon 5 and intron 5, into a *Sal*I-*Kpn*I site of pBluescript SK containing intron 1, *PGK-neo* and *PGK-tk*.

Generation of knockout mice

The targeting vector was electroporated into E14-1 embryonic stem (ES) cells derived from 129/Ola mice, and selected with G418 (0.4 mg/mL; Invitrogen, Carlsbad, CA, USA) and gancyclovir (2 μM/L; F. Hoffmann-La Roche, Basel, Switzerland). Resistant colonies were selected, expanded, and screened for homologous recombination by conventional Southern blot analyses (Yagi *et al*. 1998). The ES cells obtained were injected into E3 blastocysts taken from C57BL/6J and transferred into the uteri of pseudopregnant ICR females. Chimeric mice were mated with C57BL/6J to generate heterozygous mutants.

RT-PCR

Total RNA was prepared from an E14 mouse brain, and 1 μg of the total RNA was reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase (Roche diagnostics, Basel, Switzerland) in a 20-μL reaction volume, using random hexamers. PCR amplifications were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) or LA-Taq DNA polymerase (Takara Bio) in a 50-μL reaction volume, using 1 μL of the cDNA solution. The following primers were used with AmpliTaq DNA polymerase. For *Mass1.1* (606–1175) amplification: forward primer 5′-TCTGAGCTGATGTGCTGTGG-3′ and reverse primer 5′-TTGTATCTGATTGACACTTGGG-3′; *Vlgr1* (306–1031): forward 5′-TACTGCCATTGTGTCGCTGAG-3′ and reverse 5′-TTGTATCTGATTGACACTTGGG-3′, the same primer used for the reverse primer for *Mass1.1* (606–1175); *Mass1.1* (8797–9168): forward 5′-AGAGAGTTCGGATCTCTAGG-3′ and reverse 5′-GTGTTACGTTCCCATAACAAC-3′; *Vlgr1* (8653–9033): forward 5′-AGAGAGTTCGGATCTCTAGG-3′, the same primer used for the forward primer for *Mass1.1* (8797–9168), and reverse 5′-CCAGTGTTACAACGCCGTCG-3′. The following primers were used with LA-Taq DNA polymerase to confirm the disruption of *Vlgr1*: forward 5′-TGTGAAGGGAGGAACGGCAGCGG-3′, reverse 5′-CCATCACTATCCTTCCCACGA-3′.

Real-time PCR

Total RNA was extracted from an E14 mouse brain and contaminated genomic DNA was removed using the DNA-free RNA kit (Zymo Research, Orange, CA, USA). A 1 μg aliquot of this total RNA was reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase in a 20-μL reaction volume, using random hexamers. The real-time PCR reactions were carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The SYBR green assay was performed to detect the amount of product using the SYBR Green Master Mix (Applied Biosystems). Each SYBR green reaction (30-μL reaction volume each) contained 2 μL of diluted cDNA as a template and 50 nM gene-specific primers (described below). The thermal cycle parameters used were the universal thermal cycling parameters of the Applied Biosystems'

guideline. To verify that the SYBR green dye detected only one species of the PCR product, the samples were subjected to a heat-dissociation protocol (Applied Biosystems) after the final cycle of the PCR. The following primers were designed by Primer Express software (Applied Biosystems): for *GAPDH* detection, forward 5′-CCTGGAGAAACCTGCCAAGTAT-3′ and reverse 5′-GGTCCTCAGTGTAGCCCAAGAT-3′; for *Vlgr1* detection, forward 5′-CCGGAGCCTGATGAAACCT-3′ and reverse 5′-TGGCCATCCAAGTTTCACAAT-3′; for *Mass1.1* detection, forward 5′-ACCAAGTCCTCCCTCTCTCTGA-3′ and reverse 5′-GGTTCAATCACCGAGATTGATGA-3′.

Constructing the plasmids of EYFP (enhanced yellow fluorescent protein)-tagged molecules and its observation

Partial cDNA fragments of *Vlgr1* and *Mass1.1* were obtained using RT-PCR with the following primers. For partial Vlgr1 of 1–615 amino acid residues: forward 5′-GGGTCGACGGGCACGCAGATGTCGGTGA-3′ and reverse 5′-ATGGATCCTGCACTAGGAAGTGGGC-3′; for N-terminal region of Mass1.1 (corresponds to 179–615 amino acid residues of Vlgr1b): forward 5′-CGTCGACAATGGTCACCGTGACTT-3′ and reverse 5′-GAATTCAGGAATCCTATCTGGG-3′. Amplified PCR products were subcloned into pEYFP-N1 vector (BD Biosciences, Palo Alto, CA, USA) to produce proteins tagged with EYFP at those C-terminals. The constructed vectors were transfected into COS-7 cells using FuGene 6 transfection reagent (Roche Diagnostics). The EYFP signals were observed using the confocal laser scanning microscope, LSM5 PASCAL (Carl Zeiss, Göttingen, Germany) 48 h after transfection.

Neurosphere assay

Details of the procedures are described elsewhere (Ben-Hur *et al.* 1998). To generate neurospheres, 1×10^5 viable cells derived from forebrains of E14 mouse embryos were plated in a 35 mm diameter culture dish in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (FGF2). To estimate the potential for generating neurospheres, the number of generated neurospheres was counted as follows. Viable cells (2.5×10^4 cells per well) were cultured in a 24-well culture dish in 0.5 mL of the hemisolid culture medium. The hemi-solid culture medium contained EGF and 1.5% methylcellulose gel matrix (Methocel A4M premium grade, gift from Dow Chemical Japan Tokyo, Japan; Gritti *et al.* 1999).

Histological examination

For histological examination, whole embryos or whole brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Frontal sections were cut at 14 μm with a cryostat. For immunohistochemical examination, endogenous peroxidases were inactivated in advance by 30 min incubation in 0.01 M phosphate-buffered saline (PBS, pH 7.4) with 0.3% hydrogen peroxidase, followed by three rinses of 5 min each in 0.01 M PBS. Sections were incubated with 0.01 M PBS containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), 1% normal goat serum (Sigma-Aldrich) and 0.25% Triton X-100 for 30 min. Sections were incubated overnight at 4°C in rabbit anti-dopamine beta-hydroxylase (DBH) antisera (DiaSorin, Stillwater, MN, USA) at 1 : 2000. Primary antisera were diluted in 0.01 M PBS containing 1% bovine serum albumin, 1% normal goat serum and 0.25% Triton X-100. The sections were rinsed three times for 5 min in 0.01 M PBS, and then incubated in biotinylated goat anti-rabbit IgG antibody (Vector laboratories, Burlingame, CA, USA) for 30 min at room temperature. The sections were rinsed again three times and were incubated in avidin-biotin complex for 30 min at room temperature using Vectastain Elite ABC kit (Vector laboratories). After rinsing, immunoreactivities were visualized with diaminobenzidine (DAB) using Histofine Simple Stain DAB solution (Nichirei, Tokyo, Japan).

