Differential vulnerability of interneurons in the epileptic hippocampus

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INTRODUCTION

Interneurons play a crucial role in balancing neuronal activity in the brain. In epilepsy, the loss of inhibitory interneurons has been associated with the emergence of epileptic seizures (for review, see Lopes da Silva et al., 1994; Olsen and Avoli, 1997; Frandsen, 2008). In temporal lobe epilepsy (TLE) interneurons show differential vulnerability to an epileptogenic insult (Bouilleret et al., 2000a,b, Magloczky and Freund, 2005; Kuruba et al., 2011) characteristic for their particular expression pattern of interneuron markers: interneurons commonly express glutamic acid decarboxylase (GAD), the key enzyme for synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; Ribak et al., 1978; Freund and Buzsaki, 1996), but they can be differentiated according to the selective expression of calcium-binding proteins [calbindin, parvalbumin (PV), calretinin] and/or co-transmitters (e.g., somatostatin, neuropeptide Y (NPY), cholecystokinin) (Freund and Buzsaki, 1996).

Among the most vulnerable interneurons in human TLE and in animal models for TLE are those expressing PV (Bouilleret et al., 2000a), but they can be differentiated according to the selective expression of calcium-binding proteins [calbindin, parvalbumin (PV), calretinin] and/or co-transmitters (e.g., somatostatin, neuropeptide Y (NPY), cholecystokinin) (Freund and Buzsaki, 1996). The loss of hippocampal interneurons has been considered as one reason for the onset of temporal lobe epilepsy (TLE) by shifting the excitation-inhibition balance. Yet, there are many different interneuron types which show differential vulnerability in the context of an epileptogenic insult. We used the intrahippocampal kainate (KA) mouse model for TLE in which a focal, unilateral KA injection induces status epilepticus (SE) followed by development of granule cell dispersion (GCD) and hippocampal sclerosis surrounding the injection site but not in the intermediate and temporal hippocampus. In this study, we characterized the loss of interneurons with respect to septotemporal position and to differential vulnerability of interneuron populations. To this end, we performed intrahippocampal recordings of the initial SE, in situ hybridization for glutamic acid decarboxylase 67 (GAD67) mRNA and immunohistochemistry for parvalbumin (PV) and neuropeptide Y (NPY) in the early phase of epileptogenesis at 2 days and at 21 days after KA injection, when recurrent epileptic activity and GCD have fully developed. We show that SE extended along the entire septotemporal axis of both hippocampi, but was stronger at distant sites than at the injection site. There was an almost complete loss of interneurons surrounding the injection site and expanding to the intermediate hippocampus already at 2 days but increasing until 21 days after KA. Furthermore, we observed differential vulnerability of PV- and NPY-expressing cells: while the latter were lost at the injection site but preserved at intermediate sites, PV-expressing cells were gone even at sites more temporal than GCD. In addition, we found upregulation of GAD67 mRNA expression in dispersed granule cells and of NPY staining in ipsilateral granule cells and ipsi- and contralateral mossy fibers. Our data thus indicate differential survival capacity of interneurons in the epileptic hippocampus and compensatory plasticity mechanisms depending on the hippocampal position.

Keywords: kainate injection, glutamic acid decarboxylase, parvalbumin, neuropeptide Y, temporal lobe epilepsy, septotemporal axis
Vulnerability of interneurons has also been described in the intrahippocampal kainate (KA) mouse model for TLE, which shows histological changes closely resembling human TLE including hippocampal sclerosis and granule cell dispersion (GCD; Suzuki et al., 1995; Bouilleret et al., 1999; Riban et al., 2002). Most investigations in this model focused on the septal hippocampus (close to the injection site) as it shows prominent cell loss and strong GCD; however, our previous study revealed that status epilepticus (SE) and recurrent epileptiform activity involve the whole septotemporal extent of both hippocampi (Haussler et al., 2012). This indicates that structural changes in the network beyond the septal hippocampus are highly likely. Indeed, we described that the intermediate hippocampus constitutes a transition zone where GCD reduces to normal width, but where neurogenesis, which is lost in the septal hippocampus (Heinrich et al., 2006), is even increased compared to controls (Haussler et al., 2012). It is, however, still unknown whether and how the interneuron network is affected in this area—this might be crucial for network balance and seizure generation.

To address this question, we investigated whether there is a location-dependent vulnerability of interneurons along the whole septotemporal axis of both hippocampi early and late after KA injection. We present quantitative data showing a substantial loss of GAD-expressing interneurons beyond the area where GCD occurred, with differential vulnerability of PV- and NPY-expressing interneurons. In addition, NPY was upregulated in granule cells and mossy fibers in a time- and position-dependent manner most likely reflecting particular compensatory mechanisms at different septotemporal sites.

**MATERIALS AND METHODS**

**ANIMALS**

Experiments were carried out with adult (8–10 weeks) male C57Bl/6N mice (Charles River, Sulzfeld, Germany). Mice were kept in a 12 h light/dark cycle at room temperature (RT; 22°C). C57Bl/6N mice (Charles River, Sulzfeld, Germany). Mice were kept in a 12 h light/dark cycle at room temperature (RT; 22±1°C) with food and water ad libitum. All animal procedures were carried out in accordance with the guidelines of the European Community's Council Directive of 22 September 2010 (2010/63/EU) and approved by the regional council.

