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Research Report

Unilateral hippocampal CA3-predominant damage and short latency epileptogenesis after intra-amygdala microinjection of kainic acid in mice

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ABSTRACT

Mesial temporal lobe epilepsy is the most common, intractable seizure disorder in adults. It is associated with an asymmetric pattern of hippocampal neuron loss within the endfolium (hilus and CA3) and CA1, with limited pathology in extra-hippocampal regions. We previously developed a model of focally-evoked seizure-induced neuronal death using intra-amygdala kainic acid (KA) microinjection and characterized the acute hippocampal pathology. Here, we sought to characterize the full extent of hippocampal and potential extra-hippocampal damage in this model, and the temporal onset of epileptic seizures. Seizure damage assessed at four stereotaxic levels by FluoroJade B staining was most prominent in ipsilateral hippocampal CA3 where it extended from septal to temporal pole. Minor but significant neuronal injury was present in ipsilateral CA1. Extra-hippocampal neuronal damage was generally limited in extent and restricted to the lateral septal nucleus, injected amygdala and select regions of neocortex ipsilateral to the seizure elicitation side. Continuous surface EEG recorded with implanted telemetry units in freely-moving mice detected spontaneous, epileptic seizures by five days post-KA in all mice. Epileptic seizure number averaged 1–4 per day. Hippocampi from epileptic mice 15 days post-KA displayed unilateral CA3 lesions, astrogliosis and increased neuropeptide Y immunoreactivity suggestive of mossy fiber rearrangement. These studies characterize a mouse model of unilateral hippocampal-dominant neuronal damage and short latency epileptogenesis that may be suitable for studying the cell and molecular pathogenesis of human mesial temporal lobe epilepsy.

1. Introduction

Mesial temporal lobe epilepsy (MTLE) is the most common and intractable seizure disorder in adults (Chang and Lowenstein, 2003; Engel, 2001). The causal factors in the pathogenesis of human MTLE remain unknown. Its most common pathological hallmark is asymmetric hippocampal neuron loss within the endfolium (hilus and CA3) and CA1, with relative sparing of the

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Abbreviations: CA, cornu ammonis; DAPI, 4′,6 diamidino-2-phenylindole; EEG, electroencephalogram; FjB, FluoroJade B; GFAP, glial fibrillary acidic protein; KA, kainic acid; LSN, lateral septal nucleus; MTLE, mesial temporal lobe epilepsy; NeuN, neuron-specific nuclear protein; NPY, neuropeptide Y

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dentate granule neurons and CA2 subfield (Mathern et al., 1997; Meldrum and Bruton, 1992; Najm et al., 2006). Extra-hippocampal neuron loss within cortical regions and amygdala have been reported in some (Du et al., 1993; Hudson et al., 1993; Pitkanen et al., 1998), but not all (Bothwell et al., 2001; Dawodu and Thom, 2005) clinical studies. Acutely-incurred damage following status epilepticus (SE) in patients is found within hippocampus, amygdala, thalamic nuclei and piriform and entorhinal cortices (DeGiorgio et al., 1992; Fujikawa et al., 2000).

Convulsive SE in rodents has been used extensively to model seizure-induced neuronal death and MTLE pathology (Morimoto et al., 2004). In rats, systemic pilocarpine or kainic acid (KA) are most commonly used, but produce bilateral and widespread extra-hippocampal damage with limited or variable hippocampal

