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Genetic Mapping and Evaluation of Candidate Genes for Spasmodic, a Neurological Mouse Mutation with Abnormal Startle Response

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Spasmodic (*spd*) is a recessive mouse mutation characterized by a prolonged righting reflex, fine motor tremor, leg clasping, and stiffness. Using an intersubspecific backcross that segregates *spd*, we placed *spd* on Chr 11 with the following gene order: *Adra-1*–3.8 ± 2.1 cM–*Pad-1*–6.3 ± 2.7–(*spd, Anx-6, Csfgm, Glr-1, Il-3, Il-4, Il-5, Sparc*)–9.1 ± 2.4–*D11 Mit5*–2.2 ± 1.5–*Asgr-1*. This localization eliminated the α_1-adrenergic receptor (*Adra-1*) and the α_1 and γ_2 subunits of the GABA$_A$ receptor as candidate genes. Two other promising candidate genes, annexin VI (*Anx-6*) and a glutamate receptor (*Glr-1*), were mapped to within 2.1 cM of the *spd* locus. Although no recombination was observed between *spd* and *Anx-6* or *Glr-1*, no evidence was obtained for a lesion in either gene. The presence of normal *Anx-6* and *Glr-1* mRNA transcripts was confirmed by Northern blot analysis, *in situ* hybridization, and DNA sequence analysis. The localization of *Anx-6* and *Glr-1* extends the known synteny homology between human chromosome 5q21–q31 and mouse Chr 11 and reveals the probable chromosomal location of the human counterpart to *spd*. Synteny homology and phenotypic similarities suggest that spasmodic mice may be a genetic model for the inherited human startle disease, hyperekplexia (STHE). © 1993 Academic Press, Inc.

INTRODUCTION

Spasmodic (*spd*) is a spontaneous mouse mutation that arose on an A/HeJ background in 1979 at The Jackson Laboratory (Lane *et al.*, 1987). *spd* segregates as a recessive single gene defect with a complex neurological phenotype. Mice homozygous for this mutation exhibit a fine motor tremor and clasp their hind legs when held by the tail. Several other aspects of their phenotype, including stiffness, a toe-walking spastic gait, and prolonged righting reflex, vary in severity among individual mice and are exacerbated when the mice are disturbed. In fact, undisturbed *spd/spd* mice can appear normal. Preliminary neuroanatomical studies reveal no obvious abnormalities in brains of *spd/spd* mice and no alterations in the major myelin proteins and lipids (Lane *et al.*, 1987).

Pharmacological studies suggest that the defect in *spd/spd* mice could be a deficiency in inhibitory neurotransmission or an excess of excitatory neurotransmission. GABA (γ-aminobutyric acid) and glycine are the major inhibitory neurotransmitters in the brain, and normal mice given subconvulsive doses of the glycine receptor antagonist strychnine display a phenotype that resembles that of spasmodic mice (Heller *et al.*, 1982). Moreover, the phenotype of the *spd/spd* mutants is ameliorated with aminooxyacetic acid (AOAA), an agent that inhibits the degradation of the inhibitory neurotransmitter GABA. The *spd* mutation may directly or indirectly affect a component of the glycinergic or gabaergic systems. The interdependence of neurotransmitter systems and the pleiotropic action of many drugs make it difficult to narrow the field of candidate genes with additional pharmacological studies. A genetic approach offers a powerful mechanism for unambiguously assessing candidate genes.

Classical genetic studies placed *spd* on Chr 11 (Lane *et al.*, 1987). Three neurotransmitter receptor genes warranted further consideration as candidates for *spd* by their localization on Chr 11 (Buckwalter *et al.*, 1991; 1992; Oakey *et al.*, 1991). *Adra-1*, the α_1-adrenergic receptor gene, is involved in noradrenergic neurotransmission. The leg-clasping and muscle rigidity characteristic of spasmodic mice (Fig. 1) can be produced in normal rodents by alterations in noradrenaline levels (Mogilnicka *et al.*, 1983). *Gabra-1* and *Gabrg-2*, the α_1 and γ_2 subunit genes of the GABA$_A$ receptor, are excellent candidates because of the amelioration of the spasmodic phenotype by AOAA (Lane *et al.*, 1987). The ambiguity in comparing independent maps necessitated testing these genes for cosegregation with *spd*.

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamate receptors are postulated to play roles in excitation-induced neuronal cell death and neurodegenerative disorders (Michae-

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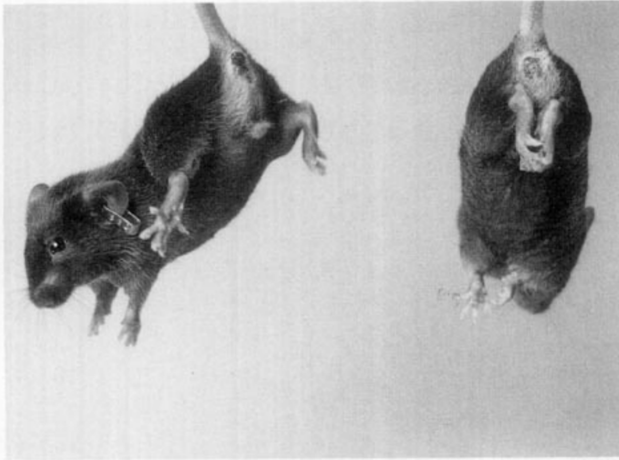