Bromodeoxyuridine (BrdU) labeling

To observe the dividing cells, cells were labeled with BrdU using the cell proliferation kit (Amersham Biosciences). Pregnant mice were injected intraperitoneally with the labeling reagent, which contains 5-bromo-2′-deoxyuridine and 5-fluoro-2′-deoxyuridine. Two hours after injection, embryos were taken out of their dams. They were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and conventional paraffin embedding procedures were used. Sections were then cut at 6 μm and immunohistochemical visualization of BrdU was carried out according to the manufacturer's protocol.

Auditory stimuli

To investigate susceptibility to seizures triggered by sound, mice were exposed consecutively four times to 11 kHz sound at an intensity of 105 dB for 20 s, with a 2 s interval between each exposure. Mice were not subjected to more than one series of auditory stimulation.

Electroencephalography

Between P22 and P27, silver bead electrodes were implanted chronically onto the surface of the cortex under anesthesia with sodium pentobarbital. After recovery from the surgery (usually 3 days later), electroencephalogram (EEG) recordings were performed while the animals were subjected to the auditory stimuli as described above.

Statistical analysis

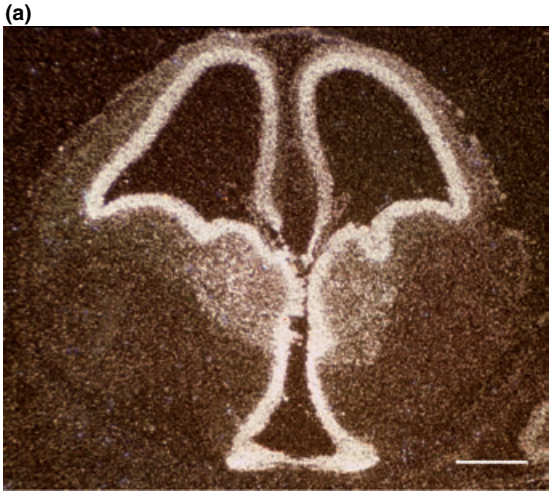
To analyze the body weight distribution of mutated mice and data from the neurosphere assays, we used Student's *t*-test and Welch's *t*-test.

Results

A novel gene, which is expressed predominantly in the ventricular zone of the rat developing central nervous system, was identified

We identified a DNA fragment, tentatively designated rat-*Neurepin* (*neuroepithelium-notable*), through our search for novel genes that are expressed transiently in the ventricular zone of the developing rat cortex. Our differential display screen revealed that rat-*Neurepin* mRNA was rich in the forebrain at E11, but poor at E18 (data not shown). Its limited expression in the ventricular zone (neuroepithelium) of the developing nervous system was confirmed by a subsequent screen carried out using *in situ* hybridization (Fig. 1a).

(a)



(b)

MSVTSEPGMISSFLLVYLSTLFISFVFGEAEIRFTGQTEFFVNETSTTVIRLVIERIGEPANVTAIVSLSGEDTGDFFDT
YAAAFIPARGTNRTVYIAVCDDDLPEPDETFTFHLTLQKPSANVKLGWPRAASVTILSNDNAFGIISFSTPSSISVIEPR
SRNASVPLTLIREKGTYGMVTVTFDVSGGPNPPEEDLNPVRGNITFPPGRATVIYNVTVLDDEVPENDELFLIQLRSVEG
GAEINASXSSVEIIVKKNDSPVNFMQSVYVVPEDDHVLTIPVLRGKDSDGNLIGSDETQVSIRYKVMTWDSTAHAQQNVD
FIDLQPDTTLVFPPFVHESHLKFQIIDDLIPEIAESFHIXLLKNTLQGDAVLMGPSTVQVTIKPNDKPYGVLSFNSILFE
RPVIIDEDTASSSRFEEIAVVRNGGTHGNVSVSWVLTRNSSDPSPVTADITPASGTLQFAQGQMLAPISLVVFDDDLPEE
AEAYLLTILPHTIQGGAEVSEPAQLLFYIQDSDNVYGEISFFPGESQKIESSPSERSLSLSLARRGGSKGDVRVIYSALY
IPAGAMDPLRAKDGILNTSRRSSLLFPEQNQQVSIKLPIRNDAFLQNGAHFLVQLEAVVLVNIFPPIPPVSPRFGEIRNI
SLLVTPAIANGEIGFLSNLPIILHEPKDSSAEVVSIPLHRDGTDGQATVYWSLRPSGFNSKAVTLDDAGPFNGSVVFLSG
QNETSINITVKGDDIPELNETVTLSLDRVSVDSDVLKSGYTSRDLIILENDDPGGIFEFSYDSRGPYVIKEGDAVELRIT
RSRGSLVKQFLRFHVEPRESNEFYGNMGVLEFTPGEREVVITLLTRLDGTPELDEHFWVILSSHGERESKLGCATLVNIT
ILKNDYPHGIIEFVSDGLSASIKESKGEDIYHAVYGVIRTRGNFGAVNVSWMVSPDFTQDVFPVQGTVCFGDQEFFKNIT
VYTLVDEIPEEMEEFTIILLNATGGAQTGIRTTASLRILRNDDPVYFAEPCVLRVQEGETANFTVLRNGSVDGACTVQYA
TVDGKASGEEGDFAPVEKGETLVFEVGSREQSISVHVKDDGIPETDEPFYIVLFNSTGDTVVYEYGVATVIIEANDDPNG
VFSLEPIDKAVEEGKTNAFWILRHRGHFGNVSVAWQLFQNASLQPGQEFYETSGTVNFTDGEETKPVILRAFPDRIPEFN
EFYILRLVNISGTNLSHL

(c)

