The Type 1 Inositol 1,4,5-Trisphosphate Receptor Gene Is Altered in the *opisthotonos* Mouse

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The *opisthotonos* (*opt*) mutation arose spontaneously in a C57BL/Ks-db^{2J} colony and is the only known, naturally occurring allele of *opt*. This mutant mouse was first identified based on its ataxic and convulsive phenotype. Genetic and molecular data presented here demonstrate that the type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1) protein, which serves as an IP₃-gated channel to release calcium from intracellular stores, is altered in the *opt* mutant. A genomic deletion in the IP₃R1 gene removes two exons from the IP₃R1 mRNA but does not interrupt the translational reading frame. The altered protein is predicted to have lost several modulatory sites and is present at markedly reduced levels in *opt* homozygotes. Nonetheless, a strong calcium release from intracellular stores can be elicited in cerebellar Purkinje neurons treated with the metabotropic

glutamate receptor (mGluR) agonist quisqualate (QA). QA activates Group I mGluRs linked to GTP-binding proteins that stimulate phospholipase C and subsequent production of the intracellular messenger IP₃, leading to calcium mobilization via the IP₃R1 protein. The calcium response in *opt* homozygotes shows less attenuation to repeated QA application than in control littermates. These data suggest that the convulsions and ataxia observed in *opt* mice may be caused by the physiological dysregulation of a functional IP₃R1 protein.

Key words: *opisthotonos*; seizures; genomic deletion; Purkinje neurons; inositol 1,4,5-trisphosphate receptor; metabotropic glutamate receptor; quisqualate; mouse chromosome 6; alternative splicing

The autosomal recessive *opisthotonos* (*opt*) mutant is a single-gene mouse mutation displaying epileptic-like behaviors. Homozygote *opt* pups are generally smaller than their littermates, and they never acquire normal locomotor abilities. At ~14 d postnatal (P14), *opt* homozygotes begin to display visible seizures. The seizure intensity and duration can range from subtle leg and body tremors lasting 2–3 sec to marked tonic-clonic convulsions in which the pup flips from side to side with a stiffly arched head, tail, and back, with the event lasting up to 30 sec. After a prolonged episode, the pup will lie panting, gripping the bedding or a littermate. The seizure intensity and frequency are progressive (Street et al., 1996), a feature of some human epilepsies. Older pups may demonstrate severe convulsions every 5–10 min. The seizures can occur spontaneously or in response to handling or agitation by a littermate. Although the pups do seem to nurse normally, they die by 3–4 weeks of age. Classical genetic techniques were used to localize *opt* to distal chromosome 6 approximately 6 centimorgans (cM) telomeric

to the mouse mutant *Microphthalmia-white* (*Mi^{wh}*) (Lane, 1972). Although three voltage-gated potassium channel genes are in this region (Lock et al., 1994), molecular genetic studies that refined the position of *opt* eliminated the potassium channel genes as candidates for the *opt* mutation (Street et al., 1995).

The type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1) gene locus (*Itpr1*) is also located on the distal portion of mouse chromosome 6 (Furuichi et al., 1993) in the vicinity of *opt*. The IP₃R1 protein binds the intracellular second messenger IP₃, which is generated by phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (Berridge, 1993). Ligand binding triggers the efflux of calcium from intracellular stores, suggesting that IP₃R1 is both a receptor for IP₃ and a calcium channel. Recently, a targeted disruption of the IP₃R1 gene was shown to exhibit a phenotype similar to that of *opt* (Matsumoto et al., 1996). In this report, we demonstrate tight genetic linkage between *opt* and *Itpr1* and define a genomic deletion that alters the IP₃R1 protein in *opt* mice.

MATERIALS AND METHODS

Genetic mapping. To establish the intersubspecific intercross segregating for *opt*, CAST/Ei-+/+ males were mated to C57BL/Ks-+/+ *opt* females. F1 hybrids carrying *opt* were intercrossed. Fifty-six F2 *opt/opt* mice were collected. Tail DNA was isolated, and microsatellite analysis was performed as described previously (Street et al., 1995). Segregation of IP₃R1 in the 56 DNAs was followed by a PCR-based approach using the primer pair 5'-TGCATAGCTGCCCCCTAGG-3' and 5'-TTCCTGTGATACT-TTGACCC-3' (annealing temperature of 59°C), which amplify a 126 base pair (bp) product from the 3' untranslated region of the IP₃R1 gene. The CAST/Ei-+/+ PCR product was cleaved by *Bsa*AI to generate a 22

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and 104 bp restriction fragment, whereas the C57BL/Ks-*opt/opt* amplification product lacked the *Bsa*AI restriction endonuclease site. Statistical analysis of the intersubspecific intercross was performed using Map Manager (Manly, 1993).

The *opt* colony is maintained by crossing *Mi^{wh}/opt* heterozygotes, allowing the genotype of nonrecombinant progeny to be predicted by coat color. The interstrain restriction fragment length variation (RFLV) was detected using Southern blot hybridization analysis (Street et al., 1995) on *Bgl*II-restricted liver DNAs with a ³²P-labeled 276 bp fragment derived from PCR primers 5'-GCTGTTTCTCGTTGCTAATGG-3' and 5'-GAGATGACACTGACTGGTCAGC-3'. The probability of heterozygosity was calculated (contact authors for details) using the generation-matrix method (Green, 1981).

