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Absence epilepsy in *apathetic*, a spontaneous mutant mouse lacking the h channel subunit, HCN2

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Abstract

Analysis of naturally occurring mutations that cause seizures in rodents has advanced understanding of the molecular mechanisms underlying epilepsy. Abnormalities of I_h and h channel expression have been found in many animal models of absence epilepsy. We characterized a novel spontaneous mutant mouse, *apathetic* (*ap/ap*), and identified the *ap* mutation as a 4 base pair insertion within the coding region of *Hcn2*, the gene encoding the h channel subunit 2 (HCN2). We demonstrated that *Hcn2^{ap}* mRNA is reduced by 90% compared to wild type, and the predicted truncated HCN2^{ap} protein is absent from the brain tissue of mice carrying the *ap* allele. *ap/ap* mice exhibited ataxia, generalized spike-wave absence seizures, and rare generalized tonic-clonic seizures. *ap/+* mice had a normal gait, occasional absence seizures and an increased severity of chemoconvulsant-induced seizures. These findings help elucidate basic mechanisms of absence epilepsy and suggest HCN2 may be a target for therapeutic intervention.

Introduction

Absence epilepsy is a common generalized epilepsy syndrome of childhood and adolescence. Several familial generalized epilepsy syndromes have been mapped in humans, and in a majority of cases, the affected gene encodes a voltage- or ligand-gated ion channel (for review see; (Steinlein, 2004)). In addition to heritable generalized epilepsies in humans, many animal models of generalized epilepsy, including absence epilepsy, have been described. Similar to human cases, generalized epilepsy syndromes in animals (arising as either spontaneous mutations in inbred colonies (Noebels, 2006), or resulting from targeted gene deletions (Burgess, 2006)), have overwhelmingly involved genes encoding ion channels.

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Of the spontaneous monogenic mouse epilepsy models, several of the implicated genes are also mutated in human epilepsy syndromes (Noebels, 2003). Mouse mutations lacking a currently appreciated human counterpart are also critical for epilepsy research as they may shed light on basic mechanisms of epileptic neuronal synchronization, offer opportunities to study genetic interactions in epilepsy, and point toward genes causing epilepsy in humans.

Here we report a novel spontaneous mouse mutation, which we have named *apathetic* (*ap*). *Apathetic* (*ap/ap*) mice are ataxic and exhibit brief behavioral arrests and tonic-clonic convulsions phenotypically resembling generalized absence and tonic-clonic convulsions, respectively. EEG studies reveal frequent generalized spike-wave seizures sensitive to ethosuximide, consistent with absence epilepsy. While otherwise appearing neurologically normal, a fraction of mice heterozygous for the *ap* allele also exhibited absence seizures, and *ap/+* mice showed enhanced sensitivity to chemoconvulsive seizures. We positionally identified the mutation in the *ap/ap* mice as a 4 base pair insertion in the gene encoding the hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel (h channel) subunit 2 (*Hcn2*). The *ap* mutation is predicted to introduce 41 novel amino acids and a novel stop codon that truncates the HCN2 protein within the cyclic nucleotide binding domain, but no truncated HCN2 protein was observed in mice harboring the *ap* allele. HCN2 was absent from the brain of *ap/ap* mice and reduced in *ap/+* animals, but we observed no changes in other h channel subunits. Our studies add to the growing body of data implicating h channels in epilepsy, and suggest that the human *HCN2* gene should be considered in future studies of familial epilepsy as well as a potential target for development of new therapeutics.

Materials and Methods

Mice

The *apathetic* (*ap*) mutation arose spontaneously in a colony of DW/Pas mice being studied for obesity/diabetes phenotypes by WKC and RLL in the vivarium at the Rockefeller University (Laboratory Animal Resource Center). Affected male and female *ap* mice were always born from unaffected mating pairs, and were observed approximately 25% of the time in litters with affected animals, suggesting autosomal recessive inheritance. A carrier female was outcrossed to a normal C57BLKS/J male to facilitate initial mapping. Pairs of male and female F1 mice were test crossed until obligate carriers were identified producing affected mice. After initial chromosomal localization, carrier DW/Pas females were crossed to CAST/Ei wild type (+/+) males to increase the number of polymorphic microsatellite markers available for fine mapping. Obligate F1 carriers were identified through test crosses and then used to generate affected F2 animals. *Ap/ap* mice were maintained in federally approved animal facilities at The Rockefeller University, Columbia University, and Northwestern University with 12L/12D cycle and *ad libitum* access to food and water. Animals were weaned at 21 days of age. All animal usage in these studies was approved by The Rockefeller University, Columbia University (IUCAC), and Northwestern University Animal Care and Use Committees (NUACUC).

Mapping the *apathetic* locus

Genome wide linkage analysis was performed using 12 affected F2 DW/Pas C57BLKS/J animals and 44 polymorphic MapPair markers (Research Genetics; Huntsville, AL) spaced at approximately 30 cM intervals across the genome. All mice were phenotyped by a single observer (WKC). Genomic DNA was extracted from spleens using proteinase K digestion and phenol chloroform extraction. Amplicons of polymorphous microsatellite markers were amplified by polymerase chain reaction and separated on a 5% agarose gel and stained with ethidium bromide. After the *ap* locus was linked to mouse chromosome 10 at approximately 40.4 cM from the centromere, further fine mapping was performed in the affected F2 Dw/PAS

× CAST/Ei with an additional 24 markers on chromosome 10 in a total of 473 F2 mice to define a minimal non-recombinant interval between D10Mit139/D10Mit260/D10Mit175 and D10Mit21/D10Mit23 containing the *ap* locus. A complete mouse BAC contig was built across the non-recombinant interval by screening the RTCO-23 C57BL/6J mouse BAC library (http://www.roswellpark.org/Research/Shared_Resources/Microarray_and_Genomics_Resource) for clones containing genes or DNA sequences in the non-recombinant interval. By comparing the sequences of the mouse BAC ends and genes contained within the mouse BACs to human sequence databases, we identified a human cosmid containing the gene *BSG* as well as the 5' end of *Hcn2*. We tested *Hcn2* as a candidate gene, and the coding regions of *Hcn2* were sequenced on genomic DNA by dideoxy sequencing on an ABI 377 according to the manufacturer's instructions in *ap/ap* and control mice.

