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Persistent seizure control in epileptic mice transplanted with GABA progenitors

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Abstract

Objective—A significant proportion of the more than 50 million people world-wide currently suffering with epilepsy are resistant to antiepileptic drugs (AEDs). As an alternative to AEDs, novel therapies based on cell transplantation offer an opportunity for long-lasting modification of epileptic circuits. To develop such a treatment requires careful preclinical studies in a chronic epilepsy model featuring unprovoked seizures, hippocampal histopathology, and behavioral comorbidities.

Methods—Transplantation of progenitor cells from embryonic medial or caudal ganglionic eminence (MGE, CGE) were made in a well-characterized mouse model of status epilepticus-induced epilepsy (systemic pilocarpine). Behavioral testing (handling and open field), continuous video-electroencephalographic (vEEG) monitoring and slice electrophysiology outcomes were obtained up to 270 days after transplantation (DAT). *Post hoc* immunohistochemistry was used to confirm cell identity.

Results—MGE progenitors transplanted into the hippocampus of epileptic mice rescued handling and open field deficits starting at 60 DAT. In these same mice, an 84–88% reduction in seizure activity was observed between 180 and 210 DAT. Inhibitory postsynaptic current frequency, measured on pyramidal neurons in acute hippocampal slices at 270 DAT, was reduced in epileptic mice but restored to naïve levels in epileptic mice receiving MGE transplants. No reduction in seizure activity was observed in epileptic mice receiving intra-hippocampal CGE progenitors.

Interpretation—Our findings demonstrate that transplanted MGE progenitors enhance functional GABA-mediated inhibition, reduce spontaneous seizure frequency and rescue behavioral deficits in a chronic epileptic animal model more than six months after treatment.

Introduction

Epilepsy is a spectrum of neurological disorders with heterogeneous origins characterized by unprovoked seizures, behavioral and cognitive comorbidities. Available epilepsy treatments

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include AEDs which provide pharmacological modulation of receptors (NMDA, AMPA and GABA) or ion channels (Na^+ and Ca^{++})[1], vagal nerve stimulation[2] or surgical resection[3]. Unfortunately, therapies are associated with side effects and nearly one-third of all epilepsy patients remain refractory.

Interneurons are specialized cells in the central nervous system that release an inhibitory neurotransmitter (GABA) and share a common embryonic origin in medial and caudal ganglionic eminences (MGE and CGE, respectively)[4–6]. In acquired and genetic forms of epilepsy inhibitory interneuron loss or dysfunction has been reported[7–9]. Because embryonic interneuron progenitor cells migrate following transplantation[10–13], they offer a potentially exploitable cell source for an interneuron-based cell therapy. Transplanted MGE-derived cells integrate and exhibit mature electrophysiological properties consistent with fast-spiking parvalbumin (PV)-positive and regular-spiking somatostatin (SOM)-positive interneuron sub-types[11,14,15]. MGE-derived interneurons receive sub-type appropriate functional excitatory synaptic inputs from host brain neurons[11,16] and synaptic contacts between transplant-derived interneurons and host neurons have been observed using electron microscopy[17], optogenetics[13,18,19], and dual recordings[16,20]. Most importantly, MGE-derived interneurons appear to selectively innervate excitatory neurons resulting in functional enhancement of GABA-mediated synaptic inhibition[11,15–17,19,21]. CGE-derived interneurons can also migrate following transplantation, differentiate to vasoactive intestinal polypeptide (VIP)- and reelin-positive interneurons, and make functional inhibitory connections onto either pyramidal cells or inhibitory interneurons[22,23].

MGE progenitor transplantation was first shown to suppress unprovoked seizures in a potassium channel mutant mouse (Kv1.1) representing an ion channel mutation associated with severe tonic-clonic seizures in humans[17]. Starting at 30 DAT, video-electroencephalographic (vEEG) monitoring demonstrated a 90% reduction in seizure frequency in these mice. Using a well-characterized pilocarpine model of acquired epilepsy, we also demonstrated that MGE progenitors transplanted into adult hippocampus reduced unprovoked seizures by 90% starting at 60 DAT[14]. While our studies strongly support continued preclinical development of MGE progenitor transplantation as a therapy for intractable epilepsies, other groups suggested these effects may be transient[18,24,25] and CGE-based cell transplantation for epilepsy has not been studied.

To address discrepancies, and provide a more complete understanding of interneuron sub-populations that could be used for therapeutic, we transplanted embryonic mouse MGE or CGE progenitors into pilocarpine mice exhibiting behavioral deficits and epilepsy. Our results demonstrate that intra-hippocampal MGE progenitor transplantation elicits a long-lasting suppression of unprovoked seizures, rescues behavioral deficits, and enhances GABA-mediated synaptic inhibition in epileptic mice. CGE progenitors were not therapeutic in epileptic animals and resulted in abnormal, and in some cases frank epileptic, electrographic activity in wild-type animals.

Materials and Methods

Animals

Experiments were done with male adult CD1 mice (http://www.criver.com/products-services/basic-research/find-a-model/cd1-e-mouse) housed under a standard 12 hr light cycle with food and water provided *ad libitum*. Epilepsy was induced by pilocarpine injection as described previously[14,26]. Briefly, adult (P50-P60) CD1 mice were first injected with methyl scopolamine (1 mg/kg), after 30 min pilocarpine (289 mg/kg) was injected i.p. to induce status epilepticus (SE). Injection of pilocarpine produced generalized electrographic activity characterized by high amplitude and high frequency paroxysmal discharges consistent with SE[27] (inset in Fig. 1A). This electrographic activity corresponded with continuous, generalized tonic-clonic seizures associated with loss of postural control. After experiencing at least 3 seizures Grade 3 or greater on a modified Racine scale[26], mice were allowed to recover for 10 days. Mice were then continuously video monitored for up to 20 days for detection of spontaneous convulsive seizures and classified as “epileptic” only after video confirmation of at least 2 seizures Grade 4 or 5. All procedures were done under the approval of the Institutional Animal Care and Use Committee and following the guidelines of the Laboratory Animal Resource Center at the University of California, San Francisco and in accordance with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals.

Interneuron progenitor isolation and grafting

MGE and CGE dissection were performed as described[11-14]. Tissue was harvested from E13.5 β-actin GFP mice for MGE transplants and from E14.5 Nkx2.1-Cre/R26-GDTA mice for CGE transplants. Diphtheria toxin alpha subunit (DTA) is a potent intracellular toxin that induces programmed cell death within 48 h of expression by blocking protein synthesis. The R26-DTA allele facilitates identification of donor cells (through GFP expression) and ablation of specific cell types through DTA expression is controlled in a Cre-dependent manner. For CGE transplants, we minimized potential MGE contamination by a combination of anatomical micro-dissection (caudal-dorsal CGE) and Nkx2.1 (MGE-specific) driven ablation. Concentrated cell suspensions (~10^3 cells nl^{-1}) were front loaded into beveled glass micropipettes for injection (3×10^4 cells per injection). Stereotaxic coordinates and hippocampal cell grafting procedures were done, as described[14]. Estimates for initial cell viability (70-80%) and cell survival following transplantation (15-20%) matched values previously reported by our laboratory[11,14].