**KAINEATE INJECTION AND ELECTRODE IMPLANTATION**

Unilateral, intrahippocampal KA injections were performed as previously described (Heinrich et al., 2006; Haussler et al., 2012). In brief, mice were anesthetized (100 mg/kg ketamine hydrochloride, 5 mg/kg xylazine, and 0.1 mg/kg atropine; i.p.) and placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) in flat skull position. 50 nl (1 nmol) of a 20 mM KA solution for 2–3 h at RT. Sections were coverslipped with anti-fading Glutamic acid decarboxylase 67 mRNA was localized by hybridization (ISH) with digoxigenin (DIG)-labeled cRNA probes (Tocris, Bristol, UK) in the dark after injection. Some mice were immediately implanted with custom-made platinum-iridium wire electrodes (Ø = 125 μm, Teflon insulated, World Precision Instruments, Sarasota, FL, USA) at four positions along the septotemporal axis of the ipsilateral and at one position in the contralateral hippocampus (coordinates in mm): (1) AP = −2.0, ML = −1.4, DV = −1.9; (2) AP = −2.8, ML = −2.0, DV = −2.0; (3) AP = −3.4, ML = −2.75, DV = −2.75. (4) AP = −3.8, ML = −2.5, DV = −4.0, (contra) AP = −2.0, ML = −1.4, DV = −1.9. Stainless steel jeweler’s screws positioned in the skull above the prefrontal cortex served as ground and reference. Electrodes were fixed to the skull with cyanoacrylate and dental cement and soldered to a connector which was permanently mounted on the skull with dental cement. After recovery from anesthesia, mice with implanted electrodes were recorded for several hours to monitor SE. Mice, destined for histological analyses were not implanted with electrodes to ensure better preservation of the tissue, but it was ensured that behavioral manifestation of SE was comparable to the recorded group.

**IN VIVO INTRAHIPPOCAMPAL RECORDINGS**

For recordings of hippocampal local field potential (LFP) activity in vivo, mice were connected to a miniature preamplifier [Multi Channel Systems (MCS), Reutlingen, Germany]. Signals were amplified (1000-fold, band-pass 1 Hz–5 kHz, MCS) and digitized (sampling rate 10 kHz, Power1411 analog-to-digital (A/D) converter, Spike2 software, Cambridge Electronic Design, Cambridge, UK). Animals were recorded during the initial SE and at 3 and 21 days after KA injection to ensure that they developed chronic TLE. Electrode positions were verified in Nissl-stained sections as described previously (Haussler et al., 2012).

**PERFUSION AND TISSUE PREPARATION**

Mice were deeply anesthetized at 2 and 21 days after KA injection and transcardially perfused with 0.9% NaCl solution followed by paraformaldehyde (PFA, 4% in 0.1 M phosphate buffer (PB), pH 7.4). The brains were post-fixed in the same fixative for 4 h at 4°C, and either cryoprotected (20% sucrose, overnight, 4°C), frozen in isopentane and sectioned with a cryostat (30 μm, coronal plane) or transferred into PB and cut on a vibratome (Leica, VT1000S, Bensheim, Germany, 50 μm, coronal plane).

**IMMUNOCYTOCHEMISTRY**

For immunocytochemistry, sections were processed using a free-floating procedure. They were preincubated in 0.25% Triton X-100 and 10% normal serum in PB for 30 min and incubated with the primary antibody (4 h at RT + overnight at 4°C). The following primary antibodies were used: rabbit anti-PV (1:2500, Swant, Bellinzona, Switzerland), rabbit anti-NPY (1:3000, Abcam, Cambridge, UK), rabbit anti-GADB5/67 (GADB5/67, 1:4000, Millipore, Temecula, CA, USA). Secondary antibodies were coupled to Cy3ss2 (1:200) or Cy5ss1 (1:400), Jackson Immunoresearch Laboratories, West Grove, PA, USA) and counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, 1:10000) in the dark for 2–3 h at RT. Sections were coveredumped with anti-fading mounting medium (IMMU-Mount, Thermobandons, Dreischen, Germany).

**IN SITU HYBRIDIZATION**

Glutamic acid decarboxylase 67 mRNA was localized by in situ hybridization (ISH) with digoxigenin (DIG)-labeled cRNA probes generated by in vitro transcription as described earlier (Kulik et al., 2003). Cryostat sections were pretreated in hybridization buffer (50% formamide, 4× SSC (1× SSC = 0.15 M NaCl, 0.1% Tween 20) at 37°C for 30 min before adding a DIG-labeled cRNA probe (50 μg/ml) and incubating for 12–16 h at 50°C. DIG-labeled cRNA probes were detected by alkaline phosphatase-coupled antibody against DIG (1:3000, Roche Applied Science, Mannheim, Germany). Blue precipitate was developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Roche Applied Science, Mannheim, Germany). Sections were counterstained with toluidine blue O (Häussler et al., 2012). This indicates that structural changes in the network beyond the septal hippocampus are highly likely. Indeed, we described that the intermediate hippocampus constitutes a transition zone where GCD reduces to normal width, but where neurogenesis, which is lost in the septal hippocampus (Heinrich et al., 2006), is even increased compared to controls (Haussler et al., 2012). It is, however, still unknown whether and how the interneuron network is affected in this area—this might be crucial for network balance and seizure generation.

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0.015 M sodium citrate, pH 7.0), 50 mM NaH2PO4, 250 μg/ml heat-denatured salmon sperm DNA, 100 μg/ml tRNA, 3% dextran sulfate and 1% Denhardt’s solution) diluted with 2× SSC (1×) for 15 min and prehybridized in hybridization buffer for 60 min at 45°C. Hybridization was performed in the same buffer including DIG-labeled GAD67 anti-sense or sense cRNA probes (50 ng/ml) at 45°C overnight. After hybridization, the brain sections were washed in 2× SSC (2× 15 min, RT), 2× SSC and 50% formamide (15 min, 55°C), 0.1× SSC and 50% formamide (15 min at 55°C), 0.1× SSC (2× 15 min, 55°C) and finally in Tris-buffered saline (TBS, 2× 10 min, RT). Blocking was performed in blocking buffer (1% blocking reagent in TBS, 60 min, RT). Immunological detection of DIG-labeled hybrids was performed with an anti-DIG antibody conjugated with alkaline phosphatase (1:1500), raised in sheep, Roche, Mannheim, Germany) following standard protocols. Sections were coveredslipped in Kaiser’s glycerol gelatin.

FLUORO-JADE B STAINING

The success of KA injections was monitored by Nissl (data not shown) or Fluoro-Jade B stainings to monitor cell death. Sections were mounted on gelatine-coated microscope slides, air-dried and transferred into 0.06% potassium permanganate solution (15 min), followed by 0.004% Fluoro-Jade B solution (30 min). Sections were cleared in xylene and coverslipped with Permount.