Genotyping the *Hcn2*^{ap} locus

Genomic DNA was purified from 1mm-length tip of mouse tails using Sigma REDEExtractNamp Tissue Kit (Sigma, MO) following the manufacturer's protocol. The mutation in exon 6 of the *Hcn2* gene containing nucleotide 1669 was amplified by PCR using 5'-primer; 5'-ATGGCTTTCTCCAGT-3' and 3'-primer; 5'-CCCATGCTGGATGAAG-3'. Amplified PCR products were separated in 15% non-denaturing PAGE gels, and visualized by ethidium bromide staining. The PCR fragment generated by the wild type allele was 90 bp and by the *ap* allele was 94 bp.

Behavioral testing

Adult mice 8-16 weeks old were assessed with a simple neurological screen for motor and sensory responses (Paylor et al., 1998). In this screen, a mouse was weighed and measured, then placed into an empty cage and observed for 1 min. The presence of several behavioral responses was recorded (i.e., wild running, freezing, licking, jumping, sniffing, rearing, movement throughout the cage, and defecation). Postural reflexes were then evaluated by determining if the mouse would splay its limbs when in a cage that is quickly lowered and moved from side to side. The righting reflex, whisker touch response, eye blink, and ear twitch were then evaluated. A platform test was conducted by placing the animal in the center of a platform (20 cm), then recording the latency to move to the edge, as well as the number of times the mouse reaches its head over the edge (nose pokes). Two-way (genotype × gender) analyses of variance (ANOVA) were used to analyze the platform test data. Nonparametric statistical tests were used to analyze the remaining data. The rotarod test was performed by placing a mouse on a rotating drum (Ugo Basile) and measuring the time each animal was able to maintain its balance on the rod. The speed of the rotarod accelerated from 4 to 40 rpm over a 10-min period. Mice were given four trials with a 30- to 60-min intertrial rest interval. Rotarod data were analyzed with a three-way (genotype × gender × trial) ANOVA with repeated measures. Significant interactions were subsequently analyzed by use of simple effects tests.

Animal surgery and EEG recording

At 3 months of age, *ap/ap* and *+/+* mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (9 mg/kg). An incision was made on the scalp to expose the skull. Four holes were drilled through the skull to dura, two placed 1mm anterior to the bregma and two placed 7mm anterior to the bregma, each being 1.5 mm lateral to the central sulcus. These holes accommodated a prefabricated mouse headmount (Pinnacle Technologies) which was fastened to the skull with stainless steel screws (Small Parts, Miami Lakes, FL). The headstage was secured to the skull using dental acrylic. Loose skin was sutured around the implant and at least 7 days were allowed before beginning data collection. Following the recovery period, mice were placed individually in a cylindrical (diameter, 10 in.) recording

chambers and allowed *ad libitum* access to food and water. EEG data were collected using a wire tether/commutator system (Plastics One, Roanoke, VA). All signals are amplified 100X at the preamplifier before the tether and swivel arrangement, and 100X at the main amplifier stage (10,000X total). Signals were filtered at 0.5 Hz high pass and 50 Hz low pass, and a 60 Hz digital notch filter was applied to all channels. Sampling at 400 Hz was digitized using a 14-bit A/D converter (Texas Instruments, Dallas, TX), collected and stored on a MacBook Pro (Apple, Cupertino, CA) computer running a PC emulator (VMware Fusion, Palo Alto, CA) and Sirenia software (Pinnacle, Austin, TX). Video recording of EEG sessions was performed with a Canon Optura Xi camera. EEG analysis was performed offline by manually scrolling through 60 second epochs of EEG data to detect repetitive rhythmic (2-8 Hz), high amplitude (>2 fold above background) sharp-wave activity lasting longer than 1 sec.

RT-PCR and qRT-PCR

Total RNA from one hemisphere of *+/+* and *ap/ap* mouse brain was purified using Trizol Reagent and the PureLink Micro-to-midi Total RNA Purification System (Invitrogen, Carlsbad, CA), following manufacturer's protocol. On-column DNA digestion was performed using amplification grade DNase I (Invitrogen, Carlsbad, CA). For qualitative RT-PCR, first strand cDNA synthesis was performed from 1 µg of total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) with random hexamers and oligo(dT), following manufacturer's protocol. From the first-strand synthesis reaction, 3.5 µl were used as template for PCR amplification of full-length *Hcn2* coding mRNA, which was performed under the following conditions: 1 min at 94°C, then 30 cycles of 20 s at 95°C, and 2 min at 68°C, followed by 3 min at 68°C using forward primer 5'-TTCTGCAGCCCCGGCGTCAACAAG-3' and reverse primer 5'-AGGGTGGCAATGGCCGATGTGA-3' yielding a ~1.67 kb amplicon. As a control, PCR was performed with no template as well as from reactions containing no reverse transcriptase. Amplified PCR products were separated in 2% agarose gels and visualized with ethidium bromide staining. To compare relative *Hcn2* transcript levels between *ap/ap* mice and *+/+* controls, quantitative RT-PCR was performed on RNA samples prepared as described above using the QTag One-Step qRT-PCR SYBR Kit (Clontech, Mountain View, CA) and a real-time PCR LightCycler (Roche, Indianapolis, IN). Reactions were performed in triplicate using intron-spanning primers to amplify *Hcn2* message (forward 5'-GCGTGCCTTTGAGACCGT-3' and reverse 5'-CTGAACCTTGTGTAGCAAGATGGA-3'). *Hcn2* transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which was amplified using forward primer 5'-GTCGTGGATCTGACGTGCC-3' and reverse primer 5'-TGCCTGCTTCACCACCTTC-3'. Melting curves were used to confirm amplification of a single product.