Northern analysis. Total brain RNA was isolated by guanidine isothiocyanate extraction (Chomczynski and Sacchi, 1987). Twenty micrograms of total brain RNA were electrophoresed at 0.83 V/cm for 21 hr, blotted, and hybridized with the ³²P-labeled open reading frame (ORF) of the rat IP₃R1 homolog (Mignery et al., 1990), in modified Church Gilbert buffer [0.34 M Na₂HPO₄ heptahydrate, pH 7.2, 7.0% SDS, 0.001 M EDTA, and 1.0% BSA (Fraction V)] at 62°C and washed the next day in 0.04 M Na₂HPO₄, 0.001 M EDTA, and 1.0% SDS at 62°C.

Reverse transcription (RT)-PCR analysis. RT-PCR was performed according to the manufacturer's (Boehringer Mannheim, Indianapolis, IN) specifications on 0.2 µg of total RNA with an annealing temperature of 60°C. 11F/12R RT-PCR products were analyzed by Southern blot hybridization using the ³²P-labeled type 3 *opt* RT-PCR product (see Fig. 3C). RT-PCR products were subcloned with the pGEM-T Vector System (Promega, Madison, WI) and sequenced using fluorescent dyedexy terminator chemistry (Applied Biosystems, Foster City, CA). Five subclones representing four different sequences were isolated from the *opt* 11F/12R RT-PCR products, whereas one subclone was isolated from the *Mi^{wh}* RT-PCR amplification.

Western analysis. Microsomes were prepared from frozen mouse cerebella by Potter-Elvehjem homogenization in ice-cold buffer (40 ml/g tissue) of 50 mM NaH₂PO₄, pH 7.3, 50 mM KCl, 1.0 mM EDTA, 15% (w/v) sucrose, 10 µg/ml aprotinin, 1.0 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 5000 × *g* for 6 min at 4°C, and the pellet was resuspended in the original volume and recentrifuged. The two supernatants were combined and centrifuged at >100,000 × *g* for 1 hr at 4°C, and the resulting pellet was washed in sucrose-free buffer and recentrifuged for 30 min. The final pellet was resuspended in 100 µl of sucrose-free buffer/30 mg pellet and homogenized with a Dounce homogenizer. Microsomal membrane proteins (50 µg protein/lane for antibody SP-3A, 8 µg protein/lane for SP-2A) were separated by SDS-PAGE on a 3–15% gradient gel and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The IP₃R1 protein was visualized with affinity-purified rabbit polyclonal antisera (SP-3A at 1:250 dilution, SP-2A at 1:1000 dilution), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Cappel, Organon Teknica, Durham, NC), and the ECL detection system (Amersham, Arlington Heights, IL). Protein expression levels were quantified by means of ¹²⁵I-labeled Protein A (1 µCi/ml) (Amersham) in place of the secondary antibody, a Fujix Bas 1000 Phosphorimager, and accompanying MacBAS v2.2 software.

Genomic structure analysis. B6/CBAF1J-+/+ (Stratagene 946304; Stratagene, La Jolla, CA) and *opt* homozygote (Stratagene) mouse genomic libraries (λ FIX II) were screened with the *Mi^{wh}* 11F/12R amplification product (see Fig. 3C), and exon D (see Fig. 5A). Restricted λ phage insert DNAs were subcloned into the pBlueScript SK vector (Stratagene) and sequenced. Five PCR primer pairs (see *1–*5 in Fig. 5A) were developed from this sequence and used to confirm the extent of the genomic deletion in *opt* DNA: *1 primers (5'-AGTGATCCTTCCAGCTCG-3' and 5'-CCAATGATGAGAATTTCG-3') generate a 172 bp product from intron 1; *2 primers (5'-CCATTGGAAAGCACAGATGC-3' and 5'-CTTTTGATCTGTGATATCTCG-3') generate a 176 bp product from intron 1; *3 primers (5'-GGAAACATCAGACCTTCAGG-3' and 5'-GGTCTCTGTTGATAGTGG-3') amplify a 73 bp product from exon B; *4 primers (5'-GGATCTAGTTCCACAAGCAGG-3' and 5'-TGCCTCTTCCAGAGTGG-3') generate a 165 bp product from exon C; and *5 primers (5'-GCTGAGCCTTGGTCTCAGG-3' and 5'-CGTAGTTACAGAGCTACG-3') generate a 269 bp product from intron 3. PCR was performed as described previously (Street et al., 1995), with an annealing temperature of 55°C for primer pair 1 and 60°C for primer pairs 2–5. The deletion was also apparent from Southern blot hybridization analysis on *Nsi*I-restricted

liver DNAs that were probed with a ³²P-labeled 3.4 kb *Nsi*I genomic B6/CBA-+/+ DNA fragment.

Purified λ DNA was used as a template for expand PCR. Expand PCR with primers 1F/6R and 7F/8R was used to establish or confirm distances in introns 1 and 3, respectively. The exact size of intron 3 (>8 kb) was not determined, because no single wild-type phage clone was isolated that spanned from exon C to 4F/5R sequence. Expand PCR was performed according to the manufacturer's (Boehringer Mannheim) specifications, with the following sequences: 1F (see Fig. 5B); 6R (5'-CACAGTATGGACATAATGGC-3'); 7F (5'-CACCAGTCTTGGAGATGG-3'); and 8R (5'-GTTTCCCAAGTCGCTGGTG-3').