MSVTSEPGMISSFLLVYLSTLFISFVFGEAEIRFTGQTEFFVNETSTTVIRLVIERIGEPANVTAIVSLSGEDTGDFFDT
YAAAFIPARGTNRTVYIAVCDDDLPEPDETFTFHLTLQKPSANVKLGWPRAASVTILSNDNAFGIISFSTPSSISVIEPR
SRNASVPLTLIREKGTYGMVTVTFDVSGGPNPPEEDLNPVRGNITFPPGRATVIYNVTVLDDEVPENDELFLIQLRSVEG
GAEINASRSSVEIIVKKNDSPVNFMQSVYVVPEDDHVLTIPVLRGKDSDGNLIGSDETQVSIRYKVMTWDSTAHAQQNVD
FIDLQPDTTLVFPPFVHESHLKFQIIDDLIPEIAESFHIMLLKNTLQGDAVLMGPSTVQVTIKPNDKPYGVLSFNSILFE
RPVIIDEDTASSSRFEEIAVVRNGGTHGNVSVSWVLTRNSSDPSPVTADITPASGTLQFAQGQMLAPISLVVFDDDLPEE
AEAYLLTILPHTIQGGAEVSEPAQLLFYIQDSDNVYGEISFFPGESQKIESSPSERSLSLSLARRGGSKGDVRVIYSALY
IPAGAMDPLRAKDGILNTSRRSSLLFPEQNQQVSIKLPIRNDAFLQNGAHFLVQLEAVVLVNIFPPIPPVSPRFGEIRNI
SLLVTPAIANGEIGFLSNLPIILHEPKDSSAEVVSIPLHRDGTDGQATVYWSLRPSGFNSKAVTLDDAGPFNGSVVFLSG
QNETSINITVKGDDIPELNETVTLSLDRVSVDSDVLKSGYTSRDLIILENDDPGGIFEFSYDSRGPYVIKEGDAVELRIT
RSRGSLVKQFLRFHVEPRESNEFYGNMGVLEFTPGEREVVITLLTRLDGTPELDEHFWVILSSHGERESKLGCATLVNIT
ILKNDYPHGIIEFVSDGLSASIKESKGEDIYHAVYGVIRTRGNFGAVNVSWMVSPDFTQDVFPVQGTVCFGDQEFFKNIT
VYTLVDEIPEEMEEFTIILLNATGGAQTGIRTTASLRILRNDDPVYFAEPCVLRVQEGETANFTVLRNGSVDGACTVQYA
TVDGKASGEEGDFAPVEKGETLVFEVGSREQSISVHVKDDGIPETDEPFYIVLFNSTGDTVVYEYGVATVIIEANDDPNG
VFSLEPIDKAVEEGKTNAFWILRHRGHFGNVSVAWQLFQNASLQPGQEFYETSGTVNFTDGEETKPVILRAFPDRIPEFN
EFYILRLVNISGTNLSHL

Fig. 1 *Neurepin1* (*Vlgr1d*) and *Neurepin2* (*Vlgr1e*), which were expressed notably in the neuroepithelium of the developing rodent brain, are identified. (a) A fragment of *Neurepin*, obtained by our differential display, revealed the limited expression of its mRNA in the developing rat ventricular zone (neuroepithelium) of the central nervous system. Coronal section of the Wistar rat embryonic day 13 (E13) brain. *In situ* hybridization. (b) and (c) Amino acid sequences of mouse Neurepin1 (Vlgr1d) (b) and Neurepin2 (Vlgr1e) (c). Predicted Calx-β domains are underlined. Scale bar in (a) = 100 μm.

Mouse *Neurepin1* and *Neurepin2* genes were cloned

By screening a mouse cDNA library, two apparent full-length mouse orthologues of the rat-*Neurepin* gene were identified. These two cDNA had polyadenylation on their 3′ end after the putative polyadenylation signals (GTAATAAAAA for *Neurepin1* and TACTTAAATA for *Neurepin2*). These two genes were named *Neurepin1* (2642 bp) and *Neurepin2* (3839 bp). The first 1952 nucleotides of these two genes are identical, but the remaining composition is different, indicating that these two genes are alternatively-spliced variants. The predicted translated products of these two genes comprised 616 and 1218 amino acid residues, respectively (Figs 1b and c). Our domain/motif search revealed that *Neurepin1* has three Calx-β domains and *Neurepin2* has five Calx-β domains; the Calx-β domain has been reported to bind calcium ions in the Na^+/Ca^{2+} exchanger (Schwarz and Benzer 1997).

Neurepins and *Vlgr1b* are alternatively spliced variants

The expression of *Neurepin* mRNAs in the developing mouse brain was examined by northern blot analyses. Four obvious bands were observed with the *Neurepin* probe (see Materials and methods) and these four bands decreased in intensity compared with *GAPDH* as the development advanced. The intensity of the largest sized band at lane E16, approximately 20 kb in size, decreased to 30% of that at lane E12, and those of the other three bands (approximate 10 kb, 2.7 kb and 1.7 kb) at lane E16 decreased to about 15% of those at lane E12. These four bands implied that at least four different alternatively-spliced variants or homologous genes are expressed in the developing mouse brain (Fig. 2a).

Although no such genes had been found in our earlier searches, two mouse genes partially harboring the identical nucleotide sequence of *Neurepin1* were identified in the DNA database. One was the very large G protein-coupled receptor-1b (*Vlgr1b*) and another was the monogenic audiogenic seizure-susceptible (*Mass1*) gene (Skradski *et al*. 2001; McMillan *et al*. 2002). Mouse *Vlgr1b* (*Vlgr1b*) is 19329 bp in size, while murine *Mass1.1* contains 9439 nucleotides (Skradski *et al*. 2001). McMillan *et al*. have suggested that *Mass1.1* is the alternatively-spliced variant of *Vlgr1b*, which is derived from the same locus, mass1 (McMillan and White 2004; see DNAdatabase enquiries in the Materials and methods section).

It was suspected that the largest sized band detected in our previous northern blot analyses with the *Neurepin* probe, which was approximately 20 kb in size, was *Vlgr1b*. To confirm this, we performed northern blot analyses using a *Vlgr1b*-specific probe (see Materials and methods) and detected an approximately 20 kb-sized band, the same size as that detected by the *Neurepin*-probe (Fig. 2b). Furthermore, a band of 2.7 kb, corresponding to *Neurepin1*, was clearly identified with the *Neurepin*-probe. In addition to the four bands shown in Figs 2a and 2b, three more weak bands were visualized after a long exposure amongst which a band of 3.8 kb, the actual size of *Neurepin2*, was included.

Moreover, it was likely that *Vlgr1b* and our newly identified *Neurepin1* and *Neurepin2* were transcribed from the same locus but were differentially spliced, since 3747 bp of 5′-*Neurepin2* cDNA was identified to the 5′ region of *Vlgr1b*, and only 92 bp in the 3′ terminal was different from *Vlgr1b*. To confirm this, a partial sequence of the human *Neurepin* orthologue was obtained and then a human radiation hybrid mapping study was carried out (see Materials and methods). Later, enquiries into the human and mouse