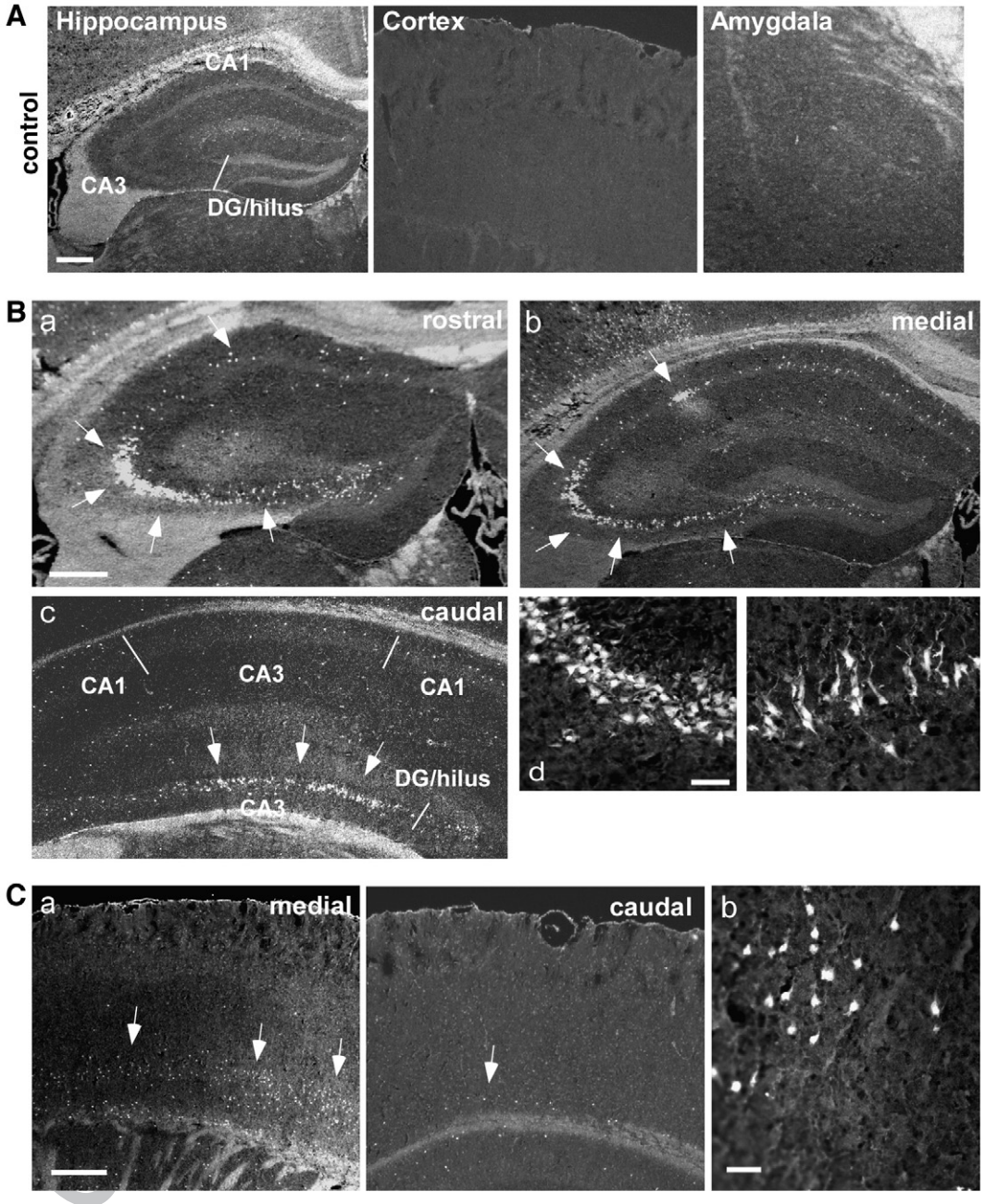

Fig. 1 – Hippocampal and cortical injury following seizures evoked by intra-amygdala KA microinjection. Photomicrographs show representative FluoroJade B (FjB) staining of ipsilateral fields from control and KA-treated mice at 24 h. (A) Staining (4X lens) in control mice for the hippocampus, cortex and amygdala. Note absence of FjB positive cells. (B) Representative images of ipsilateral hippocampus from seizure-damaged mice at rostral (AP, –1.22 mm), medial (AP, –1.82) and caudal (AP, –2.92 mm) levels, respectively. Arrows highlight FjB positive cells. Images in d show higher power views (40X lens) of FjB-positive cells in CA3 from (left panel) middle and (right panel) caudal levels. (C) Representative images in a show seizure damage in ipsilateral temporal cortex at medial and caudal levels. Panel b is a higher power image (40X lens) of representative cells at the level of medial temporal cortex. Scale bars, A, Ba, Ca, 500 μm; Bd, Cb, 50 μm.

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pathology (Sloviter, 2005; Sloviter et al., 2007). In contrast, focally-evoked seizures by electrical stimulation of the perforant path in rats or mice replicate closely the unilateral pathology of MTLE (Harvey and Sloviter, 2005; Kienzler et al., 2006; Sloviter, 1983).

Intra-cerebral KA injection offers an alternate approach to focal elicitation of seizures and induction of epileptogenesis. This includes intra-hippocampal KA (Gouder et al., 2003; Vezzani et al., 1999) and evoking SE by intra-amygdala KA microinjection. In the latter case, the seizures produce a mainly unilateral hippocampal lesion with limited extra-hippocampal damage (Ben-Ari et al., 1980; Henshall et al., 2002a,b). This model has been adapted with similar results to cats (Tanaka et al., 1985) and baboons (Menini et al., 1980). In mice, hippocampal damage is also largely unilateral, with damage to hippocampal CA3 and the hilus and minor cell death in CA1 (Araki et al., 2002; Murphy et al., 2007; Shinoda et al., 2004a). Whether hippocampal pathology extends from septal (dorsal) to temporal (caudal) pole has not been fully explored, nor has the extent of extra-hippocampal damage.

