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ACUTE AND CHRONIC RESPONSES TO THE CONVULSANT PILOCARPINE IN DBA/2J AND A/J MICE

M. R. WINAWER^a, N. MAKARENKO^b, D. P. McCLOSKEY^b, T. M. HINTZ^b, N. NAIR^b, A. A. PALMER^c, and H. E. SCHARFMAN^{b,d,*}

^aDepartment of Neurology and G.H. Sergievsky Center, Columbia University, New York, NY 10032, USA

^bCNRRR, Helen Hayes Hospital, Route 9W, West Haverstraw, NY 10993-1195, USA

^cDepartment of Human Genetics, University of Chicago, Chicago, IL 60637, USA

^dDepartments of Pharmacology and Neurology, Columbia University, New York, NY 10032, USA

Abstract

Characterizing the responses of different mouse strains to experimentally-induced seizures can provide clues to the genes that are responsible for seizure susceptibility, and factors that contribute to epilepsy. This approach is optimal when sequenced mouse strains are available. Therefore, we compared two sequenced strains, DBA/2J (DBA) and A/J. These strains were compared using the chemoconvulsant pilocarpine, because pilocarpine induces status epilepticus, a state of severe, prolonged seizures. In addition, pilocarpine-induced status is followed by changes in the brain that are associated with the pathophysiology of temporal lobe epilepsy (TLE). Therefore, pilocarpine can be used to address susceptibility to severe seizures, as well as genes that could be relevant to TLE.

A/J mice had a higher incidence of status, but a longer latency to status than DBA mice. DBA mice exhibited more hippocampal pyramidal cell damage. DBA mice developed more ectopic granule cells in the hilus, a result of aberrant migration of granule cells born after status. DBA mice experienced sudden death in the weeks following status, while A/J mice exhibited the most sudden death in the initial hour after pilocarpine administration.

The results support previous studies of strain differences based on responses to convulsants. They suggest caution in studies of seizure susceptibility that are based only on incidence or latency. In addition, the results provide new insight into the strain-specific characteristics of DBA and A/J mice. A/J mice provide a potential resource to examine the progression to status. The DBA mouse may be valuable to clarify genes regulating other seizure-associated phenomena, such as seizure-induced neurogenesis and sudden death.

Keywords

epilepsy; mossy fiber sprouting; neurogenesis; neuropeptide Y; seizure; status epilepticus

Temporal lobe epilepsy (TLE) is a common, complex disorder, with heterogeneous clinical manifestations and multiple genetic and non-genetic factors (Mathern et al., 1996; Cendes et al., 1998; Engel, 2001; Fuerst et al., 2001; Kobayashi et al., 2001; Vadlamudi et al., 2003; Scharfman and Pedley, 2006). Despite a number of anticonvulsant drug therapies, as well as the option of surgery, many individuals with TLE continue to have seizures that resist

*Correspondence to: H. E. Scharfman, The Nathan Kline Institute, 140 Old Orangeburg Road, Orangeburg, NY 10962, USA. Tel: +1-845-398-5427; fax: +1-845-398-5422. E-mail address: hscharfman@nki.rfmh.org or helensch@optonline.net (H. E. Scharfman).

medication, or return after surgical resection. Identifying genes for this common refractory epilepsy has become a priority in order to develop new therapeutic options.

One important approach is to use quantitative trait locus (QTL) mapping in animal models of epilepsy (Neumann and Collins, 1991, 1992; Martin et al., 1995; Clement et al., 1996; Buck et al., 1997; Ferraro et al., 1997, 1999, 2001; Gershenfeld et al., 1999; Hain et al., 2000; Buck and Finn, 2001; Fehr et al., 2002). This powerful strategy can provide a framework for identifying genetic influences for complex human disorders like TLE (Palmer and Phillips, 2002; Phillips et al., 2002; Biola et al., 2003). Indeed, inbred strains have been compared, and they have provided many examples for potential genetic differences underlying different responses to experimental insults that cause limbic seizures (Ferraro et al., 1995, 1997; Schauwecker and Steward 1997; Cantalops and Routtenberg, 2000; Borges et al., 2003; McKhann et al., 2003; Schauwecker 2003) or other types of seizures (Neumann and Collins 1991, 1992; Ferraro et al., 1998; Hain et al., 2000).

Recent years have provided greater resources for studies of strain differences, because mouse strains have been sequenced. We chose to compare the DBA and A/J mouse strains, and specifically their response to the chemoconvulsant pilocarpine, because this comparison had never been studied, and because both strains are sequenced. Pilocarpine was of interest for several reasons. First, induction of seizures in mice by pilocarpine has been studied less often than other chemoconvulsants, such as kainic acid (Cantalops and Routtenberg, 2000; McKhann et al., 2003; Schauwecker, 2003), despite its widespread use as a rat model for TLE (Turski, 2000). Second, pilocarpine can elicit status, which is of interest because it is a condition that occurs in humans, and appears to have a genetic predisposition (Corey et al., 1998). Third, pilocarpine-induced status is followed by a sequence of changes that are potentially relevant to TLE. For example, a pattern of neuronal damage develops within days of pilocarpine-induced status, and it resembles the pathology in many patients with TLE. The pathology generally involves neuronal loss of CA1 and CA3 pyramidal cells and hilar neurons, with relative sparing of granule cells and area CA2 (Scharfman and Pedley, 2006). Whether the pathology may be influenced by genetic factors can be addressed by comparing different mouse strains.

In rodents that have had status, animals develop recurrent seizures after several weeks, and these last for the rest of the lifespan (Turski et al., 1989; Turski, 2000). This timing resembles TLE, because patients often report a delay between an initial precipitating event and the first seizure. Therefore, the pilocarpine model also provides an opportunity to examine genetic factors that influence the delay, or changes occurring during the delay. Some of the changes that might be important include mossy fiber sprouting, a growth of dentate gyrus granule cell axons into an abnormal target zone (Sutula and Dudek, 2007); another change is the emergence of ectopic granule cells in the hilar region, reflecting mismigration of granule cells that are born after status (Scharfman, 2004; Scharfman and Hen, 2007). Even if mossy fiber sprouting and seizure-induced ectopic granule cells are not critical to epileptogenesis, genetic regulation of them is of interest, because they are interesting examples of plasticity, and may influence cognitive deficits in TLE, even if they do not cause seizures per se. Although some studies of the differences between mouse strains, for example in mossy fiber sprouting, have been studied (Cantalops and Routtenberg, 2000; Schauwecker et al., 2000; Borges et al., 2003; McKhann et al., 2003), relatively little is known about hippocampal pathology in the DBA and A/J strains in the pilocarpine model. Therefore, we compared pilocarpine-induced status in the DBA and A/J mouse strains, and also examined hippocampal pathology many weeks after status, at a time when neuronal damage, mossy fiber sprouting and other changes, would have developed.