Immunofluorescence

Mice were deeply anesthetized and perfused with cold 4% paraformaldehyde solution. The fixed brains were sliced in 50 μm coronal sections using a free-floating vibratome (Leica). Sections were double stained with a primary antibody against GFP (Aves Labs) and GAD67 (Abcam; ab97739)/TUJ (synaptic system; 302302)/SOM (Santa Cruz; sc7819)/PV (Abcam; ab11427)/CR (Millipore; AB5054)/nNOS (Millipore; AB5380)/VIP (Abcam; AB22736)/Reelin (Millipore; MAB5364). Secondary antibodies were anti-chicken Alexa 488 and anti-mouse or anti-rabbit Alexa 594 (Vector; 11039, 11005, 11012 respectively). Nuclei staining

were done for 5 min as a final step (Sigma; H33342). Sections were mounted onto charged slides (Fisher Scientific, Superfrost Plus) using Fluoroshield solution (Sigma, F6937).

Cell quantification

Images were obtained with a Leica TCS SP5 confocal or Nikon Eclipse Ni-E epifluorescence microscope. Labeled cells were counted using Fiji (http://imagej.net). Cell quantification was done by t-test analysis using GraphPad Prism 7. Data is expressed as mean ± standard error of the mean (SEM).

Behavior

Behavioral tests were done by the Neurobehavioral Core for Rehabilitation Research at UCSF. Briefly, for the Open Field test mice were placed in the center of the arena (Kinder Scientific) and recorded for 10 min using a computer operated tracking system (MotorMonitor Kinder Scientific). Handling test was done as described[14]. Non-stressful handling was performed by rubbing slowly along the back of the mouse in a petting motion in the direction of the grain of fur with a latex-gloved hand, and stressful handling was performed by rubbing vigorously against the grain of fur, pinching at the tail tip with a plastic-tipped hemostat and pinching at the tail base. Each task was performed for 15 s. Reaction to handling was scored for each task using a four-point rating scale in which 1 indicated initial struggle, but calmed within 15 s, 2 indicated struggle for more than 15 s, 3 indicated struggle for more than 15 s and exhibiting one or more defensive reactions (piloerection, flattening of the ears against the head, attempt to bite or back away from the experimenter), and 4 indicated struggled for more than 15 s and exhibited flight behavior (loud vocalization or wild running). Behavioral test analysis was done using GraphPad Prism 7 software.

Chronic video-EEG (vEEG)

EEG recordings were obtained using a time-locked and tethered vEEG monitoring system (Pinnacle Technologies). Each mouse was anesthetized with ketamine and xylazine (10 mg/kg and 1 mg/kg i.p.) so that there was no limb-withdrawal response to a noxious foot pinch. Sterile, stainless steel bone screw recording electrodes were placed epidurally through burr holes drilled in the skull (one electrode on either side of the sagittal suture, approximately halfway between bregma and lambda sutures and ≈1 mm from the midline) using surface head-mount EEG hardware (Pinnacle Technologies). Electrodes were cemented in place with a fast-acting adhesive and dental acrylic. Two wires were laid on the shoulder muscles for electromyographic (EMG) recording. Animals were allowed to recover for 5 days before EEG monitoring was initiated. Electrographic seizures were defined as progressive high-frequency (minimum of 5 Hz), high-voltage synchronized polyspike or paroxysmal sharp waves with amplitude >2-fold background that lasted for a minimum of 15 sec. All EEG files were coded and analyzed by an investigator blinded to the recording condition. Electrographic EEG seizures were analyzed using SireniaScore software (Pinnacle Technologies) at one minute per frame. All mice included in vEEG analysis were monitored for 14 consecutive days (24 h/day) e.g., a period sufficient to capture at least one seizure cycle[18,27].

To determine the number of mice necessary for EEG monitoring a power analysis was performed using SISA software (http://www.quantitativeskills.com/sisa/). Spontaneous seizure frequency for pilocarpine-treated CD1 mice was estimated at 2.4 seizures per day with a standard deviation of ± 0.46 seizures/day[14,26]. Using 10% as a value for β (1 – β = power or 90%), 0.01 as a value for α and an expected 80% change in seizure frequency[14] it is estimated that sample size should be 5 animals per experimental group.

Electrophysiology

Coronal brain slices (300 μm thickness) were prepared from mice at 270 DAT. Mice were deeply anesthetized with ketamine and xylazine (10 mg/kg and 1 mg/kg i.p.) until unresponsive to toe pinch. Mice were decapitated and brains were submerged into ice-cold oxygenated sucrose cutting solution, containing (in mM): 150 sucrose, 50 NaCl, 25 $NaHCO_3$, 10 dextrose, 2.5 KCl, 1 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$. Brains were adhered to a cutting platform on a vibratome in this same solution. Slices were then transferred into a holding chamber containing artificial cerebrospinal fluid (ACSF), containing (in mM): 124 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl. The ACSF temperature was held at 35° C for 30 min, then allowed to equilibrate to room temperature. For recordings, slices were transferred into a recording chamber mounted to an upright microscope (Olympus) and constantly perfused with oxygenated ACSF. Borosilicate glass patch pipettes (3-5 MΩ) were guided to CA1 pyramidal neurons visually identified under IR-DIC optics. Pipettes contained a cesium chloride-based internal solution for optimal recording of inhibitory postsynaptic currents (IPSCs), containing (in mM): 140 CsCl, 11 EGTA, 10 HEPES, 1 $MgCl_2$, 2 ATP-Mg, 0.5 GTP-Na. After whole cell configuration was achieved, cells were held at −70 mV. Spontaneous IPSCs (sIPSCs) were recorded for a 5-min epoch. A 5-min epoch was recorded during which slices were perfused with ACSF containing kynurenic acid (3 mM) and tetrodotoxin (0.1 μM) to block glutamatergic synaptic transmission and action potential-mediated IPSCs. Miniature IPSCs (mIPSCs) were then recorded during a third 5-min epoch. Only cells in which input resistance and holding current were stable were used for data analysis. s- and mIPSCs were analyzed using MiniAnalysis v6 (Synaptosoft) and Prism v6 (Graphpad) software.

Statistics

Data is expressed as mean ± SEM and compared by repeated measures one- or two-way ANOVA with Tukey's multiple comparison test, except as otherwise indicated. Significance in all cases was set at $p < 0.05$.

Results

MGE progenitors transplanted in adult epileptic mice rescue behavioral comorbidities

Adult male CD-1 mice (n = 134) were administered pilocarpine (P50-P60) to elicit a series of generalized tonic–clonic limbic seizures progressing to status epilepticus (SE)[26,28]. After recovering from SE, mice were video monitored for evidence of spontaneous seizure behavior between 10 and 20 days after pilocarpine (n = 70). Only mice that survived acute SE and were subsequently observed to exhibit at least two unprovoked tonic-clonic seizures of Racine Grade 4 or greater were designated as "epileptic" (n = 63; 90%) and became

candidates for transplantation. The open field (OF) test was used to monitor the severity and progression of epilepsy co-morbidities in these animals (Fig. 1A). The first OF test was performed ~P50 before SE (OF1) with subsequent tests at P80 after the 20th day of post-pilocarpine video monitoring but prior to transplantation (OF2) and then at P120 (1 day before transplantation, DAT; OF3) and P180 (60 days after transplantation, DAT; OF4); age-matched naive male CD-1 mice served as controls at equivalent time-points. As expected[29,30], epileptic mice exhibited progressive OF behavior deficits compared to controls (Figs. 1B1-B2). Increased time spent in the periphery of the arena was observed at P120 (OF3) and P180 (OF4) for epileptic mice but not controls (Fig. 1B1). Decreased time standing on their hind legs (rearing) was also observed at P80 (OF2), P120 (OF3) and P180 (OF4) for epileptic mice but not controls (Fig. 1B2). Next, we examined whether MGE cell transplantation could rescue the OF deficit. Epileptic mice receiving bilateral intra-hippocampal MGE progenitor cell transplantation (n = 26) spent significantly less time in the periphery of the arena (Fig. 1B3) and showed no change in rearing behavior (Fig. 1B4). As an additional behavioral evaluation, a handling test was performed[14]. Epileptic mice exhibited increased reaction to handling that was reduced in mice receiving bilateral MGE transplants (Fig. 1C). All epileptic mice displayed aggressive behavior to touch and a “less groomed” overall appearance (Fig. 1D).