FIG. 1. Muscle rigidity and leg-clasping are characteristic of spasmodic mice. When suspended in the air normal mice arch their back, spread their feet and attempt to right themselves (left). Spasmodic mice become rigid and clasp their hind legs (right).

lis *et al.*, 1992). At least 20 glutamate receptor genes are known to be expressed in the CNS. The human *Glr-1* gene, also known as GluR-K1 (Hollmann *et al.*, 1989), GluHI and GluR1 (Puckett *et al.*, 1991), and GluR-A (Keinänen *et al.*, 1990), has been mapped to chromosome 5q33 (Puckett *et al.*, 1991), and synteny homology suggests that the mouse gene would map on mouse Chr 11 or 18 (Westbrook *et al.*, 1992). Thus, the role of *Glr-1* in excitatory neurotransmission and the likelihood of linkage conservation suggested *Glr-1* as a candidate gene for *spd*.

The important role of calcium in neurotransmission suggested the merit of examining candidate genes such as annexin VI (*Anx-6*). Anx-6 exhibits calcium-dependent binding to membranes, and it is implicated in endocytosis (Lin *et al.*, 1992) and regulation of the ryanodine-sensitive Ca^{2+} channel (Díaz-Muñoz *et al.*, 1990). *Anx-6* is expressed in many cell types, including lymphocytes and neurons, but not glial cells (Owens *et al.*, 1984; Woolgar *et al.*, 1990). As a membrane binding protein probably involved in calcium-dependent endocytosis, Anx-6 may be, either directly or indirectly, involved in release of neurotransmitter vesicles from neurons or neurotransmitter uptake from synapses. Prior to this study, *Anx-6* had been assigned to Chr 11 with the use of somatic cell hybrids, under the name of p68 calcium-binding protein (*Cabm*), but not localized (Davies *et al.*, 1989).

In this paper we report additional characterization of the *spd/spd* phenotype, localization of *spd* with respect to 11 molecular markers, assessment of *Adra-1, Gabrg-2, Gabra-1, Glr-1,* and *Anx-6* as candidates for *spd,* and we discuss evidence that *spd* may be a mouse model for the human disease hyperekplexia (STHE).

MATERIALS AND METHODS

Mice. Mice carrying the spasmodic mutation (B6C3Fe-*a/a-spd/spd*) and *Mus musculus castaneus* mice (inbred strain *CASA/Rk*) were purchased from The Jackson Laboratory (Bar Harbor, ME). Female B6C3Fe-*a/a-spd/spd* mice were mated to *CASA/Rk* males and the male F1 progeny were backcrossed to B6C3Fe-*a/a-spd/spd* females. Progeny of the backcross were killed at 4–6 weeks of age. Backcross progeny were classified as *spd/spd* if they displayed any of the following characteristics: a fine motor tremor, clasping of the hind legs when suspended by the tail, or delayed righting reflex.

C57BL/6J and C3HeB/FeJ mice, purchased from The Jackson Laboratory, were bred to produce F1 mice to serve as normal controls.

All experiments were approved by the University of Michigan Committee on Use and Care of Animals and all animals were housed and cared for according to NIH guidelines.

Electrocortical recordings. Mice were anesthetized with avertin (1.25% tribromoethanol/amyl alcohol solution) by intraperitoneal injection (0.02 ml/g). Silver wire electrodes (0.001-in. diameter) soldered to a microminiature connector were implanted bilaterally into the subdural space over frontal and parietal cortex. Electrocortical activity was recorded 24 h after surgery on mice moving freely in the test cage using a Grass Model 6 electroencephalograph. Ketamine was administered at 100 mg/kg.

Isolation of DNA and RNA. Approximately 3 cm of tail tissue was biopsied from 145 backcross animals, and a salt extraction procedure (Miller *et al.*, 1988) was used to isolate high-molecular-weight genomic DNA. Liver, kidney, lung, and spleen from each animal were snap-frozen in liquid nitrogen and stored at −70°C. High-molecular-weight genomic DNA was prepared from frozen organs as described (Buckwalter *et al.*, 1991). RNA was isolated from whole brains by homogenization in guanidinium thiocyanate and centrifugation through a CsCl gradient (Chirgwin *et al.*, 1979). Polyadenylated RNA was affinity purified by oligo(dT)-cellulose chromatography.

Locus mapping. Allele typing at *Anx-6, Glr-1, Adra-1, Csfgm/Il-3* (granulocyte–macrophage colony stimulating factor and interleukin 3), *Il-5* (interleukin 5), *Pad-1* (a mouse mammary tumor virus insertion site), *Sparc* (osteonectin), and *Asgr-1* (asialoglycoprotein receptor-1) was performed with Southern blots (Buckwalter *et al.*, 1991). CF4.2, a full-length murine annexin VI cDNA, was donated by Stephen E. Moss, University College, London (Moss *et al.*, 1988). To generate a *Glr-1* probe, cDNA was synthesized from B6C3Fe-*a/a-spd/spd* and (C57BL/6J × C3HeB/FeJ)F1 brain RNAs with reverse transcriptase (avian myeloblastosis virus reverse transcriptase, Seikakagu America, Inc.) and amplified with the primers K1 and K2, 5′-TCAGGCAGATTCCAAGCAG-3′ and 5′-CTGTGGGTAGCCCAAGTCAT-3′ (Puckett *et al.*, 1991). Amplified product, corresponding to amino acids 135 to 305 of the 889 amino acid protein, was excised from an agarose gel and used directly for labeling. Probes used for typing *Adra-1, Csfgm/Il-3, Sparc,* and *Asgr-1* have been described previously (Buckwalter *et al.*, 1991). Allele typing at *Il-4* (interleukin 4), *Glns* (glutamine synthetase), *D11Mit1,* and *D11Mit5* was accomplished by PCR (Buckwalter *et al.*, 1992; Love *et al.*, 1990).