Status epilepticus is also an effective experimental approach to trigger epileptogenesis, with spontaneous seizures emerging in the subsequent days or weeks. Characteristic hippocampal changes implicated as causative factors include neuronal death, rearrangement of dentate granule neuron axons (mossy fibre sprouting) (Wuarin and Dudek, 1996), astrogliosis (Li et al., 2008) and neurogenesis (Parent et al., 1997). Unilateral intra-amygdala KA microinjection can precipitate epilepsy within two weeks in rats and cats (Tanaka et al., 1988, 1985). Intra-cerebral recordings in mice three weeks after intra-amygdala KA microinjection have shown the insult is epileptogenic, with spontaneous seizures originating from the hippocampus (Li et al., 2008).

The purpose of the present study was to characterize the extent of hippocampal and extra-hippocampal damage following seizures evoked by intra-amygdala KA microinjection in mice, and to determine the onset time of subsequent spontaneous seizures. Our data show seizure damage in this model is principally unilateral and mainly confined to the hippocampus, and that the injury precipitates epilepsy after a short latent period.

2. Results

2.1. *Behavior during seizures evoked by kainic acid*

Mice showed normal activity immediately after KA microinjection. Polyspike seizure EEG typically started 5–10 min later, with mice showing immobility. Mice began to develop continuous seizures after a further 20 min. These episodes were characterized by immobility, followed by tail extension (Straub-tail). Whole body clonus, head bobbing and rearing and falling (mouse Racine scale 6) were also commonly observed. Short periods of tonic–clonic seizures with loss of posture and jumping also occurred during the seizures.

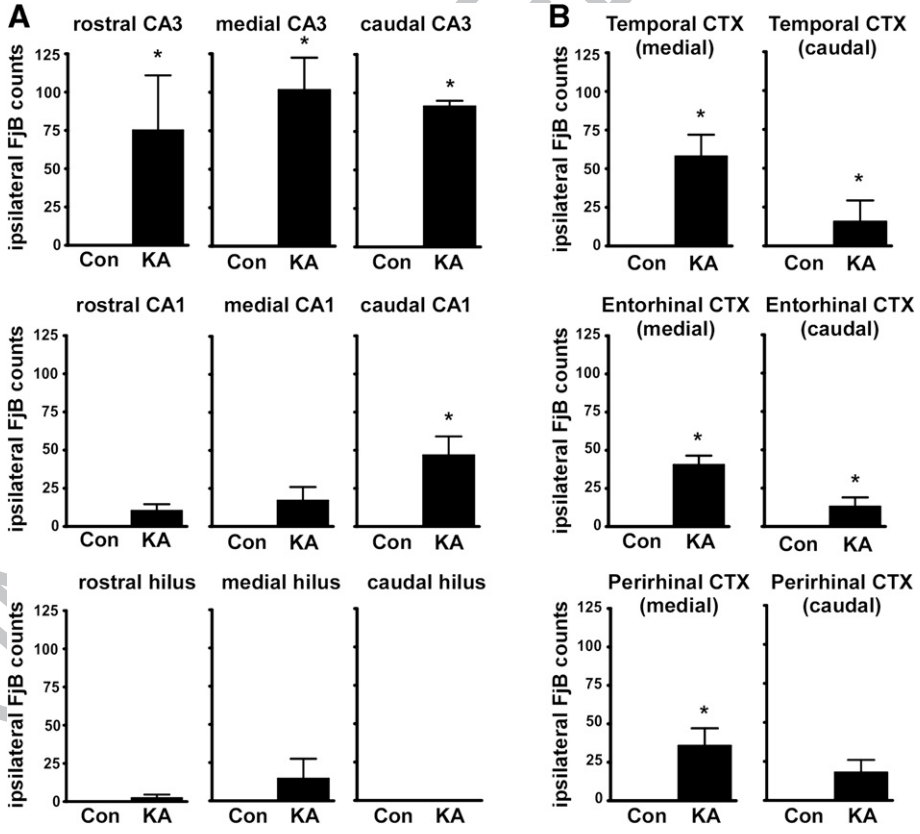

Fig. 2 – Quantification of seizure damage in ipsilateral hippocampus and cortex. (A) Graphs show mean FjB-positive cell counts at three stereotaxic levels for (top) CA3, (middle) CA1 and (bottom) hilus, in control (Con) and KA-treated mice (KA) at 24 h. (B) Graphs show FjB-positive cell counts in temporal, entorhinal and perirhinal cortex at two stereotaxic levels in control and KA-treated mice. Data are mean ± SD from $n = 4$ per group. $^{*}p < 0.05$ versus control.