To identify a fragment containing aberrant *opt* genomic DNA, oligonucleotides 1F and 7F, which flanked the altered area, were hybridized to panels of restricted B6/CBA-+/+ and *opt* homozygote λ phage insert DNAs. 1F and 7F hybridized to the same size 3.0 kb *Xba*I fragment in *opt* DNA, whereas 1F detected a 1.6 kb wild-type *Xba*I band. Both *Xba*I fragments were sequenced and aligned, which revealed that they diverged 298 bp 3' of exon A (see Fig. 3B). For the analysis, oligonucleotides 1F and 7F were end-labeled with [γ-³²P] ATP and hybridized in 6× SSC, 20 mM Na₂HPO₄ heptahydrate, pH 7.0, 2× Denhardt's solution, 0.05% Na₂P₂O₇ decahydrate, 0.1% SDS, 0.1 mg/ml salmon sperm DNA at 55°C for 16 hr, and then washed in 6× SSC.

Histology. P4 and P23 mice were decapitated, and the brains were removed into Millonig's fixative (1.86 gm NaH₂PO₄, 0.42 gm NaOH, 4% formaldehyde in 100 ml of water). The brains were embedded in paraffin, and 10 µm sections were taken through the cerebellum. After they were mounted on glass slides, sections were stained with cresyl violet and coverslipped. The number of Purkinje cell bodies per 525 µm wide field was compared between *opt/opt* and +/+ mice at P23. The sections were aligned horizontally to maximize cell number, and the Purkinje cell bodies were counted in 16 *opt/opt* and 14 +/+ fields.

Physiological analysis. P4 mice were decapitated, the brains were removed quickly into ice-cold Gey's Balanced Salt Solution (GBSS, Life Technologies, Gaithersburg, MD), and the cerebellum was dissected free. The cerebellum was placed on a block of 2% agar and sliced into sagittal 200 µm sections using a brain slicer (Katz, 1987). The slices were cleaned of membranes and choroid plexus, and they were incubated for 30 min at 37°C in a bicarbonate-containing Ringer's solution containing (in mM): 150 NaCl, 2.5 KCl, 3 CaCl₂, 1 MgCl₂, 10 HEPES, 5 NaHCO₃, 8 glucose, pH 7.4, with 10 µM fura-2 AM and 10 µM pluronic acid. Slices were then mounted on the stage of an inverted microscope (Nikon Diaphot, Melville, NY) and held in place with an overlying screen. The bath was perfused continuously with 95% O₂/5% CO₂-bubbled Ringer's solution. Neurons were imaged by alternate flashes of 340 and 380 nm light every 10 sec, except during peaks of Ca²⁺ changes when sampling occurred every 3 sec. Fura-2 fluorescence emissions were collected at 510 nm by an intensifying CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ); 340/380 ratios and images were analyzed and displayed by Image-1/Fluor software (Universal Imaging, West Chester, PA). Ca²⁺-free (Mg replaced) Ringer's solution was applied 1–2 min before and during quisqualate (QA) to isolate the release of intracellular stores from sources of extracellular calcium. Between applications of QA, Ca²⁺-containing Ringer's solution flowed for 2–3 min to replenish intracellular stores. Analysis of data and statistics was performed using Excel (Microsoft, Redmond, WA) and Cricket Graph (Computer Associates, San Diego, CA).

PCR assays for *opt* and *Mi^{wh}* alleles. The number of *opt* and *Mi^{wh}* alleles carried by the pups was determined by PCR analysis on tail-clip DNA. For the *opt* analysis, primer pair 1F/3R generated a product from animals carrying at least one *opt* IP₃R1 allele, whereas primers from exon C (see *4, Fig. 5A) amplified a band in DNA from mice carrying at least one wild-type IP₃R1 allele. By using both primer pairs on the same DNAs, the +/+, +/opt, and *opt/opt* mice could be distinguished from one another.

The *Mi^{wh}* analysis used a nested PCR approach and the T to A point mutation at the *Mi^{wh}* locus (Steingrimsson et al., 1994). The A residue (bp 764) in *Mi^{wh}* is the first nucleotide of exon 7 (Tassabehji et al., 1994). Primers located 5' of A(764) were designed from an unpublished intron sequence provided by E. Steingrimsson. The first PCR round used primer pair MiF1/MiR1, which perfectly matched the wild-type sequence and flanked the nucleotide residue altered in the *Mi^{wh}* mice to generate a 120 bp product. Oligonucleotide MiF1 matches unpublished intronic sequence, and primer MiR1 corresponds to exon 7 sequence 5'-GATCATTTGACTTGGGGATC-3' (Hodgkinson et al., 1993). PCR was performed as described previously (Street et al., 1995), with an annealing temperature of 60°C. This amplification product was diluted 1:100, and 2

μ l of this dilution served as the template for the second 25 μ l PCR reaction, which was also performed with an annealing temperature of 60°C. The second PCR round used primers MiF2/MiR2. Primer MiR2 matches exon 7 sequence 5'-TCATTGACTTGGGGATCAGAG-TACC-3' (Hodgkinson et al., 1993), whereas oligonucleotide MiF2 was designed based on unpublished intronic sequence immediately 5' of residue A(764); however, two nts of the first six at the 3' end of MiF2 did not match the wild-type sequence, but instead formed the first portion of an *Xba*I recognition site (TCTAG). Primers amplifying the *Mi*^{wh} allele create an *Xba*I site (T/CTAGA), whereas primers using wild-type DNA as a template do not create the endonuclease recognition site (TCTAGT). By directly digesting 9 μ l of the second-round PCR amplification product with *Xba*I and running it on a 3% Metaphor gel, the 26 bp size shift between *Mi*^{wh} and wild-type DNA could be visualized, allowing +/+, +/*Mi*^{wh}, and *Mi*^{wh}/*Mi*^{wh} mice to be distinguished from one another.