Statistical analysis. Genetic distances are given in centimorgans with the estimated standard deviation and 95% confidence intervals calculated as described previously (Buckwalter *et al.*, 1991). When no recombination events are observed, the 95% confidence interval of the distance between the two markers is calculated as in Lyon and Searle (1989).

Cloning and sequencing. *Anx-6* cDNA was amplified in two overlapping parts. Nucleotides 17 to 1308 of the cDNA were amplified with the oligonucleotides 5′-CCGGATCCTAGCGCGTGGTTTCTGCTGC-3′ and 5′-GGTCTAGAGAGCCCCAGAATCAGCCTTGC-3′, and nucleotides 1211 to 2355 were amplified with the primer pair 5′-GGGGATCCCATCGACATCGTCACTCACC-3′ and 5′-GGTCTAGAGTGACAAAGCTCAAGTCAGG-3′ (Moss *et al.*, 1988). Amplified products were purified by agarose gel electrophoresis, digested with *Bam*HI and *Xba*I restriction endonucleases (Bethesda Research Laboratories), and ligated into pUC18 plasmid. Sequencing was performed with M13 forward and reverse primers (Boehringer Mannheim Biochemicals) and 15-bp primers corresponding to nucleotides 224–238, 284–298, 514–528, 730–744, 1083–1097, 1489–1503, 1770–1784, and 2153–2167.

Segments of the *Glr-1* cDNA corresponding to nucleotides 206 to

1142 and 1135 to 2870 of the rat cDNA (GenBank Accession No. M36418) (Hollmann *et al.*, 1989) were amplified using the oligonucleotide pairs 5′-TTCGCCAATGTAAAAAGGAATATGCCGTA-3′ and K2 (see locus mapping) and 5′-TCCCAAAGCCTGCGGAGGCAG-AGGATTGA-3′ and 5′-GGGCATCCCTGAACTGTGACTCATGCA-GGG-3′, respectively. The alternatively spliced exon was cloned using primers spanning nucleotides 2450–2965, 5′-GCGGATCCGGCTATG-GCATTGCGACACC-3′ and 5′-GGTCTAGAGGCACTGAAGGGC-TTGGGATGGGGCTG-3′. To clone the 5′ untranslated region, primers 5′-CCTCTAGACGAGCTCGGCTCCCCTTCC-3′ and 5′-CGGGTACCTAAAAGCCGCATGTTCC-3′ were used to amplify nucleotides 29–306. After amplification, the products were separated from primers on 1% agarose/1× TAE gels and purified by centrifuging gel slices through glass wool. The DNA was digested with restriction enzymes or incubated with T4 polymerase to create blunt ends and cloned into pUC18. Sequencing reactions were performed using Sequenase T7 polymerase (United States Biochemicals) and the primers used for amplification as well as M13 forward and reverse primers and additional 15-mers, representing nucleotides 609–623, 1366–1381, 1619–1633, 1865–1879, and 2291–2305 of *Glr-1*.

In situ hybridization. The specific oligonucleotides for the neurotransmitter receptor isoforms GluR-A flip, GluR-A flop, and GluR-B flip were as described (Sommer *et al.*, 1990). The NMDAR1 (*N*-methyl-D-aspartate receptor 1) oligonucleotide probe corresponded to amino acids 138–152: 5′-AAACAAGACGCTGGACTGGTGGGA-GTAGGGCGGCACCGTGCGAAG-3′ (Moriyoshi *et al.*, 1991). Probes were 3′-end labeled with [^{35}S]dATP (sp act >1000 Ci/mmol, Dupont-NEN) and terminal deoxynucleotidyl transferase using a Dupont-NEN kit (NEP 100).

