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Sound-induced seizures in serotonin 5-HT_{2C} receptor mutant mice

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The epilepsies are a heterogeneous collection of seizure disorders with a lifetime expectancy risk rate of 2–4%[1]. A convergence of evidence indicates that heritable factors contribute significantly to seizure susceptibility[2,3]. Genetically epilepsy-prone rodent strains have been frequently used to examine the effect of genetic factors on seizure susceptibility. The most extensively studied of these have been strains that are susceptible to sound-induced convulsions (audiogenic seizures, or AGSs). Early observations of the AGS phenomenon were made in the laboratory of Dr. Ivan Pavlov; in the course of appetite-conditioning experiments in mice, the loud bell used to signal food presentation unexpectedly produced seizures in some animals[4]. In 1947, DBA/2 (D2) mice were found to exhibit a genetic susceptibility to AGSs stimulated by a doorbell mounted in an iron tub[5]. Since this discovery, AGSs have been among the most intensively studied phenotypes in behavioural genetics[6,7]. Although several genetic loci confer susceptibility to AGSs, the corresponding genes have not been cloned. We report that null mutant mice lacking serotonin 5-HT_{2C} receptors are extremely susceptible to AGSs. The onset of susceptibility is between two and three months of age, with complete penetrance in adult animals. AGS-induced immediate early gene expression indicates that AGSs are subcortical phenomena in auditory circuits. This AGS syndrome is the first produced by a known genetic defect; it provides a robust model for the examination of serotoninergic mechanisms in epilepsy.

Several lines of evidence indicate that there is an inverse correlation between serotoninergic activity and AGS susceptibility in the two most widely used AGS models, the D2 mouse and the genetically epilepsy-prone rat (GEPR)[8–14]. The 5-HT receptor subtype(s) through which these effects are mediated have been difficult to ascertain owing to the marked diversity of these receptors and to limitations in the selectivity of pharmacological probes of receptor function. We have observed an epilepsy syndrome in a line of 5-HT_{2C} receptor null mutant mice[15]. This syndrome is characterized by infrequent spontaneous tonic-clonic seizures and normal interictal EEGs, indicating the absence of subclinical seizure activity (Noebels, unpublished observations). In light of evidence for the serotoninergic modulation of AGS susceptibility, we sought to determine whether a sound stimulus could serve as a noninvasive precipitant of seizures in the mutants.

Mutant and wild-type animals were exposed to a sound stimulus that was designed to incorporate features that had been effective in eliciting AGSs in other susceptible strains[16]. A modulated auditory stimulus consisting of frequencies in the 5–19-kHz range was presented at a volume of 108 dB. Whereas no wild-type animals (at any age) displayed behavioural evidence of seizures, mutants manifested dramatic and highly stereotyped seizure responses. At 2–14 seconds after sound stimulation, the mutants suddenly exhibited wild running and erratic leaping (Fig. 1). This behaviour persisted for 1–2 s and was immediately followed by a tonic phase, which consisted of a loss of righting ability and hindlimb flexion, followed abruptly by 15–25 seconds of hindlimb extension, extensor rigidity of the body and respiratory arrest. Death ensued if no intervention was made; however, animals could be resuscitated by artificial ventilation applied during the onset of the respiratory arrest phase (see Methods). The seizures did not represent enhanced startle responses; seizures were not produced by startling tactile stimulation or brief 108-dB noise bursts. In addition, seizures in 5-HT_{2C} receptor mutants were not readily induced by other means, such as the jangling of keys, handling or the application of stressors.

AGS susceptibility in the mutants was age dependent. Most of the mutants displayed AGSs by 75 days of age, and complete penetrance was observed by 120 days (Fig. 2). Seizure responses appeared to occur in an all-or-none manner, so that all mutant animals that displayed behavioural evidence of seizures (except one at 60 days of age) progressed to the tonic extension phase. These results indicate that sound administration provides a reliable, noninvasive method to provoke generalized seizures in adult 5-HT_{2C} receptor mutant mice. The relatively late onset of susceptibility contrasts with D2 mice, which display maximal AGS sensitivity at 20–24 days of age[17,18]. The severity of AGSs in 5-HT_{2C} receptor mutants is par-