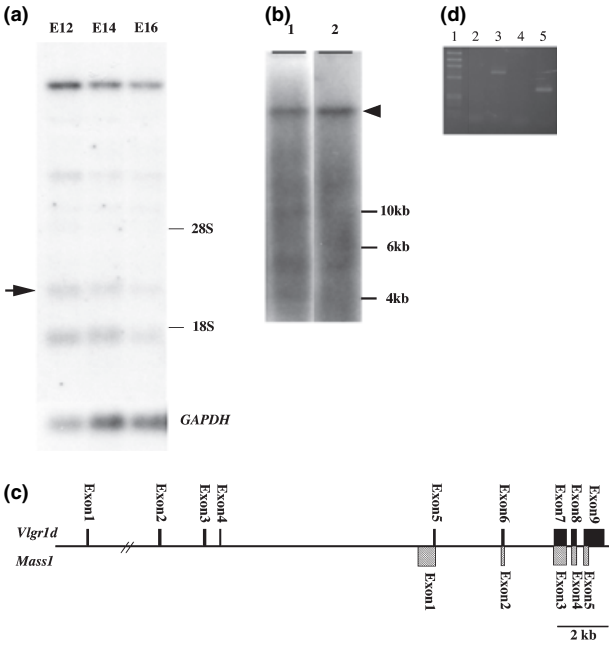


Fig. 2 mRNA profiles of *Vlgr1d* (*Neurepin1*), *Vlgr1e* (*Neurepin2*) and their related genes. (a) mRNA expression of *Vlgr1d* and their alternatively-spliced variants was shown by northern blot analysis using the *Neurepin*-probe. In each lane of E12, E14 and E16, 2 μg of the poly(A)+ RNA extracted from ICR mice embryonic brain at E12, E14 and E16, respectively, were separated on 1% agarose-formaldehyde gel. Rehybridization with the *GAPDH* probe is shown at the bottom. The arrow indicates the bands' apparent V*lgr1d*. The positions of 28S and 18S ribosomal RNA (rRNA) are indicated. (b) *Vlgr1b* was detected using northern blot analysis. In each lane, 6 μg of the poly(A)+ RNA extracted from E14 ICR mouse embryos were separated on 0.9% agarose-formaldehyde gel. Lane 1 was hybridized with the *Neurepin*-probe and lane 2 was rehybridized with the *Vlgr1b*-specific probe after stripping the radiolabeled *Neurepin*-probe from the membrane. The arrowhead indicates the bands that are applicable to Vlgr1b. Molecular size markers (10 kb, 6 kb, 4 kb) were run simultaneously and their positions are indicated. (c) Genomic structure of *Vlgr1d* (exons 1–9) compared with *Mass1.1* (exons 1–5). The distance from exon 1 to exon 2 of *Neurepin1* was approximately 36 kb. (d) Expression levels of *Mass1.1* were lower than those of *Vlgr1* in E14 mouse brains. Lane 1: DNA marker PhiX174 *Hae*III digest; lane 2: *Mass1.1* 606–1175; lane 3: *Vlgr1* 306–1031; lane 4: *Mass1.1* 8797–9168; lane 5: *Vlgr1* 8653–9033.

genomic DNA database were also made. The results of these searches indicated that the *Neurepin* gene is located on the mass1 locus of human chromosome 5 and mouse chromosome 13. Consequently, the *Neurepins* are new alternatively-spliced variants that are derived from the same gene from which *Vlgr1b* is transcribed. We then re-named *Neurepin1* as *Vlgr1d* and *Neurepin2* as *Vlgr1e*.

Semi-quantitative analyses of Vlgr1 variants expression

Although the nucleotide sequences of the *Vlgr1d*, *Vlgr1e* and *Vlgr1b* are identical in their 5′-terminal, the first 710 bp of *Vlgr1* (*Vlgr1b*, *Vlgr1d*, *Vlgr1e*) differ from that of *Mass1.1*. Enquiries into the genomic DNA database revealed that the first exon of *Mass1.1* is 820 bp in size, and this consists of a partial fragment of the fourth intron and the whole exon 5 of the *Vlgr1* (Fig. 2c). We compared the expressions of *Mass1.1* and the *Vlgr1* by RT-PCR using sets of paired primers that were designed to amplify exons 1–3 of *Mass1.1* and exons 2–7 of *Vlgr1* (exon 3 of *Mass1.1* and exon 7 of *Vlgr1* are identical). The intensity of the *Mass1.1* mRNA band was much lower than that of *Vlgr1* (Fig. 2d). This was confirmed by real-time PCR using a set of primers designed by Primer Express. The expression of *Mass1.1* was estimated at less than 1/100 of that of *Vlgr1* in amount.

Subcellular localization of Vlgr1d and Vlgr1e

Vlgr1b and Mass1.1 are alternatively-spliced variants. The putative translated product of Vlgr1b comprises 6298 amino acid residues and that of Mass1.1, 2780 amino acid residues. Because the N-terminal region of Vlgr1 is different from Mass1.1, their subcellular localizations may not be the same. To examine this, the N-terminal regions of Vlgr1 and Mass1.1, tagged with EYFP at their C-terminal ends, were overexpressed in COS-7 cells and their subcellular distribution was studied. The N-terminal of Mass1.1 of 437 amino acid residues corresponding to 179–615 amino acid residues of Vlgr1, tagged with EYFP, was distributed ubiquitously in a cell, while Vlgr1 of 615 amino acid residues corresponding to 1–615 amino acid residues of Vlgr1, tagged with EYFP, was confined to the endoplasmic reticulum and the Golgi apparatus (Fig. 3). Therefore, Vlgr1 is secreted and/or is a membrane-bound protein. Moreover, since no membrane-spanning region is found in Vlgr1d or Vlgr1e, it is likely that they are secreted from a cell, while Vlgr1b is a receptor (McMillan *et al*. 2002).

Generation of *Vlgr1* knockout mice

To disrupt *Vlgr1*, a targeting vector was constructed to replace exons 2–4 of *Vlgr1* with the neomycin-resistant gene (*Neo*)

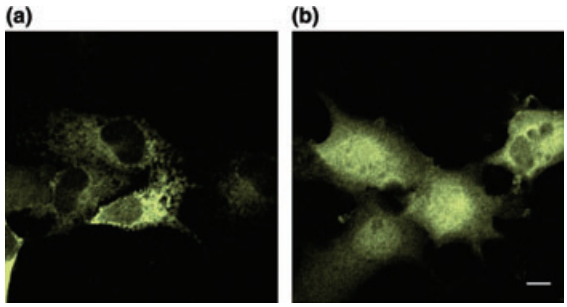


Fig. 3 The N-terminal region of Vlgr1 was critical to their subcellular distribution. (a) Vlgr1 (1–615 amino acid residues) tagged with EYFP was expressed in COS-7 cells. EYFP was confined to the endoplasmic reticulum and the Golgi apparatus. (b) The N-terminal region of Mass1.1 (this is identical to N-terminal deleted Vlgr1b, 179–615 amino acid residues of Vlgr1b) tagged with EYFP was expressed in COS-7 cells. EYFP was distributed ubiquitously in COS-7 cells. Scale bar = 10 μm.