MGE progenitors transplanted in adult epileptic mice suppresses seizure activity

Our previous studies reported a 92% reduction in electrographic seizures following bilateral intra-hippocampal MGE transplantation in epileptic mice starting at 60 DAT[14]. Henderson et al., (2014), however, reported a much smaller overall reduction in the total number of spontaneous recurrent seizures following bilateral intra-hilar MGE transplantation (35% at 47-67 DAT), and the effect reported was transient with no reduction seen at 68-97 DAT. Therefore, to further investigate the long-term disease modifying effects of intra-hippocampal MGE progenitor cell transplantation, adult mice (P300) tested in the OF and handling assays underwent 14 days of continuous video-EEG (vEEG) monitoring starting at 180 DAT (Fig. 1A). Mice were separated into 3 groups and coded for off-line detection of seizures (Fig. 2A) by an investigator blind to the status of the experiment: (i) epileptic mice with bilateral intra-hippocampal vehicle injection (Pilo; n = 5), (ii) epileptic mice with bilateral intra-hippocampal MGE transplantation (Pilo + MGE; n = 7), and (iii) age-matched naïve mice (Con; n = 5). All vehicle-injected epileptic mice displayed spontaneous electrographic seizures greater than 15 s in duration, defined by high-frequency, high-voltage, rhythmic activity with clear onset, progression and termination. A representative example is shown in Figure 2A. Electrographic seizures were never observed in naïve mice. Epileptic mice with bilateral intra-hippocampal MGE transplantation exhibited an 84% reduction in mean seizure frequency (Figs. 2B-C) and an 88% reduction in the total number of seizures detected (Fig. 2D); one mouse was seizure free during the entire 14-day monitoring period. Transplantation had no effect on seizure duration (Fig. 2E), as reported previously[14].

At 210 DAT, a sub-set of epileptic mice with bilateral intra-hippocampal MGE transplantation, behavioral testing, and EEG monitoring were sacrificed for immunohistochemistry (Fig. 1A; n = 5). Antibodies recognizing green fluorescent protein

(GFP) and markers of MGE-derived interneurons (e.g., GAD67, parvalbumin, somatostatin and nNOS) were used to confirm cell identity. Consistent with previous results[14,15,21], we found that GFP+ MGE cells migrate following transplantation (Fig. 3A), co-express neuronal markers such as β3 tubulin (Tuj1) and GAD67 (Figs. 3B-C), as well as parvalbumin, somatostatin or nNOS (Fig. 3D). To confirm the long-lasting integration of MGE-derived interneurons in pilocarpine mice, we processed a separate group of epileptic animals at 12 months after transplantation (360 DAT; n = 3) and confirmed that GFP+ MGE cells remain positive for parvalbumin, somatostatin or nNOS (Fig. 3E).

At 270 DAT, a separate pool of naïve (n = 6), untreated epileptic (n = 7), and epileptic mice receiving bilateral intra-hippocampal MGE progenitor cell transplantation (n = 6) previously tested on behavioral and EEG assays were sacrificed for slice electrophysiology. To confirm a functional enhancement of GABA-mediated inhibition[11,16,17], we obtained voltage-clamp recordings from pyramidal neurons in regions of hippocampus containing GFP+ cells. CA1 pyramidal neurons were held at -70 mV and spontaneous and miniature inhibitory postsynaptic current (sIPSC and mIPSC, respectively) was measured (Fig. 4A). By one-way ANOVA followed by Tukey's multiple comparisons test ($p = 0.0011$), event frequency was significantly reduced for sIPSCs in epileptic animals compared with naïve, as previously reported[28] (naïve: 9.36 ± 1.15 Hz, n = 6 cells; pilocarpine: 3.62 ± 0.69 Hz, n = 14 cells) but restored to levels comparable to age-matched naïve controls in epileptic mice receiving intra-hippocampal MGE cells (MGE: 6.86 ± 1.14 Hz, n = 9 cells; Fig. 4B). Similar statistically significant reductions ($p = 0.0135$) were observed for mIPSC frequency (naïve: 7.79 ±1.84 Hz, n = 6 cells; pilocarpine: 3.09 ± 0.84 Hz; n = 12 cells) and restored to levels comparable to age-matched naïve controls in epileptic mice receiving intra-hippocampal MGE cells (MGE: 7.65 ± 1.42, n = 6 cells; Fig. 4E). No change in sIPSC decay tau kinetics ($p = 0.296$; Figs. 4C) or rise time ($p = 0.705$; naïve: 1.98 ± 0.24 ms, n = 6 cells; pilocarpine: 2.16 ± 0.87 ms, n = 14 cells; MGE: 2.35 ± 0.31 ms, n = 9 cells) were apparent between groups. No change in mIPSC decay tau kinetics ($p = 0.425$; Figs. 4F) or rise time ($p = 0.052$; naïve: 2.19 ± 0.34 ms, n = 6 cells; pilocarpine: 1.76 ± 0.21 ms, n = 12 cells; MGE: 2.72 ± 0.26 ms, n = 9 cells) were apparent between groups.

CGE progenitor transplantation is not therapeutic in mice

CGE progenitors transplanted into adult mouse hippocampus also migrate and integrate as GABAergic neurons[12,13]. In contrast to MGE, CGE transplanted progenitors primarily differentiate to calretinin, vasoactive intestinal protein (VIP) and reelin-positive interneuron sub-populations shown to form functional synapses with excitatory and inhibitory neurons. Here we investigated the transplantation of CGE progenitors in adult mice using *Nkx2.1-Cre;R26-GDTA* donor embryos to genetically ablate MGE-derived interneurons[13,31]. We followed two transplant strategies: (i) intra-hippocampal transplantation into wild-type neonatal mice to evaluate CGE progenitors in healthy animals and (ii) intra-hippocampal transplantation into adult epileptic mice to evaluate potential therapeutic effects (Fig. 5A). CGE-derived neurons from *Nkx2.1-Cre;R26-GDTA* donor mice contain virtually no PV or SOM interneurons[13] but co-express VIP (Fig. 5A). At 30 DAT, we observed brief "interictal" bursts lasting 2 to 5 s in duration in all CGE-transplanted mice (n = 3; Fig. 5B). At 90 DAT, EEG events with high-frequency, high-voltage, rhythmic activity with clear

onset, progression and termination were observed in all mice (n = 3; Fig. 5B). Next, a separate group of adult epileptic mice (~P50) receiving bilateral CGE progenitor cell transplantation 10-20 days after pilocarpine SE underwent 14 days of continuous vEEG monitoring starting at 60 DAT (n = 5). In stark contrast to epileptic mice receiving MGE progenitor cells (Fig. 3), the pattern and frequency of electrographic seizure activity did not diminish (Fig. 5C-D). No statistical difference between seizure frequency ($p > 0.05$ Kruskal-Wallis one-way ANOVA on ranks; Pilo + CGE: 3.1 ± 1.1 seizures/day; Pilo: 4.6 ± 0.7 seizures/day) or the total number of seizures detected ($p > 0.05$ Kruskal-Wallis one-way ANOVA on ranks; Pilo + CGE: 43 ± 16 seizures; Pilo: 71 ± 26 seizures) was noted.