EXPERIMENTAL PROCEDURES

Animal care and housing

All methods met the guidelines of the New York State Department of Health and the U.S. National Institutes of Health. Every effort was made to minimize the number of animals used and their suffering. Animals were housed for 1 week prior to use, in order to allow them to acclimatize to the new environment. Mice were housed individually in a temperature and humidity-controlled environment, with a 12-h light/dark schedule (lights on at 7:00 a.m.) and food and water were available *ad libitum*. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Convulsant administration

Adult male DBA2/J and A/J mice (10 weeks old; “J” for Jackson Laboratories, Bar Harbor, ME, USA) were administered atropine methylbromide (1 mg/kg i.p.) 30 min before pilocarpine hydrochloride (200, 220, 250, or 300 mg/kg i.p.). Doses were chosen to bracket a range from minimal (no animal ever had status at doses below 200 mg/kg in pilot studies) to maximal without mortality (mortality was increased if dose was higher than 300 mg/kg in pilot studies). Controls received the same treatment except that a similar volume of 0.9% saline was substituted for pilocarpine. The onset of status epilepticus was defined as the time of a stage 4 to 5 seizure which did not terminate in the subsequent 3 min. Seizure stage was defined by the Racine scale (Racine, 1972). Typically status developed after initial mild seizure behavior, such as trembling of the limbs, tail, body, and head, facial movements, and salivation.

Animals that had status epilepticus were administered diazepam (5 mg/kg i.p., Henry Schein, Melville, NY, USA) 1 h after the onset of status epilepticus, to decrease seizure severity. There was no detectable difference among any animals/strains in the severity of behaviors associated with status once it began, or the response to diazepam. Animals that did not have status epilepticus had some signs of milder seizures, such as repetitive chewing, salivation, head/body trembling, limb stiffening, and clonic movements. They were administered the same dose of diazepam at approximately the same time as the animals that had status.

Mice were housed in clear cages and randomly observed over the weeks after status. After 3–4 weeks, all animals were observed to have seizures that were spontaneous. Observations were made randomly between 9:00 a.m. and 5:00 p.m. from Monday to Friday by investigators who intermittently entered the room. When at least two spontaneous stage 5 limbic seizures were witnessed, an animal was considered to have entered the period of recurrent seizures, and was therefore “epileptic.” After the initial two seizures were noted, more seizures were observed in the same animal until it was killed. We recognize that 24 h video EEG would be required to assess seizure frequency definitively; this discussion merely is presented to explain why we believe the animals were epileptic.

Neuroanatomical examination

General methods—Animals were anesthetized with an overdose of urethane (2.5 mg/kg, i.p.) and transcardially-perfused with 30 ml of 4% paraformaldehyde (pH 7.4). Brains were postfixed for approximately 1–3 days and then sectioned (50 μ m) using a vibratome (Ted Pella, Redding, CA, USA). Sections were stained with Cresyl Violet, or immunocytochemistry was conducted using antibodies to NeuN, neuropeptide Y (NPY), or Prox1 as described below (see also Scharfman et al., 2000, 2002; McCloskey et al., 2006). Sections were dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped using Permount (Fisher, Hampton, NH, USA). They were viewed and photographed with a brightfield microscope (BX51, Olympus America, Melville, NY, USA) attached to a motorized stage (Optiscan, Prior, Rockland, ME, USA) and video camera (Optronics, Goleta, CA, USA).

Immunohistochemistry—Free-floating sections were processed concurrently to optimize comparisons. Sections from each animal were placed in separate compartments of plastic trays, floating in approximately 8 ml solution per compartment. Sections from each compartment were transferred in the same order so that sections from each animal would be exposed to solutions for the same periods of time. Sections were initially washed twice (5 min each) in 0.1 M Tris buffer (pH 7.6) and then were treated with 1% H₂O₂ (Fisher) diluted in 0.1 M Tris buffer (pH 7.6; 30 min). During washes and incubations, the trays were placed on a rotator at room temperature. Next, sections were washed in 0.1 M Tris buffer (pH 7.6; 5 min) and incubated in 0.1% Triton X-100 diluted in 0.1 M Tris buffer (Tris A; 10 min), followed by 0.1% Triton X-100 and 0.005% bovine serum albumin (BSA) in 0.1 M Tris buffer (Tris B; 10 min). Sections were subsequently transferred to a Tris B solution containing 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 45 min. Afterward, sections were washed in Tris A (10 min) and then Tris B (10 min) and incubated in antisera (diluted in Tris B) for 48 h at 4 °C on a rotating shaker. There were three antisera used: antisera to a neuronal nuclear marker (NeuN, monoclonal, 1:5000; Chemicon, Temecula, CA, USA), antisera to NPY (polyclonal, 1:2000; Chemicon), or antisera to Prox1, a transcription factor that has been used to distinguish granule cells from other cell types in the hippocampus (polyclonal, 1:30,000; Chemicon).

On the following day, sections were treated with Tris A (10 min) followed by Tris B (10 min), and then incubated for 45 min with a biotinylated secondary antibody against rabbit IgG made in goat (1:1000; Vector). Sections were washed in Tris A (10 min), then 0.1% Triton X-100 and 0.005% BSA in 0.5 M Tris buffer (Tris D; pH 7.6; 10 min), and finally incubated for 1 h in avidin-biotin horseradish peroxidase complex diluted in Tris D (ABC kit, 1:1000; Vector). Sections were developed in diaminobenzidine (DAB; 50 mg/100 ml Tris) plus 200 mg β-D-glucose, 0.3 mg glucose oxidase, and 40 mg ammonium chloride, and were subsequently washed in Tris buffer.

Quantitative evaluation of ectopic hilar granule cells—Starting at the most dorsal part of the hippocampus, where the blades of the dentate gyrus become fully established (approximately 2.2 mm posterior to Bregma; Paxinos and Watson, 1986), 50 μm-thick sections were cut, and one section was selected for stereology every 300 μm. Up to 10 sections were collected. At this point collection stopped because the dentate gyrus began to curve as the hippocampus descended in the temporal and ventral direction. At these levels, the basis for defining the hilus (described below) can no longer be used. Therefore, counts were confined to the dorsal hippocampus. However, we do not believe that the estimations of counted cells were biased by the emphasis on dorsal hippocampus, because in a previous study there were no apparent septotemporal differences in ectopic hilar granule cells (McCloskey et al., 2006). In addition, there was no obvious septotemporal difference upon inspection of ventral levels in the current study.

For the dorsal sections, the hilus was outlined by tracing an area on a computer screen showing the stained section, at 20× magnification. The hilus was defined as the area between the blades of the dentate gyrus and the lateral tips of the suprapyramidal (upper) and infrapyramidal (lower) blades, excluding 20 μm below the granule cell layer. The 20 μm exclusion zone was used to ensure that cells at the border of the granule cell layer and hilus would not be included, because they might be normal granule cells that were slightly shifted from their normal position. Although the hilus does not include the part of CA3c that enters the dentate gyrus, it was included in the area that was assessed because the CA3c border with the hilus was difficult to discriminate with confidence. However, the inclusion of CA3c was unlikely to influence the resultant counts of ectopic granule cells, because ectopic granule cells have not been detected in the CA3c layer (Scharfman et al., 2000).