Antibody preparation

cDNA encoding amino acids 45-135 of mouse *Hcn2* was generated by PCR using primers (5'-CGCGAATTCACGACCCCCTCGCAC and 3'-CGCGTCGACGAAGCTAGCCTGGCT), followed by subcloning the PCR product into the *EcoRI* and *BamHI* sites of the glutathione-S-transferase-producing vector, pGEX-4T1 (Pharmacia, Piscataway, NJ). Fusion protein was expressed in BL21 bacteria (Stratagene, La Jolla, CA) and purified by Glutathione-sepharose affinity chromatography according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ). The purified HCN2 (45-135) fusion protein was used to immunize guinea pigs to produce antisera specific to the amino (N)-terminus of mouse HCN2 (α -N'-HCN2) (Affinity Bioreagents, Golden, CO).

Cos-7 cell culture, transfection, and generation of protein extracts

Cos-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (10 units/ml), and streptomycin (10 µg/ml). Cells were transfected at 30% confluence in serum-free media using Lipofectamine plus reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). After 24-36 hours, cells were washed with ice-cold PBS and protein extracts were generated in Teen-Tx (0.1M Tris, 1mM EDTA, 1mM EGTA, 1% Triton-X100).

Immunoprecipitation and Western blotting

+/, *ap*/+ and *ap/ap* mice were anesthetized by isoflurane inhalation and sacrificed by decapitation. Brains were rapidly removed and homogenized in 10 vol (w/v) of buffer containing 10 mM HEPES, pH 7.4, and 320 mM sucrose, and centrifuged at $1000 \times g$ to remove nuclei and insoluble material. The post-nuclear homogenate was centrifuged at $50,000 \times g$ for 40 min to yield a cytosolic fraction (S2) and crude membrane pellet, which was resuspended in Teen-Tx (S3). Protein extracts were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Primary antibodies were diluted in block solution containing 5% milk and 0.1% Tween-20 in TBS (TBST) and incubated with membranes overnight at 4 °C or 1 hr at room temperature (RT) [guinea pig (gp) α -HCN2, 1:1000 (Shin et al., 2006); gp α -N'-HCN2, 1:500, rab α -HCN2, 1:200, Alomone, Israel; gp α -HCN1, 1:2000 (Shin and Chetkovich, 2007); gp α -HCN4, 1:2000, (Shin et al., 2008) and Supplementary Figure 1; mouse α -tubulin, 1:2000, Millipore, Bedford, MA]. Blots were washed 3×10 min with TBST, and species-appropriate secondary antibody conjugated to horseradish peroxidase (Amersham, Piscataway, NJ) was added in TBST containing 5% milk at a dilution of 1:2500. Labeled bands were visualized using Supersignal chemiluminescence (Pierce, Rockford, IL), and densitometric analysis performed using NIH Image J software.

Immunohistochemistry

For immunohistochemistry of fixed brain tissues, mice were perfused with fixative, 4% freshly depolymerized paraformaldehyde in PBS. Brains were removed and post-fixed overnight at 4°C in fixative, and parasagittal free-floating sections (50 µm) were cut on a microslicer (VT1000 S, Leica, Wetzlar, Germany). DAB staining was performed with gp α -N'-HCN2 (1:500) followed by species-appropriate secondary antibody in an avidin-biotin-peroxidase system (ABC Elite; Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Peroxidase staining was developed using 3,9-diaminobenzidine as the chromogen.

Light microscopy

Digital images of DAB-stained sections were taken with 3X or 8X objectives affixed to a Nikon SMZ 1000 microscope with SPOT Advance software, equipped with RT slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI). All images were exported and analyzed using NIH image J software.

4-AP induced seizure and measurement of seizure severity

4-aminopyridine (4-AP) was administered intraperitoneally (10mg/kg, i.p) to 8-10 week old +/+ or *ap*/+ mice. Animals were returned to their cages, and behavior was observed continuously for up to 90 min. A stereotypical progression of seizures following 4-AP administration was scored according to prior studies (Weiergraber et al., 2006). Briefly, phase I was characterized as hypoactivity, phase II as partial clonus (including head bobbing and forelimb clonus), phase III as generalized myoclonus (including the whole body clonus, wild running, and loss of motor control), and phase IV as generalized tonic-clonic seizures (terminating in hindlimb extension upon the death of the animal).

Results

The *apathetic* phenotype

We discovered a new neurological mutant mouse, *apathetic* (*Hcn2^{ap/ap}*, *ap/ap*), that arose spontaneously on a DW/Pas background. The mutation segregated as an autosomal recessive trait, and homozygous affected mice (*ap/ap*) were identifiable as early as postnatal day 14 by their diminished body length and weight (Figure 1 and Table 1). When forced to swim, *ap/ap* mice had difficulty in coordinating their extremity movements to orchestrate stroking motions, and would drown if allowed to do so. The *ap/ap* mice also demonstrated difficulty with balance and fell to one side when standing on their hind legs to feed. Furthermore, *ap/ap* but not heterozygous (*ap/+*) or wild type (*+/+*) animals exhibited striking motor incoordination (See Supplementary Video 1, showing *+/+* and *ap/ap* mouse ambulating)). In a series of simple neurological screening tests (Paylor et al., 1998) that is summarized in Table 1, *ap/ap* mice showed evidence of ataxia, with unsteady gait and intention tremors as they ambulated. For example, when placed in a cage that was quickly lowered and moved from side to side *+/+* and *ap/+* animals splayed their legs and remained upright, whereas *ap/ap* animals exhibited postural instability and usually fell over. When placed in the center of a suspended platform, *+/+* and *ap/+* mice easily negotiated the platform, whereas the ataxic *ap/ap* mice were often unable to check themselves from falling at the edges. Motor coordination and balance were also tested using an accelerating rotarod. *ap/ap* mice were completely unable to walk on the rotarod without falling. Comparison of *ap/+* and *+/+* mice revealed that both genotypes spent increasing time on the rotarod with training [$F(3,90) = 2.91$, $P < 0.05$] (Figure 1). Both *ap/+* and *+/+* mice spent equal amounts of time on the rotarod [$F(1,90) = 0.0007$, $P = 0.979$]. Regardless of genotype, female mice spent significantly more time on the rotarod than male mice [$F(1,90) = 8.88$, $P = 0.0001$]. Other neurological reflexes such as the righting reflex, whisker touch response, eye blink, and ear twitch were indistinguishable between genotypes (Table 1). In summary, neurological testing revealed that *ap/ap* mice exhibit a profound motor defect that is most consistent with ataxia, whereas the motor function of *ap/+* animals is indistinguishable from *+/+* animals.