During continuous 14-day monitoring of EEG activity in naïve control, epileptic and epileptic mice with MGE/CGE transplants we noted the presence of prominent and sustained oscillations with a peak frequency around 8 Hz (Fig. 6A-B). These oscillations were not associated with any overt behaviors or EMG activity. Analysis of a random sampling of 100 oscillatory events per animal group showed that the duration of these events was similar in all mice (Fig. 6C). These oscillatory events, though greater than 2 s in duration, characterized by an abrupt onset and termination, and appearing to be both high-amplitude and rhythmic, were not classified as seizures or considered pathological.

Discussion

We found that MGE progenitor cells transplanted into the hippocampus of adult epileptic mice exert a long-lasting therapeutic effect. Studies described here were performed between 1 and 12 months after transplantation in a well-characterized clinically-relevant mouse model of acquired epilepsy[29,32–34]. These studies independently confirm and significantly extend our earlier report of seizure suppression and rescue of behavioral comorbidities in this model starting at 2 months after transplantation using MGE progenitors[14]. We also showed, for the first time, that CGE progenitors transplanted into the hippocampus of adult animals lead to abnormal electrographic seizure-like activity in healthy mice but have no effect on seizure activity in epileptic mice.

Although our primary goal was to demonstrate an effect of MGE progenitor cell transplantation on spontaneous recurrent seizures, the pilocarpine model used here also exhibits well-established behavioral comorbidities[32–34]. TLE patients exhibit comorbidities such as cognitive decline, anxiety and depression[35]. While seizure control is an important consideration in the treatment of these patients, a therapy that also modifies comorbidities would be of great value. Epileptic pilocarpine-treated mice allowed us to efficiently study both issues in a pre-clinical research setting. Unlike other studies putatively designed to analyze the effects of cell transplantation on epilepsy comorbidities[36,37], we first confirmed a seizure phenotype in all test animals using continuous video monitoring following pilocarpine-induced status epilepticus. With this strategy, we avoid transplanting mice that recover from status epilepticus and remain seizure-free[30] thus maintaining an experimental protocol analogous to the clinical situation wherein a patient would seek treatment *after* the emergence of spontaneous recurrent seizures. Once identified as epileptic, mice were periodically monitored for open field and handling deficits[29,32,34]. Consistent with previous studies[29,30], epileptic pilocarpine-treated mice progressively spent less time in the

unprotected open environment section of the arena. Monitoring open field and handling behavior in epileptic mice receiving MGE progenitor cells, and age-matched controls, we observed a rescue of these behaviors that persisted to 60 DAT, as reported previously[14]. Most importantly, following these animals to 180 DAT and using a two-week 24/7 video EEG monitoring period we observed an 84% reduction in mean seizure frequency and an 88% reduction in the total number of seizures. Given that we assessed animals around 250-280 d after pilocarpine-induced status epilepticus, and in mice with verified epilepsy and behavioral deficit, it seems plausible to conclude that MGE progenitor cell transplantation has significant and long-lasting benefits.

Additionally, when the identity of transplanted cells was confirmed in these animals at 210 DAT, we noted that neurons were GABAergic and expressed markers (SOM, PV and nNOS) consistent with an MGE lineage[4,6,10,11]. A similar expression pattern was observed 12 months after transplantation further confirming stable long-term integration of MGE-derived interneurons in the host brain (see Fig. 3E). Although most GFP-positive neurons co-labeled with an antibody to the GABA synthesizing enzyme GAD67 these cell counts may underestimate the true number of MGE-derived GABAergic interneurons because (i) tissue for these studies was processed from adult mice undergoing various procedures that could impact activity-dependent enzyme levels, and (ii) GAD67 expression was confirmed in only 75% of GFP-expressing cells in studies using GAD67-GFP mice[51]. It has previously been shown that IPSC frequency, a physiological measure of GABA-mediated inhibition, is enhanced 20-40% in regions containing MGE progenitor derived interneurons[11,15,17,38]. This increase would be expected to enhance interneuron-mediated “surround inhibition” in the epileptic hippocampi[39–41] and provides the most parsimonious explanation for seizure suppression observed with MGE progenitors. Consistent with this mechanistic interpretation, we initially observed a decrease in IPSC frequency in epileptic mice comparable to that reported previously[28,42,43], and then an increase of miniature and spontaneous IPSCs back toward control levels in mice receiving MGE progenitor cells. Although sample sizes are relatively modest for adult slice electrophysiology experiments, the small variability and statistical significance of these findings are consistent with this interpretation. Future studies using optogenetics[19] or dual-patch recordings[16] will be necessary to more fully evaluate integration of MGE-derived interneurons in epileptic animals. Taken together, these functional studies demonstrate that transplanted interneurons integrate into neural circuits affected by epilepsy and argue against earlier suggestions that transplanted interneurons may lose their ability to establish inhibitory synapses, weaken endogenous inhibition, or elicit functional reorganization in the host brain[18,44,45]. As these electrophysiology studies were performed at 270 DAT in mice already confirmed to exhibit handling and open field behaviors comparable to controls, as well as a significant reduction in electrographic seizures, they provide direct preclinical evidence that transplanted MGE-derived interneurons integrate into neural networks affected by long-standing epilepsy where they exert a long-lasting effect on seizure phenotypes and comorbidities.

Although it has been suggested that seizure suppressive effects of MGE progenitor cell transplantation may be transient[18,25], here starting at 180 DAT, and in our earlier publications at 45 and 60 DAT[14,17] we have found absolutely no evidence to support this conclusion. There are at least two potential explanations for this discrepancy. First, in

Henderson et al., (2014)[18] MGE cell transplantation occurred two weeks after pilocarpine-induced status epilepticus (SE) in mice that were not monitored for the emergence of an epileptic phenotype. Because of the variability inherent in this acquired epilepsy model[30,46], some pilocarpine-treated mice will never progress to exhibit spontaneous recurrent seizures, making it possible that Henderson et al. (2014)[18] MGE treatment groups, which used a milder form of initial SE and then failed to monitor post-SE animals to confirm a seizure phenotype prior to transplantation, include mice that were not epileptic[30,33,34]. In contrast, our studies utilize continuous 24 hr video pre-monitoring to confirm seizure phenotype during the latent period in all pilocarpine-treated SE animals before inclusion into treatment (or control) groups, followed by a progressive monitoring of epilepsy behavioral comorbidities. Second, our protocol utilizes bilateral transplantation of freshly dissociated MGE cells (suspended in Leibovitz L-15 medium) into three intra-hippocampal sites to achieve wide distribution of new inhibitory interneurons at multiple locations[14]. Henderson et al. (2014)[18] used bilateral transplantation of MGE cells (transfected with a neural stem cell nucleofector kit and pre-incubated in media containing growth factors) into a single hilar site in the dentate gyrus. Freshly harvested or *in toto* cryopreserved MGE progenitors have significantly better cell viability and migratory properties than growth factor treated preparations[47] and this protocol difference, combined with a single injection site strategy, may contribute to the relatively small area of new interneuron integration observed in Henderson et al. (2014)[18]. Given the widespread hippocampal damage and circuit re-organization characteristic of the systemic pilocarpine model[29,32–34] it would be difficult to achieve a significant therapeutic effect with such a limited distribution of MGE-derived interneurons.