MICROSCOPY AND COUNTING PROCEDURES

Histological sections were analyzed with a microscope equipped with appropriate fluorescence filters (Axiooplan 2, Zeiss, Göttingen, Germany), photomicrographs were taken with a digital camera and processed with Axvision software (Zeiss). Identical exposure times were used for sections that were compared. ISH sections were analyzed using brightfield microscopy with the same equipment. In the photomicrographs of ISH sections, GAD67 mRNA-positive cells were counted using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) with the integrated Cell Counter plugin. For quantifications, representative coronal sections were selected at four positions along the septotemporal axis according to the following positions given in the Allen Mouse Brain Atlas (http://mouse.brain-map.org): AP relative to bregma (in mm); (1): −1.35, (2): −2.15, (3): −2.98, (4): −3.55. As regions of interest (ROI) we marked the hilus or the complete hippocampus in each section, respectively. Only mice for which sections at all septotemporal positions of the ipsilateral hippocampus and the contralateral hippocampus were available were used for quantification. To compare densities of GAD67 mRNA-positive cells in epileptic and control mice at these particular positions, all GAD67 mRNA-positive interneurons within each ROI were manually marked, counted and the density was calculated using the area of the ROI (values are given in cells/mm²). The densities were then averaged across animals for each position. The same method was applied to quantify PV-positive interneurons in immunocytochemically stained sections.

For densitometric analysis of Fluoro-Jade B-positive cells images with equal illumination were taken and the same threshold was applied for every image. The total area of the hippocampus was divided by the area of pixels with brightness exceeding threshold resulting in the relative area of positive pixels as a measure for the amount of cell death.

DATA ANALYSIS

For visualization of different densities of GAD67 mRNA-positive cells in all hippocampal areas, a custom-made C# program (Microsoft® Visual Studio Professional 2010, Redmond, WA, USA) was developed to create heatmap images in false-color along the septotemporal axis. Images from representative ISH-stained sections were selected for each of the four positions for controls and 2 and 21 days after KA. Using the GAD67 mRNA-positive cells marked in ImageJ, where the position of each marked cell defines an x- and y-coordinate in a coordinate system, a basic matrix was placed centrally onto the position of each cell. The matrix consisted of radially decreasing intensity values (radius 100 pixels in the original image, values linearly decreasing from 100 in the center to 0 in the periphery). Overlapping basic matrix values of different cells were summed resulting in high intensity values where cells were located close to each other. To allow comparison, the values were normalized to the maximal intensity value for each septotemporal position. Finally, the matrices were transferred into grayscale (i.e., scaled to values between 0 and 255) and allocated to a new RGB color space (gray indicates no cells, red indicates highest cell density).

STATISTICAL ANALYSIS

For all values, mean and standard error of the mean (SEM) are given. Statistical comparison was made with a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Significance thresholds were as follows: * p < 0.05, ** p < 0.01, *** p < 0.001. Statistical analysis was performed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

RESULTS

STATUS EPILEPTICUS IS FOLLOWED BY EXTENSIVE CELL DEATH

To monitor SE, a series of mice was implanted with hippocampal electrodes and LFPs were recorded for 2–3 h after mice had awakened from anesthesia. SE activity, consisting of a repetitive pattern of high amplitude population spikes and spike-and-wave discharges with intermittent short depression periods, was recorded at all septotemporal positions of the ipsilateral hippocampus and in the contralateral hippocampus. At the time point of recording, amplitudes were largest in the ipsilateral intermediate and the contralateral hippocampus in all mice (n = 6, Figures 2A,B). This is in accordance with our previous study (Blasius et al., 2012), where quantification revealed a significant increase in power in the theta band (3–8 Hz) in the intermediate, temporal and contralateral hippocampus during SE. Simultaneous with pathophysiological activity, mice showed behavioral SE, including rotations, chewing or convulsive movements of the forelimbs, alternating with immobility. This pattern continuously persisted for 6–10 h after KA injection and subsided spontaneously.

One day after KA injection prominent cell death occurred mainly close to the injection site in CA1, CA3 and the hilus of the ipsilateral hippocampus, as shown by Fluoro-Jade B stainings (n = 5, Figures 2B,D,E). The contralateral hippocampus was...
FIGURE 1 | Representative LFP recording during status epilepticus (SE). (A, B) LFPs were recorded at 4 positions (1: most septal; 4: most temporal) in the ipsilateral and at one position in the contralateral (c) hippocampus (for illustration of electrode positions see schematic brain drawing starting from ~2 h after awakening from surgery. SE was characterized by a repetitive pattern of spike-and-wave discharges with short intermittent depression periods, accompanied by behavioral signs such as convulsive movements, rotator or immobility. (B) Enlargement of (A). SE activity was strongest in the intermediate and contralateral hippocampus at this time point.

devoid of cell loss (Figures 2A,C) or a group of dying cells was observed in distal CA1 (3/5 mice). Ipsilateral cell loss following focal KA injection extended to the intermediate hippocampus (Figure 2D) but the temporal hippocampus was unaffected. Den- sitometric analysis revealed prominent differences between cell loss in the ipsilateral and contralateral hippocampus, which, however, only were significant at position 2 (Figure 2F). ANOVA: p < 0.001, Tukey’s post-test: p < 0.001, most likely due to variable extent of cell death mainly in CA1 across mice. In addition to strong cell loss in the pyramidal cell layer in CA3 and CA1 and hilar mossy cells, Fluoro-Jade B-positive cells were also positioned in strata oriens and radiatum of CA3 and CA1 and in the inner and outer portion of the granule cell layer. An enlargement of the hilus and granule cell layer, displaying the shape of Fluoro-Jade B-positive cells is shown in (E). Fluoro-Jade B-positive cells were also visible in the intermediate hippocampus but the temporal hippocampus was devoid of dying cells. The arrow marks the most temporally located Fluoro-Jade B-positive cell group. (F) Denitometric analysis of Fluoro-Jade B-positive areas relative to the area of the whole hippocampus in the same section at four positions along the septotemporal axis (see scheme in Figure 1) in the ipsilateral and contralateral hippocampus. Values are displayed as mean ± SEM. A significant difference was observed at position 2 (n = 5, p < 0.001, ANOVA, ***p < 0.001 Tukey’s post-test). Scale bars: A-D, 100 μm; E, 50 μm; GCL, granule cell layer; H, hilus; CA1, CA2, CA3, cornu ammonis; I1, ipsilateral position 1; C1, contralateral position 1.