Brains from two spasmodic mice and two littermate controls (age 10 weeks) were frozen on dry ice and stored at −70°C. Twelve-micrometer brain sections from the midsagittal region were mounted onto poly-L-lysine (Sigma)-coated slides. Slides were fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4 (10 min), rinsed in three changes of 0.1 *M* phosphate buffer with 0.9 g per liter NaCl, pH 7.4 (5 min each), acetylated in 0.1 *M* triethanolamine, pH 8.0, with 0.25% acetic anhydride (10 min), rinsed in PBS (5 min), dehydrated through graded ethanol solutions (2 min each), defatted in 100% chloroform (5 min), and partially rehydrated through 100 and 95% ethanol (1 min each). The hybridization solution was 50% formamide, 0.3 *M* NaCl, 10 m*M* Tris, pH 8.0, 1 m*M* EDTA, 10% dextran sulfate, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin; Sigma), and 100 m*M* dithiothreitol. Radiolabeled oligonucleotides were added to a concentration of approximately 30,000 dpm per milliliter of hybridization solution. Sections were incubated in 50 μl hybridization solution overnight at 37°C. The slides were washed to a final stringency of 0.5× SSC (40 min, 50°C), rinsed with 70% ethanol, air-dried, and exposed to film (βmax, Amersham) for 2 to 3 weeks. Specificity of hybridization was tested by pretreating sections with ribonuclease A (Sigma; 50 mg/ml in 100 m*M* Tris, 25 m*M* EDTA, pH 8.0, 37°C for 30 min) or adding a 10-fold excess of unlabeled DNA probe to the hybridization buffer. These treatments abolished the hybridization signal.

RESULTS

Electrocortical activity in spd homozygotes. Direct electrocortical recordings from chronically implanted spasmodic mice showed that the amplitudes and frequencies of the baseline resting EEG activity in adult homozygotes were unremarkable when compared with those from normal control mice, and no abnormal spontaneous discharges were noted during exploratory behavioral activity (Fig. 2). We observed an unusual reaction during anesthesia in spasmodic mice. Administration of ketamine dramatically enhanced several neurological features of spasmodic mice, including a greatly exaggerated tremor and stiffness. Brain waves were unchanged following ketamine injection; however, in response to an auditory stimulus the mice demonstrated vigorous body tremor at 12–14 Hz lasting from less than 1 s to several seconds. Although it was not possible to record from the unrestrained mouse without movement artifact during this period, cortical activity was entirely normal immediately following the cessation of movement (Fig. 2), and the same stimulus presented to the mouse when it was manually restrained revealed normal EEG rhythms during auditory stimulation. Repetitive stimulation induced brisk startle responses with no evidence of habituation.

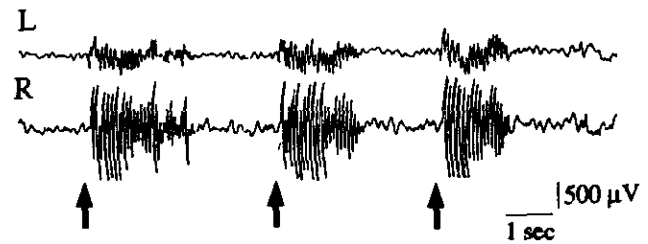

FIG. 2. Normal EEG activity during violent auditory-induced myoclonus in adult *spd/spd* mice distinguishes these events from epileptic seizures. Bilateral neocortical EEG tracings (L, left cortex; R, right cortex) recorded from chronically implanted mice show response to repeated auditory stimuli following injection of ketamine. Each stimulus (arrow) immediately evokes a violent burst of 12–14 Hz tremor (rapid oscillating movement artifact in trace), followed by an instant return of the normal baseline EEG activity. Unchanged amplitude of episodes reveals lack of habituation to repeated startle stimuli.

Genetic mapping of the spd locus eliminates three candidate genes, Adra-1, Gabra-1, and Gabrg-2. Since the discovery of the spasmodic mutation in A/HeJ mice at The Jackson Laboratory in 1979, it has been maintained by breeding carriers to B6C3Fe-*a/a* F1 mice, which were constructed from C57BL/6JLe and C3HeB/FeJLe-*a/a* mice. Thus, the alleles for *spd* and closely linked loci are likely to be derived from A/HeJ, whereas C57BL/6JLe or C3HeB/FeJLe alleles will be present at most other loci. We constructed a backcross between the stock carrying *spd* and *M. m. castaneus* [(B6C3Fe-*a/a-spd/spd* X CASA/Rk) F1 X B6C3Fe-*a/a-spd/spd*]. Of the 145 progeny collected, 73 were typed as *spd/spd* and 72 as *spd*/+, based on their phenotype. Tremor, delayed righting reflex, and leg clasping were noted among all of the animals classified as *spd/spd*, except one mildly affected individual that exhibited only leg clasping. The classification of this animal was confirmed genetically by demonstrating that it was homozygous for B6C3Fe alleles at all loci tested. Two animals were phenotypically classified as *spd*/+, but were homozygous for B6C3Fe alleles at seven loci on Chr 11, suggesting that they may represent examples of incomplete penetrance. These 2 animals were excluded from analysis. One hundred forty-three backcross progeny were analyzed at 11 loci in addition to *spd*. *M. castaneus* alleles of all 11 loci were readily distinguished from the 3 alleles potentially present in the *spd* stock.

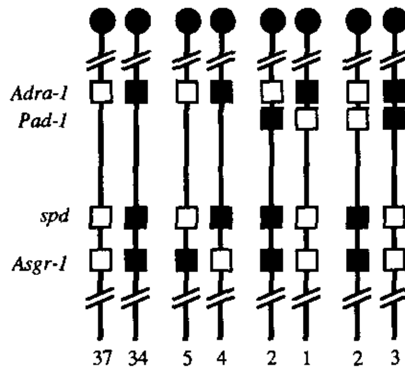

FIG. 3. Haplotype analysis of 88 backcross progeny demonstrate that *spd* is between *Adra-1* and *Asgr-1*, distal to *Pad-1*. Analysis of the individuals exhibiting recombination between *Adra-1* and *spd* resulted in the unambiguous gene order *Adra-1-Pad-1-spd-Asgr-1*. All other gene orders result in multiple double crossover events. The number of animals observed with each haplotype is listed below the schematic of the chromosome. The genotypes *spd/spd* and *spd/+* are depicted at each locus tested (left) by open and closed boxes, respectively.