Because interneurons derived from the CGE can migrate and integrate when transplanted in the neonatal or adult brain[12,13,48], we also tested whether CGE progenitors could be beneficial for epilepsy therapy. As small numbers of migrating MGE progenitors can contaminate anatomical CGE transplants[48] we used a genetic manipulation to ablate $Nkx2.1^{+}$ MGE progenitor cells from our CGE transplants[13]. This strategy was recently used to demonstrate CGE-derived interneurons integrate as primarily VIP-, CR- or reelin-positive interneurons but do not induce cortical plasticity[13]. Here we showed that healthy WT mice receiving CGE progenitors at postnatal day 2 began to exhibit abnormal network excitability as early as 30 DAT and frank seizure-like electrographic activity by 60 DAT. Moreover, when CGE progenitors were transplanted into adult epileptic mice, we failed to detect any suppression of seizure activity. Because CGE-derived interneuron sub-types include those that can form functional inhibitory synapses onto inhibitory neurons (i.e., VIP-or calretinin-expressing cells)[6,13] the additional CGE-derived neurons could result in disinhibition. In a wild-type mouse, new CGE-derived interneurons would increase inhibitory tone onto fast-spiking and non-adapting non-fast-spiking inhibitory neurons that innervate excitatory neurons, resulting in reduced inhibitory-to-excitatory neuron function, a disinhibitory microcircuit and generation of abnormal electrographic activity (see Fig. 5B). In an epileptic hippocampus with reduced inhibitory-to-excitatory neuron connections these CGE-derived interneuron-innervating interneurons and the additional disinhibition would not serve to reduce abnormal levels of excitability associated with epilepsy (see Fig. 5C). In contrast, MGE-derived interneuron sub-types include those that are strategically located to control

excitatory neuron spiking and burst firing (i.e., PV- and SST-expressing cells)[6] thus acting to enhance inhibitory tone in epileptic animals (see Fig. 4) and suppress seizures (see Fig. 2). Taken together, these findings suggest that "MGE-like" interneurons generated from human embryonic or induced pluripotent stem cells containing significant fractions of VIP- or calretinin-positive CGE-like interneurons[49,50] are not appropriate for cell therapy-based applications in epilepsy.

We conclude that MGE, but not CGE, progenitor cell transplantation is associated with a long-lasting therapeutic effect in pre-clinical epilepsy models. Highlighting the importance of appropriate disease model, interneuron location, distribution and sub-population identity in understanding the therapeutic potential of progenitor cell transplantation.

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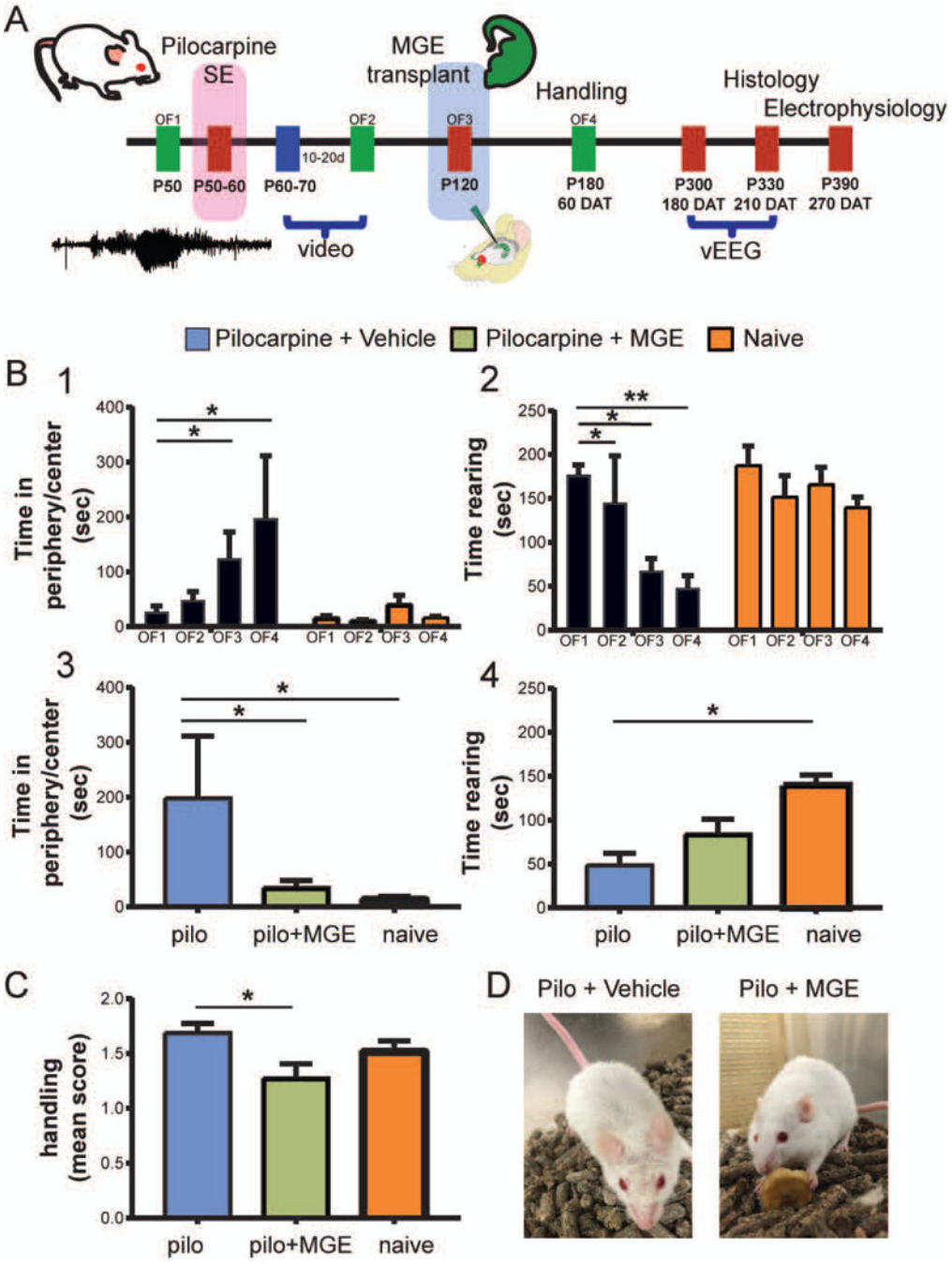

Fig. 1. MGE transplant rescues behavioral comorbidities associated with epilepsy

(A) Timeline of the experimental design. (B) Open field testing. Bar plots show the mean time spent in the periphery/center of the arena (in sec; B1) or time rearing (in sec; B2) for pilocarpine + vehicle or naïve animal groups. Bars represent the mean ± s.e.m. *p < 0.05 or **p < 0.001, One-way ANOVA. Bar plot shows the mean time spent in the periphery/center of the arena (in sec; B3) for all groups. One-way ANOVA with Tukey post hoc comparisons: pilocarpine + vehicle vs. pilocarpine + MGE (*$p = 0.006$); pilocarpine + MGE vs. naïve ($p = 0.232$), and pilocarpine + vehicle vs. naïve (*$p = 0.003$). Bar plot shows time rearing (in sec;

B4) for all groups. One-way ANOVA with Tukey post hoc comparisons: pilocarpine + vehicle vs. pilocarpine + MGE ($p = 0.273$); pilocarpine + MGE vs. naïve ($p = 0.085$), and pilocarpine + vehicle vs. naïve (*$p = 0.0057$). Bars represent the mean ± s.e.m. (C) Handling test. Bar plot shows the mean handling score for all groups. One-way ANOVA with Tukey post hoc comparisons: pilocarpine + vehicle vs. pilocarpine + MGE (*$p = 0.0327$); pilocarpine + vehicle vs. naïve ($p = 0.4418$); and pilocarpine + MGE vs. naïve ($p = 0.205$). (D) Grooming and general appearance is also restored to naïve levels in animals receiving MGE transplant. Representative images of a pilocarpine + vehicle or pilocarpine + MGE mouse are shown.