performed ISH for GAD67 mRNA expression at 2 and 21 days after injection. In control mice GAD67 mRNA-expressing interneurons were distributed in all hippocampal areas but accumulated in or close to the pyramidal cell layer, the granule cell layer and in the hilus at all septotemporal positions (controls: n = 7 mice, Figures 3A-D). The increased density of GAD67 mRNA-positive cells in the temporal hippocampus (Figure 3D) matches previous studies

FIGURE 2 | Status epilepticus is followed by prominent neuronal death in the septal ipsilateral hippocampus. (A–E) Representative Fluoro-Jade B-stained sections of a KA-injected mouse at one day after KA injection to monitor neuronal degeneration. (A, C) The contralateral hippocampus was devoid of any cell death in the septal (A) and intermediate and temporal hippocampus (C). The ipsilateral hippocampus prominently showed neuronal death in the pyramidal cell layer of CA3 and CA1 and in the hilus, but single Fluoro-Jade B-positive cells were also located in strata oriens and radiatum of CA3 and CA1 and in the inner and outer portion of the granule cell layer. An enlargement of the hilus and granule cell layer, displaying the shape of Fluoro-Jade B-positive cells is shown in (E). Fluoro-Jade B-positive cells were also visible in the intermediate hippocampus but the temporal hippocampus was devoid of dying cells. The arrow marks the most temporally located Fluoro-Jade B-positive cell group. (B) Enlargement of (A). SE activity was strongest in the intermediate and contralateral hippocampus at this time point.

Glutamic acid decarboxylase 67 is expressed in nearly all GABAergic interneurons (Freund and Buzsaki, 1996). To monitor the distribution of hippocampal interneurons along the septotemporal axis after focal KA injection into the septal hippocampus, we performed ISH for GAD67 mRNA expression at 2 and 21 days after injection. In control mice GAD67 mRNA-expressing interneurons were distributed in all hippocampal areas but accumulated in or close to the pyramidal cell layer, the granule cell layer and in the hilus at all septotemporal positions (controls: n = 7 mice, Figures 3A-D). The increased density of GAD67 mRNA-positive cells in the temporal hippocampus (Figure 3D) matches previous studies.
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FIGURE 3 | Continued
In the contralateral hippocampus of KA-injected mice the distribution of GAD67 mRNA-positive cells was comparable to controls at all septotemporal levels at 2 days (Figures 3E–H) and 21 days after KA (data not shown), except for a small reduction in density in distal CA1 of the septal hippocampus in some mice. Quantification revealed that the number of GAD67 mRNA-expressing cells was comparable to controls at all septotemporal levels (Figures 3Q,R). GAD67 mRNA expression in granule cells was comparable to controls or only slightly upregulated in the septal hippocampus (Figures 3E,F) but unchanged in the intermediate and temporal hippocampus (Figures 3G,H).

In contrast, already at 2 days after KA injection, GAD67 mRNA expression was almost completely lost in the ipsilateral septal hippocampus, except for a group of interneurons clustered in CA3a/CA2 and a few scattered cells in the dentate gyra (KA 2 days; n = 7 mice; Figures 3J,L). Quantification of GAD67 mRNA-positive cell bodies revealed a significant reduction of cell density in the ipsilateral septal hippocampus compared to controls and to the contralateral hippocampus (n = 5 for each group; position 1: ANOVA: p = 0.024, pairwise Tukey’s post-test: ipsilateral-control p < 0.001, ipsilateral-contralateral p < 0.01; position 2: ANOVA: p = 0.001, pairwise: ipsilateral-control p < 0.001, ipsilateral-contralateral p < 0.001; Figure 3Q). In the intermediate hippocampus GAD67 mRNA-expressing cells were only partially lost in the dorsal parts of the section but preserved in ventral parts (Figure 3K). In the temporal hippocampus they were mostly preserved at 2 days after KA injection (Figure 3L). No significant differences in cell density of GAD67 mRNA-positive cells were observed in the intermediate and temporal hippocampus when we considered the area of the whole hippocampus for quantification (position 3: ANOVA: p = 0.53; position 4: ANOVA: p = 0.32; Figure 3Q). When considering only the area of the hilus, we found a significant reduction of interneuron density compared to controls and to the contralateral side at the level of the septal and the intermediate hippocampus but not at temporal sites (n = 5 each group; position 1: ANOVA: p < 0.001, pairwise: ipsilateral-control and ipsilateral-contralateral p < 0.01; position 2: ANOVA: p = 0.0003, pairwise: ipsilateral-control and ipsilateral-contralateral p < 0.001; position 3: ANOVA: p = 0.0008, pairwise: ipsilateral-control and ipsilateral-contralateral p < 0.01; position 4: ANOVA: p = 0.036, pairwise: not significant, Figure 3R). Committed with the loss of interneurons, we observed a strong upregulation of GAD67 mRNA expression in ipsilateral granule cells at all positions except for the temporal hippocampus (Figures 3J,L), which is in agreement with studies in rats (Schwartz and Sprek, 1993), but in contrast to Makiura et al. (1999) who showed a transient increase of GABA but not of GAD67.