The microscope, video camera, and motorized stage already described were used at 20× magnification to visualize Prox1-immunoreactive neurons. This magnification allowed the stereotypical, spherical Prox1-immunoreactive profile to be recognized with confidence. Only cells that were within the part of the section between the cut surfaces were included. A “guard zone” (5 μm^2) was used for the top and bottom surfaces of the sections to avoid counting cut cells.

Neuronal density of ectopic granule cells was estimated using an optical fractionator stereological probe using StereoInvestigator software (Microbrightfield, Inc., Colchester, VT, USA). A blinded investigator evaluated all sections. Cells were counted from a randomly selected hippocampus (the left hippocampus) for each animal. The probe used a counting frame of 25 μm^2 , and a randomly-oriented, 50 μm^2 XY grid. Therefore, cells were counted in 1/4 of the total hilar region. Manual counts (at least one section per animal) confirmed stereological estimations. The number of counted cells in each section was multiplied by 4, divided by the estimated hilar volume, and multiplied by thickness of the mounted section to calculate cell density. The estimated hilar volume was calculated as the area of the traced hilus multiplied by the thickness of the mounted section. The thickness of a section was determined by focusing through the granule cell layer with a calibrated stage, and using the computerized calibration to measure the distance from the point at the upper surface where the first cell came into focus to the point at the bottom surface where focus was lost. Mean section volumes were not statistically different (DBA, $0.000911 \pm 0.000214 \text{ mm}^3$ per section, $n=6$ mice; A/J: $0.001148 \pm 0.00026 \text{ mm}^3$, $n=4$ mice; Student's *t*-test, two-tailed, $P=0.0501$), indicating that any strain difference in neuronal density was not due to differential tissue shrinkage.

Hippocampal recordings *in vivo*

Surgical implantation of electrodes—A different group of animals than those tested for seizure susceptibility was used for EEG recordings. Animals were anesthetized with isoflurane (Henry Schein) and placed in a stereotaxic apparatus with a mouse adaptor (David Kopf, Tujunga, CA, USA). The skull was exposed after a midline incision to the skin overlying the scalp, and a jeweler's screw (Braintree Scientific, Braintree, MA, USA) was implanted immediately rostral and lateral to bregma. A second hole, approximately 1-2 mm wide, was drilled over the left hippocampus. Lacquer-coated, stainless steel wire (75 μm , Braintree Scientific) was twisted to make a bipolar electrode, approximately 2 mm long. Lacquer was removed from each tip, and each tip, was soldered to a gold pin. The electrode was placed vertically into the dorsal hippocampus under stereotaxic control. A reference electrode was made from a single strand of wire, and one end was removed from its lacquer coating and wrapped around the jeweler's screw. All electrodes were cemented to the skull dental cement (Braintree Scientific). The skin was sutured over the edges of the dental cement, alcohol was applied to the wound, and the animal was allowed to recover for at least 1 week prior to pilocarpine injection.

Recordings—Electrographic recordings were made using an amplifier and computer system (MP150, Biopac, Inc., Goleta, CA, USA). Simultaneous video was recorded using a digital camera attached to the side of the cage. After recordings, animals were killed by CO_2 anesthesia followed by decapitation. The brain was removed and immersed in 4% paraformaldehyde for 1-2 days. The location of electrodes in the hippocampus was verified by cutting the brain coronally at the level of the electrode track, and then using a dissecting microscope to inspect the location of the tip of the electrode track. The location was either in the area CA1 stratum radiatum, CA3b stratum radiatum, or the dentate gyrus.

Statistics

Statistical comparisons were made using SPSS software for Windows (v.11, 2001; SPSS Inc., Chicago, IL, USA), StatView (v.5.0; SAS Institute, Cary, NC, USA), or Microsoft Excel (Microsoft Office 2000; Microsoft, Redmond, WA, USA). Statistical significance was set at $P < 0.05$ prior to all experiments.

RESULTS

Status epilepticus: incidence and latency

There were 108 mice used in this study, 60 DBA mice and 48 A/J mice. Forty-eight animals had status after pilocarpine administration (24 DBA and 26 A/J). Table 1 and Fig. 1 show the number of animals that received each dose of pilocarpine.

The incidence of status was higher for the A/J strain (Table 1; Fig. 1). Despite the higher incidence of status in A/J mice, the latency to status was usually very long relative to the DBA mice (Table 1; Fig. 1). For some doses, such as the lowest dose (200 mg/kg), few DBA mice entered status, so statistical comparisons were precluded. However, sufficient animals entered status at the 250 mg/kg dose to allow a statistical comparison, and the latency difference was significant (see Table 1 for data and statistics). For all mice that entered status (all doses pooled), the mean latency was also distinct between strains (DBA: 30 ± 2 min, $n=24$; A/J: 133 ± 6 min, $n=26$; Student's *t*-test, two-tailed, $P < 0.0000001$).

Mortality

There were acute and chronic differences in mortality that were distinct in the DBA and A/J strains. In the initial 60 min after pilocarpine injections, 18/68 (26%) of mice died during a sudden tonic-clonic seizure (these mice are not included in calculations of incidence or latency in Table 1 and discussed above, because they did not meet the definition of entering status, i.e. 3 min continuous seizures). The severe tonic-clonic events were almost exclusively in the A/J mice (14/18 mice, or 78%, were A/J mice). The latency from pilocarpine administration to death was not statistically different between the two strains (DBA: 18 ± 3 min, $n=4$; A/J: 27 ± 5 min, $n=14$; Student's *t*-test, two-tailed, $P=0.218$). For A/J mice, the latency from pilocarpine injection to sudden death was not related to dose (ANOVA for linearity, $P=0.302$; Table 1), but incidence increased with dose (ANOVA for linearity, $P < 0.00001$). For DBA mice, statistical examination of the relationship between dose and death was not possible due to the low incidence of death.

Although mortality was high in A/J mice in the first hour after pilocarpine administration, the opposite appeared to be true when strains were compared several weeks after status. In other words, mortality was relatively high in DBA mice compared with A/J mice during the chronic period, i.e. weeks after status, and during the time when spontaneous recurrent seizures had developed. Of 15 DBA mice and 14 A/J mice that were followed after they had status, 9 of 15 (60%) DBA mice died in the 3 months after status, but none of the A/J mice died during this period of time (0/14; 0%). This difference was significant (Fisher's exact test, $P < 0.05$). The mean latency to death for DBA mice was 11.6 ± 1.4 weeks after status (range, 5-16 weeks; $n=9$).