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2.2. *Hippocampal damage following intra-amygdala KA microinjection in mice*

We recently described induction of seizures by intra-amygdala KA microinjection in mice, seizure termination effects of two anticonvulsants and hippocampal injury at the level of the dorsal hippocampus (Shinoda et al., 2004a). In the present study we sought to characterize the extent of hippocampal damage and extra-hippocampal damage resulting from seizures evoked by intra-amygdala KA microinjection, using FluoroJade B (FjB) staining to mark irreversibly damaged cells. Four stereotaxic levels were examined at 24 h, representing the level of KA injection, and the rostral, medial (septal pole) and caudal (temporal pole) extent of the hippocampus.

Control mice displayed no significant FjB staining in the hippocampus ipsilateral or contralateral to the side of vehicle injection (Figs. 1A,2A and data not shown). In contrast, mice that received intra-amygdala KA microinjection had prominent FjB staining in ipsilateral CA3 at the levels of the rostral, medial and caudal hippocampus (Figs. 1B and 2A). Numbers of FjB-positive CA3 cells in KA-injected mice were significantly higher than controls at each level examined (Fig. 2A). Counts in KA-injected mice were somewhat higher at medial and caudal levels than rostral hippocampus (Fig. 2A). A variable number of FjB-positive cells were present within the hilar region of the dentate gyrus in these mice (Figs. 1B and 2A).

All KA-injected mice had small numbers of FjB-positive cells present in ipsilateral CA1 at one or more of the three hippocampal levels studied (Fig. 1B). Counts were only significant compared to control at the level of caudal hippocampus (Fig. 2A). Occasional FjB-positive cells were found within contralateral CA1 and CA3 in KA-injected mice but numbers were not significant (data not shown).

2.3. *Seizure damage in ipsilateral temporal, entorhinal and perirhinal cortices*

We next examined damage within three cortical regions at levels corresponding to the medial and caudal hippocampus. No FjB-positive cells were found in vehicle-injected controls (Figs. 1A and 2B). Small but significant numbers of FjB-positive cells were present in KA-injected mice in temporal, entorhinal and perirhinal cortex at the level of medial hippocampus (Figs. 1C and 2B). Numbers of cortical FjB positive cells at the level of the caudal hippocampus were smaller than at the medial level but were significant in two of the three cortical regions examined (Fig. 2B). No significant FjB staining was found in cortical regions in the contralateral hemisphere of KA-injected mice (data not shown).

2.4. *FjB staining in the amygdala and lateral septal nucleus*

As expected, FjB-positive cells were present within the ipsilateral amygdala at the level of injection site in both KA-injected and control mice (Fig. 4B). Counts of FjB-positive cells were significantly higher in KA-injected mice compared to controls in the ipsilateral amygdala at levels corresponding to rostral and medial hippocampus (Figs. 3A,B and 4B). No FjB-