In the chronic phase at 21 days after KA injection, when GCD and recurrent epileptic activity had fully developed, GAD67 mRNA expression was even further decreased: in the septal hippocampus a few GAD67 mRNA-expressing cells remained in the dentate gyra but GAD67 mRNA-expressing interneurons in CA3a/CA2 were lost at this time point (KA 21 days; n = 5 mice, Figures 3M,N). The reduction of interneurons in...
expressing interneurons we generated heatmaps which display fiber sprouting. Notably, when comparing the septotemporal extent of GCD and interneuron loss, we observed that GAD67 mRNA-positive interneurons were mostly lost at sites where GCD was present and their full density was only established temporally to the transition zone from GCD to normal granule cell layer width (Figures 3S–U). The upregulation of GAD67 mRNA expression in granule cells was still visible at 21 days but interestingly only in dispersed granule cells (Figure 3P). Immunocytochemical analysis with an antibody against GAD65/67 confirmed the region-selective loss of GAD65/67-expressing interneurons and upregulation of GAD65/67 in granule cells in the septal and intermediate, but not temporal hippocampus on the protein level (data not shown). In addition, it revealed a strong upregulation of GAD65/67 mRNA expression in ipsilateral mossy fiber terminals in the hilus and CA3 at 2 days after KA and throughout the granule cell layer, molecular layer and CA3 at 21 days after KA, reflecting mossy fiber sprouting.

For visualization of the distribution of GAD67 mRNA-expressing interneurons we generated heatmaps which display the cell density in false color (Figure 4). These maps illustrate the progressive loss of GAD67 mRNA expression between 2 and 21 days in the intermediate and temporal hippocampus (positions 3 and 4, Figures 4G,H,K,L). Furthermore, they show that reduced interneuron density extended to more temporal sites than GCD (Figures 4G,L), indicating the high sensitivity of GABAergic interneurons to KA injection and SE.

**Parvalbumin-Positive Interneurons are Reduced Beyond the Transition Zone.**

To more specifically characterize the loss of interneurons along the septotemporal axis, we immunocytochemically stained PV-expressing interneurons since they are highly vulnerable in KA-injected mice surrounding the injection site (Bouilleret et al., 2000a). In controls, the majority of PV-expressing cells were located close to or within the principal cell layers (controls: n = 6 mice, Figure 5A), representing most likely axo-axonic and basket cells. A few PV-positive interneurons were also located in the hilus and strata radiatum and oriens of the CA region. The distribution of PV-positive cells was comparable at all septotemporal sites (Figures 5A–C), which was confirmed by quantification (Figures S5A,P) and matched previous results (Kosaka et al., 1987; Somogyi and Klausberger, 2003). In the contralateral hippocampus of KA-injected mice the density of PV-expressing cells was comparable to controls at all sites (Figures S5O,P). Due to the high similarity of positions 1 and 2, as shown for GAD67 mRNA expression, we only display positions 2–4 in the following photomicrographs.

Already at 2 days after KA injection PV-positive cell bodies were strongly reduced in the septal hippocampus except for a few cells in CA3 and distal CA1 (KA 2 days: n = 4 mice, Figures 5D,E). The PV-positive axon plexus in CA1 and in the inner molecular layer was also vanishing (Figures 5F,I). The strong fluorescence in the pyramidal cell layer of CA1 is comparable to what can be observed for many different antibodies and is most likely due to unspecific staining of degenerating cells. Quantification of PV-expressing interneurons revealed that the loss of these cells was significant compared to controls and the contralateral hippocampus for the hilus (n = 6 controls, n = 4 KA-injected mice; position 1: ANOVA: p = 0.0011, pairwise Tukey’s post-test: ipsilateral-control p < 0.05; position 2: ANOVA: p < 0.0001, pairwise: ipsilateral-control and ipsilateral-contralateral p < 0.001) as well as the whole hippocampus (position 1: ANOVA: p = 0.0002, pairwise: ipsilateral-contralateral p < 0.05; position 2: ANOVA: p < 0.0001, pairwise: ipsilateral-control p < 0.001 and ipsilateral-contralateral p < 0.01; Figures S5O,P).

In the intermediate hippocampus a characteristic pattern was observable: In the dorsal part the reduction of PV-positive cell bodies and partial preservation of the PV-positive plexus was comparable to the septal hippocampus (Figures 5G–I), while in the ventral part PV-positive cell bodies and their axon plexus seemed preserved (Figures 5J,K). This was confirmed by quantification which revealed a significant loss of PV-expressing cells in the hilus (position 3: ANOVA: p = 0.0013; pairwise: ipsilateral-contralateral and ipsilateral-contralateral p < 0.05) but no changes when the whole hippocampus was regarded (ANOVA: p = 0.30; Figures S5O,P). In the temporal hippocampus PV-positive cells and axons were only lost in the very dorsal parts of the section but preserved elsewhere (Figures 5L–N) and quantification showed comparable densities for the hilus (position 4: ANOVA: p = 0.10) and hippocampus (ANOVA: p = 0.02; pairwise: not significant; Figures S5O,P).

At 21 days after KA, PV-expressing cells in the septal hippocampus were nearly completely lost except for a very small group of dysmorphic neurons in CA3 (Figures 6A,B); furthermore, the PV-stained axon plexus in CA1 and the dentate gyrus was no longer visible (KA 21 days: n = 5 mice, Figure 6A). This reduction was significant when regarding the whole hippocampus (position 1: pairwise Tukey’s post-test: ipsilateral-control and ipsilateral-contralateral p < 0.01; position 2: pairwise: ipsilateral-control and ipsilateral-contralateral p < 0.01; Figure 5Q) as well as the hilus (position 1: pairwise: ipsilateral-control p < 0.01; position 2: pairwise: ipsilateral-control p < 0.001, ipsilateral-contralateral p < 0.01; Figure 5P). In the intermediate hippocampus the loss of PV-positive cells and axons also progressed to slightly more ventral areas (i.e., toward the temporal hippocampus) than 2 days after KA injection (Figure 6C). Yet, this difference was only significant for the hilus (position 3: pairwise: ipsilateral-control p < 0.01) but not for the whole hippocampus (Figures 5O,P). At the most temporal site (position 4), the progression of the loss of PV labeling between 2 and 21 days after KA was also apparent, in particular in the hilus (Figure 6D), however, this reduction was not significant (Figure 5P). Notably, PV-positive cell bodies were preserved only at sites temporal to the transition zone from GCD to normal granule cell layer width (Figures 6C,D).
FIGURE 4 | Spatial distribution of GAD67 mRNA-positive interneurons. (A–L) Density of GAD67 mRNA-expressing interneurons in representative hippocampal sections along the septotemporal axis displayed as heatmaps in false-color, relative to the maximal density within each position. The outer border of the hippocampus and the hilus are marked with white traces. Brighter color indicates higher, darker color lower relative cell density. (A–D) Control mouse, septal (A, B), intermediate (C) and temporal hippocampus (D). (E–H) Ipsilateral hippocampus, 2 days after KA injection, septal (E, F), intermediate (G) and temporal level (H). A gradient of cell loss along the septotemporal axis is visible. (I–L) Ipsilateral hippocampus, 21 days after KA injection, septal (I, J), intermediate (K) and temporal level (L). Note that the loss of GAD67 mRNA-expressing cells extends to further temporal areas than at 2 days after KA and the reduction of interneuron density can be observed beyond the transition zone from GCD to normal granule cell layer width (L, arrows). Scale bars: 200 μm.