RESULTS

Genetic mapping

To test whether a mutation in IP₃R1 could be causally related to the *opt* mouse, we mapped the IP₃R1 gene on an intersubspecific intercross segregating for *opt* (Fig. 1*A*). In this cross, IP₃R1 was not separated from *opt* by a recombination event in 112 meioses (Fig. 1*B,C*), indicating that IP₃R1 and *opt* are tightly linked genetically.

Genetic linkage between IP₃R1 and *opt* was also suggested by analyzing mice from the heterozygous intercross (*Mi*^{wh}, +/+, *opt* × *Mi*^{wh}, +/+, *opt*) used to propagate the *opt* mutation (Fig. 2*A*). The presence of the *Mi*^{wh} mutation, which arose in a cross between DBA and C57BL, allows the *opt* colony to be maintained in part by coat color selection (Grobman and Charles, 1947). When a 3' untranslated IP₃R1 DNA fragment was hybridized to Southern blots containing *Mi*^{wh} homozygote, *Mi*^{wh}/*opt* heterozygote (*Mi*^{wh}, +/+, *opt*), and *opt* homozygote DNAs, the probe detected an RFLV between *Mi*^{wh} and *opt* homozygotes (Fig. 2*B*). Given that the *opt* colony has been maintained in our laboratory by brother–sister intercrossing for 50 generations (N50), unselected loci would have been driven to homozygosity unless the gene was tightly linked to either of the two selected mutations, *Mi*^{wh} or *opt*. The probability of heterozygosity at an unselected locus such as IP₃R1 in this cross at N50 would be 2.2×10^{-5} . Therefore, our finding of heterozygosity at the IP₃R1 locus in all N50 *opt*-carrying mice that were analyzed ($n = 3$) supports the previous finding that this gene and *opt* are tightly linked genetically.

Expression of IP₃R1 in *opt*

Expression of IP₃R1 mRNA in the brains of *opt* mice was examined by Northern blot analysis. *Mi*^{wh} homozygote mice displayed a transcript of ~10 kb (Fig. 3*A*, lane 2), whereas the *opt* homozygotes contained a slightly smaller transcript (lane 5), with *Mi*^{wh}/*opt* heterozygotes demonstrating both mRNA species (lanes 3, 4). Although size differences were observed, the intensity of each IP₃R1 transcript in the *Mi*^{wh}/*opt* heterozygotes was similar, as was the band intensity between age-matched *Mi*^{wh} and *opt* homozygotes, suggesting that IP₃R1 mRNA levels are not reduced dramatically by the *opt* mutation.

To further characterize the altered IP₃R1 transcript, RT-PCR was performed on whole-brain RNA from a *Mi*^{wh} homozygote, a *Mi*^{wh}/*opt* heterozygote, and an *opt* homozygote pup, with overlapping PCR primer pairs spanning the 8247 bp IP₃R1 ORF and immediate 5' and 3' flanking regions. Aberrant *opt* RT-PCR products were detected with only one primer pair (11F/12R) (Nakagawa et al., 1991) positioned within the modulatory and transducing domain (Furuichi and Mikoshiba, 1995). Primers

Figure 1. Location of the IP₃R1 gene relative to the *opt* locus. *A*, An intersubspecific intercross was established segregating for *opt*. *B*, Four microsatellites and a novel PCR marker derived from the IP₃R1 gene were mapped in the 56 progeny from the intersubspecific intercross. The number of recombination events observed among the 112 intercross chromosomes analyzed, and genetic distances in centimorgans ($\pm$ SE) separating adjacent loci, are represented by the numbers to the left of the chromosome. The human homolog of the mouse IP₃R1 gene maps to the short arm of chromosome 3, as noted in parentheses to the right of the mouse gene (Yamada et al., 1994). *C*, One hundred twelve chromosomes from the 56 intersubspecific intercross progeny were scored for parental C57BL/Ks-*opt/opt* (open boxes) and CAST/Ei-+/+ DNAs (filled boxes) at five loci. The number of chromosomes for each haplotype is shown below the columns.

11F/12R amplified a 648 bp product from *Mi*^{wh} homozygote RNA (Fig. 3*B*, lane 2), which was slightly smaller because of alternative splicing in the SII region (Nakagawa et al., 1991) than the 699 bp area spanned by these oligonucleotides in the published cerebellar IP₃R1 cDNA nucleotide sequence (Furuichi et al., 1989a). Although 11F/12R generated one product from *Mi*^{wh} homozygote RNA, the same primer pair amplified at least three smaller products in *opt* homozygotes (lane 5), with *Mi*^{wh}/*opt* heterozygotes seeming to generate all of the products (lanes 3, 4). Cloning and analysis of these RT-PCR products indicated that at least four

Figure 2. Heterozygosity at the IP₃R1 locus. *A*, Maintenance of the *opt* colony. *B*, Southern blot hybridization analysis demonstrates an interstrain RFLV with a probe derived from the 3' untranslated region of the IP₃R1 gene.

alternatively spliced RNA species were present in the brains of *opt* homozygotes, with all transcripts lacking the 5194–5517 nucleotide (nt) area encoding amino acids 1732–1839 (Fig. 3C). Several regulatory sites are contained within this area: a cGMP-dependent (GKA) (Komalavilas and Lincoln, 1994) and cAMP-dependent protein kinase (PKA) (Ferris et al., 1991) phosphorylation site at amino acid residue 1755, and at least one potential ATP binding domain at 1773–1778 (Fig. 3C). Although the *opt* transcript type 2 lacks only nucleotide residues 5194–5517, the *opt*

type 1, 3, and 4 transcripts have additional domains deleted 5' of this area (Fig. 3C). The splice junctions to remove these domains are also used in pre-mRNA from wild-type mice, as shown previously (Nakagawa et al., 1991), and are referred to as the SII region (nt residues 1692–1731) containing segment A (1692–1714), B (1715), and C (1716–1731). The exon skipping displayed by all four *opt* transcript subtypes does not result in translational frameshifts or premature stop codons, leaving the transcript unaltered 3' of exon D.