In contrast to acute and chronic differences, there was no evidence of a strain difference in mortality during the 24 h after status: 0/26 A/J mice died, and 3 of 24 DBA mice died (0% vs. 12%, Fisher's exact test, $P > 0.05$). In summary, A/J mice appeared predisposed to acute tonic-clonic seizures ending in death, within 60 min of pilocarpine administration, whereas DBA mice demonstrated mortality in the period of chronic seizures.

Hippocampal electrographic recordings *in vivo*

The data described above indicate that A/J mice have a longer latency to status than DBA mice, regardless of dose. However, these data were based on behavioral observation. It is possible that the behavioral signs of status in the A/J mice were misleading, since A/J has not been well-studied with respect to status. Therefore, we considered the possibility that A/J mice had electrographic status earlier than behavioral signs would suggest. To address this possibility, we evaluated electrographic status in a new group of A/J mice. For these experiments, animals were implanted with a hippocampal electrode 1 week before pilocarpine administration (see Experimental Procedures). A 300 mg/kg dose was used to maximize the number of mice that would have status. Concurrent video and electrographic recordings showed that no electrographic manifestations of seizures were detected before, or in the 30 min following atropine administration (Fig. 2A; $n=3$). In all mice that were tested ($n=3$), EEG seizures did not begin until there were behavioral seizures (Fig. 2B). When the behaviors associated with the seizure ceased, so did the electrographic events. When behavioral status began, status began at the electrographic level as well (Fig. 2C). Electrographic status was defined by uninterrupted seizure activity in the EEG recording (Fig. 2C). The results suggest that the long latency to behavioral status of A/J mice was also the onset of electrographic status, and use of behavioral observation to identify the latency to status was valid for A/J mice.

Hippocampal changes resulting from status epilepticus in DBA vs. A/J mice

Animals who survived status and had spontaneous seizures were randomly selected for anatomical evaluation at a time when recurrent, spontaneous seizures had begun (at least 4 weeks after status). To minimize potential variability that might be related to the initial dose of pilocarpine, only animals that received 250 mg/kg pilocarpine were used.

Neuronal damage—To determine whether status led to a different degree of hippocampal pyramidal cell loss in the two strains, animals were perfused at least 4 weeks after status, and sections were evaluated semi-quantitatively using the neuronal marker NeuN. Specifically, DBA mice were perfused 62 ± 20 days after status (range, 30-120 days), and A/J mice were killed 83 ± 25 days after status, (range, 30-150); time to status was not statistically different between strains (Student's *t*-test, $P>0.05$). In this analysis, we assumed that the majority of hippocampal pyramidal cell damage had occurred by the time animals were perfused, an assumption based on the evidence that the majority of cell death after status in the rodent occurs in the days after status (Covolani and Mello, 2000; Wall et al., 2000; Meldrum, 2002; Fujikawa, 2005), and that status, not spontaneous seizures, is primarily responsible for damage (Pitkänen et al., 2002).

In the DBA strain, it appeared that a pattern of damage occurred that was typical of Ammon's horn sclerosis, because most of the CA1 and CA3 pyramidal cell layers were devoid of NeuN immunoreactivity, but granule cells and area CA2 appeared to be spared (Fig. 3B). In contrast, there was greater preservation of the pyramidal cell layers in the A/J mouse (Fig. 3C). The loss of NeuN reflected loss of neurons, rather than a loss of NeuN immunoreactivity, because it was confirmed by Cresyl Violet staining (data not shown).

As shown in Fig. 3, there were large sections of the CA3 pyramidal cell layer of DBA mice that were lost. This was present in all DBA mice examined ($n=7/7$; 100%), but not in any of the A/J mice ($n=0/5$, 0%; Mann-Whitney *U* test, $P<0.05$). For these evaluations, at least three sections, chosen from different anterior-posterior levels of the dorsal hippocampus, were evaluated for all animals, and results from the three sections were the same. All subfields were examined, and the damage in the DBA mouse was only evident in CA3a/b (Fig. 3).

In the same mice that were used to evaluate CA3, CA1 also demonstrated neuronal loss. In four of seven DBA mice there were sections of the cell layer that were devoid of immunoreactivity throughout its entire width (from stratum oriens to stratum radiatum; Fig. 3). Cresyl Violet staining showed that the loss of immunoreactivity was associated with a loss of neurons. At least three sections were examined per animal, like the examination of CA3. In contrast to DBA mice, none of the five A/J mice demonstrated neuronal loss in CA1. The difference between DBA and A/J mice was significant by non-parametric evaluation (DBA, 4 of 7, 57%; A/J, 0/5, 0%; Mann-Whitney *U* test, $P < 0.05$). The results suggest a distinct pattern of neuronal damage in the DBA strain after status relative to the A/J strain.

NPY—It has been shown that adult rats and mice which have had recurrent spontaneous seizures after status demonstrate de novo expression of NPY protein in the axons of granule cells, the mossy fibers (Sperk et al., 1996; Borges et al., 2003). However, this does not occur after an individual seizure (Sperk et al., 1996). Therefore, mossy fiber expression of NPY can be used to confirm that each strain had recurrent seizures prior to being killed. Therefore, we used mossy fiber NPY expression as a tool to confirm that each strain had recurrent seizures. Mossy fiber NPY expression was robust in the hilus and stratum lucidum, as well as the inner molecular layer, indicating de novo expression of NPY and mossy fiber sprouting in both strains (DBA, $n=5$ mice; A/J, $n=5$; Fig. 4). None of the saline-treated control mice had mossy fiber NPY expression ($n=7$; data not shown). We therefore conclude that both strains developed recurrent seizures (epilepsy), indicating that status leads to spontaneous seizures in both the DBA and A/J mouse. This is significant because some mouse strains do not appear to develop epilepsy after status (McKhann et al., 2003).

Some qualitative differences are evident in Fig. 4 upon comparison of the section from the DBA and A/J mouse. In the A/J mouse, hilar NPY immunoreactivity was greater than in the DBA mouse (Fig. 4). There also appeared to be de novo expression of NPY in some CA1 pyramidal cells (Fig. 4). In the DBA mouse, CA1 stratum radiatum immunoreactivity appeared greater than in the A/J mouse (Fig. 4). However, these differences were not observed in all animals.

Ectopic hilar granule cells—Status epilepticus increases the rate of neurogenesis in the dentate gyrus dramatically, and the effect is consistent across animal models of status (Parent et al., 1997; for review, see Scharfman, 2004; Scharfman et al., 2007). Many of the new neurons enter the hilar region (Parent et al., 1997, 2006; Scharfman et al., 2000). This also occurs in mice after status (Jung et al., 2004; Jessberger et al., 2005). The development of these “ectopic” hilar granule cells depends on a number of the factors, and genetic influence is likely given that the rate of baseline neurogenesis in the normal adult mouse varies with strain (Hayes and Nowakowski, 2002; Kempermann et al., 2006). Therefore, we examined the numbers of ectopic granule cells in the hilus in the two strains. Animals were perfused at least 4 weeks after status, a time after status when the population of hilar ectopic granule cells appears stable, based on data from the pilocarpine model in the rat (McCloskey et al., 2006). As shown in Fig. 5, cell density was greater in DBA mice (DBA: $32,984 \pm 8676$ cells/mm³, $n=6$; A/J: 6860 ± 1015 cells/mm³, $n=4$; Student's *t*-test, two-tailed, $P=0.0304$).