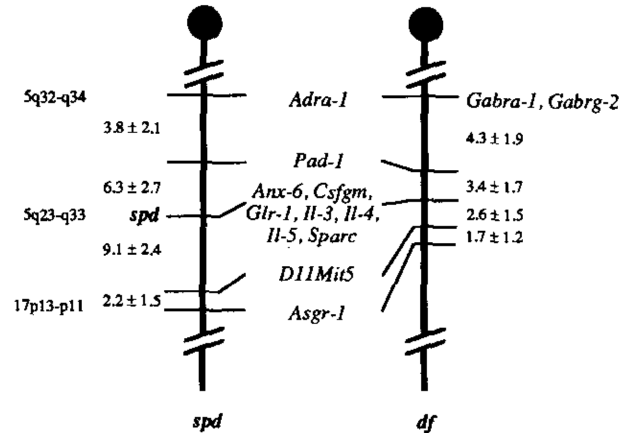

FIG. 4. Comparison of genetic maps of mouse chromosome 11. Haplotype analysis and minimization of crossover frequency were used to determine genetic distances in backcrosses segregating *spd* (left) and *df* (right) (Buckwalter *et al.*, 1991). Eleven individual loci were mapped on both crosses (center). *Gabra-1* and *Gabrg-2* were mapped on the *df* cross (Buckwalter *et al.*, 1992). No recombination was observed between *Adra-1*, *Gabra-1*, and *Gabrg-2* in 96 animals. Genetic distances and the estimated standard deviation (cM) are reported for each interval. The human chromosomal locations of each locus group are listed (GDB), except for *Pad-1*, *spd*, and *D11Mit5*, which are unknown.

We tested the first few backcross progeny with several markers on Chr 11 to determine which interval contained the *spd* locus. After localizing *spd* to the region between *Adra-1* and *Asgr-1*, we employed an interval mapping strategy that focused on recombinants between these two loci (Fig. 3). Results of this analysis unambiguously placed *spd* distal to *Pad-1*.

To increase efficiency, later typings were performed by PCR amplification of simple sequence repeat polymorphisms at a different pair of flanking markers, *Glns* and *D11Mit5*, which map 19.5 ± 6.2 cM proximal and 9.1 ± 2.4 cM distal to *spd*, respectively. Thirty-one recombinant progeny identified by interval mapping with this pair and the previous pair of flanking markers were typed with additional markers. No recombinants were observed between *spd* and *Csfgm/Il-3*, *Il-4*, *Il-5*, and *Sparc*. The gene order and genetic distances observed in this cross (Fig. 4) are consistent with those generated in another intersubspecific backcross with *M. m. castaneus* (Buckwalter *et al.*, 1991) and with the consensus map of Chr 11 (Buchberg *et al.*, 1992).

Three neurotransmitter genes were considered as candidate genes for *spd: Adra-1*, *Gabra-1*, and *Gabrg-2*. *Adra-1* was mapped 9.3 ± 1.9 cM proximal to *spd*. The detection of numerous recombination events between *Adra-1* and *spd* demonstrates that they are distinct loci and eliminates *Adra-1* as a candidate gene. *Gabra-1* and *Gabrg-2* were considered especially good candidates for *spd* because they are part of the gabaergic neurotransmitter system. We mapped these two genes 4.3 ± 1.9 cM proximal to *Pad-1* (Buckwalter *et al.*, 1992), and *spd* distal to *Pad-1* (Fig. 4). The placement of *spd* in a different interval of the chromosome from that containing *Gabra-1* and *Gabrg-2* eliminated them as candidates for *spd*.

Analysis of Anx-6 as a candidate gene for spd. We used Southern blots to genotype animals at *Anx-6* (Fig. 5). No recombination was observed between the *spd* locus and this gene in 143 backcross progeny, localizing *Anx-6* to within 2.1 cM (95% confidence interval) of *spd*. Northern blots of whole brain RNA from spasmodic and control (C57BL/6J × C3HeB/FeJ)F1 mice demonstrated that *Anx-6* mRNA was present in spasmodic mice in normal quantities (Fig. 6). Transcript size appeared to be 2.4 kb, unaltered relative to normal. Therefore, it is unlikely that spasmodic mice carry a defect in *Anx-6* that affects gene transcription, mRNA stability, splicing, or polyadenylation.