REDUCTION OF NPY-POSITIVE INTERNEURONS, BUT LOCATION-DEPENDENT UPREGULATION OF NPY IN MOSSY FIBERS AND GRANULE CELLS

In controls, NPY-positive cells were abundant in the hilus and many NPY-expressing cell bodies were visible in stratum oriens of CA3 and CA1 at all septotemporal levels (controls: n = 4 mice, Figures 7A–D), in line with previous reports in mice (Makriyannis et al., 1999) and rats (Deller and Leranth, 1990; Speck et al., 2007; Kuruba et al., 2011). Some NPY-positive cells were present in strata radiatum and pyramidale of CA3 and CA1. In addition, a weakly stained NPY-positive axon plexus was visible throughout the hippocampus (Figures 7A–D).

At 2 days after KA injection, the contralateral hippocampus showed considerably enhanced NPY staining in cell bodies mainly in stratum oriens, pyramidale and radiatum of CA1 of the septal hippocampus (n = 5 of 5 mice, Figures 7E,F) and in dorsally located parts of the intermediate and temporal hippocampus (4/5 mice, Figures 7G,H). In addition, NPY was slightly (2/5 mice) or even strongly upregulated in contralateral mossy fibers at all septotemporal levels (3/5 mice, Figures 6E–H). This upregulation spatially overlapped with NPY-positive interneurons and did not allow reliable quantification of NPY-positive cells. NPY expression in granule cells was comparable to controls.
In contrast, in the ipsilateral hippocampus, NPY-positive interneurons were lost in the septal hilus and strongly reduced in CA1, but mostly preserved in CA3 (KA 2 days: 5/5 mice, Figures 7L,J). In the intermediate and temporal hippocampus NPY-positive interneurons were preserved in the hilus and CA region (Figures 7K,L). More conspicuous, however, was the strong upregulation of NPY in septal granule cells and in dorsally located granule cells of the intermediate hippocampus and in their dendrites at 2 days after KA injection (3/5 mice, Figures 7I–K). In addition, mossy fibers showed a strongly increased NPY staining in the septal hippocampus (3/5 mice, Figures 7I,J), which extended to the intermediate and temporal hippocampus (2/5 mice, Figures 7K,L).

At 21 days after KA, the upregulation of NPY expression in interneurons in the contralateral hippocampus was no
FIGURE 7 | Ectopic pattern of NPY staining at 2 days after KA injection. (A–L) Representative sections of immunocytochemical staining for NPY (red with DAPI counterstaining blue) at 2 days after KA injection. (A–D) Control mouse, septal, intermediate (C) and temporal hippocampus (D). NPY-expressing cells were located mainly in the hilus and in stratum oriens and their connections were visible throughout the hippocampus. (E) Cutout of (A) with hilus and granule cell layer enlarged. NPY-positive cells were mostly located in the hilus and their axons were visible throughout the dentate gyrus but granule cells and mossy fibers were not stained. (E–H) Contralateral hippocampus. (E) In the septal hippocampus an upregulation of NPY expression in interneurons was visible in the CA region in strata oriens, pyramidale and radiatum. In addition, NPY was strongly upregulated in the mossy fibers. NPY-positive interneurons in the hilus were preserved but mostly outshined by the strong mossy fiber staining in the whole hilus (see enlarged in F). (G, H) In the intermediate and temporal hippocampus, staining of NPY-positive interneurons was slightly upregulated in the dorsal part of the section but comparable to controls in the ventral part. NPY expression in the mossy fibers was strongly increased in the hilus, CA3 and CA2 at all septotemporal levels. (I-L) Bilateral hippocampus. (I) In the septal hippocampus NPY-expressing interneurons were lost except for a few remaining, strongly stained cells in CA3 stratum oriens. NPY expression was upregulated in the mossy fibers. In contrast to the contralateral side, NPY immunostaining was strongly enhanced in a large portion of granule cell bodies and dendrites (see enlarged in J, arrow). (K) In the intermediate hippocampus NPY-expressing cells were preserved in the hilus and expression in the CA region was comparable to controls. Upregulation of NPY in the mossy fibers was only visible in the very dorsal parts of the section. (L) In the temporal hippocampus NPY expression was comparable to controls. Scale bars: 200 μm; B, F, J, insets 100 μm.

longer visible and staining of interneurons was comparable to controls in all mice (n = 6, Figures 8A–E). In contrast, the enhanced NPY labeling in mossy fibers was maintained at all septotemporal positions (Figures 8A–E), however, the intensity of labeling was variable across mice (3/6 mice with weak NPY upregulation, 3/6 mice with strong NPY upregulation) in agreement with Aradadzisz et al. (2005). In addition, in the septal hippocampus NPY-positive terminals were apparent in the inner molecular layer close to the granule cells (Figure 8A).
In the ipsilateral septal hippocampus the progressive loss of NPY-positive cells was more pronounced than at 2 days after KA and also affected CA3 (KA 21 days: $n = 6$ mice, Figure 8F). In the ipsilateral intermediate and temporal hippocampus NPY expression in cell bodies was preserved in the hilus and CA region in all mice (Figures 8G,H). Remarkably, NPY-positive cell bodies were visible in the hilus at sites where GCD was present (i.e., septal to the transition zone, Figures 8H,I), which is in contrast to PV.