DISCUSSION

Summary

This study compared two strains that had not been previously characterized for their response to the chemoconvulsant pilocarpine: DBA and A/J mice. We found differences in the acute response to pilocarpine in that there were differences in the incidence and latency to status. A/J mice had a higher incidence, but longer latency. A/J mice also appeared to have a

predisposition to a sudden severe seizure that ended in death in the acute period after pilocarpine administration, whereas DBA mice demonstrated sudden death occurring in the chronic phase after recovery from status.

We also compared changes in hippocampus that developed after status. The pattern of neuronal damage was distinct in the CA1 and CA3 cell layers of the two strains, with a pattern of damage in the DBA strain that resembled Ammon's horn sclerosis. A/J mice lacked this pattern of damage, and showed fewer patches of neuronal loss in the pyramidal cell layers. There was greater mortality in the DBA strain, but only during the period when there were recurrent, spontaneous seizures. There were more ectopic hilar granule cells in the DBA strain.

Both strains developed spontaneous seizures and mossy fiber sprouting, suggesting some common sequelae to status despite the differences discussed above. However, there could have been differences in the degree spontaneous seizures developed. In other words, we cannot exclude a difference in the frequency of spontaneous seizures, or their severity and duration. Long-term, quantitative EEG would be required to determine if such differences exist. There could also have been differences in the extent of mossy fiber sprouting that NPY expression did not detect, and again, further experiments would be required to prove differences exist.

Susceptibility to status epilepticus induced by pilocarpine

Upon initial consideration, one might expect a “susceptible strain” would have both a high incidence and short latency to seizures, and more mortality, but the results suggest that susceptibility is not so straightforward. The higher incidence of status in the A/J strain, but longer latency to status, supports this perspective. Moreover, a higher incidence does not appear to predict long-term outcome. These conclusions are consistent with those previously discussed by others for kainic acid-induced seizures (McKhann et al., 2003; Schauwecker, 2003). They are also consistent with the suggestion that incidence, severity, onset, and duration of audiogenic seizures might be under separate genetic control (Seyfried et al., 1980).

Susceptibility has been examined by others, but to our knowledge there have been no studies of status, limbic seizures, or pilocarpine responses using A/J mice, and no comparisons between A/J and DBA mice have been reported. However, others have examined other types of experimentally-induced seizures in DBA and A/J mice, such as electrically-induced seizures (Frankel et al., 2001) and cocaine-induced seizures (Ferraro et al., 2001). The results of these studies, and the present results, indicate that the relative sensitivity of DBA and A/J mice appears to depend on the mode of seizure induction. For example, A/J are more resistant to maximum electroshock (MEST) seizures compared with DBA, whereas A/J were more susceptible than DBA to cocaine-induced seizures. Kosobud and Crabbe (1990) studied the DBA and A/J strains to compare the ED50 for seizure behavior (not necessarily status), using many different convulsants drugs. Based on the ED50 for convulsions, there were similarities in the ED50 for the DBA and A/J strains for some drugs, such as pentylenetetrazol, but not for others, such as DMCM (methyl-6,7-dimethoxy-4-ethyl beta carboline-3-carboxylate). One explanation could be that there are different genetic factors that contribute to different types of seizures (e.g. a single stage 5 seizure vs. status), and distinct genes control seizures induced by electrical vs. pharmacological methods.

Consequences of status in DBA and A/J mice

The DBA mouse appeared to develop a pattern of hippocampal pathology reminiscent of classic Ammon's horn sclerosis, with a widespread loss of CA1 and CA3 pyramidal cells, yet sparing of CA2 and the dentate gyrus. This did not appear to be the case in the A/J mouse, suggesting that the DBA strain provides a useful tool to address the hippocampal pathology common in TLE.

One reason for the difference might be a different severity of status in DBA and A/J mice, although there was no evidence of a difference in behavioral seizures during status. This possibility also seems unlikely given that studies of kainic acid-induced seizures and hippocampal pathology have shown that duration and severity of seizure activity during status did not predict hippocampal pyramidal cell death (McKhann et al., 2003). McLin and Steward (2006) have also demonstrated that the behavioral manifestations of seizures in different mouse strains are not related to subsequent neurodegeneration.

Differences in ectopic hilar granule cells

In adult rats or mice that have had status, neurogenesis in the dentate gyrus increases (Parent et al., 1997). Some of these new cells migrate “ectopically” to the hilus, and become granule cells (Parent et al., 1997; Scharfman et al., 2000; Scharfman, 2004), and this occurs in some patients with intractable TLE (Parent et al., 2006). It appears that the same is true for the DBA and A/J mouse, especially the DBA mouse, because there were more ec-topic hilar granule cells in the DBA mice that had status.

One potential explanation for the greater number of ectopic hilar granule cells in the DBA mouse is a higher rate of neurogenesis under all conditions. However, in a comparison between DBA and A/J mice, A/J mice had the higher rate of neurogenesis (Kempermann and Gage, 2002). Therefore, a higher basal rate of neurogenesis in the DBA strain is unlikely to have contributed to the results. Another potential explanation is that there was more hippocampal neuronal damage in the DBA mouse, and this may have induced a greater increase in neurogenesis. However, the relationship between seizures, hippocampal neuronal damage, neurogenesis, and formation of ectopic granule cells is not clear. It may not be a linear relationship. Thus, severe seizures appear to increase neurogenesis and ectopic granule cells more than single seizures, but extremely severe seizures that cause more neuronal cell death are accompanied by reduced survival of newly-born granule cells (Mohapel et al., 2004).

Other explanations for the difference in DBA and A/J mice could be a difference in seizure-induced changes in gene expression. For example, there could be greater loss of reelin in the DBA hilus, because a loss of reelin after status is thought to contribute to granule cell migration into the hilus after status (Gong et al., 2007).

Implications

The data presented here support the body of evidence that the best experimental designs to evaluate genes related to epilepsy would be those that measure multiple parameters. The results also provide the first data comparing the DBA and A/J mouse using the pilocarpine model, and suggest the A/J mouse might be useful to examine latency to status, given its unusually long latency to onset. A/J mice might also provide insight into seizures that lead to death, and particularly death after cholinergic seizures, which has potential relevance to seizures that occur after exposure to neurotoxins that are cholinesterase inhibitors. In contrast to A/J mice, the DBA strain might be informative in studies that address pathology in TLE, such as Ammon's horn sclerosis. The DBA mouse could lead to a better understanding of genes that influence seizure-induced neurogenesis. DBA mice could also be valuable in studies of sudden unexplained death in epilepsy (SUDEP). Taken together, this comparative study provides information that can be used to gain greater insight into the genetic factors that influence seizures, as well as mechanisms of TLE.