In addition to ataxia in the *ap/ap* mice, young (postnatal day 21-60) *ap/ap* mice, but not *ap/+* or *+/+* mice, demonstrate abnormal episodic motor behavior; 1) frequent episodes of brief behavioral arrest lasting 2-3 seconds 2) occasional repeated and rhythmic jumping toward the top of the cage on their hind legs, and 3) rare frank tonic-clonic convulsions. The latter rhythmic jumping and tonic-clonic convulsions are occasionally followed by a period of quiescence (postictal period), and animals have been observed to die immediately after these episodes. As *ap/ap* animals age, they become less active and interact less with their littermates and their environment by six months of age, hence their designation as *apathetic* (*ap*). As animals age, they continue to exhibit episodes of behavioral arrest, but jumping spells are no longer observed after 6 weeks of age, and older animals (>4 months) frequently assume an unusual posture for mice in which they curl up on their sides. *ap/ap* mice show increased mortality of 13.5% between the ages of 21-28 days of life with subsequent mortality of approximately 10% per month until 12 months of age by which time virtually all animals are dead. The cause of death has not been determined despite numerous post-mortem pathological examinations of affected animals, suggesting a neurological or cardiac electrical cause of death.

Epilepsy in *apathetic* mice

The abnormal “spells” observed in *ap/ap* mice resemble epileptic seizures, but in mice as in humans, it can often be difficult to distinguish between epileptic and nonepileptic events (Noebels, 2006). In order to evaluate whether *ap/ap* mice are epileptic, we performed cortical surface EEG recordings. Table 2 summarizes the characteristics of seizures noted through observation and EEG recording. From analysis of 50 hours of EEG recording, *ap/ap* animals

exhibited frequent (4.2 ± 0.7 events per minute, $n=3$), large amplitude (>2 fold baseline amplitude), runs of spike-wave discharges (Figure 2). The frequency of spike-wave discharges ranged from 3-7 Hz and individual runs of spike-wave lasted 1-3 seconds in duration. They were often, but not always, associated with a brief head twitch or behavioral pause. No EEG spike-wave discharges were observed in $+/+$ animals ($n=3$) in over 89 hours of recording. Because the spike-wave discharges coupled with behavioral pauses resembled absence seizures, we administered the Ca^{++} channel antagonist, ethosuximide, while recording EEG in ap/ap mice. Ethosuximide, a drug that specifically prevents absence seizures in humans and rodents (Browne, 1983; Heller et al., 1983; Sasa et al., 1988; Snead, 1988), completely eliminated the spike-wave discharges and episodes of behavioral arrest in ap/ap mice (Figure 2). We were unable to record the EEG during jumping spells (because the younger animals (<6 weeks) that exhibit jumping spells do not survive anesthesia and surgery for the EEG headmount), but strongly suspect they are epileptic because animals exhibit post-ictal quiescence and have been observed to die following these spells. EEG and video recorded during tonic-clonic convulsive events revealed these events to be epileptic as well (examples shown in Supplementary Figure 2 and Supplementary Video 2, respectively). In summary, behavioral observations together with findings of ethosuximide sensitive spike-wave discharges strongly suggest ap/ap mice have a generalized epilepsy syndrome that resembles human absence epilepsy.

Although $ap/+$ mice showed no overt behavioral arrest episodes, exhibited no jumping behaviors and had normal longevity, we nonetheless performed EEG monitoring of $ap/+$ mice to look for seizures. Indeed in >500 hours of recording in 10 mice we observed that two $ap/+$ mice exhibited the spontaneous, paroxysmal 1-3 second runs of spike-wave discharges that were observed in all of the ap/ap mice. Similar to ap/ap animals, retrospective video/EEG analysis revealed that these seizures were associated with behavioral arrest, however the seizures were much less frequent, occurring only 2-3 times per hour in the two $ap/+$ mice exhibiting seizures as opposed to 4-5 times per minute in each of the ap/ap mice.

Increased severity of provoked seizures in $ap/+$ mice

In contrast to ap/ap mice, only a small fraction of $ap/+$ mice exhibited spontaneous generalized absence seizures and spike-wave EEG abnormalities. We wondered whether the ap mutation might lead to increased seizure propensity in $ap/+$ animals not exhibiting spontaneous seizures. Thus, we investigated whether $ap/+$ mice are more susceptible to the chemoconvulsant-induced generalized seizures. Using the K^+ channel blocker, 4-aminopyridine (4-AP, 10 mg/kg, i.p.), we induced seizures in $+/+$ and $ap/+$ mice. 4-AP blocks A-type K^+ channels and delayed rectifier K^+ channels, which induces burst firing and leads to generalized-tonic clonic seizures in mice and rats (Komagiri and Kitamura, 2003; Russell et al., 1994; Thompson, 1982). After 4-AP injection, we found that 14 out of 15 $+/+$ animals, and 17 out of 17 $ap/+$ animals developed generalized tonic clonic seizures (phase IV) leading to death. Interestingly, we found that the latency between injection of 4-AP and initiation of limb clonus (phase II) and generalized myoclonus (phase III) was significantly shorter in $ap/+$ compared to $+/+$ animals (Figure 3; phase II= 148.5 ± 11.5 sec vs 397.3 ± 23.3 sec, $n=17$ for $ap/+$, $n=14$ for $+/+$, $** P<0.001$; phase III= 365.3 ± 36.4 sec vs 616.2 ± 50.2 sec, $n=17$ for $ap/+$, $n=14$ for $+/+$, $** P<0.001$). Moreover, the duration of phase III plus phase IV seizures was markedly shorter in $ap/+$ compared to $+/+$ mice, (Figure 3; 192.5 ± 50.4 sec vs 1150.8 ± 257.7 sec, $n=17$ for $ap/+$, $n=14$ for $+/+$, $* P<0.01$). These data demonstrate that mice heterozygous for the ap allele have an accelerated epileptic response to the chemoconvulsant, 4-AP, and are consistent with neuronal hyperexcitability in the $ap/+$ mice.