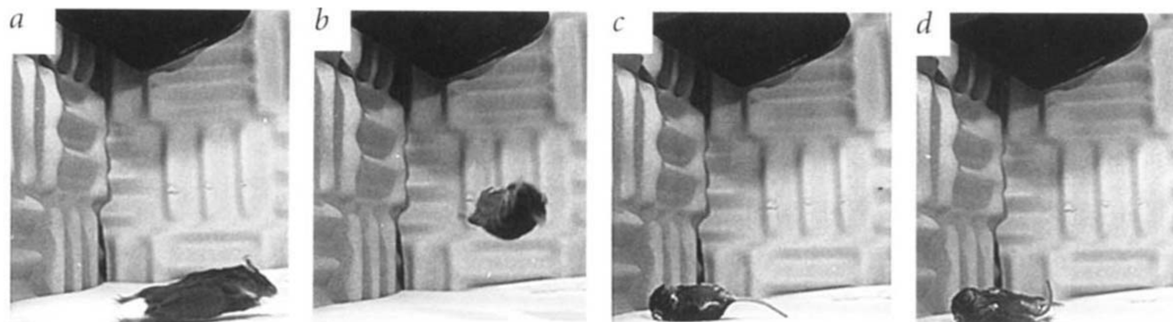

Fig. 1 Sequence of AGS behaviour in a 5-month-old 5-HT_{2C} receptor mutant mouse. The first image in the sequence was taken during the first second of the AGS response. The interval between each frame is 0.5 s. ***a,b,*** Phase of wild running/erratic leaping. ***c,*** Onset of tonic phase, with forelimb and hindlimb flexion. ***d,*** Tonic phase, with characteristic rigid hindlimb extension.

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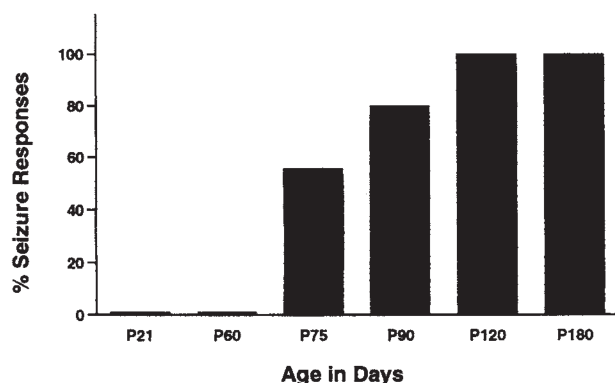

Fig. 2 Developmental time course of AGS susceptibility in 5-HT_{2C} receptor mutant mice. Naive male mice were exposed once to the 108-dB sound stimulus for 60 s, or until the onset of AGS behaviour. Percentage of animals exhibiting the complete AGS response is indicated. All AGS responses progressed to tonic extension except one P60 mutant mouse, which exhibited wild running only. No wild-type animals exhibited any phase of the AGS response. The number of mutant mice tested at each age were P21=7, P60=9, P75=9, P90=10, P120=7, P180=6.

ticularly striking given that the null mutation was bred onto a C57BL/6 (B6) background that is normally AGS resistant[5,19,20].

Expression of the immediate early gene transcription factor c-fos is rapidly induced in the central nervous system (CNS) in response to seizure activity and has been used as a marker of neuronal activation[21,22]. To identify brain regions that are activated by AGSs in the mutants, we examined the induction of c-fos–like immunoreactivity (FLI) in the CNS. AGSs consistently produced robust induction of FLI in the inferior colliculus (IC), predominantly in its external cortical and dorsal cortical regions, sparing the central nucleus (Fig. 3). Staining was also observed in the lateral lemniscal nuclei, which have reciprocal connections with the IC. In the thalamus, staining was observed in the medial portion of the medial geniculate nucleus and in the posterior intralaminar nucleus, regions that receive afferents from the IC. Additionally, FLI was consistently observed in deep layers of the superior colliculus and in the central grey. This pattern of FLI closely matches those seen in other AGS-susceptible strains and indicates that AGSs engage subcortical auditory and brain-stem structures[23,24]. In contrast, wild-type animals exposed to the same stimulus consistently showed little FLI in these auditory/brain-stem regions.