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Abbreviations

BSA, bovine serum albumin; NeuN, neuronal nuclear; NPY, neuropeptide Y; TLE, temporal lobe epilepsy.

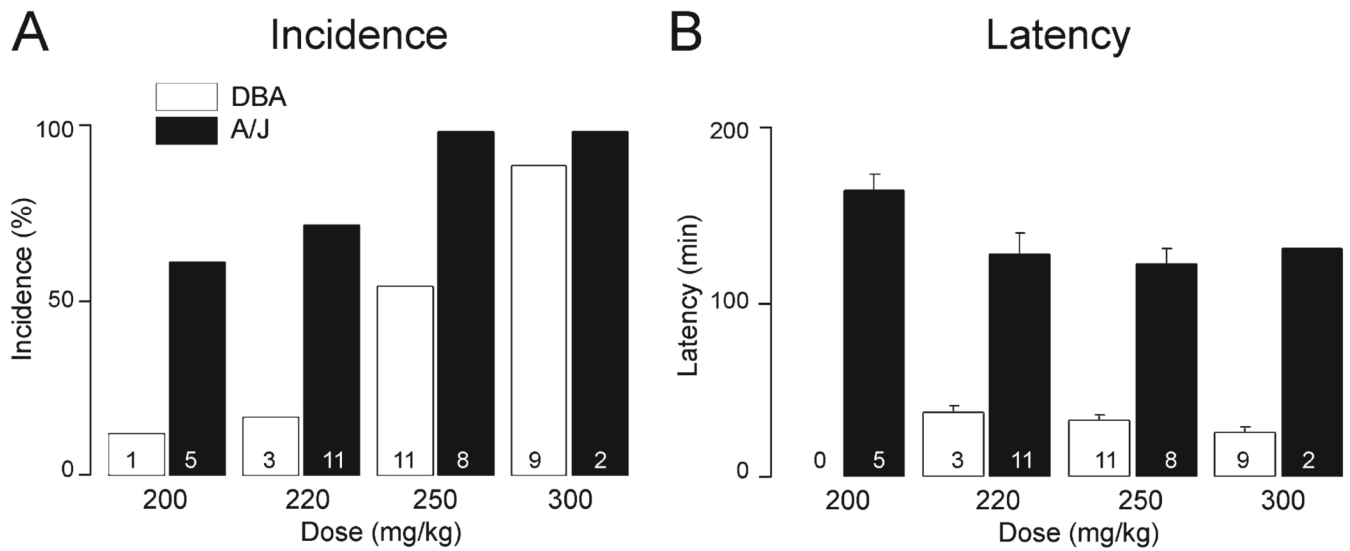
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**Fig. 1.**

Incidence and latency to pilocarpine-induced status in DBA and A/J mice. (A) The incidence of status is shown for DBA (white bar) and A/J (black bar) mice. Incidence was defined as the number of animals that entered status out of those that were administered pilocarpine and is expressed as a percent. Data for all doses are shown, and the sample sizes are designated by the numbers at the base of each bar. (B) The mean latency to status is shown for the same mice as used for part A. Statistics are provided in Table 1 and in the text.

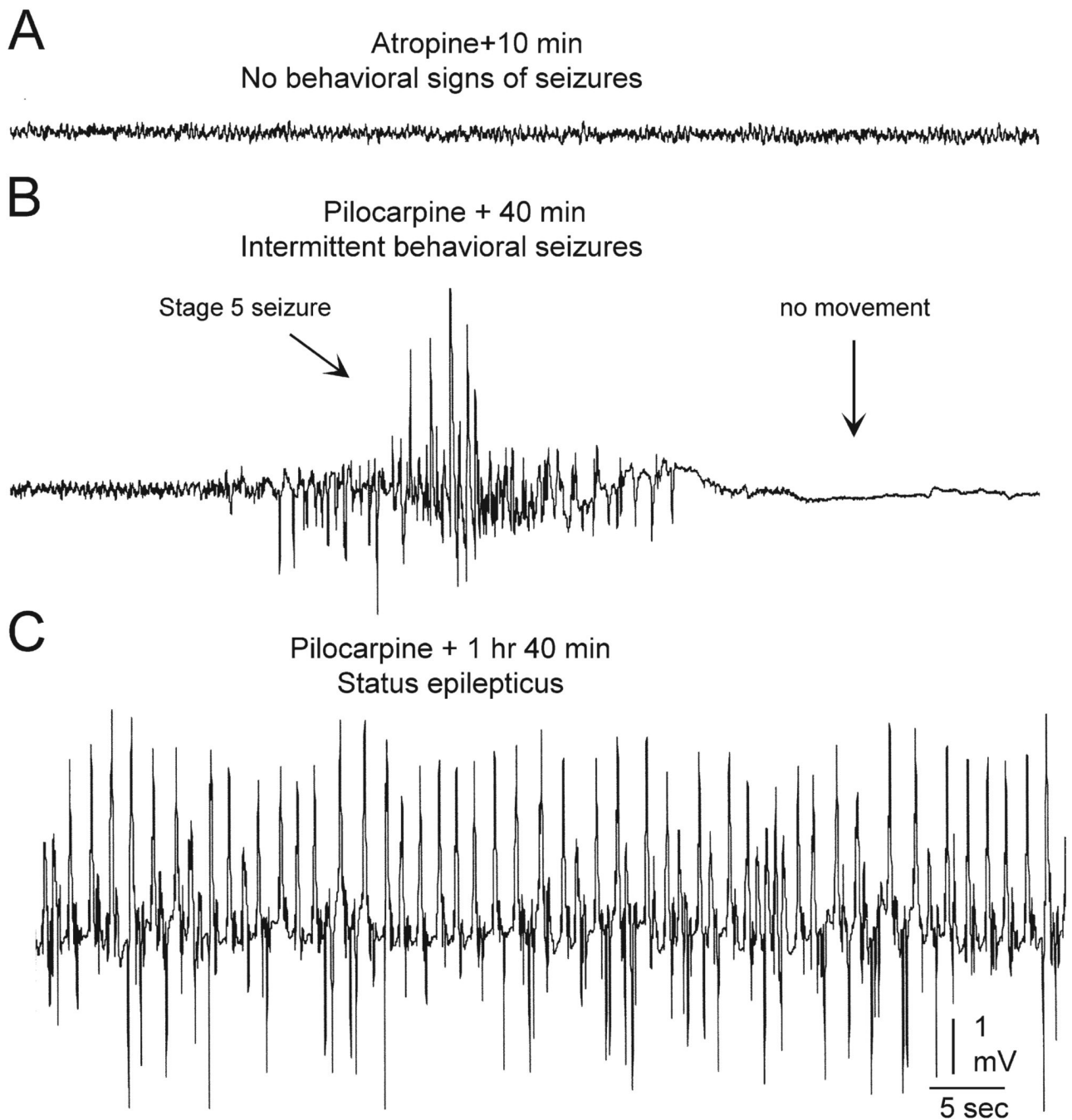


Fig. 2. EEG recordings from hippocampus of A/J mice. (A) A representative recording from dorsal hippocampus of an awake, behaving A/J mouse, using a bipolar electrode implanted in hippocampus. The trace was recorded 10 min after administration of atropine, 20 min prior to pilocarpine administration (see Experimental Procedures). There were no behavioral signs of seizures at the time, and no signs of seizures electrographically. (B) In the same animal as used for part A, a behavioral seizure occurred 40 min after pilocarpine injection. The recording that was taken during this time is shown. The stage 5 seizure occurred at the same time as the high-amplitude voltage deflections. After the seizure was over, the animal ceased all motor behaviors, and there was a decrease in EEG amplitude below the amplitude that was observed