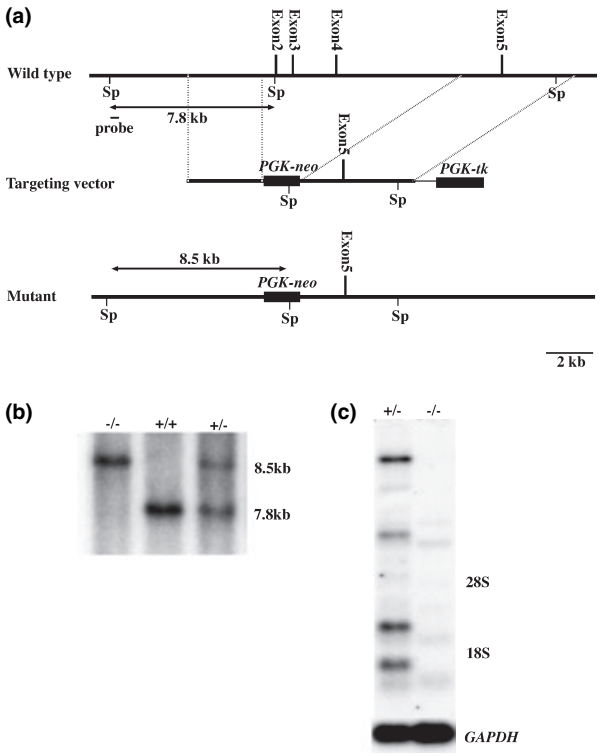


Fig. 4 Targeting strategy of *Vlgr1* and confirmation of targeted disruptions in *Vlgr1* knockout mice. (a) Maps of the targeting vector, wild-type allele and mutant allele of *Vlgr1* knockout mice. Sp indicates the restriction enzyme site of *Spe*I. (b) Genomic DNA extracted from the tail tips of *Vlgr1* knockout mice and littermates was digested with *Spe*I and hybridized with the probe shown in Fig. 3(a). Indicated bands correspond to the wild-type (7.8 kb) and mutant (8.5 kb) gene. +/+, wild-type mouse; +/-, *Vlgr1* heterozygous knockout mouse; -/-, *Vlgr1* homozygous knockout mouse. (c) Northern blot analyses. A 5 μg aliquot of the poly(A)+ RNA extracted from *Vlgr1* heterozygous knockout (+/- lane) and *Vlgr1* homozygous knockout (-/- lane) mouse brains was separated in 1% agarose-formaldehyde gel. Rehybridization with the *GAPDH* probe is shown at the bottom of the figure. The positions of 28S and 18S rRNA are indicated.

cassette (Fig. 4a). After intercrossing of heterozygous mice ($Vlgr1^{+/-}$ mice), the genotypes of their litters were examined at 4 weeks of age by Southern blot analyses (Fig. 4b); homozygous mice ($Vlgr1^{-/-}$ mice) were then bred. Once fully grown, the $Vlgr1^{-/-}$ mice could not be distinguished from their normal littermates in terms of appearance. There was no apparent difference in their body weight (Student's *t*-test, $p > 0.05$); the average body weight of P28 $Vlgr1^{-/-}$ mice was 12.9 ± 4.7 g (mean ± SD, $n = 10$) for males and 13.2 ± 2.6 g ($n = 12$) for females, while that of their normal littermates was 15.2 ± 1.9 g ($n = 5$) and 13.3 ± 2.0 g ($n = 11$), respectively. After the intercrossing of homozygous mice, the litters were born and their mothers suckled their litters as normal.

The disruption of *Vlgr1* mRNA was confirmed by northern blot analyses with the *Neurepin*-probe. While the largest sized band of apparent *Vlgr1b* could not be clearly seen in the lane representing the homozygous mice, other bands, apparently derived from the wild-type in the lane representing the heterozygous mice, were noticeably shorter in size in the lane representing the homozygous mice (Fig. 4c). We also checked the disruption of *Vlgr1* mRNA by RT-PCR using the primers spanning exons 1 and 7 of *Vlgr1*. The PCR product of the homozygous animals showed that the *Vlgr1* knockout mice lacked exons 2–4 of *Vlgr1*; exon 1 was ligated to exon 5 (data not shown). Although the initiation codon of *Vlgr1* is located in the first exon, the stop codon for translation is induced by frame shifting in the resultant truncated mRNA, which is transcribed from directly connected exon 1–exon 5, resulting in the generation of a 14 amino acid peptide, if any.

No obvious histological abnormalities were observed in the embryonic brains of the *Vlgr1* knockout mice

As the expression of *Vlgr1* was observed in the neuroepithelium (ventricular zone), *Vlgr1* knockout mice were studied to establish whether there were any morphological abnormalities in the central nervous system of these animals during development. At E14 and E16, no obvious morphological abnormalities were detected in the developing brains of *Vlgr1* knockout mice by thionine staining. Since no apparent morphological abnormalities were observed in the ventricular zone where *Vlgr1* mRNA is located, the location of proliferative cells was studied further. Two hours before they were killed, a solution of BrdU was injected into pregnant mice at 14 days post-coitus. The distribution of BrdU-positive cells was then studied. In the cortex, no significant difference was observed in BrdU-positive cells, in terms of their number and location, between the *Vlgr1* knockout mice and their wild-type littermates (Figs 5a–f).

No obvious difference was detected by the neurosphere assay

Since there were no apparent histological abnormalities in the developing brain of $Vlgr1^{-/-}$ mice, we checked the ability of neuroepithelial cells to generate neurospheres *in vitro*. The neurosphere assay was performed at E14. The average number of total cells derived from the forebrain of each embryo was as follows: 3.1×10^6 ($Vlgr1^{-/-}$ mice), 3.2×10^6 ($Vlgr1^{+/-}$ mice) and 3.1×10^6 ($Vlgr1^{+/+}$ mice). No statistical differences were recognized in terms of the average number of cells among $Vlgr1^{-/-}$, $Vlgr1^{+/-}$ and the $Vlgr1^{+/+}$ mice. The neurosphere assay was then performed using these forebrain cells in the presence of the growth factors EGF and FGF2. The number of neurospheres was counted using the hemi-solid medium. No significant differences were observed among $Vlgr1^{-/-}$, $Vlgr1^{+/-}$ or their wild-type littermates.

Mutated *Vlgr1* ($Vlgr1^{-/-}$) mice were highly susceptible to audiogenic seizure

Since it has been reported that the mutation of *Mass1.1*, which was one of the alternatively-spliced variants of the

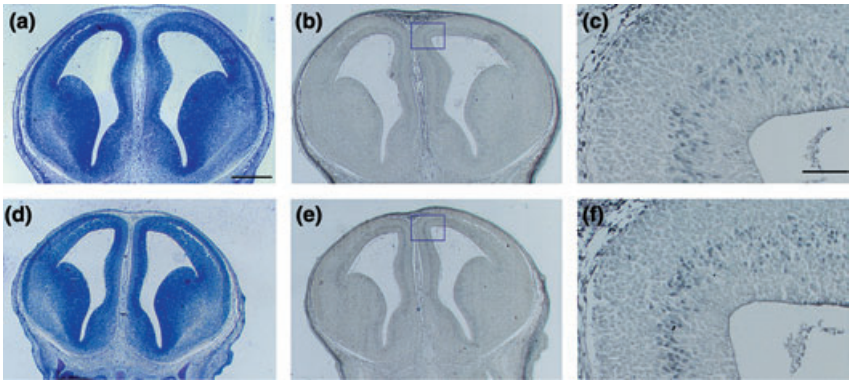


Fig. 5 No apparent abnormalities in proliferating cells were detected by BrdU labeling in E14 mice. (a, b and c) Wild-type: (d, e and f) *Vlgr1* homozygous knockout mouse. (a, d) Sections were stained with thionine for conventional histological examination. (b, e) BrdU-labeled cells were observed in serial sections. (c, f) High-magnification views of the boxes shown in (b) and (e). Scale bar = 0.5 mm (a, b, d, e), 50 μm (c, f).