Several lines of evidence indicate that AGSs reflect a disuse super-sensitivity of the IC resulting from a hearing impairment during a critical period of development[25,26]. To determine whether the absence of 5-HT_{2C} receptors produces a hearing loss, we examined the response properties of IC neurons in 15–16-week-old mutant and wild-type mice. Characteristic frequency, response threshold, response latency and receptive field bandwidth were determined at 100-μm intervals throughout the dorsoventral extent of the IC contralateral to the sound source (Fig. 4). A generalized elevation in threshold for high-frequency sounds was observed in both mutant and wild-type animals, consistent with the moderate age-related hearing loss characteristic of the B6 strain. However, no phenotypic differences were observed in the distribution and range of characteristic frequencies, excitatory bandwidths or response thresholds, indicating that the mutation did not produce a hearing loss beyond that normally seen in B6 mice. In addition, no after-discharge activity (sustained excitatory discharges persisting >50 ms beyond stimulus termination) was observed in mutant or wild-type mice. The main phenotypic differences detected were moderately elevated response latencies in mutant mice. This alteration was associated with a decreased ability to respond to repetitive auditory stimuli, a measure of temporal coding capacity[27]. It is unclear how this finding relates to AGS susceptibility. Additional studies will be required to determine whether these changes also occur in proximal sites in the auditory pathway.

Although AGS-associated behaviour and FLI induction in the mutants are similar to those seen in D2 mice and GEPR rats, the AGS syndrome of 5-HT_{2C} receptor mutant mice is distinct with regard to its mode of inheritance. The genetic determinants of AGS sensitivity in D2 mice and GEPRs are complex. For example, in D2 mice much of the heritable variation in AGS susceptibility is attributable to three loci, termed *Asp1, 2* and *3* (audiogenic seizure prone)[28–30]. Unlike the X-linked 5-HT_{2C} receptor gene[31], these *Asp* loci are autosomal, suggesting that the AGS susceptibility of D2 mice is not produced by a polymorphic variant of the 5-HT_{2C} receptor gene. Thus, the AGS phenomenon may be produced by various independent genetic mechanisms.

The 5-HT_{2C} receptor mutant AGS syndrome also differs from the D2 mouse and GEPR rat models with regard to the developmental time course of AGS susceptibility. Whereas sensitivity develops within the first three weeks of life in D2 mice and GEPR rats[17,18,32], 5-HT_{2C} receptor mutants do not become susceptible until adulthood. It is possible that this late onset reflects an inter-

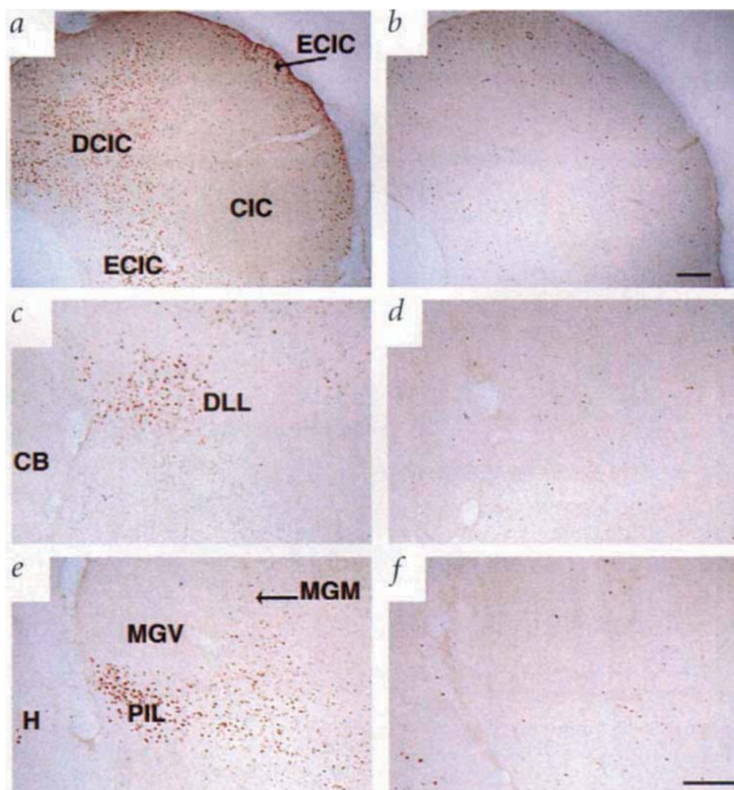

Fig. 3 Coronal brain sections demonstrating patterns of FLI produced by sound exposure in 5-HT_{2C} receptor mutant (*a,c,e*) and wild-type (*b,d,f*) mice. ***a,b,*** Staining in the external cortex (ECIC) and dorsal cortex (DCIC) of the inferior colliculus. ***c,d,*** Dorsal lateral lemniscal nucleus (DLL). ***e,f,*** Staining in the medial portion of the medial geniculate nucleus (MGM) and posterior intralaminar thalamic nucleus (PIL). CIC, central nucleus of the inferior colliculus; H, hippocampus; CB, cerebellum; MGV, ventral portion of medial geniculate nucleus. Scale bar=200 μm.