To screen for point mutations, mRNA from spasmodic and control (C57BL/6J × C3HeB/FeJ)F1 mice

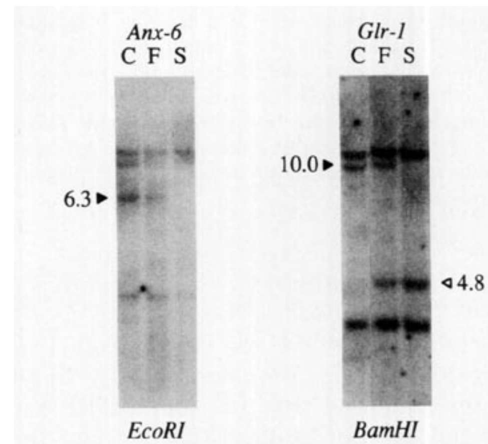

FIG. 5. Southern blot identification of RFLPs in the *Anx-6* and *Glr-1* genes. Genomic DNA from CASA/Rk mice (C), (CASA/Rk X B6C3Fe-*a/a-spd/spd*)F1 mice (F), and B6C3Fe-*a/a-spd/spd* mice (S) was digested with the indicated restriction enzyme (bottom) and hybridized with cDNA probes for *Anx-6* (left) or *Glr-1* (right). Closed arrowheads indicate the molecular weights, in kb, of hybridizing restriction fragments unique to CASA/Rk DNA, and the open arrowhead indicates a fragment unique to B6C3Fe-*a/a-spd/spd* DNA.

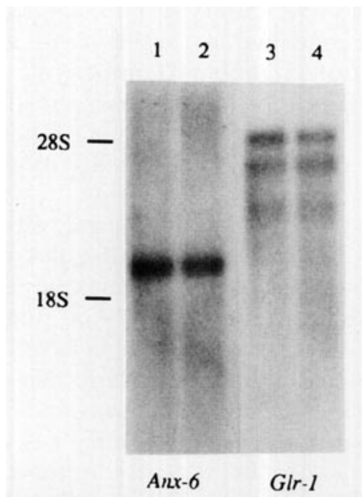

FIG. 6. Northern blots demonstrate the presence of *Anx-6* and *Glr-1* transcripts in the brains of spasmodic mice. Whole brain poly(A)+ RNA was analyzed by Northern blotting. Filters from gels containing 1 and 3 μg of RNA were hybridized with cDNA probes for *Anx-6* and *Glr-1*, respectively. The locations of 28S and 18S RNA are indicated on the left. Lanes **1** and **3**, B6C3Fe-*a*/*a*-*spd*/*spd*; lanes **2** and **4**; (C57BL6/J X C3HeB/J)F1 hybrids.

was reverse transcribed and amplified with *Anx-6*-specific primers. Clones comprising the entire coding region and all but 17 bp of the 5′ and 45 bp of the 3′ untranslated regions of *Anx-6* were isolated and sequenced from each mRNA sample. Sequences were compared to the published mouse sequence (Moss *et al.*, 1988; Accession No. X13460). Four potential basepair substitutions in the cloned sequences were found to be PCR or cloning artifacts by directly sequencing a pool of PCR products. One silent change was noted in the coding sequence, a G to an A at nucleotide 831. This change probably represents strain variation from the published *Anx-6* sequence because it was also present in the sequence we obtained from the F1 control mice. In the 3′ untranslated region, five base differences were noted between *spd* and the published sequence of *Anx-6,* including G to A changes at nucleotides 2056, 2083, and 2149, an insertion of a G after nucleotide 2213, and an A to C change at nucleotide 2284. These differences are attributable to strain variation because they were found in the F1 control mice. Thus, there is no evidence for mutations in the coding region or 5′ and 3′ untranslated regions of *Anx-6*.

Evaluation of Glr-1 as a candidate gene for spd. We developed a mouse cDNA probe for *Glr-1* (Materials and Methods) and used it to genotype backcross progeny at *Glr-1* (Fig. 5). No recombinants were observed between *spd* and *Glr-1* in 143 chromosomes examined, indicating that the two genes are located within 2.1 cM (95% confidence interval) of each other. Northern blots performed on whole brain RNA from spasmodic mice and control (C57BL/6J × C3HeB/FeJ)F1 mice demonstrated that the steady-state levels of *Glr-1* transcripts were normal (Fig. 6). In addition, normal *Glr-1* transcripts of approximately 5.0, 3.9, and 2.7 kb were present in the spasmodic RNA (Fig. 6) (Hollmann *et al.*, 1989). Because the alternatively spliced forms of *Glr-1* are known to be functionally different (Sommer *et al.*, 1990), a physiologically significant change in cell-specific expression might be undetectable by Northern blot. We examined the distribution of *Glr-1* mRNA in brain of spasmodic mice and littermate controls by *in situ* hybridization (Fig. 7). The expression of both isoforms of *Glr-1* mRNA was identical in *spd*/*spd* and normal mice, indicating that cell-specific expression and alternate splicing of *Glr-1* are normal in spasmodic mice.