Vlgr1, causes audiogenic seizure susceptibility in mice (Skradski *et al*. 2001), the *Vlgr1*$^{-/-}$ mice were studied to establish whether or not they exhibit susceptibility to audiogenic seizures. Audiogenic seizures consist of three phases: the preconvulsive phase, the convulsive phase and the postconvulsive phase (Frings *et al*. 1952). Typically, mice show wild running (which was easily judged by simply examining their movement) when they are in the preconvulsive phase. From P21 to P27, 95% of *Vlgr1*$^{-/-}$ mice exhibited wild running and 68% exhibited convulsions during the first 20 s of stimulation (Table 1). Numbers of mice that showed wild running and convulsions during a series of exposures to auditory stimuli, consisting of four consecutive exposures of 20 s with 2 s intervals between, were also counted (Table 1). Twenty-six percent of *Vlgr1*$^{-/-}$ mice died following tonic convulsion during the examination. On the other hand, approximately 10% of the wild-type and heterozygous littermates exhibited wild running. With the same experimental design, we tested susceptibility to audiogenic seizures of DBA/2J mice. At postnatal 3 weeks, all of the 17 tested DBA/2J mice exhibited wild running and convulsion during the first 20 s stimulation, and 15 out of 17 tested mice died following tonic convulsion. In contrast, auditory stimuli did not induce any convulsions in 8-week-old DBA/2J mice. In the case of *Vlgr1*$^{-/-}$ mice, three out of eight tested mice showed wild running and one out of three showed convulsion, even at 6 months after birth.

Table 1 Vlgr1$^{-/-}$ mice show high susceptibility to audiogenic seizures

	Total number of tested mice	Mice exhibiting components of audiogenic seizure	
		Wild running	Convulsion
Wild-type	16	**1**	**0**
		1	*0*
Heterozygous	18	**2**	**0**
		2	*0*
Homozygous	19	**18**	**13 (5)**
		18	*14 (6)*

Numbers of mice that showed wild running or convulsions by the first 20 s of auditory stimulation are indicated by boldface. Italicized figures indicate the numbers of mice that showed wild running or convulsions during a series of four consecutive auditory stimuli. Figures in parenthesis indicate the number of dead mice after tonic convulsion.

We examined the cortical EEG of *Vlgr1*$^{-/-}$ mice during auditory stimuli and found that it was suppressed during the convulsive phase; there was a reduction in the amplitude of the trace and no obvious spikes were detected (Fig. 6).

The genetic background of the *Vlgr1*$^{-/-}$ mice is C57BL/6J and 129/Ola hybrid. To exclude the possibility that susceptibility to audiogenic seizure was influenced by this genetic background, the *Vlgr1* knockout mice were backcrossed to C57BL/6J mice for at least six generations. It has been reported that the strain of mouse influences the susceptibility to audiogenic seizures, and the C57BL/6J strain is known to be resistant to audiogenic seizures (Fuller and Sjursen 1967). We examined susceptibility to audiogenic seizures of this *Vlgr1* knockout mouse which had a genetic background of C57BL/6J. After being exposed to auditory stimuli from P21 to P27, all 14 C57BL/6J-backcrossed *Vlgr1*$^{-/-}$ mice exhibited wild running, seven exhibited convulsions, and four out of the seven mice died after the tonic convulsions (Table 2). Particularly from P21 to P23, all seven C57BL/6J-backcrossed *Vlgr1*$^{-/-}$ mice exhibited wild running and convulsions.

No obvious histological abnormalities were observed in the brainstem of *Vlgr1*$^{-/-}$ mice

Since the *Vlgr1*$^{-/-}$ mice exhibited audiogenic seizures of which cortical EEG was suppressed during the convulsive phase, morphological examination was performed on the central nervous system, especially on the brainstem, in *Vlgr1*$^{-/-}$ mice of 3 weeks old and older. We could not detect any morphological abnormalities by thionine staining (Figs 7a and b). As it has been reported that norepinephrine-deficient mice have increased susceptibility to audio-

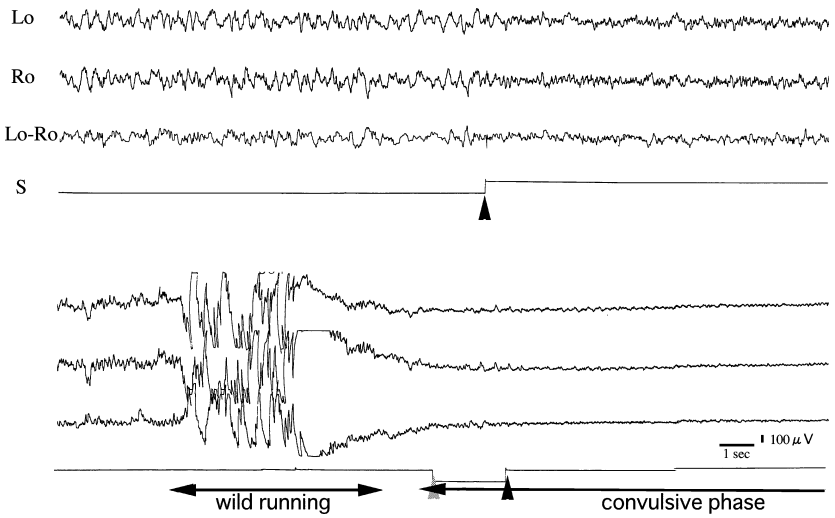


Fig. 6 The cortical EEG was suppressed during the convulsive phase induced by auditory stimuli. Before exposure to the auditory stimuli, the EEG of *Vlgr1*$^{-/-}$ mice was identical to the normal cortical EEG traces of the quiet state. Just after being exposed to the stimulus, the mouse was aroused. The EEG indicated that the mouse was in the arrest state. At 2 s before the onset of wild running, the mouse began to walk and theta waves were observed in the traces of EEG. During wild running, the EEG was obscured by movement artefacts. The electrodes were positioned at the left occipital cortex (2.5 mm lateral to the sagittal suture and 1 mm anterior to the lambdoid suture), the right occipital cortex (1 mm lateral to the sagittal suture and 1 mm anterior to the lambdoid suture) and the left olfactory bulb (reference electrode, 0.5 mm anterior to Bregma and 0.5 mm lateral to the sagittal suture). Lo, left occipital cortex; Ro, right occipital cortex; Lo-Ro, left and right occipital cortex bipolar recording; S, indicator of the acoustic stimulus. Black arrowheads represent the onset of the acoustic stimulus and the grey arrowhead represents the offset of the acoustic stimulus.