Genetic analysis of *apathetic* mice

By linkage analysis, we mapped this new murine neurological mutation to mouse chromosome 10 at 40.4 cM (Figure 4A), constructed a complete mouse BAC contig across the non-recombinant interval and identified human genomic clones mapping to the orthologous region on human 19p13.3 (Puttagunta et al., 2000). By identifying and sequencing positional candidate genes mapping to this region in humans and mouse, we identified a 4 base pair insertion at nucleotide position 1669 in the cyclic nucleotide binding domain of the hyperpolarization-activated cation channel *Hcn2* in the *ap/ap* mouse. This 4 base pair insertion, TTCA, is located in the middle of exon 6 of the *Hcn2* gene as a tandem repeat, likely resulting from duplication of the same 4 bp 5' to the insertion (Figure 4B). We designed a genotyping PCR primer pair for detecting a 90 bp fragment for the wild type *Hcn2* allele and 94 bp fragment for the *Hcn2^{ap}* allele. By separating PCR amplicons on non-denaturing gels we were able to confirm the genotypes of *ap/ap*, *+/+* and *ap/+* animals (Figure 4C).

Apathetic mRNA is transcribed

mRNA stability is governed by a complex and incompletely understood process, and even small insertions and deletions within mRNA sequences can lead to instability or decreased production of mRNA (Hollams et al., 2002). To evaluate expression of *Hcn2^{ap}*, we performed reverse-transcription PCR (RT-PCR) of *Hcn2* mRNA in *+/+* and *ap/ap* animals. We amplified full-length HCN2 mRNA along with no template and no reverse transcriptase controls and found that HCN2 mRNA is detected in the brain of both *+/+* and *ap/ap* mice (Figure 5A). To determine whether *ap/ap* mice have decreased quantity of *Hcn2* mRNA, we performed quantitative real-time RT-PCR and found that *Hcn2* mRNA is reduced by ~90% in *ap/ap* compared to *+/+* mice (Figure 5B), suggesting that although the *Hcn2^{ap}* mutation does not abolish transcription of *Hcn2* mRNA, the *ap* mutation may affect production or stability of the *Hcn2* mRNA.

Apathetic protein is undetectable

The *Hcn2^{ap}* allele is a 4 base pair insertion located within exon 6 of the *Hcn2* gene, which, if translated, is predicted to produce a frame-shift and insertion of 41 new amino acids in the cyclic nucleotide binding domain of HCN2 before a stop codon is introduced (Figure 4D). Thus, whereas wild type HCN2 protein has a molecular weight of 100 kD (~110 kD with glycosylation), transcription and translation of *Hcn2^{ap}* is predicted to produce a partial C-terminus-truncated HCN2 protein with expected mass of 70 kD (HCN2^{ap} protein) by early termination of translation.

In order to detect *ap* protein, we generated an antibody that recognizes the N-terminus of mouse HCN2, an epitope predicted to be shared by wild type and HCN2^{ap} protein. We subcloned, expressed, and purified the GST fusion protein, GST-HCN2(43-135), and then submitted the epitope for commercial generation of gp polyclonal antisera. Western blotting with gp- α -N'-HCN2(43-135) detected 100 kD and 110 kD bands in *+/+* mouse brain extract, which correspond to glycosylated and non-glycosylated HCN2, respectively (Figure 5C and data not shown). We next demonstrated ability to detect both wild type HCN2 and HCN2^{ap}. To do this we generated cDNA constructs encoding these HCN2 isoforms and transfected them into Cos-7 cells. As expected, western blotting of transfected Cos-7 cells using our α -N'-HCN2(43-135) recognized both proteins, whereas an antibody prepared against the C-terminus of mouse HCN2, α -HCN2(761-863) (Shin et al., 2006), detected wild type but not the truncated *ap* protein which has wild type sequence through amino acid 557. We then looked for the presence of *ap* HCN2 protein in brain by western blotting using the membrane extract of brains from *+/+*, *ap/+* and *ap/ap* mice. Surprisingly, no bands were detected in the brain extract of *ap/ap* mice with α -HCN2 antibodies recognizing either the N-terminus (gp α -N'-HCN2) or C-terminus of HCN2 (gp α -HCN2) (Figure 5C-E). In the brain extracts of both *+/+* and *ap/+* mice, bands of

100 kD and 110 kD were detected with either antibody, corresponding to the predicted size of the unglycosylated and glycosylated wild type HCN2 protein (Figure 5D); however, HCN2 protein was 50% decreased in *ap/+* compared to *+/+* brain (50.0 ± 6.4 % compared to *+/+*, $n=4$, * $P<0.01$, Figure 5D). We did not detect any smaller bands in *ap/+* brain using the antibody that recognizes the HCN2 N-terminus, α -N'-HCN2(43-135). We also performed immunoprecipitation of HCN2 from brain extract using a commercial antibody prepared against the HCN2 N-terminus (rab α -HCN2, Alomone, Jerusalem), followed by detection with α -N'-HCN2(43-135). Although this antibody immunoprecipitated HCN2^{ap} when expressed in Cos-7 cells, and increased the detection of wild type HCN2 protein in brain extract of *ap/+* animals, we did not detect any HCN2^{ap} expression in *ap/+* or *ap/ap* animals (Figure 5E). We also used our sensitive and specific α -N'-HCN2(43-135) antisera to evaluate HCN2 immunoreactivity in immunohistochemical studies. Similar to what was observed using another specific HCN2 polyclonal antibody (Notomi and Shigemoto, 2004), α -N'-HCN2 (43-135) demonstrated enhanced immunoreactivity in the stratum lacunosum moleculare of hippocampal area CA1, the granule cell layer of cerebellum and in the thalamus of both *ap/+* and *+/+* mice (Figure 6). Consistent with biochemical studies, we found no HCN2 immunoreactivity in *ap/ap* mouse brain (Figure 6). Thus, it is likely that *Hcn2*^{ap} mRNA is not translated into protein or that the mutant *ap* protein is preferentially degraded. Taken together, these data suggest that the *Hcn2*^{ap} allele is a functionally “null” mutation.