To determine whether the altered transcripts seen in *opt* are translated, Western blot analysis was performed on cerebellar microsomes with two polyclonal antibodies made against IP₃R1 peptide antigens specific to IP₃R1 (Lin, 1995) (Fig. 4). The first antibody, SP-3A, was raised against amino acid residues 2485–2501, which are located 3' of the area deleted in the IP₃R1 mRNA from *opt*. This antibody recognized a protein migrating at ~255 kDa in the 3-month-old C57BL/6-+/+, P12 *Mi^{wh}* homozygote, and P12 *Mi^{wh}/opt* heterozygote lanes, and a protein migrating slightly faster at 245 kDa in the lanes from two *opt* homozygotes (P12 and P19) (Fig. 4). Staining with SP-3A confirms that the ORF of the IP₃R1 protein is maintained in *opt* homozygotes, although the level of expression was markedly reduced. P12 and P19 *opt* homozygotes show similarly decreased expression levels of cerebellar IP₃R1 protein. Quantification of IP₃R1 protein levels using SP-3A and iodinated Protein A indicated that the P12 *opt* homozygote and *Mi^{wh}/opt* heterozygote expression was 10% and 67%, respectively, of their *Mi^{wh}* homozygote littermate (data not shown).

The second antibody, SP-2A, recognizes residues 1745–1760 (Fig. 3C), which fall within the region deleted from all of the *opt* transcripts. This antibody detected a 255 kDa band in the C57BL/6-+/+, *Mi^{wh}* homozygote, and *Mi^{wh}/opt* heterozygote lanes, but not in the two *opt* homozygote lanes. These protein and RNA blots, combined with the RT-PCR and sequence data, indicate that *opt* mice contain aberrantly spliced IP₃R1 mRNA transcripts, which lead to the production of IP₃R1 protein missing several potential regulatory sites, with the altered protein being present at markedly reduced levels.

Genomic structure of IP₃R1 in *opt*

To investigate the underlying cause of the aberrant splicing of the *opt* IP₃R1 mRNA, the genomic structure of the gene from exon A to exon D was compared in wild-type and *opt* homozygote DNA (Fig. 5A). The comparison indicated that *opt* homozygotes contain a >10 kb genomic DNA deletion that begins 298 bp 3' of exon A (Fig. 5B) and ends in intron 3 (size unknown) at a site >1 kb 3' of exon C and >7 kb 5' of exon D (Fig. 5A). Hybridization of the wild-type 3.4 kb *Nsi*I genomic fragment, spanning exon B and part of exon C, to Southern blots containing *Nsi*I-digested DNAs from C57BL/6J-+/+, CBA/Ca-+/+, *Mi^{wh}* homozygote, *Mi^{wh}/opt* heterozygote, and *opt* homozygote DNA, confirmed that a large region was deleted in the *opt* genome. The probe hybridized to an appropriately sized fragment in all of the DNAs except for the *opt* homozygote, where no hybridization signal was detected (Fig. 5C). Five PCR primer pairs were designed from the wild-type nucleotide sequence suspected to be deleted in *opt* (Fig. 5A). All of the primers amplified the expected size product from C57BL/6J-+/+ and *Mi^{wh}* homozygote DNA, but failed to generate a product when *opt* homozygote DNA was used as a template. PCR primer pairs were also generated from the nucleotide sequence of a 3.0 kb *Xba*I *opt* genomic fragment

Figure 3. Expression of IP₃R1 mRNA in *opt* brain. *A*, IP₃R1 transcript size is reduced in *opt* homozygote mice. From left to right, lanes 1–5 represent total brain RNA from a P13 C57BL/6J-+/+, P13 *Mi^{wh}* homozygote, P11 *Mi^{wh}/opt* heterozygote, adult *Mi^{wh}/opt* heterozygote, and P13 *opt* homozygote mouse. An identically loaded Northern gel was electrophoresed for a shorter duration in parallel to the one shown here to retain smaller size transcripts. This blot was probed with EF 1 α (Xiang and Werner, 1989) to control for lane loading. *B*, RT-PCR analysis of IP₃R1 mRNA with primers 11F/12R; lanes 1–5 as above. *C*, Primary structure of altered IP₃R1 mRNA species in *opt* homozygotes generated with primers 11F/12R. Primer 12R is underlined by an arrow. Primer 11F located at nt 4875–4897 is not shown. The first and second line contain the amino acid (Furuichi et al., 1989b) and nucleotide sequence (Furuichi et al., 1989a) of the cerebellar IP₃R1 cDNA, respectively. The nucleotide sequence is numbered from the 5' to 3' direction where +1 was assigned to the first base of the predicted initiation codon. Line three displays a nucleotide sequence of the RT-PCR product from *Mi^{wh}* homozygote RNA, and lines 4–7 display the four different products from *opt* homozygote RNA, labeled 1–4. Exon borders determined by genomic sequence analysis are indicated by vertical lines. A GKA and PKA phosphorylation site is circled, and a putative ATP-binding domain is boxed. Asterisks denote nucleotide changes used to generate antibody SP-2A.