Glr-1 cDNA was cloned from spasmodic whole brain RNA, sequenced, and compared to the cDNA sequences of rat and human (GenBank Accession Nos. M36418 and M64752, respectively) and selected portions of our control F1 mouse. The predicted amino acid sequence of the mouse cDNA was identical to the rat sequence in all but three amino acids. Amino acid 257 is an alanine in the rat and a serine residue in the mouse. This conservative difference is not significant because the human GLR1 cDNA predicts a serine in this position (Puckett *et al.*, 1991). The other two differences between the spasmodic mouse and rat *Glr-1* cDNA sequences were amino acid 843 (tryptophan in the mouse and arginine in rats and humans) and amino acid 853 (serine in the mouse and glycine in rats and humans). These two differences represent species variation because we also observed them in the cDNA from (C57BL/6J × C3HeB/FeJ)F1 mice. Several glutamate receptor mRNAs undergo RNA editing within the second transmembrane region, resulting in alteration of receptor properties (Sommer *et al.*, 1991). *Glr-1* mRNA is not normally edited, and we found no evidence for editing in the *Glr-1* cDNA of spasmodic mice. The 3′ untranslated region of *Glr-1* transcripts varies between approximately 50 and over 1000 nucleotides. The first 42 nucleotides of the 3′ untranslated region were examined and the spasmodic and F1 control mouse sequences were identical. The rat *Glr-1* cDNA

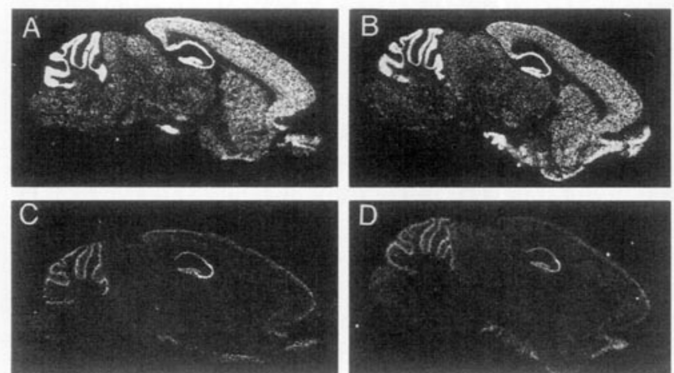

FIG. 7. The cellular distribution of alternatively spliced *Glr-1* isoforms is normal in *spd*/*spd* mouse brain. *Glr-1* is an α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA)-type glutamate receptor. Two functionally different isoforms, flip and flop, generated by alternative splicing of two distinct 115-bp exons, are differentially expressed in well-defined regions of the brain (Sommer *et al.*, 1991). The expression and distribution of two alternatively spliced forms of *Glr-1*, flop (**A** and **B**) and flip (**C** and **D**), were analyzed by *in situ* hybridization. All hybridizations were performed on midsagittal brain sections in two separate experiments, with the same result. (**A, C**) Normal littermate control; (**B, D**) *spd*/*spd* mouse.

clone contains a 5′ untranslated region of approximately 197 nucleotides, with out-of-frame ATGs located 14 and 43 nucleotides upstream of the consensus AUG. The mouse *Glr-1* 5′ untranslated region also contained these additional ATGs, and the sequences 168 bp upstream of the consensus ATG were identical in spasmodic and F1 control mice. Thus, the *Glr-1* cDNA from spasmodic mice has no significant differences in the coding region or 5′ untranslated region.

DISCUSSION

Mapping *spd* relative to other genes known to be on Chr 11 allowed us to rule out three promising neurotransmitter candidate genes. *Adra-1* was eliminated after direct observation of multiple crossover events between it and *spd*. *Gabra-1* and *Gabrg-2* map proximal to *Pad-1* and were eliminated by our localization of *spd* distal to *Pad-1*.

We report the first localization of *Anx-6* and *Glr-1* in mice. Both genes mapped within 2.1 cM of *spd* on MMU 11. Since the human counterparts to *Anx-6* and *Glr-1* have been localized to chromosome 5q32–q34 (Davies *et al.*, 1989) and 5q33 (Puckett *et al.*, 1991), our mapping extends the known synteny homology between human chromosome 5q and mouse Chr 11 (Buckwalter *et al.*, 1991, 1992). These loci were considered as candidates for *spd*; however, we did not detect any evidence for rearrangement or deletion of *Anx-6* or *Glr-1* in spasmodic mice by Southern blot analysis of genomic DNA (data not shown). In spasmodic mouse brains, the abundance and molecular weight of *Anx-6* and *Glr-1* mRNA transcripts were normal, and the two alternatively expressed forms of *Glr-1* exhibited appropriate cell-specific expression. Sequence analysis revealed no significant changes in the coding region or 5′ untranslated region of either *Anx-6* or *Glr-1* cDNAs. While functional analysis of the proteins has not been carried out, the lack of significant nucleotide changes in the cDNA sequences suggests that alteration of *Glr-1* or *Anx-6* function in spasmodic mice is unlikely. Altered transcriptional regulation has not been directly examined but seems unlikely in view of the normal steady-state levels of both transcripts. However, subtle differences in regulation of gene expression in the mutant cannot be ruled out. The normal size of the transcripts indicates that the lesion in *spd* mice does not result from a gross rearrangement of *Anx-6* or *Glr-1* or from errors in RNA splicing or polyadenylation. Thus, there is no evidence for mutations in either *Glr-1* or *Anx-6* in spasmodic mice.

It is not unique to have a number of candidate genes closely linked to a mutant locus. For example, the familial adenomatosis polyposis (FAP) gene was identified after several candidate genes, including the *MCC* gene, were analyzed. The sex-determining gene *Sry* was uncovered after evidence accumulated against the neighboring candidate *Zfy*. In some cases clustering of related genes may reflect evolutionary history and/or shared regulatory elements, as shown for the clustered hemoglobin and homeobox gene families. While *Anx-6* and *Glr-1* are both members of large gene families, all members of these two families that have been mapped to date are unlinked. Nevertheless, the mutation in spasmodic mice may be in an uncharacterized annexin or glutamate receptor gene.