Expression of HCN1 and HCN4 is unchanged in *apathetic* mice

HCN subunit expression may be regulated by activity (Bender et al., 2003; Fan et al., 2005; van Welie et al., 2004). As such, it is possible that loss of HCN2 or seizures in the *ap/ap* mouse might change expression of other HCN subunits. Thus, in addition to HCN2, we also explored the expression levels of the other major brain HCN subunits, HCN1 and HCN4. Because we and others have noted that commercial anti-HCN preparations vary in sensitivity and specificity, we utilized custom antisera directed against HCN1 (Shin and Chetkovich, 2007) and HCN4 (Shin et al., 2008) to perform western blotting of extracts prepared from cortex of adult *+/+*, *ap/+* and *ap/ap* mice. In contrast to HCN2 which was reduced in *ap/+* and absent in *ap/ap* mice, levels of HCN1 and HCN4 protein in *ap/+* and *ap/ap* mice were unchanged as compared to *+/+* mice (Figure 7; HCN1: *ap/+* 86 ± 19 , *ap/ap* 87 ± 13 % of *+/+* levels, $n=3$, $P=0.75$; HCN4 *ap/+* 95 ± 26 , *ap/ap* 85 ± 5 % of *+/+* levels, $n=3$, $P=.14$). These data suggest that the *ap* mutation specifically reduces HCN2 protein levels without resulting in compensatory overexpression of HCN1 or HCN4.

Discussion

We report that a spontaneously arising 4 base pair insertion in the mouse gene encoding the h channel subunit, *Hcn2*, eliminates HCN2 protein production, and that homozygosity for the mutation produces an early onset neurological phenotype that includes ataxia and generalized absence seizures. The epilepsy, reduced size, and decreased viability of the homozygous *ap/ap* mice, which we have named *apathetic*, are similar to that reported previously for a targeted knockout of the *Hcn2* gene (Ludwig et al., 2003). The initial report of the *Hcn2* knockout mouse specifically described reduced locomotor activity (Ludwig et al., 2003); however, the *Hcn2* knockout mouse was noted to be ataxic in a review of h channels (Herrmann et al., 2007). Our present study utilizes numerous behavioral observations to characterize the predominant *ap/ap* motor abnormality as ataxia. Seizures in mice heterozygous for the *Hcn2* knockout allele have not been observed, but detailed analysis of these mice by EEG recording has not been performed (Personal communication, Andreas Ludwig). In contrast, we found spontaneous seizures and enhanced chemoconvulsant-induced seizures in the *ap/+* mouse, suggesting the *ap* allele confers increased seizure propensity even in heterozygosity. We furthermore showed reduction of HCN2 protein levels, but not levels of HCN1 or HCN4 in brain of *ap/ap* and *ap/*

+ mice, suggesting that the abnormalities in mice harboring the *ap* allele are due to loss of HCN2. Our findings are significant in that 1) we describe a novel, spontaneous mutation of the HCN2 gene that results in increased propensity to generalized seizures, 2) we identify a naturally-occurring *Hcn2* mutation that results in an epilepsy phenotype similar to that observed in an engineered *Hcn2* knockout mouse, and 3) we characterize the molecular and physiological consequences of the *ap* mutation of *Hcn2* to expand our understanding of the relationship between HCN2 protein, ataxia, and epilepsy.

HCN2 is one of four subunits (HCN1-4) of the h channel, which mediates the current, I_h , in heart and brain (Ludwig et al., 1998; Santoro et al., 1998). Whereas all subunits are expressed in brain, HCN1, HCN2, and HCN4 are the predominant subunits. HCN1 and HCN2 are expressed together in neurons of the cortex, hippocampus and cerebellum, while HCN2 and HCN4 are the major h channel subunits expressed in the thalamus (Abbas et al., 2006; Notomi and Shigemoto, 2004; Santoro et al., 2000). I_h is critical for the rhythmic activity of thalamic neurons and the periodicity of thalamocortical network oscillations as a pacemaker (Maccaferri and McBain, 1996; Pape and McCormick, 1989). We report here that HCN2 (but not HCN1 or HCN4) levels are undetectable in *ap/ap* and significantly reduced in *ap/+* mice. In the homozygous *Hcn2* knockout animal, absence epilepsy results from a near complete loss of I_h in thalamocortical relay neurons, resulting in hyperpolarization of the resting membrane potential, an altered response to depolarizing inputs and an increased susceptibility to oscillations (Ludwig et al., 2003). Given the similar reduction in HCN2 without changes in HCN1 or HCN4, we reason that seizures present in mice harboring the *ap* mutation are likely due to specific loss of HCN2, and likely share a similar mechanism with the *Hcn2* knockout animal.