containing the fusion point (Fig. 5B). Primers 1F/3R, which span the fusion point, amplified a product from *opt* but not wild-type genomic DNA under standard PCR amplification conditions (data not shown). Primer pairs positioned immedi-

ately 5' (1F/2R) or 3' (4F/5R) of the fusion point amplified the expected size product in both C57BL/6J-+/+ and *opt* homozygote genomic liver DNA. PCR analysis with primers 4F/5R indicate that the *opt* sequence 3' of the fusion point is from

Figure 4. Expression of IP₃R1 protein in *opt* cerebellum. Western blot analysis of cerebellar microsomal membrane fractions from a 3-month-old C57BL/6-+/+, P12 *Mi^{wh}* homozygote, P12 *Mi^{wh}/opt* heterozygote, P12 *opt* homozygote, and P19 *opt* homozygote with antibodies SP-3A and SP-2A (Lin, 1995). P12 pups are littermates.

intron 3, because the expected size product is generated from a B6/CBA-+/+ wild-type phage genomic insert lacking exon C but containing intronic DNA from between exons C and D, and exon D. This genomic DNA analysis data indicates that the aberrant splicing of the IP₃R1 mRNA in *opt* homozygotes is caused by a large genomic deletion that removes the majority of intron 1, all of exon B, intron 2, and exon C, and a portion of intron 3, and leaves exons A and D intact.

Comparison of the wild-type genomic nucleotide sequence with that of the published cerebellar IP₃R1 cDNA (Furuichi et al., 1989a) allows the exon/intron boundaries (Padgett et al., 1986) to be determined for this section of the IP₃R1 gene, as noted in Figure 3C. This analysis indicates that exons A and D can be fused in *opt* homozygote mRNA without creating a premature stop codon or translational frameshift, because introns 1 and 3 are both phase 0 introns (Sharp, 1981) interrupting the reading frame between codons (Fig. 5D). Therefore the original ORF is shortened because it is missing exons B and C but otherwise is maintained.

Cerebellum morphology

The cerebella of control and *opt* homozygote mice compared at P4 and P23 show no apparent differences at the light microscope level (Fig. 6). At P4, the mature architecture of the cerebellum is not yet seen, and the Purkinje neuron layer is only a relatively narrow band between the granular and molecular layers (Fig. 6A–D). The gross appearance of the P4 tissue is similar between control and *opt* pups. At P23, a mature appearance is seen in both control and *opt* cerebella (Fig. 6E–H). The normal, ordered arrangement of the Purkinje cell layer to molecular and granular layers is observed (Fig. 6E,D). At a higher magnification (Fig. 6G,H), the cell bodies of Purkinje neurons appear similar in size and density when compared between genotypes. The average number of Purkinje cell bodies/field in a P23 mouse was 19.1 ± 0.6 for *opt* homozygotes and 19.2 ± 0.9 for control pups. These anatomical findings suggest that the 10-fold reduction in cerebellar IP₃R1 protein expression seen in the P12 and P19 *opt* homozygotes is not attributable to Purkinje cell death. The architecture of

the cerebella from P4 and P23 *Mi^{wh}* homozygote pups and from P4 *Mi^{wh}/opt* heterozygotes was indistinguishable from that of the cerebella shown here.

Physiological analysis

As a first step toward investigating the physiological consequences of having exons B and C deleted from the IP₃R1 protein, a cerebellar slice preparation was used to ask whether any biological differences in release from IP₃-sensitive calcium stores could be detected between P4 *opt* mutants and wild-type littermates when the inositol-phospholipid pathway was activated by an extracellular signal. A slice preparation was chosen to maintain cellular components that might be critical in modulating the response. Cerebellar slices were used because cerebellar Purkinje cells show enriched levels of IP₃R1 (Furuichi et al., 1993), with IP₃R1 expression accounting for ~94% of the IP₃R mRNA in the cerebellum. The remaining 6% is constituted by expression of mRNA for IP₃R2 and IP₃R3, and putative isoforms IP₃R4 and IP₃R5 (De Smedt et al., 1994). Cerebellar slices were taken from P4 pups for several reasons. First, the somata of the P4 Purkinje neurons are ~15 μ m in diameter and is ordered in a characteristic array (Fig. 6), allowing individual Purkinje cell bodies to be resolved easily and distinguished clearly from other non-Purkinje cells within the slice preparation at the light microscope level. Second, IP₃R1 and metabotropic glutamate receptor 1 (mGluR1) are both expressed on the somata of mouse Purkinje neurons by P3 (Ryo et al., 1993), with mGluR5 being expressed in the Purkinje cell layer at the same developmental time (Bettler et al., 1990). Within the mGluR family, mGluR1 and mGluR5 comprise Group I, which is characterized by linkage to the phospholipase C/IP₃ cascade pathway, and by the following potency rank order for agonists: QA > L-glutamate > ibotenate > (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine > (1S,3R-ACPD) (Pin and Duvoisin, 1995). By the equivalent of P4, the percentage of Purkinje neurons exhibiting metabotropic responses to QA application is maximal, with the response being localized primarily to the soma (Yuzaki and Mikoshiba, 1992). We therefore chose to use QA to activate the inositol-phospholipid pathway in the P4 cerebellar slice preparations. Although QA is most effective at activating the Group I mGluR, it is also active on the ionotropic AMPA-type GluRs (Pin and Duvoisin, 1995); therefore, to ensure that QA was mediating only Ca²⁺ release from intracellular stores, the compound was always applied in Ca²⁺-free extracellular solution.