At 21 days the staining of mossy fibers has substantially changed compared to 2 days: in the septal hippocampus NPY-labeled mossy fibers were observed throughout the granule cell layer and molecular layer and at a small spot in CA3/CA2 (Figures 8F–H), resembling the spatial pattern of Timm staining (Bouilleret et al., 1999) and thus most likely reflects mossy fiber sprouting. This pattern was present at all positions where GCD occurred and extended even slightly beyond the transition zone (Figures 8G,H).

**DISCUSSION**

In the current study we compared the intrahippocampal spread of KA-induced SE with the spatial pattern of interneuron loss along the septotemporal axis of the hippocampus. We show that in the ipsilateral septal hippocampus most GAD67 mRNA-expressing interneurons were lost already at 2 days after KA injection except for a small population of interneurons in CA3 including PV- and NPY-positive cells. Cell loss extended to the intermediate hippocampus already at 2 days, but progressed further toward the intermediate hippocampus and to CA3 until 21 days. In the intermediate hippocampus we observed a differential vulnerability of the different interneuron populations: PV-expressing cells were lost at much more temporally located areas than NPY-expressing cells. In addition to the loss of interneurons, we show that GAD67 mRNA was exclusively upregulated in dispersed granule cells, whereas upregulation of NPY expression occurred in ipsilateral septal granule cells and in mossy fibers throughout the hippocampus, most likely representing a compensatory mechanism to the loss of inhibition.

**INTERNEURONS WERE LOST BEYOND THE SCLEROTIC AREAS**

In our previous study, we demonstrated that recurrent epileptiform activity is not strongest in the most sclerotic areas of the septal hippocampus, but instead in the intermediate hippocampus, which shows only minor histological changes (Häussler et al., 2012). Here, we thus analyzed how inhibition could contribute to differential strength of epileptiform activity by mapping the distribution of GAD67 mRNA-expressing interneurons along the septotemporal axis. To this end, we quantified the distribution of GAD67 mRNA-expressing cells and PV-expressing interneurons along the septotemporal axis of the hippocampus. In our quantification PV-positive interneurons make up for 8–13% of the total density of GAD67-positive interneurons in the hilus and 12–15% in the whole hippocampus which is lower than what has been shown in the literature (14–20% in the hilus, 20–24% in the hippocampus; Freund and Buzsaki, 1996). A higher yield and better signal-to-noise ratio in *in situ*
hybridization for GAD67 mRNA compared to immunocytochemistry for PV expression might lead to this underestimation of the relation. However, as the relation is comparable for controls and KA-injected mice for all four positions, it does not influence our results on changes in the distribution of PV-positive cells in TLE.

In addition to the nearly complete loss of GAD67 mRNA-expressing interneurons in the septal hippocampus at 21 days after KA injection, which has been previously shown (Bouilleret et al., 2000a,b), our analysis revealed that the loss of interneurons is not restricted to the sclerotic area, but extends to the intermediate hippocampus. More precisely, the loss of interneurons extends even beyond the transition zone from GCD to normal granule cell layer width. In particular interneurons expressing PV showed a high vulnerability: PV-positive somata, as well as the dense PV-positive axon plexus were also only visible at sites temporal to this transition zone.

We are aware that the loss of PV expression might not be equivalent to the death of these interneurons and might reflect only a transient down-regulation of PV synthesis as suggested previously (Sloviter et al., 1991; Scotti et al., 1997; Wittner et al., 2001), or a transient reduction of inhibitory function followed by structural or functional reconstruction, as shown in other epilepsy models (Houser and Esclapez, 1996; Hellier et al., 1999; Bernard et al., 2000; Holkamp et al., 2005; Sovie et al., 2006). However, given the loss of GAD67 mRNA expression at the same sites and neuronal death mainly in the hilus, close to the granule cell layer and in the CA region, as shown by Fluoro-Jade B staining, it is highly likely that the loss of PV expression reflects the death of these inhibitory interneurons instead of a transient loss of function. This is supported by a recent study in which granule cell responses upon perforant path stimulation were recorded in vivo in intrahippocampally KA-injected mice: granule cells showed oscillatory responses and reduced paired pulse inhibition already at 3 days after KA and the number of population spikes was even increased at 21 days after KA while paired pulse inhibition did not recover (Rougier et al., 2005), indicating the loss of inhibitory neurons.

Conflicting results on vulnerability of PV-expressing interneurons in human TLE and animal models exist in the literature (de Lanerolle et al., 1989; Sloviter et al., 1991; Zhu et al., 1997; van Vliet et al., 2004; Andrioli et al., 2007; Wryth et al., 2010; Kuruba et al., 2011). Our data show that the almost complete loss of PV-positive interneurons explains only partially the total loss of GAD67 mRNA-positive interneurons and preliminary analyses revealed that this difference is not fully explained by the loss of NPY-expressing interneurons. This is in agreement with previous studies, in which, additionally, the loss of somatostatin-positive interneurons (Bouilleret et al., 2000b; Daygladre et al., 2007), calretinin- and calbindin-positive interneurons has been shown in the septal hippocampus of KA-injected mice (Bouilleret et al., 2000a). Mapping the contribution of these additional interneuron subtypes to total interneuron loss along the septotemporal axis is a goal for future studies.

The nearly complete loss of interneurons in the septal hippocampus in our model might be due to the strong sclerosis in this area and may represent an overestimation of what happens in human TLE. However, a correlation between the loss of pyramidal cells and the loss of interneurons in the hilus and in CA1 has also been shown in human TLE (Thom et al., 2012). In addition, that study showed that gradients of cell loss of principal cells and interneurons occur along the human longitudinal hippocampal axis in TLE with a trend toward stronger loss in the anterior and intermediate hippocampus. These data strongly support the transferability of our animal model to the human pathology. Furthermore, the expansion of cell loss toward the intermediate hippocampus and toward CA3 between 2 and 21 days in our study indicates that interneuron loss cannot be completely due to excitotoxicity of KA but that also SE and recurrent epileptiform activity have a destructive effect on interneurons (Mello and Covolan, 1996) and lead to progression of the disease as suggested by others (van Vliet et al., 2004).