We found 90% reduction of *Hcn2* mRNA expression in *ap/ap* mice, and no HCN2^{ap} protein. The absence of any detectable HCN2 protein in the brain of *ap/ap* mice indicates that the *ap* mutation is a null mutation, which is often the case when DNA insertion or deletion leads to a premature stop codon (Conti and Izaurralde, 2005). This inference is further supported by the several common phenotypes shared by the *apathetic* and *Hcn2* knockout mice, including seizures, locomotor defects, small body length and weight compared to littermates, as well as whole-body tremor and progressive hypoactivity with age (Ludwig et al., 2003). Although otherwise indistinguishable from *+/+* mice, *ap/+* mice exhibit spontaneous seizures and increased susceptibility to chemoconvulsant-induced seizure progression. Whether mice heterozygous for the *Hcn2* knockout allele have epilepsy has not been reported (Ludwig et al., 2003). We found a 50% reduction in HCN2 protein in the *ap/+* mice, and no changes in HCN1 or HCN4. The results are analogous to those of Ludwig et al, who showed reduction of HCN2 but not HCN1 and HCN4 mRNA in mice heterozygous for the *Hcn2* knockout allele (Ludwig et al., 2003). While it is possible that different strain background could influence seizure propensity differently in *ap/ap* versus *Hcn2* knockout mice, it is also possible that undetectable levels of HCN2^{ap} protein (which would not be present in mice heterozygous for the *Hcn2* knockout allele) could impact h channel or other neuronal function and increase seizure propensity in *ap/+* mice. Future studies will be aimed at exploring potential differences between the knockout and *ap* alleles with respect to epilepsy.

H channel abnormalities have been reported in several animal models of inherited generalized absence epilepsy (Di Pasquale et al., 1997; Kuisle et al., 2006; Strauss et al., 2004). In the rat models of absence epilepsy, Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats and Genetic Absence Epilepsy Rats from Strasbourg (GAERS), onset of seizures is correlated with altered I_h and h channel expression in cortex and thalamus (Budde et al., 2005; Kole et al., 2007; Strauss et al., 2004). Interestingly, Blumenfeld et al recently found that pre-morbid treatment of WAG/Rij rats with ethosuximide prevented the development of absence epilepsy and eliminated reduction of HCN1 observed in these rats (Blumenfeld et al., 2008). These

observations, coupled with our and others' findings of absence epilepsy in the *apathetic* and *Hcn2* knockout mice, respectively, suggest an important role for h channel changes in generalized absence epilepsy.

Ap/ap mice die prematurely from unknown causes. Although some *ap/ap* mice have been observed to die following myoclonic-like jumping or generalized convulsions, the cause of death is unresolved. *Hcn2* knockout mice have cardiac sinus dysrhythmia characterized by increased variability in the heart rate at rest that is due to the reduction of I_h in cardiomyocytes (Ludwig et al., 2003). We are planning future studies to determine whether a similar cardiac abnormality exists in *ap/ap* animals, and will explore whether the premature deaths are due to intrinsic cardiac dysrhythmia or perhaps result from another process analogous to human "sudden death in epilepsy" (Nashef et al., 2007).

In summary, we discovered a novel spontaneous mutant mouse, *apathetic*, with a 4 base pair insertion at nucleotide 1669 in the cyclic nucleotide binding domain of the *Hcn2* gene that eliminates HCN2 protein production. The phenotype of the *ap/ap* mouse includes ataxia and absence epilepsy that is ameliorated by ethosuximide treatment. Overall, these studies support the concept that h channels are critical for maintaining normal neuronal network oscillations, and that genetic loss of function of *Hcn2* predisposes animals to generalized absence epilepsy. Although we do not yet know whether *Hcn2* mutations cause familial epilepsy in humans, we reason that HCN2 may be an important target for future studies aimed at developing novel therapeutics to treat generalized epilepsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Apathetic mice have smaller body size and an ataxic gait. A, photograph of postnatal day 25 wild type ($+/+$), heterozygous ($ap/+$) and *apathetic* (ap/ap) mice reveals smaller body size (also note the wide-based stance of the ap/ap mouse). B, Rotarod test. The averages $\pm$ S.E.M for time spent on the rotarod across four test trials for $+/+$ (filled circles), $ap/+$ (open squares) and ap/ap (x shape) mice. $n=6$ each male and female $+/+$, $n=10$ each male and female $ap/+$ and $n=5$ each ap/ap male and female. Note the ap/ap mice are totally unable to walk on rotarod, whereas $+/+$ and $ap/+$ mice perform equally well.

Figure 2.

Epidural electroencephalographic (EEG) recordings from +/+ and *ap/ap* mice. A, Continuous two minute EEG recording in +/+ littermate (left panel) and *ap/ap* (right panel) mice. Asterisks (*) indicate typical spike-wave discharges observed in the *ap/ap* mice. B, Expanded time scale of EEG activity in +/+ (left trace) and *ap/ap* (right panel) mice demonstrates representative (3-7 Hz, 1-4 sec) spike-wave discharge recorded during behavioral arrest in an *ap/ap* mouse (right panel). C, Ethosuximide (ETX, 200 mg/kg, i.p.) injection in an *ap/ap* mouse eliminates behavioral arrests and spike-wave seizures detected by EEG.

Figure 3.

Seizure severity of *ap*/+ mice is higher than +/+ mice. Generalized seizures were induced by injecting +/+ (n=15) and *ap*/+ (n=17) mice with 4-aminopyridine (4-AP; 10mg/kg). Latency to stage II and III seizure as well as duration of stage III seizure are presented. Both latency and duration of seizures were significantly shorter in *+/ap* mice compared to +/+ mice (* $P < 0.01$, ** $P < 0.001$)

Figure 4.

Apathetic mice harbor a four base pair insertion in the *Hcn2* gene. A, Genetic map of mouse chromosome 10 at the *apathetic* locus with the homologous human chromosomes. Map based upon 946 meioses. Maximum, non-recombinant interval containing *ap* indicated. B, Schematic diagram of the 4 base pair insertion (TTCA) in *Hcn2* in *ap/ap* mice, localized to the coding region of the cyclic nucleotide binding domain of HCN2 protein. C, Electropherograms of wild type and *apathetic* *Hcn2* gene shows insertion of 4 base pairs, TTCA in *Hcn2^{ap}* gene. D, PCR amplification of the *Hcn2* gene yields a 90 and 94 base pair amplicon for wild type (+) and *apathetic* (*Hcn2^{ap}*) alleles, respectively. E, A Predicted translation products of wild type and *apathetic* *Hcn2* alleles show the frameshift and premature stop codon (red arrow) in predicted *Hcn2^{ap}*.

Figure 5.