P4 cerebellar tissue slices were loaded with the membrane-permeate Ca²⁺ indicator dye fura-2 AM and then challenged with QA in Ca²⁺-free external solution (Llano et al., 1991; Yuzaki and Mikoshiba, 1992). A cerebellar field was visualized at 400 $\times$ magnification by a light microscope and displayed on a video screen. A typical cerebellar field at this magnification contained an array of 4–12 Purkinje neuron somata. The image analysis software allowed individual Purkinje cell bodies to be selected for imaging by outlining the somata on the video screen. The slice was then alternately exposed to 340 and 380 nm wavelength light, allowing quantitation of calcium release from intracellular stores for the selected Purkinje somata. So although the entire slice was loaded with fura-2 AM, we were measuring responses only from the cell bodies of selected Purkinje neurons. Only slices in which all Purkinje neurons in the field appeared healthy based on their initial calcium content and their KCl response at the end of the experiment were considered in the analysis. In several experiments, non-Purkinje cells were also selected for imaging, and in no case did these cells respond to agonist application.

Figure 5. Genomic analysis of *opt* locus. **A**, Comparison of wild-type and mutant genomic structure reveals deletion in *opt* DNA. Wild-type composite diagram (*top line*) was constructed using restriction mapping, Southern blot hybridization, sequencing, and PCR-based approaches in B6/CBA-+/+ genomic DNA derived from a λ phage library. Exons are depicted as filled boxes and intervening introns as a solid line. Bases of the mouse cerebellar IP₃R1 cDNA corresponding to the beginning and end of each exon are as follows: A (5074–5142), B (5143–5193), C (5194–5517), and D (5518–5698). 1F, 6R, 7F, and 8R are expand PCR primers. Asterisks indicate the location of five PCR primer pairs used to confirm the extent of the genomic deletion in *opt*. The lower line depicts an *opt* homozygote genomic DNA map based on restriction analysis of subcloned fragments derived from a custom-made *opt* homozygote λ phage library. Restriction endonuclease sites are designated as follows: S, *Sac*I; X, *Xba*I; and N, *Nsi*I. Not all restriction sites are shown. **B**, The deletion in *opt* DNA fuses introns 1 and 3. Exon A is boxed, intron 3 sequence is shaded, and PCR primers are designated by arrows. **C**, Southern blot hybridization analysis demonstrates that a 3.4 kb *Nsi*I fragment is deleted in *opt* genomic DNA. **D**, The intron/exon slice boundaries for exons A, B, C, and D are compared with a consensus splice sequence.

The traces in Figure 7A (*top*) show Ca²⁺ responses in several Purkinje neurons from a wild-type (+/+; *left*) and an *opt* homozygote cerebellar brain slice (*right*) during three consecutive applications of QA. Each colored trace depicts the response from one individual Purkinje neuron. The ensemble of colored traces represents all of the Purkinje neurons monitored simultaneously in a

single experiment. The color images in Figure 7A (*bottom*) are from two Purkinje neuron somata within these same two slices [+/+ (*left*) and *opt* homozygote (*right*)] and show 340/380 nm intensity ratios in the somata at three time points (A, B, C) during the experiment. The first QA application (QA1) is able to generate a substantial calcium response from both +/+ and *opt* ho-

Figure 6. Paraffin sections of cerebellum stained with cresyl violet. P4 pups are shown in *A–D*, P23 mice in *E–H*. Control C57BL/6J-+/+ mice are shown on the left, *opt* homozygote mice on the right. Scale bars: *A, B, E, F*, 200 μ m; *C, D, G, H*, 50 μ m.

mozygote Purkinje neurons. After allowing for refilling of internal calcium stores by exposure to 2 min of Ca^{2+} -containing Ringer's solution between challenges, repeated application of the same QA dose (*QA2, QA3*) continued to elicit large responses in *opt* ho-

mozygotes, whereas +/+ mice responded poorly to both the second and third applications of QA (Fig. 7*A*). After QA application, cells from *opt* homozygote slices returned to baseline Ca^{2+} levels, whereas a subset of +/+ and heterozygote P4 cells in some

Figure 7. Physiological analysis of *opt* mutation. *A*, Examples of experiments in *+/+* (left) and *opt/opt* (right) cerebellar slices showing 340/380 ratios of each of several Purkinje neurons in the field under control conditions and in response to repeated 30 sec 100 μ M QA application in Ca^{2+} -free Ringer's solution. Each colored trace represents the response from one individual Purkinje neuron. Images were taken at points indicated in traces above where *A* is the control, and *B* and *C* are the responses to the first and second QA application, respectively. Blue bars show periods of Ca^{2+} -free Ringer's solution flow. Higher 340/380 ratios refer to higher Ca^{2+} ion levels on the color scale. *B*, Histogram of amplitudes of changes in the ratio of 340/380 signals in response to the first QA application compared between *opt/opt* ($n = 4$ animals), *opt/+* ($n = 1$), *opt/Mi^{wh}* ($n = 7$), and *+/+* ($n = 2$) pups. Numbers above SE bars are the number of neurons. The number of *+/+* and *opt/+* mice is limited, because they are produced only through recombination in the *opt/Mi^{wh}* intercross. *C*, Comparison of successive QA responses plotted relative to the intensity changes for the first response of each genotype [*opt/opt* ($n = 4$), *opt/+* ($n = 1$), *opt/Mi^{wh}* ($n = 7$ and 3 for second and third response, respectively), and *+/+* ($n = 2$)]. All genotypes were significant at >0.01 level compared with *opt* homozygotes.

slices drifted toward higher values, as seen in Figure 7*A*. The data from numerous *opt/opt*, *opt/+*, *opt/Mi^{wh}*, and *+/+* Purkinje neurons are summarized in Figure 7*B,C*. Although IP₃R1 protein expression is reduced in *opt* homozygote cerebellum, QA application is able to generate an initial calcium response from *opt* Purkinje neurons that is only moderately reduced from that seen in *+/+* mice, with pups carrying only one *opt* allele responding in an intermediate fashion (Fig. 7*B*). In addition, the calcium response in *opt* homozygotes consistently shows less attenuation to repeated QA application than in *+/+* mice, with *opt* heterozygotes (*opt/+* and *opt/Mi^{wh}*) again generating intermediate values (Fig. 7*C*).