before the seizure began. The decreased EEG amplitude after the seizure presumably reflects postictal depression. (C) In the same animal, behavioral status epilepticus began 1 h and 40 min after pilocarpine administration. During behavioral status, the electrographic activity that is shown was recorded. The continuous high voltage spikes began as the behavioral signs of status started, and are continuous, reflecting electrographic status.

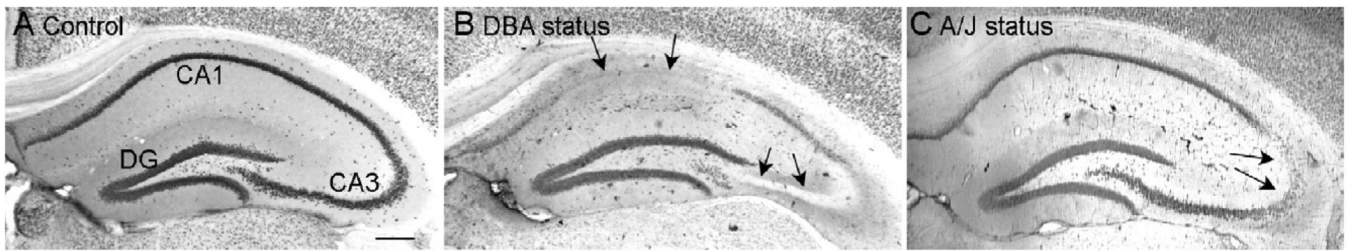


Fig. 3.

NeuN immunoreactivity in DBA and A/J mice. (A) A coronal section through the dorsal hippocampus from an A/J mouse that was injected with saline instead of pilocarpine illustrates the normal neuronal distribution in mouse hippocampus. Densely packed neurons comprise the principal cell layers: the dentate gyrus granule cell layer (DG), area CA3 pyramidal cell layer (CA3) and area CA1 (CA1). Scale bar=250 μ m. (B) A tissue section from a DBA mouse that had pilocarpine-induced status epilepticus and chronic seizures illustrates a pattern of neuronal loss similar to TLE. There is a substantial loss of area CA1 and area CA3 (arrows) pyramidal cells, as well as neurons in the hilus of the DG. Area CA2 and the DG granule cell layer are relatively preserved. Scale bar same as A. (C) A tissue section from an A/J mouse that had pilocarpine-induced status epilepticus and chronic seizures shows relatively preserved area CA1 neurons, and a small area of neuronal loss in the part of area CA3 termed CA3a (arrows). Scale bar same as A. The DBA and A/J animals had the same dose of pilocarpine (250 mg/kg), similar behavioral manifestations during status, and were killed at a similar age after status occurred (1 month). There was no evidence that either animal had more severe status or more recurrent seizures, although EEG recording may have demonstrated differences. Therefore, it is likely that there was a difference related to the strain that led to differential neuronal damage.

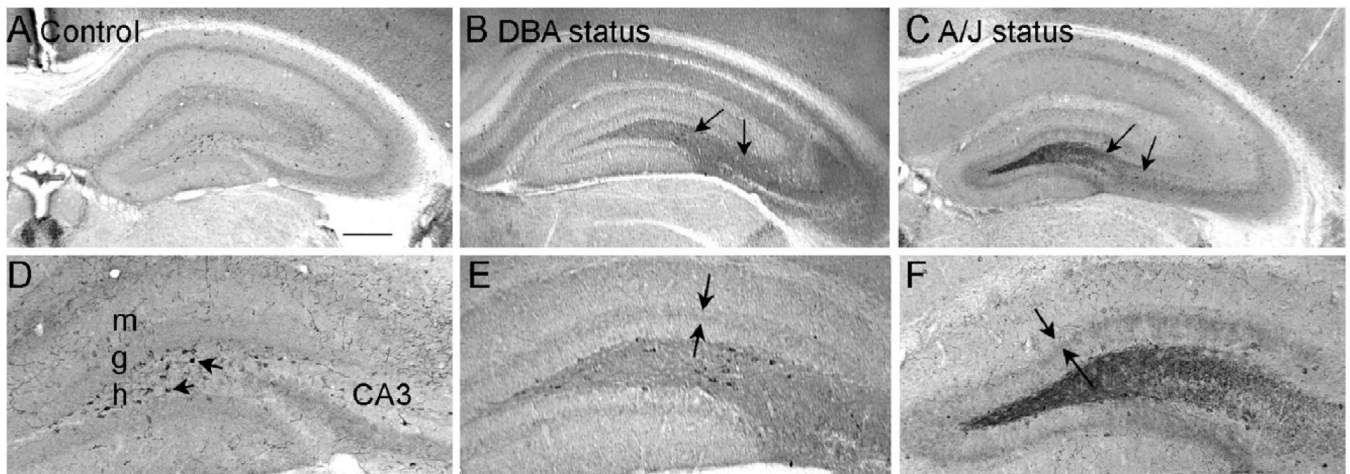


Fig. 4.

Comparison of NPY immunoreactivity in DBA and A/J mice. (A) A tissue section from an A/J mouse that was treated with pilocarpine but had no evidence of seizures, and subsequently was perfused 4 months later to evaluate NPY immunoreactivity in hippocampus. The section illustrates a pattern of NPY expression primarily in non-principal cells, similar to the normal adult rodent, which is shown at higher resolution in part D. Scale bar=250 μ m. (B) A section from a DBA mouse that had pilocarpine-induced status epilepticus and chronic seizures, and was killed 3.5 months after status. The arrows indicate the increase in expression in NPY in the mossy fiber pathway that is typical of animals with recurrent seizures. Scale bar same as A. (C) NPY immunoreactivity in an A/J mouse that had pilocarpine-induced status and recurrent seizures, and was perfused 3 months later. The arrows point to the mossy fibers, which are NPY-immunoreactive. Scale bar same as A. (D) Higher resolution images of the DG from sections illustrated in A. NPY immunoreactivity is present in neurons in the hilus (arrows). Scale bar=150 μ m (A). (E, F) Higher resolution images of the sections shown in B-C, respectively. Animals with chronic seizures exhibited de novo expression of NPY in mossy fibers within the hilus and stratum lucidum of CA3, the normal projection of mossy fibers. There also was immunoreactivity in the inner molecular layer, reflecting mossy fiber sprouting (arrows in E, F). Although the density of mossy fiber immunoreactivity appeared darker in the section shown in F relative to E, this was not consistent across animals. Scale bar=150 μ m (A). m, molecular layer; g, granule cell layer; h, hilus.