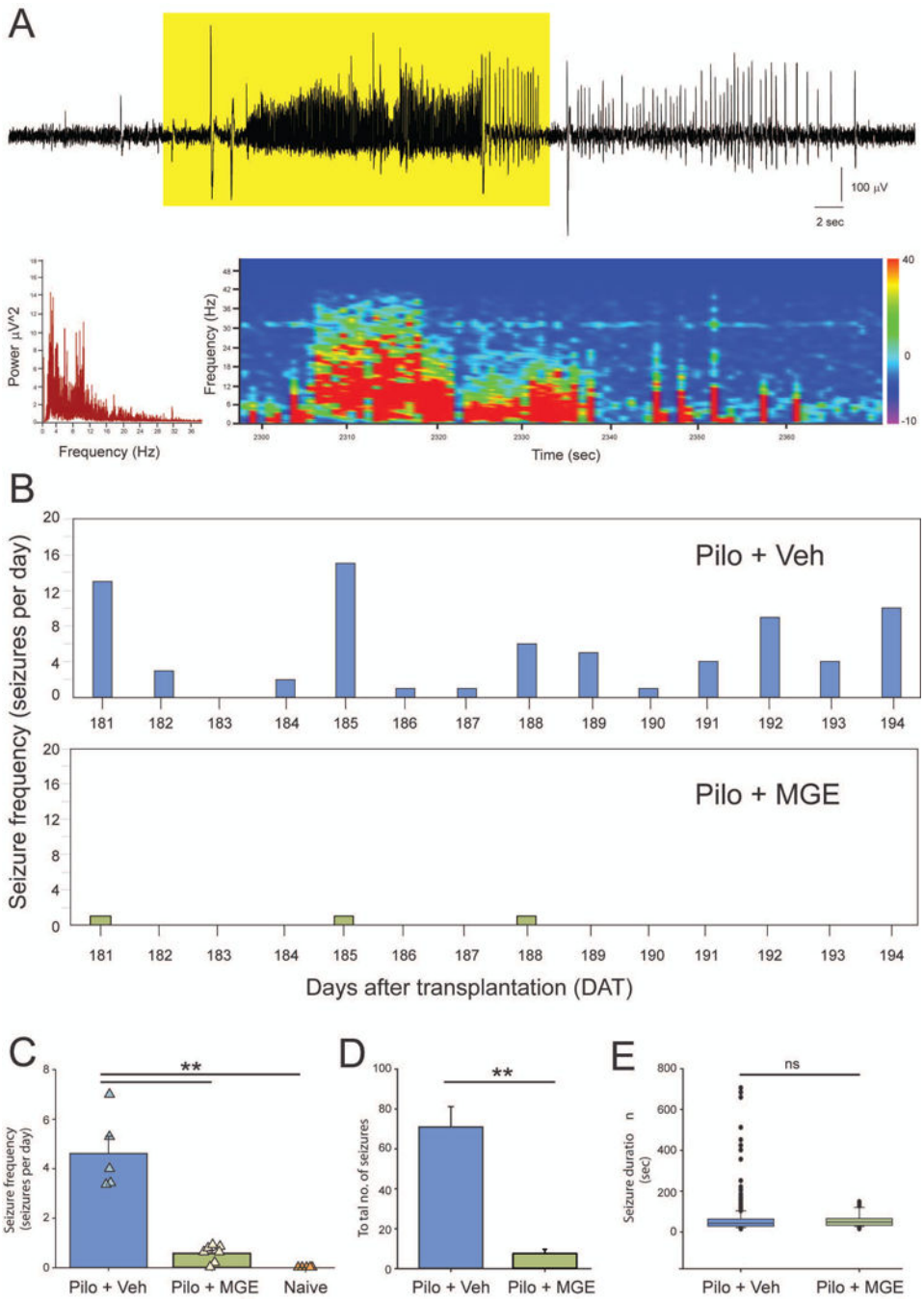

Fig. 2. Reduction in seizure frequency after MGE transplantation

(A) Representative example of an electrographic seizure event (top trace). Note the high-frequency, high-voltage, rhythmic activity with clear onset, progression and termination. Power and spectral frequency plots are shown for the event highlighted in yellow. (B) Representative seizure frequency plot in pilocarpine + vehicle (top panel) and pilocarpine + MGE transplanted (bottom panel) epileptic animals over the entire 14 day vEEG monitoring epoch. (C). Bar plots show the mean spontaneous seizure frequency (seizures per day) for each group based on continuous EEG recordings. Bars represent the mean ± s.e.m. The data

points superimposed over the bar graph each represent an individual mouse. $**p < 0.001$ (Kruskal-Wallis one-way ANOVA on ranks, Dunn's pairwise comparison) (D) Bar plots showing the total number of seizures. $**p = 0.001$ (Mann-Whitney rank sum test) (E) Box plots show seizure duration median, quartiles, and whiskers extending to the furthest point within 1.5 IQR (data points superimposed over the box plot each represent an individual mouse; $p = 0.262$ Mann-Whitney rank sum test).