Hcn2^{ap} is transcribed, but *apathetic* HCN2 protein is not detected. A, RT-PCR of full length of *Hcn2* mRNA shows that *Hcn2* mRNA is present in both wild type (+/+) and *apathetic* (*ap/ap*) mice (arrow). B, Quantitative RT-PCR shows that mRNA of *Hcn2* is decreased by ~90% in *ap/ap* mice as compared to +/+ controls. C, Protein extracts from Cos-7 cells overexpressing wild type or *apathetic* HCN2 (HCN2^{ap}), and protein extracts from +/+, *ap/+*, and *ap/ap* mouse brains were generated and separated by SDS-PAGE. Blots were labeled with gp α -HCN2 (C-terminal epitope) or gp α -N'-HCN2 (N-terminal epitope) antibodies. Gp α -HCN2 detects wild type HCN2, but not HCN2^{ap} protein, whereas α -N'-HCN2 detects both proteins in overexpressed heterologous cells. α -N'-HCN2 does not detect HCN2^{ap} in *ap/+* or *ap/ap* brain,

suggesting absence of HCN2^{ap} protein. D, Protein expression of HCN2 was decreased ~50% in the brain of *ap/+* compared to *+/+* mice (n=4, ** $P<0.01$). E, HCN2^{ap} protein was not detected even after concentrating protein extracts by immunoprecipitation. Protein extracts prepared from Cos-7 cells overexpressing wild type or HCN2^{ap}, as well as *+/+*, *ap/+*, and *ap/ap* mouse brains were immunoprecipitated with rabbit α -HCN2 antibody and probed with goat α -N'-HCN2. Whereas both wild type and HCN2^{ap} proteins in Cos-7 cells were concentrated and showed increased band intensity, no bands were detected from brain extracts of *ap/ap* mice. inp= input extract, ip= immunoprecipitated extract.

Figure 6.

Distribution of HCN2 protein in $+/+$, $ap/+$ and ap/ap mouse brain. 50 μm -thick parasagittal brain sections were generated and immunohistochemistry was performed using gp $\alpha\text{-N}^{\prime}$ -HCN2 antibody, visualized with DAB staining. HCN2 is concentrated in the stratum lacunosum-moleculare layer of hippocampal area CA1 (HP, top panel), thalamic nuclei (TH, middle panel), and granule cell layer in the cerebellum (CB, bottom panel). Note that distribution patterns of HCN2 are not significantly different between $+/+$ and $ap/+$ brains. No immunoreactivity of HCN2 was detected in ap/ap brain.

Figure 7.

HCN1 and HCN4 expression in +/+, *ap/ap*, and *ap/ap* brain. **A.** Brain extracts were generated and proteins separated by SDS-PAGE. Blots were probed with gp α -HCN1 antibody, gp α -HCN4 antibody, or ms α - β III-tubulin antibody. **B.** Density of HCN1 and HCN4 bands were normalized to the density of β III-tubulin to control for loading differences. No significant change in the protein expression levels of HCN1 or HCN4 was detected when comparing *ap/ap* or *ap/ap* to +/+ mice ($n=3$, $P=.75$ and $.14$ for HCN1 and HCN4, respectively). Data shown are mean $\pm$ S.E.M.

Table 1General motor and sensory responses of *apathetic*, heterozygous and wild type mice.

	+/+	ap/+	ap/ap
<i>Physical characteristics</i>			
Male weight (g)	28.8 (±0.6)	28.6 (±0.7)	15.1 (±0.4) ***
Female weight (g)	23.0 (±0.6)	22.5 (±0.7)	13.0 (±0.5) ***
Male length (cm)	8.9 (±0.1)	8.8 (±0.2)	7.6 (±0.1) ***
Female Length (cm)	8.7 (±0.1)	8.8 (±0.2)	7.2 (±0.1) ***
<i>General behavioral observations</i> (% subjects displaying response)			
wild running	0	0	0
freezing	0	0	0
sniffing	100	100	100
licking	0	0	0
rearing	100	100	20 ***
jumping	0	0	0
defecation	30	20	20
urination	0	0	0
move around entire cage	100	100	100
<i>Sensorimotor reflexes</i> (% subjects displaying "normal response")			
cage movement	100	100	10 ***
righting	100	100	100
whisker response	100	100	100
eye blink	100	100	100
ear twitch	100	100	100
<i>Motor responses</i>			
Elevated platform			
latency to edge (sec)	2.5 (±0.8)	4.2 (±1.1)	13.8 (±3.0) ***
no. of exploratory nose pokes	9 (±1)	10 (±1)	1 (±0) ***
falls from platform (%)	0	0	70 ***

Data represent the mean (±S.E.M.). Weight and length measurements include 13-16 mice of each gender and genotype. Behavioral testing includes 5 male and 5 female mice for each genotype.

P< 0.001

Table 2

Characteristics of seizures in *ap/ap* and *ap/+* mice

Genotype	Sz type	% with Sz	Sz frequency		Sz duration	Spike-wave frequency	
			Behavioral	EEG			
<i>ap/ap</i>	Behavioral Arrest	100	9-10/hour	200-300/hour [†]	1-3 sec	3-7 Hz	
	Jumping spells	100 [*]	2-3/hour	None [*]	N/A	N/A	
	Gen. Tonic-Clonic	100	Occasional	Occasional	5-30 sec	3-7 Hz	
<i>ap/+</i>	Behavioral Arrest	20 ^{**}	None ^{**}	1-2/hour	1-3 sec	3-7 Hz	
	Jumping spells	0	None	None	N/A	N/A	
	Gen. Tonic Clonic	0	None	None	N/A	N/A	

[†]Note that EEG recording of *ap/ap* mice is 20-fold more sensitive than behavioral observation to detect seizures

^{*}Jumping spells are present between 3 and 6 weeks of age, but we are only able to record EEG from older *ap/ap* mice owing to the small size and fragility of younger mice.

^{**}In the *ap/+* mice with spike-wave seizures on EEG recording, the motor manifestations are so infrequent and subtle that a trained observer does not score them.