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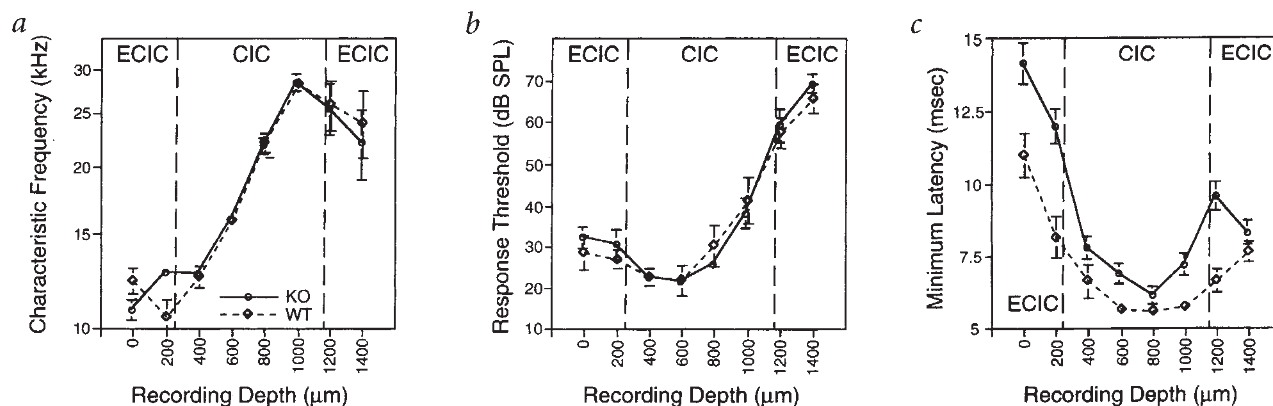

Fig. 4 Electrophysiological response properties of IC neurons in age-matched 5-HT_{2C} receptor mutant and wild-type mice. Dashed lines indicate the average recording depths corresponding to the external cortical nucleus (ECIC) and central nucleus (CIC) of the inferior colliculus. ***a***, No differences between mutant and wild-type characteristic frequencies as a function of depth of the recording site. ***b***, No differences in response thresholds between mutant and wild-type animals. ***c***, Moderately increased response latencies in mutant mice, in both the ECIC and CIC (ANOVA, Δ latency 1.44 ms; $P < 0.003$). Error bars indicate S.E.M.

action of the mutation with the B6 genetic background. Studies of B6/D2 recombinant inbred strains indicate that B6 mice may possess genes that confer adult onset AGS susceptibility and that different genetic factors contribute to early onset susceptibility[33]. Moreover, B6 mice exhibit a slowly progressive sensorineural hearing impairment, affecting high-frequency responses by two months of age[34,35]. This results in auditory system plasticity characterized by increases in the representation of middle frequencies such as those composing the auditory stimulus used in these studies. The extent to which genetic background contributes to the AGS phenotype of 5-HT_{2C} receptor mutants will be determined by crossing the mutation to a variety of inbred strains.

The AGS syndrome in 5-HT_{2C} receptor mutant mice is the first to be associated with an identified genetic defect. This model provides support for a significant contribution of 5-HT_{2C} receptors to the serotoninergic suppression of AGSs. Substantial evidence indicates that anticonvulsant effects of serotoninergic stimulation are also observed in a wide variety of non-audiogenic epilepsy models. In rodents, serotonin has anticonvulsant effects in the epileptic EL mouse[36] and in models employing electroshock and chemical convulsants[37–39]. In a primate model, seizures in the photosensitive baboon *Papio papio* are suppressed by L-tryptophan administration and enhanced by serotonin depletion[40]. These findings indicate that serotonin systems produce a generalized suppression of neuronal network excitability and seizure activity. The AGS syndrome of 5-HT_{2C} receptor mutant mice may therefore provide insights into the mechanisms through which serotoninergic systems influence susceptibility to the epilepsies.