Five loci that cosegregated with *spd* were considered poor candidate genes for the mutation and were not pursued further. Although some cytokines play a role in neuronal differentiation (Hall *et al.*, 1992), the main function of *Il-3*, *Il-4*, *Il-5*, and *Csfgm* is believed to be in hematopoiesis and the immune system, neither of which has been reported to be altered in spasmodic mice. *Sparc* codes for osteonectin, a high-affinity calcium binding protein component of the extracellular matrix (reviewed in Sage *et al.*, 1991). We consider it unlikely that *Sparc* is mutated in spasmodic mice because *Sparc* expression predominates in nonneuronal tissues (Holland *et al.*, 1987). Higher resolution genetic analysis may provide further support for elimination of the linked genes as candidates for *spd*.

Spasmodic mice provide a defined genetic model for neurological syndromes featuring an abnormal startle response. The underlying lesion in abnormal startle syndromes appears to alter the amplitude of the initial reflex response to sensory stimuli and the subsequent rate of stimulus habituation. Interactions between these two components may explain why the central pathways involved in modifying the startle and related motor reflex responses extend beyond the primary sensory relay nuclei to involve limbic, brain stem reticular, cerebellar, and other descending spinal pathways (Davis, 1989; Thompson, 1988). The associated tremor and impaired righting reflex in spasmodic mice both point toward involvement of spino–cerebellar or cerebello–thalamic pathways, suggesting possible impairment of polysynaptic cerebellar outflow pathways in the brain stem. These pathways are essential for modulating adaptive gain changes in motor reflex circuits (Thompson, 1988). Pathological startle can be modulated by selective pharmacological manipulation of signaling by multiple neurotransmitters, including glutamate, gaba, noradrenaline, serotonin, dopamine, and selected peptides (Ebert *et al.*, 1992; Liang *et al.*, 1992; Snodgrass, 1990). The CNS anesthetic ketamine used to potentiate the startle response in the *spd/spd* mutants is a noncompetitive inhibitor of excitatory glutamatergic synaptic transmission at the NMDA receptor site and has been shown to facilitate the startle response (Falls *et al.*, 1992; Mansbach *et al.*, 1991).

The diversity of these experimental phenocopies at the molecular level suggests a reasonable likelihood for genetic heterogeneity among human abnormal startle syndromes. The available evidence suggests that startle epilepsy, or hyperekplexia (STHE), is a genetically homogeneous startle disorder (Ryan *et al.*, 1992b). Several phenotypic characteristics of STHE suggest that the *spd* mutant locus provides a promising mouse model for developmental analysis of STHE. First, the absence of

EEG abnormalities, as found clinically (Ryan *et al.*, 1992a), suggests that the lesion is subcortical, while the absence of weakness or muscular wasting excludes the involvement of lower motor neuron pathways. Second, flexion hypertonia was evident in the hindlimbs, as described in the extremities of young affected members of the human pedigree, although this diminished with age. The symptoms of STHE can be effectively treated with clonazepam (Ryan *et al.*, 1992a), which, like AOAA, acts in part by stimulation of the gabaergic neurotransmitter system (Greenblatt *et al.*, 1987). Thus, STHE and *spd* are similar in several aspects of phenotype and pharmacology.

Genetic studies provide the most convincing evidence for spasmodic mice as a model for STHE. STHE has recently been mapped to a 5-cM region of human chromosome 5, which is proximal to *GABRA1* and *GABRG2*, but includes *SPARC, GLR1, RPS14,* and *CSF1R* (Ryan *et al.*, 1992b). The mouse homologues of these genes map to two small regions (0–2 cM), one on mouse chromosome 18 (*Rps14* and *Csfmr*) and the other on mouse chromosome 11 (*Sparc* and *Glr-1*). The demonstration that *spd,* like STHE, is linked to *Gabra-1, Gabrg-2, Sparc,* and *Glr-1* suggests that *spd* is the mouse counterpart to STHE. Although we cannot exclude the possibility that the mouse counterpart to STHE is located on chromosome 18, there are currently no mutants on chromosome 18 with abnormal startle response. STHE is inherited as an autosomal dominant trait and *spd* as a recessive trait, and there are some phenotypic differences between the affected mice and humans. However, many diseases have both dominant and recessive mutant alleles, and different lesions in the same gene can result in dramatically different phenotypes. Regardless of whether *spd* and STHE represent lesions in homologous genes, the similar linkages of the mutant genes suggest that *ANX6* and *GLR1* should be examined for mutations in humans with STHE and imply that efforts to identify the mutant genes will be complementary. The spasmodic mouse will provide further information about abnormal startle through future studies on the pharmacology of *spd* and identification of the mutant gene by positional cloning.

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Note added in proof. Higher resolution genetic analysis has provided support for the elimination of *Anx-6* and *Glr-1* as canidate genes for *spd.* Two recombinants were observed between *spd* and *Glr-1* in a total of 313 individuals examined, and one recombinant was noted between *spd* and *Anx-6* in 407 individuals tested (L. M. Scarlett and N. M. Tokarz).

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