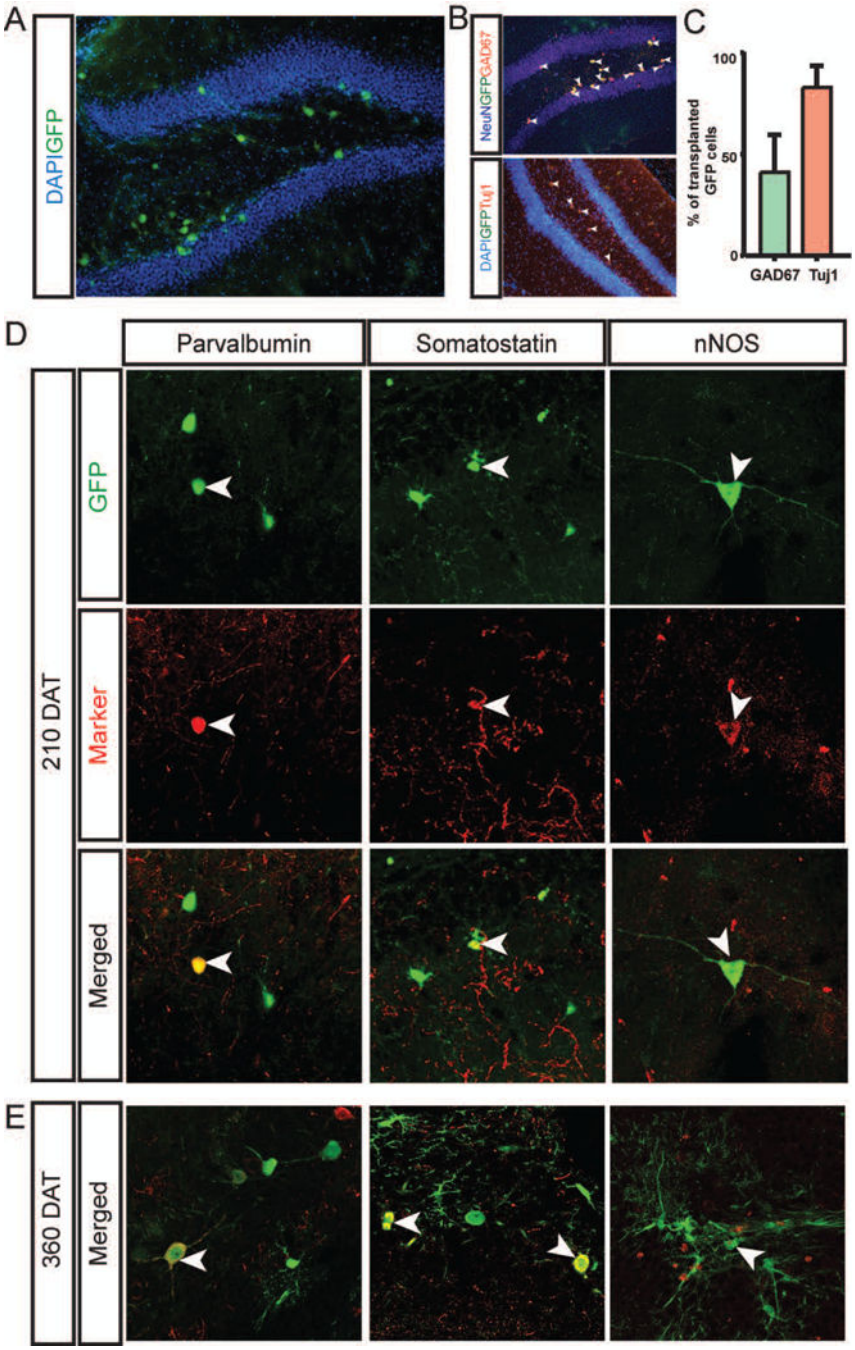

Fig. 3. Transplanted MGE cells express markers of inhibitory neurons

Representative hippocampal sections from pilocarpine mice transplanted with MGE cells (210 DAT) labeled for DAPI and GFP (A), NeuN, GFP and GAD67 (B, top) or DAPI, GFP and Tuj1 (B, bottom). (C) Quantification of marker expression in GFP^+ cells pilocarpine mice transplanted with MGE cells at 210 DAT for GAD67 and Tuj1 (n = 5 animals, each). (D) Higher resolution hippocampal images from pilocarpine mice transplanted with MGE cells (210 DAT) labeled for GFP plus parvalbumin, somatostatin or nNOS. (E) Same at 360 DAT. Arrowheads indicate double-labeled cells.

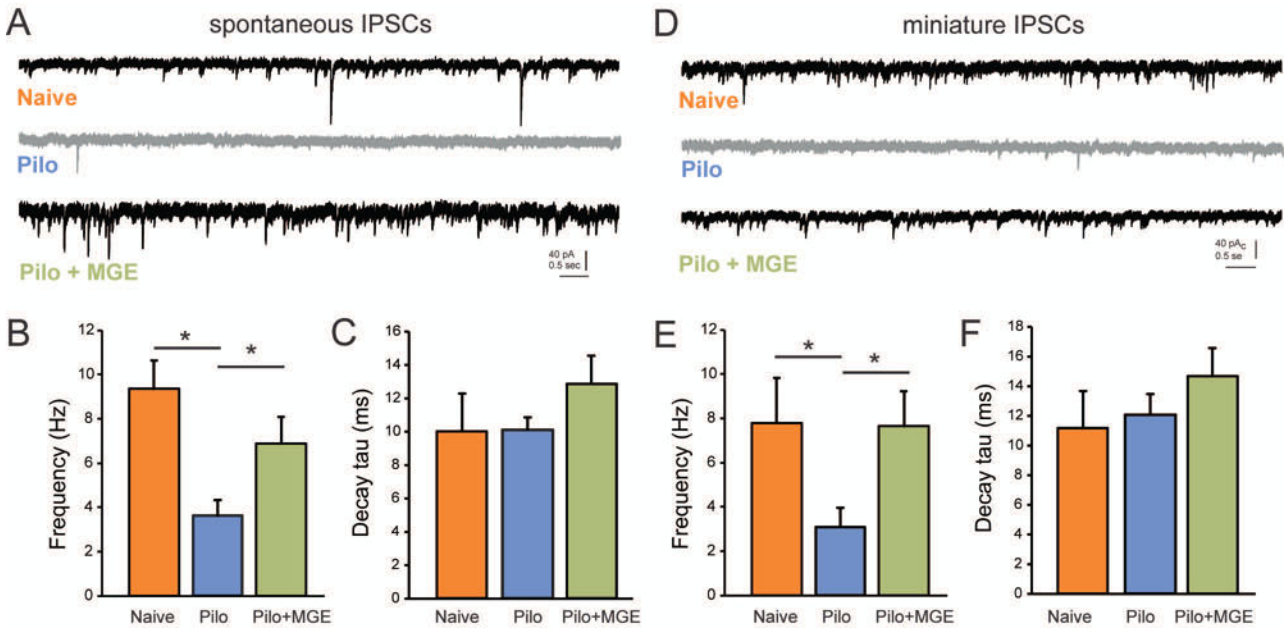

Fig. 4. Spontaneous and miniature IPSC frequencies increase after MGE transplantation

(A) Representative voltage-clamp traces of spontaneous IPSCs are shown for the three groups (from top to bottom: naïve, pilocarpine, or pilocarpine + MGE). (B) Bar plots show the mean ± s.e.m. sIPSC frequency (in Hz) for each group. One-way ANOVA followed by Tukey's multiple comparisons test indicated no statistical difference ($p < 0.05$, No) for the naïve vs. Pilo + MGE groups (6.22 Diff of Ranks, Q = 1.387). (C) Bar plots show the mean ± s.e.m decay tau constant (in ms) for each group. Bars represent the mean ± s.e.m. (D-F) Same for miniature IPSCs. $**p < 0.05$.