Since the lack of PV-expression has been associated with increased seizure susceptibility (Schwaller et al., 2004), it seems likely that the strong reduction of perisomatically inhibiting PV-positive interneurons in the septal and intermediate hippocampus indeed contributes to the increased epileptogenesis in these regions. In the septal hippocampus, where dispersed granule cells show diminished excitability (Young et al., 2009), this might result in weaker epileptiform activity than at sites adjacent to the transition zone, matching the results of our previous study (Häussler et al., 2012). Yet, hypothesizing such an effect only from our current results is difficult due to the complex function of perisomatic and dendritc inhibition in the dentate gyrus since it actually might have a depolarizing effect due to the low resting potential of granule cells (Sauer et al., 2012). Our data thus highlight the importance of studying in detail the interaction between granule cells and interneurons in the structurally preserved intermediate hippocampus.

Differential upregulation of NPY and GAD67 expression

The loss of NPY-expressing interneurons in the ipsilateral septal hippocampus at 2 days after KA, the slight progress of cell loss until 21 days, as well as the transient upregulation of NPY in septal granule cells only at 2 days are in agreement with previous results (Makura et al., 1999). However, the up-regulation of NPY in interneurons in the contralateral CA1 region at 2 days after KA has not been described in this model previously.

FIGURE 5 | Continued

Instead, NPY was strongly upregulated in mossy fibers. Note that mossy fibers had sprouted into the dispersed granule cell layer and molecular layer (white arrow). In addition a termination zone in dorsal CA3/CA2 was visible (yellow arrow). In contrast to the earlier time point, granule cell bodies were not strongly NPY-positive. In the intermediate hippocampal NPY-positive interneurons were preserved in the ventral parts of the section, however, starting from areas located more septal than the transition zone from GCD to normal granule cell layer width (arrow). In the dorsal part of the section a pattern of mossy fiber sprouting comparable to the septal hippocampus occurred whereas in the ventral parts NPY was still upregulated in mossy fibers but at their normal position (note the overlap of sprouted and normal mossy fibers (M) in the temporal hippocampus). NPY-positive interneurons were preserved slightly dorsal to the transition zone (arrow), whereas GCD was still visible and mossy fiber sprouting occurred (white box, enlarged in J). Preservation of NPY-positive cells was also seen more ventrally where enhanced NPY expression was seen in mossy fibers in the hilar (yellow box, enlarged in J). Scale bars: 200 µm, D, E, I, J, 50 µm.
A second, possibly compensatory mechanism is the upregulation of GAD67 in granule cells which mainly occurs in the septal and intermediate ipsilateral hippocampus at 2 days after KA and is restricted to dispersed granule cells at 21 days after KA. This is in agreement with results in human TLE where strong upregulation of GAD67 has been shown in granule cells (Sperl et al., 2012) and with other animal models (Schwarzer and Sperk, 1995; Ramirez and Gutierrez, 2001). It has been shown that mossy fiber boutons release glutamate and GABA under healthy conditions (Beltrán and Gutierrez, 2001), suggesting that the upregulation of GAD67 in granule cells in our mice also leads to locally increased GABAergic transmission. To clarify whether this transmission has an effect compensating hyperexcitability requires experiments in slices from different septotemporal levels since the influence on CA3 pyramidal cells and, via mossy fiber sprouting-induced backpropagation, on other granule cells might be different.

**DOES THE INTERMEDIATE HIPPOCAMPUS CONSTITUTE AN EPILEPTOGENIC NETWORK?**

Our study indicates that the intermediate hippocampus comprises multifaceted changes including the transition from GCD to normal granule cell layer width, differential vulnerability of PV- and NPY-expressing cells, and recovery of neurogenesis (Häussler et al., 2012). All these changes might be functionally linked. GCD is caused by migrating granule cells which lose their positional information through the loss and functional inactivation of Reelin (Haas et al., 2002; Heinrich et al., 2006; Müller et al., 2009; Tinevez et al., 2011) which normally acts as a position signal during development and in the adult. In the healthy hippocampus, Reelin is expressed by interneurons in the hilus, at the hippocampal fissure and in stratum oriens of the CA region (Pesold et al., 1998). In particular, the hilar interneurons are lost after KA injection as shown in our study. Interestingly, Reelin-expressing interneurons co-express NPY or somatostatin, but not PV (Pesold et al., 1999).

The loss of PV-expressing interneurons in our study extended beyond GCD, but NPY-expressing cells were visible slightly more septally indicating that the extent of NPY cell loss might influence the septotemporal extent of GCD through expression gradients of Reelin. Another important fact is that NPY promotes hippocampal neurogenesis (Hövell et al., 2003, 2005; Decressac et al., 2011) and we previously showed differential regulation of neurogenesis with respect to strength of the initial SE: neurogenesis is lost in the septal hippocampus and only present in the intermediate and temporal hippocampus (Häussler et al., 2012). In fact, in the intermediate, temporal and contralateral hippocampus neurogenesis is strongly increased compared to controls which might be stimulated by the expression of NPY in non-sprouted mossy fibers together with the expression of NPY in surviving interneurons.

Does the transition zone have a functional role? In our previous study, we have shown that epileptiform activity was not strongest at sites where hippocampal sclerosis was most pronounced but, instead, in the adjacent area of the transition zone where neurogenesis reappeared. Neurogenesis, together with the reduction of inhibitory interneurons in this region, might comprise a network...
with disturbed excitation-inhibition balance and high epileptogenicity. The functional consequences of such gradual changes might be transferred to human TLE in which gradients of cell loss along the longitudinal hippocampal axis have been shown (Thom et al., 2012). In addition, our precise quantitative analysis of neuron densities and neurogenesis in the whole hippocampus paves a way for modeling studies on the epileptogenicity of small shifts in network balance.

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