Table 2 The C57BL/6J-backcrossed *Vlgr1* knockout mice show high susceptibility to audiogenic seizures

	Total number of tested mice	Mice exhibiting components of audiogenic seizure	
		Wild running	Convulsion
Wild-type	8	**0**	**0**
		0	*0*
Heterozygous	28	**0**	**0**
		0	*0*
Homozygous	14	**14**	**7 (4)**
		14	*10* (*7*)

Numbers of mice that showed wild running or convulsions by the first 20 s of auditory stimulation are indicated by boldface. Italicized figures indicate the numbers of mice that showed wild running or convulsions during a series of four consecutive auditory stimuli. Figures in parenthesis indicate the number of dead mice after tonic convulsion.

genic seizures (Szot *et al*. 1999), we also checked the noradrenergic neurons, using anti-DBH antisera, to determine whether they are normally developed or not. The DBH-positive neurons were detected in the locus coeruleus of *Vlgr1*$^{-/-}$ mice, and no difference was observed in terms of their number and location compared with those of the wild and heterozygous littermates (Figs 7c and d).

Discussion

In the study presented here, we identified two novel alternatively-spliced variants of *Vlgr1b*, *Vlgr1d* and *Vlgr1e* which are distinct from any other alternatively-spliced variants of *Vlgr1b* and *Mass1.1*. To date, McMillan *et al*. have identified three alternatively-spliced variants (*Vlgr1*-variants) that are transcribed from the vlgr1 locus in humans: *Vlgr1a*, *Vlgr1b* and *Vlgr1c* (McMillan *et al*. 2002). In parallel, Skradski *et al*. carried out a genetic mapping study and disclosed that the mass1 locus is responsible for susceptibility to audiogenic seizures in the *Frings* mouse, and that three alternatively-spliced variants, *Mass1.1*, *Mass1.2* and *Mass1.3* (*Mass1*-variants), are transcribed from the mass1 locus in mice (Skradski *et al*. 1998, 2001). Although the nucleotide sequence of *Vlgr1b* is longer and contains additional putative motifs, the nucleotide sequences of *Vlgr1b* and *Mass1.1* are almost identical and their loci are apparently the same; they are differently transcribed siblings (Nikkila *et al*. 2000; McMillan *et al*. 2002). This apparent common locus of *Mus musculus* is found as Mass1 in the NCBI database (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid = 10090). Therefore, *Vlgr1d* and *Vlgr1e* are derived from the same mass1 locus, in accordance with our sequence comparison, radiation hybrid mapping and genome database enquires.

We disclosed the expression profiles of variants of *Vlgr1/Mass1* in the developing brain by northern blot analyses with

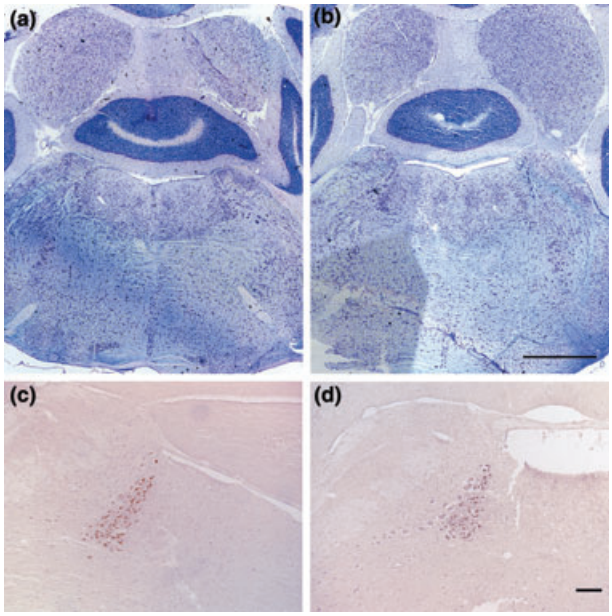


Fig. 7 Histological examinations of the brainstem. (a, b) *Vlgr1*$^{+/-}$ (a) and *Vlgr1*$^{-/-}$ mouse (b) at 4 months old. Sections were stained with thionine. Scale bar = 1 mm. (c, d) The norepinephrinergic neurons in the locus coeruleus were visualized by anti-DBH antisera. Sections were derived from a *Vlgr1*$^{+/-}$ (c) and *Vlgr1*$^{-/-}$ mouse (d) at 30 days old. Scale bar = 100 μm.

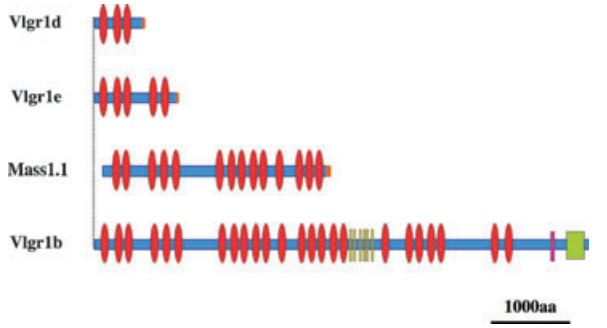


Fig. 8 Domain structures of the Vlgr1b, Vlgr1d, Vlgr1e and Mass1.1. The blue bar indicates the identical amino acid sequences. Red oval, Calx-β domain; yellow bar, EPTP repeats; green box, 7-transmembrane domain; purple box, G protein-coupled receptor proteolytic site (McMillan *et al.* 2002).

the *Neurepin*-probe that binds to *Vlgr1b*, *Vlgr1c*, *Vlgr1d*, *Vlgr1e* and *Mass1.1*. Strong expression of *Vlgr1b* and *Vlgr1d* was confirmed. Strong expression of *Vlgr1b*, approximately 20 kb in size, was also perceived with the *Vlgr1b*-specific probe, which was designed to recognize *Vlgr1a* and *Vlgr1b* although no obvious *Vlgr1a* expression was detected. This result is consistent with the report indicating that *Vlgr1a* is not expressed in the mouse (McMillan *et al.* 2002). We further measured *Mass1.1* expression quantitatively using real-time PCR and found that *Mass1.1* mRNA is expressed very faintly compared with *Vlgr1b*, *Vlgr1d* and *Vlgr1e*. This is consistent with the results of McMillan and White (2004). They have reported that the amount of Mass1 is lower than that of Vlgr1b using semi-quantitive RT-PCR and *in situ* hybridization. Two more bands were observed in our northern blot analyses with the *Neurepin*-probe, in addition to *Vlgr1b* and *Vlgr1d*. One was approximately 10 kb in size and another was approximately 1.7 kb. As the intensities of these two bands decreased during development as did those of *Vlgr1b* and *Vlgr1d*, these two bands of 10 kb and 1.7 kb were considered to be additional alternatively-spliced variants of *Vlgr1b*.