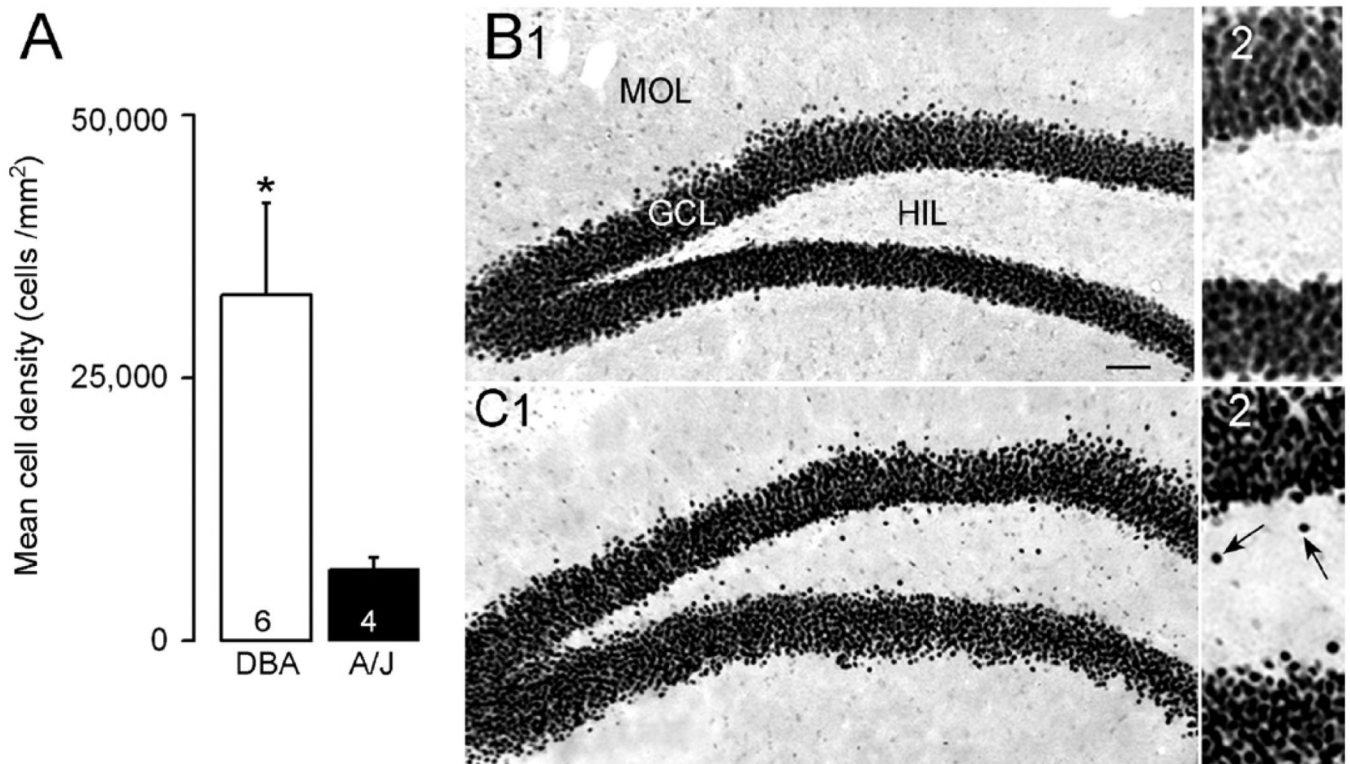


Fig. 5.

Comparison of hilar ectopic granule cells in DBA and A/J mice after status. (A) The numbers of ectopic granule cells in the DBA (white bar) and A/J (black bar) strains after status were compared using Prox1 as a marker of granule cells. Sample size (number of animals) is shown at the base of the bar. DBA mice had a greater density of hilar ectopic granule cells (asterisk; for values, statistics, and Experimental Procedures, see text). (B1) Prox1-immunoreactivity in a coronal section through dorsal hippocampus of a DBA mouse that was administered pilocarpine but did not have status epilepticus. MOL, molecular layer; GCL, granule cell layer; HIL, hilus. Scale bar=50 μ m (A, B). (B2) Higher magnification of the section shown in B1 illustrates the lack of Prox1 immunoreactivity in the hilus. Scale bar=20 μ m (B1). (C1) Prox1-immunoreactivity in a section that was selected from a similar septotemporal level as the one in part A, but was from a DBA mouse that had pilocarpine-induced status epilepticus and recurrent seizures. (C2) Arrows point to immunoreactive profiles in the hilus from part of the section shown in B2 to illustrate Prox1-immunoreactive profiles. Scale bar=20 μ m (B1).

Table 1
Incidence and latency to status epilepticus in DBA and A/J mice

Measure	Dose (mg/kg)			
	200	220	250	300
Incidence ^a				
DBA				
Status/tested ^b	1/8	3/18	11/22	9/10
%	12	17	50	90
A/J				
Status/tested	5/8	11/15	8/8	2/2
%	62	73	100	100
Latency ^c				
DBA (min)				
Mean±S.E.M.	38 ^d	36±4	32±3	25±2
<i>n</i>	1	3	11	9
A/J (min)				
Mean±S.E.M.	163±10	127±12	121±9	130 ^e
<i>n</i>	5	11	8	2
Mortality ^f				
DBA				
Deaths/total tested	1/9	0/18	1/21	2/12
%	11	0	5	17
A/J				
Deaths/total tested	0/8	2/17	6/14	6/8
%	0	12	43	75

^aThe number of animals that experienced status relative to the number that were injected with pilocarpine is listed for the four doses of pilocarpine that were used. The incidence of status was different for all doses (Fisher's exact test, $P < 0.05$).

^bThe number tested was defined here as the total number, except for those mice that died in the first 60 minutes after pilocarpine administration. These mice died of a sudden tonic-clonic seizure and it could not be determined if they would have entered status, because death occurred before the 3 min used to define status (see Experimental Procedures).

^cThe latency from pilocarpine injection to the onset of status is listed for the same animals as used to evaluate incidence of status. The latency to status was statistically different for the 250 mg/kg doses, the only dose that could be compared statistically (Student's *t*-test, $P < 0.0000001$).

^dLatency of the one DBA mouse that had status at the 200 mg/kg dose.

^eS.E.M. not applicable.

^fThe number of animals that had a severe tonic-clonic seizure within 60 min of pilocarpine administration is listed. These animals ceased respiration during the seizure. These animals are not included in incidence and latency calculations.