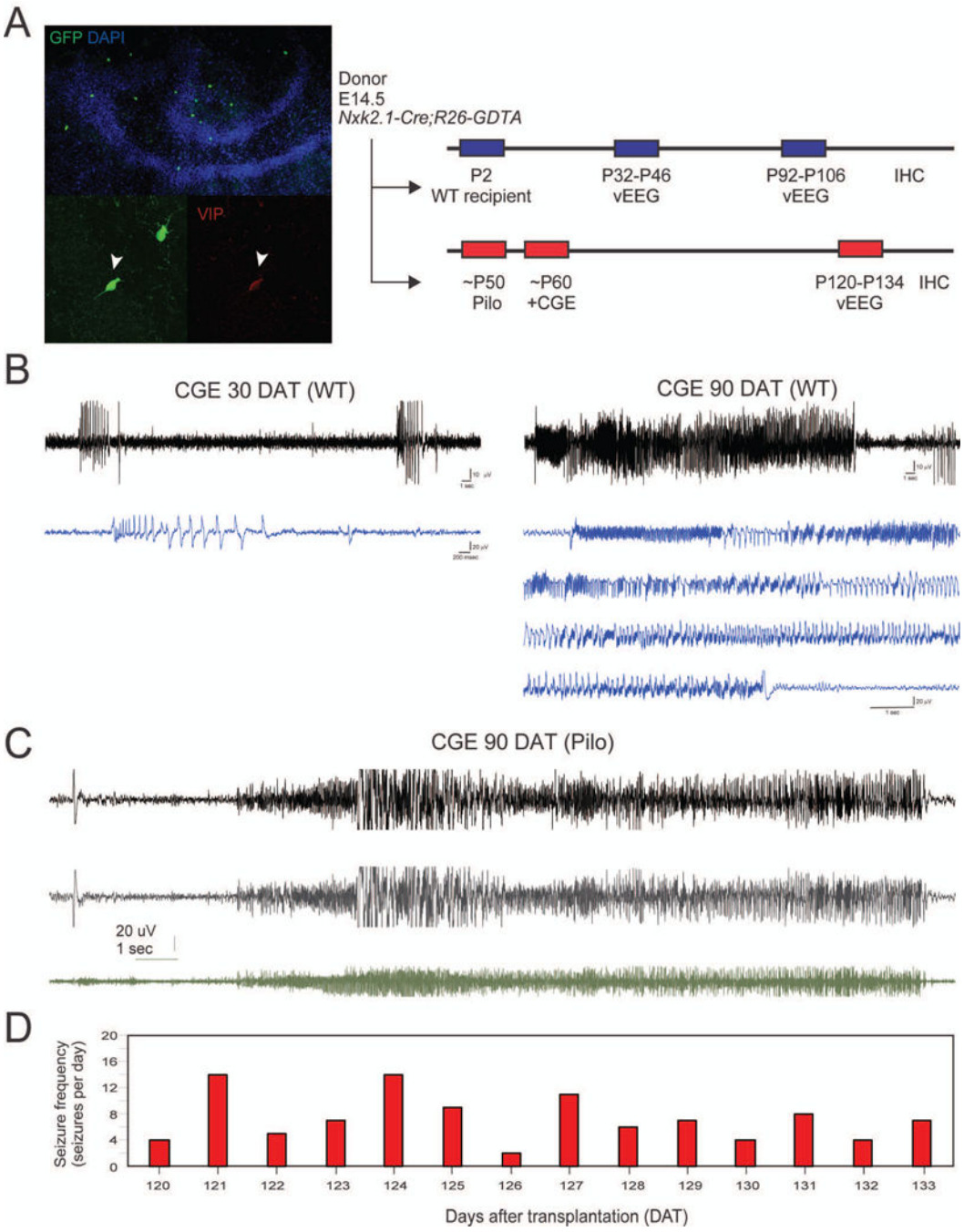

Fig. 5. CGE progenitor cell transplantation

(A) Hippocampal section from pilocarpine mouse transplanted with CGE cells (210 DAT) labeled for DAPI, VIP and GFP (at left). Experimental strategy for CGE transplantation (at right). (B) One-channel (of two) recording of abnormal EEG with a tethered monitoring system in a WT mouse (30 DAT) transplanted with CGE cells at P2 (Top, left) and (Bottom, left), expanded views of the waveforms. Abnormal ictal-like discharges are present in the signal. One-channel (of two) recording of abnormal EEG with a tethered monitoring system in a WT mouse (90 DAT) transplanted with CGE cells at P2 (Top, right) and (Bottom, right),

expanded views of the waveforms. Seizure activity > 15 sec in duration with progression, and high-frequency high-amplitude components is present in the signal. (C) Dual-channel recording of an electrographic seizure and simultaneous EMG in a pilocarpine mouse transplanted with CGE cells at P60 and monitored 60 days after transplantation. (D) Representative seizure frequency plot for this pilocarpine + CGE mouse over the entire 14 day vEEG monitoring epoch.

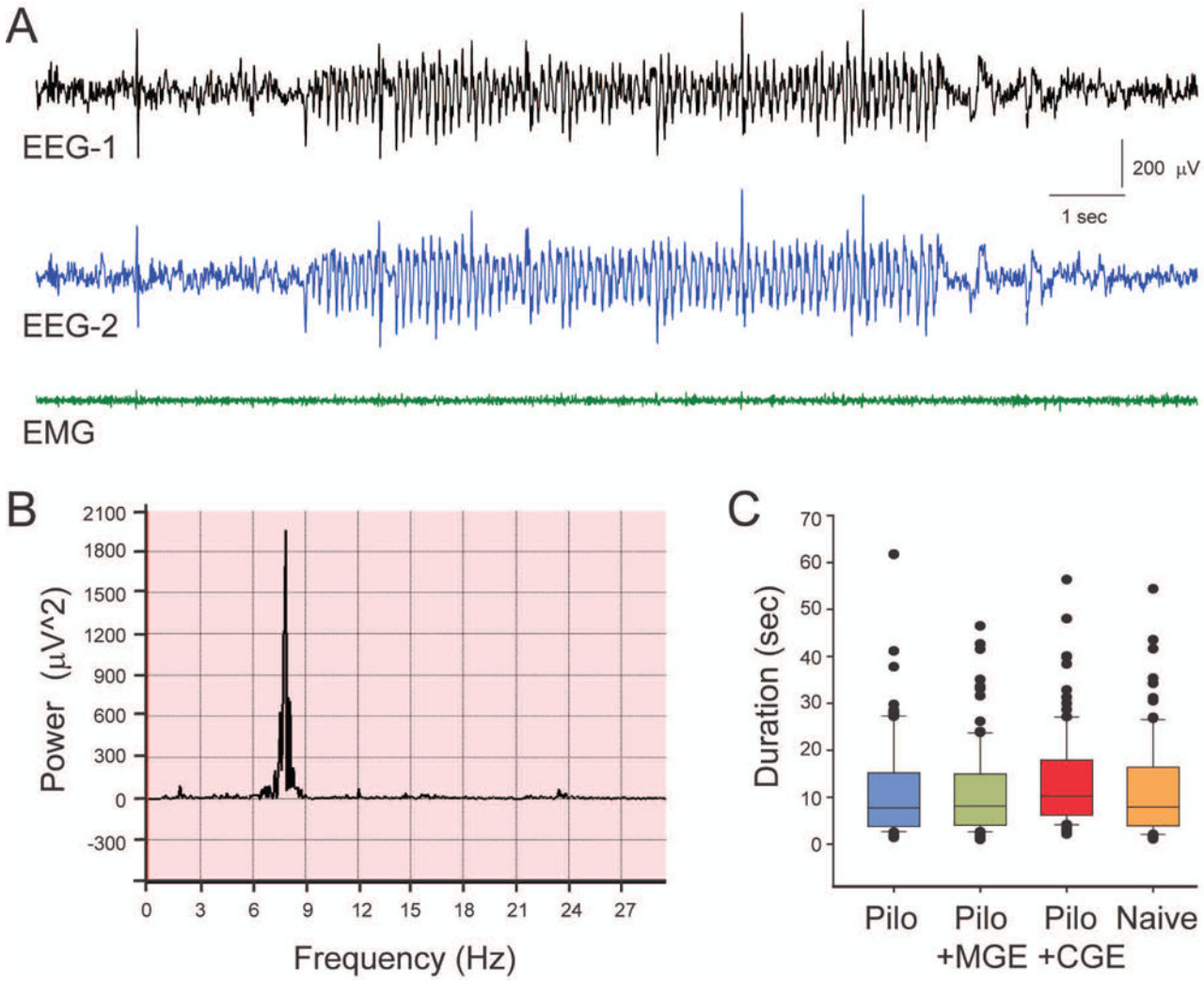

Fig. 6. Eight Hz activity

(A) Dual-channel recording of EEG activity and simultaneous EMG in a naïve mouse. Note the presence of high-amplitude rhythmic activity in the 8 Hz range with no concurrent movement in the EMG. (B) Plot of the power vs. frequency for this representative event with a peak between 7 and 9 Hz. (C) Box plots show event duration median, quartiles, and whiskers extending to the furthest point within 1.5 IQR for pilocarpine (pilo), pilocarpine + MGE (pilo + MGE), pilocarpine + CGE (pilo + CGE) and naïve groups. 100 events analyzed per group. $P = 0.051$ (Kruskal-Wallis one-way ANOVA on ranks).