DISCUSSION

By using two independent genetic analyses we have shown that *opt* is tightly linked to the IP₃R1 gene on distal mouse chromosome 6,

making IP₃R1 a candidate locus for the *opt* mutation. Northern blot analysis demonstrated that relative to controls, the size of the IP₃R1 transcript in *opt* mice was reduced. Detailed primary structure analysis revealed that all of the IP₃R1 transcripts recovered from *opt* brain RNA lacked a 324 nt region encoding 108 amino acids positioned within the putative transducing/modulatory domain of the IP₃R1 protein. Western blot analysis confirmed this deletion and showed that the ORF had not been interrupted, although the altered IP₃R1 protein was present at markedly reduced levels in the *opt* mice. These data clearly demonstrate that the IP₃R1 transcript and protein are altered in *opt*.

The underlying cause of the altered IP₃R1 protein in *opt* was shown to be a >10 kb genomic deletion that removes two exons (B and C) from the *opt* IP₃R1 locus. This deletion occurs immediately 3' of a region called SII, which produces a number of alternatively splicing transcripts in the wild-type IP₃R1 gene (Na-

kagawa et al., 1991). Our sequence analysis of intron/exon junctions (Fig. 5D) shows that splices in the SII region occur at phase 0; i.e., the exons are spliced between codons (Sharp, 1981), allowing all combinations of exon inclusion. Furthermore, we show that the glutamine residue, called SIIB by Nakagawa et al. (1991), is contiguous with exon A, the glutamine codon providing a second 3' acceptor site at that junction while maintaining the phase 0 reading frame. Finally, the observation that exon D is also phase 0 allows each of the alternate 5' donor sites from the SII region to form uninterrupted translational frames in *opt* transcripts. Although matching of reading frames around the *opt* deletion is fortuitous, phase 0 introns have been shown to occur more often than phase 1 or 2 introns, with frequencies of 48%, 30%, and 22%, respectively, in a sample of 11,117 mammalian introns analyzed (Long et al., 1995). Thus the fact that the *opt* deletion begins and ends within intronic regions statistically favors the possibility of a maintained ORF, in this case permitting the *opt* allele to produce an altered but potentially functional IP₃R1 protein.

Using a cerebellar slice preparation, we found that despite markedly reduced IP₃R1 protein levels in *opt*, a strong calcium release from intracellular stores can still be elicited in Purkinje neurons, with the calcium response showing less attenuation to repeated QA application in *opt* homozygotes compared with that in littermates. How might this physiological phenotype result from deletion of exons B and C of the IP₃R1 protein? One possible explanation is that the *opt* deletion, occurring in the cytoplasmic transducing domain, alters tertiary and quaternary protein structure in a region thought to couple the amino-terminal IP₃-binding domain with the C-terminal calcium channel domain, thereby affecting the activation, conductance, or gating properties of the IP₃R1 channel in *opt*. These possibilities could be addressed directly by studies at the single-channel level. Conformational changes in the *opt* IP₃R1 protein might also affect the ability of accessory proteins such as calreticulin (Enyedi et al., 1993) and FK506-binding protein (Cameron et al., 1995) to complex with IP₃R1 in *opt*, thus altering normal channel regulation. In addition to deletion-induced conformational changes, the removal of a specific modulatory site for phosphorylation by PKA and GKA might contribute to the physiological phenotype of *opt*. The deletion in *opt* removes serine-1755, which is phosphorylated by GKA and PKA, leaving only serine-1588 available for PKA phosphorylation in *opt* homozygotes. This change in PKA phosphorylation site availability may alter the physiological outcome of phosphorylation on channel structure or function (Bezprozvanny and Ehrlich, 1995). Finally, because IP₃R1 is reduced in *opt*, the change in the ratio of IP₃R1 to other IP₃R subtypes and to other proteins that regulate the calcium balance across the endoplasmic reticulum membrane, such as the calcium pump, luminal calcium-binding molecules, and other luminal and cytosolic accessory proteins, may affect the normal regulation of calcium uptake, storage, and mobilization (Taylor and Traynor, 1995), thereby changing the properties of release from IP₃-sensitive calcium stores. In sum, the physiological phenotype seen in the P4 cerebellar Purkinje neurons of *opt* mice could be a consequence of reduced IP₃R1 receptor number, conformational changes resulting from the deletion, and/or removal of specific modulatory sites from the IP₃R1 protein.

The visible seizure phenotype displayed by *opt* begins ~2 weeks after birth, which coincides with the formation of synapses between Purkinje cells and parallel fibers of the granule cells (Altman, 1972), and prominent expression of IP₃R1 mRNA (Furuichi et al., 1993) and protein (Maeda et al., 1989) in the cerebellum of

wild-type mice, specifically in the somata and dendritic arbors of the Purkinje neurons (Ryo et al., 1993). Although we cannot formally exclude the possibility that a second tightly linked gene mutation might also contribute to the phenotype of *opt*, data presented here strongly suggest that alteration of the IP₃R1 protein is likely to be the primary deficit responsible for the physiological and behavioral changes seen in *opt*.

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