The domain database indicated that there are three Calx-β domains in Vlgr1d and five in Vlgr1e (Fig. 8). The Calx-β domain is the Ca^{2+}-binding domain of the Na^{+}/Ca^{2+} exchanger molecule (Schwarz and Benzer 1997). It has been reported that the Calx-β domain is present in an extracellular region of some adhesion molecules, such as the sponge and sea urchin proteins, MAFp3/MAFp4 and ECM3 (Fernàndez-Busquets and Burger 1997; Fernàndez-Busquets *et al.* 1998; Hodor *et al.* 2000). These molecules are Ca^{2+}-dependent aggregating molecules, and it is suggested that the Calx-β domain plays a role in cell aggregation (Fernàndez-Busquets and Burger 1997; Hodor *et al.* 2000). Since Vlgr1b, Vlgr1d and Vlgr1e have identical N-terminals in which a putative signal peptide for secretion is located (McMillan *et al.* 2002), and only Vlgr1b has a putative membrane-spanning region, it is likely that Vlgr1d and Vlgr1e are released from cells while Vlgr1b functions as a receptor. These results suggest that Vlgr1d and Vlgr1e are extracellular proteins, and that their function is related to extracellular Ca^{2+} ion distribution with Calx-β domains.

In our *Vlgr1*$^{-/-}$ mice, we deleted exons 2–4 of *Vlgr1*, leaving intact the initiation codon of *Mass1.1* located at exon 5 of *Vlgr1*. It is therefore probable that the expression of *Mass1* variants was preserved even in the *Vlgr1*$^{-/-}$ mice. In addition, the expression of truncated mRNA, which was spliced from exon 1 and skipped to exon 5 of *Vlgr1*, was confirmed in our *Vlgr1*$^{-/-}$ mice. This alternatively-spliced variant was also expressed in the normal littermates (data not shown), but its expression was very faint compared with *Vlgr1*.

Unlike Vlgr1, a putative signal peptide for secretion was lacking in the Mass1.1 protein. In fact, we found the N-terminal of Mass1 (179–615 amino acid residues of Vlgr1b) tagged with EYFP to be distributed ubiquitously in COS-7 cells, while Vlgr1b (1–615 amino acid residues of Vlgr1b) tagged with EYFP was confined to the endoplasmic reticulum and the Golgi apparatus. This suggests that Vlgr1 is a membrane-bound and/or secreted protein, while Mass1.1 is an intracellular protein. Therefore, it is highly likely that no functional Vlgr1 is expressed on the cell surface or is released in our mutated *Vlgr1* mice, since we deleted most of the N-terminal region of Vlgr1 in which the signal peptide is present in our mutated mice.

The *Vlgr1*$^{-/-}$ mice succumbed to seizures induced by loud noise 3 weeks postnatally. We further confirmed that this behavior is typical of audiogenic seizures using EEG. Audiogenic seizures originate in the brainstem, and the cortical EEG is suppressed during the convulsive phase (Maxson and Cowen 1976).

Two bioinformatics groups have identified a new domain, Epitempin (EPTP) repeat (also known as epilepsy-associated repeat), in LGI1/Epitempin and Vlgr1b as an epilepsy-related domain by comparing the amino acid sequences of Epitempin and Vlgr1b (Scheel *et al*. 2002; Staub *et al*. 2002). Kalachikov *et al*. have demonstrated that the mutation of LGI1/Epitempin causes autosomal dominant lateral temporal epilepsy (Kalachikov *et al*. 2002). Although it was not revealed what function the LGI1/Epitempin domain has, loss of this domain was considered to be related to susceptibility to audiogenic seizures. In the *Frings* mice, which are highly susceptible to audiogenic seizures without priming, one base deletion at nucleotide 7009 of *Mass1.1* cDNA (6865 of *Vlgr1b* cDNA) was reported (Skradski *et al*. 2001). It is noteworthy that the point mutation of *Mass1.1* in the *Frings* mouse resulted in truncated Vlgr1b that lacks the EPTP repeat. McMillan and White have reported that knockout mice which lack the putative transmembrane and cytoplasmic segments of Vlgr1 show high susceptibility to audiogenic seizures (McMillan and White 2004). Based on this observation and our results reported here including Yagi *et al*. (2003), the cause of susceptibility to audiogenic seizure is suspected to be loss of the function of Vlgr1b.

Our *Vlgr1*$^{-/-}$ mice, whose genetic background was a C57BL/6J and 129/Ola hybrid, were susceptible to audiogenic seizures 3 weeks postnatally. The incidence of susceptibility to audiogenic seizures depends on the strain of mouse (Fuller and Sjursen 1967), and the 129/J mouse is known to be susceptible to audiogenic seizures at around P21 (Fuller and Sjursen 1967). To exclude the possibility that the genetic background, especially the 129 line, might influence susceptibility to audiogenic seizures, we backcrossed the *Vlgr1* knockout mice with C57BL/6J mice for more than six generations. Although the C57BL/6J strain is highly resistant to sound-induced convulsions (Fuller and Sjursen, 1967), our C57BL/6J-backcrossed *Vlgr1*$^{-/-}$ mice exhibited wild running and/or convulsions induced by auditory stimuli. It should be noted all tested C57BL/6J-backcrossed *Vlgr1*$^{-/-}$ mice showed wild running and convulsions between P21 and P23. Therefore, Vlgr1 is responsible for the susceptibility to audiogenic seizures.

It is of interest that the onset and duration of susceptibility to audiogenic seizures were significantly different among our *Vlgr1*$^{-/-}$ mice and other monogenic audiogenic epilepsy mice. Sound-induced seizures were observed in serotonin 5-HT_{2c} receptor mutant mice in adults over P60 (Brennan *et al*. 1997) and in thyroid hormone receptor β-deficient mice from an early developmental age to the adult (Ng *et al*. 2001), and disturbance of cell-surface-expressed glycosphingolipids resulted in high susceptibility to audiogenic seizures even at 2–4 months old (Kawai *et al*. 2001). It remains to be established why the onset and duration of susceptibility to audiogenic seizures differ. A period of such sensitivity has also been described with respect to human reflex seizures. Further studies on the maturation of neuronal circuit formation of our mutated mice may address these questions. Recently, it has been reported that Vlgr1 mutation is the cause of Usher syndrome type II c, which is a genetically autosomal recessive disorder and of which the phenotype is congenital hearing loss and progressive retinitis pigmentosa (Weston *et al*. 2004). Therefore, it may be possible that the reduction of susceptibility to audiogenic seizure at an older age is due to hearing loss. However, 38% of our *Vlgr1* knockout mice showed wild running even at 6 months old by auditory stimuli.

It has been demonstrated that at least some audiogenic seizures are polygenic. In the DBA/2J strain, three gene loci, located on chromosomes 4, 7 and 12, are linked to susceptibility to audiogenic seizures (Seyfried *et al.* 1980; Neumann and Collins 1991). Our *Vlgr1*$^{-/-}$ mice showed that the monogenic gene mutation on chromosome 13 was a cause of susceptibility to audiogenic seizures. We confirmed the result of the forward genetics, that the mass1 locus is responsible for audiogenic seizure susceptibility (as identified using genetic mapping), by reverse genetics.

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