Methods

5-HT_{2C} receptor mutant mice. Since their initial derivation[15], the mutants have been back-crossed for ten generations to a C57BL/6 background. Wild-type male mice were mated with heterozygous females; because the 5-HT_{2C} receptor gene is X-linked[31], the resulting males were either hemizygous mutants or wild types. Genotyping was performed by PCR analysis, with the mutant allele detected using primers derived from neomycin resistance gene (*Neo*) sequences: NeoD (5′–CACCTTGCTCCTGCCGAGAAA–3′) and NeoH (5′–AGAAGGCGATAGAAGGCGATG–3′). The wild-type allele was detected with primers derived from 5-HT_{2C} receptor gene sequences flanking the *Neo* insertion: 5N2 (5′–CAACTTGGTTGTTACACACGG–3′) and 3N2 (5′–TCTACCCTTCTCATACTAGTT–3′). Animals were group housed, three to six mice per standard cage, with free access to food and water under a 12-h light/dark cycle (lights on at 7 h).

Auditory stimulation. Test animals were removed from the colony at approximately 2–3 h after the start of the light cycle. Auditory stimulation was applied approximately 1–2 h after removal from the colony. The apparatus used for auditory stimulation consisted of a 22×16×22-inch Comatex box lined with 1/24-inch lead sheeting on the outside to minimize escape of sound. The interior of the box was lined with acoustical foam to decrease variability due to standing waves. A 110-W, 8-ohm Optimus Pro-X77 speaker was mounted to the ceiling and driven by a CD player via a 100-W amplifier. Animals were placed into an 8×8×7-inch wire-mesh cage located 10 inches below the speaker. Behaviour was monitored via a CCD camera mounted on the ceiling of the box and recorded with a VCR. After a 30–60 s period of acclimatization to the chamber, a 108-dB auditory stimulus was used that consisted of a mixture of four pure tones (5, 11, 15 and 19 kHz) whose amplitudes varied through time independently from 20 to 110 dB SPL. The stimulus was maintained for 60 s or until an overt seizure had occurred. Artificial ventilation was performed by placing a 13-mm (i.d.) tube over the snout of the animal and puffing rapidly (rate approximately 150/min) and gently into the other end. Typically, spontaneous ventilations returned within 30 s. After a 30–60-min period of postictal lethargy, the animals resumed apparently normal behaviour.

High-speed photography. To photograph AGS behavior, a Pentax C500 camera mounted to a tripod was used with a 35-mm lens with aperture set at 4.5. The shutter speed was adjusted to 1/500 s and programmed to shoot continuously with Kodak 400 ASA film. There was an interval of 0.5 s between each shutter release. Animals were placed into the auditory stimulation chamber, with one wall of the box replaced by a glass window to facilitate photography.

Immunocytochemistry. Two hours after sound exposure, animals were anaesthetized and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brain was immediately dissected and post-fixed in the same fixative for 24 h at 4 °C. The tissue was then equilibrated in 30% sucrose/PBS before 40-µm-thick sections were cut on a sliding microtome. After pre-treatment for 60 min in 3% normal goat serum, 0.3% Triton and PBS, sections were incubated for 18 h at 4 °C in rabbit polyclonal anti-fos antisera (Oncogene Sciences, 1:15,000 dilution). Antibody binding was revealed by use of the ABC-peroxidase technique (Vector Laboratories), using diaminobenzidine (0.1%) with 0.02% H_2O_2.

Electrophysiology. Neuronal activity was measured in 15–16-week-old urethane-anaesthetized animals by the introduction of parylene-coated microelectrodes into the IC contralateral to the sound source. The angle of penetration was 40° from the midline, positioned to traverse the center of the central nucleus. Stimuli were delivered via Stax earphones in a sound system calibrated with a BrŸel and KjÏr sound-level meter and a waveform analyser (General Radio). Neuronal activity was amplified, band-pass filtered and monitored on an oscilloscope and an audio monitor. Spike activity was isolated from background noise with a window discriminator (BAK DIS-1), and the time of occurrence of each spike was stored by computer.

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Characteristic frequency, response threshold, bandwidth of the tuning curve (10 dB above the minimum threshold) and onset latency were determined with repetitive 50 ms tone bursts (3-ms rise/fall time). These measurements were obtained by visual examination of the stimulus-synchronized spike record on the oscilloscope or with computer-reconstructed frequency response areas[41]. The lowest repetition rate at which a stimulus (5 ms, 40–60 dB above threshold) failed to elicit 1:1 following was used as a measure of temporal coding capacity.

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