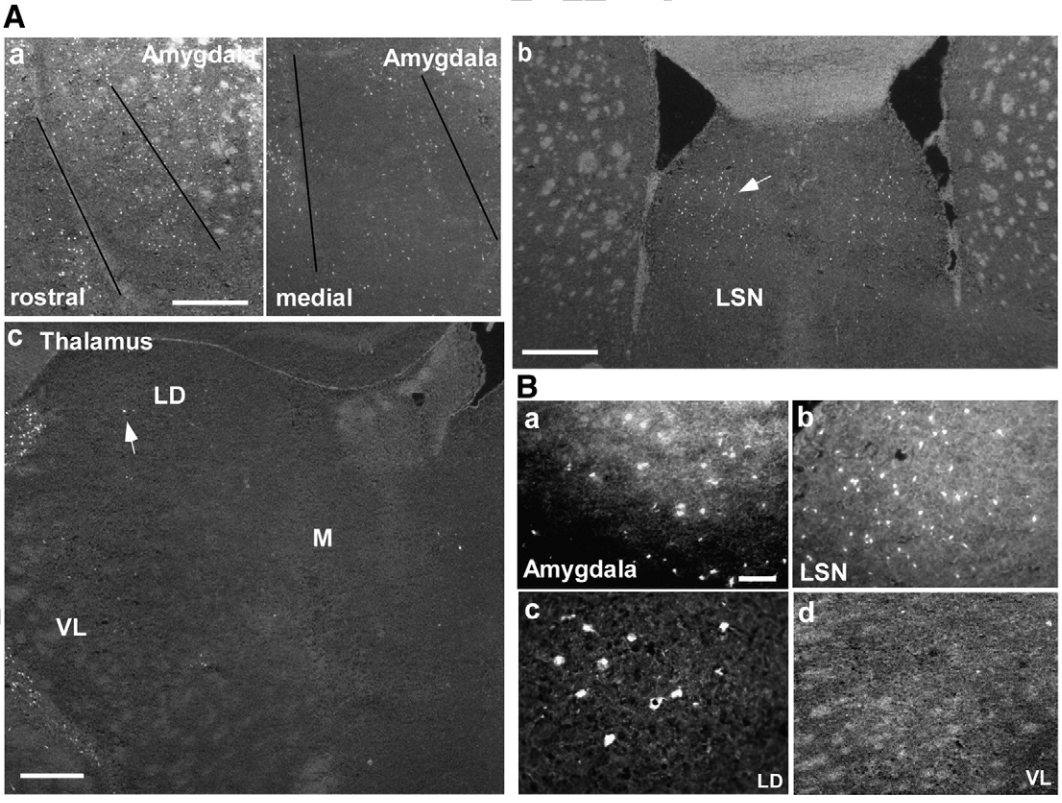

Fig. 3 – Seizure damage in amygdala, thalamus and lateral septal nucleus. (A) Representative photomicrographs from KA-treated mice at 24 h showing FjB staining within *a*, amygdala at rostral and medial levels, *b* lateral septal nucleus (LSN) and *c* thalamus. LD, lateral dorsal; VL, ventrolateral; M, medial. (B) Higher power (20× lens) images of FjB-stained cells in *a*, amygdala, *b*, lateral septal nucleus and *c*,*d* thalamic nuclei. Scale bars in *A*, 500 μm; *B*, 100 μm.

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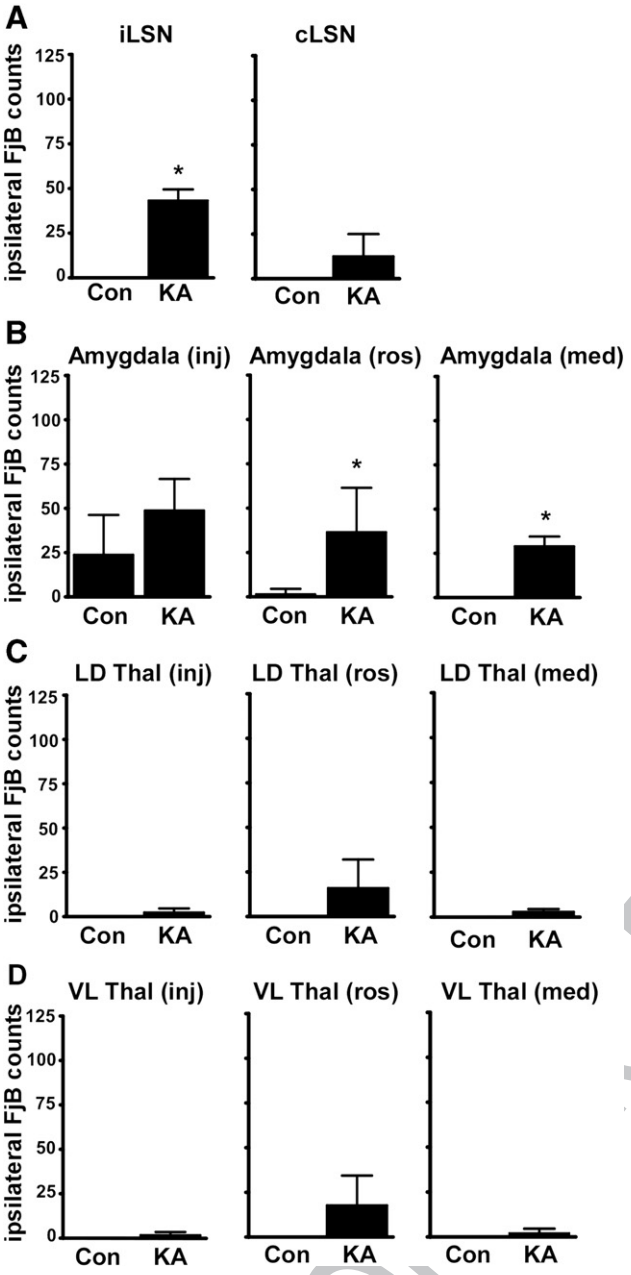

Fig. 4 – Quantification of seizure damage in lateral septal nucleus, amygdala and thalamus. (A) Counts of FjB-positive cells in control (Con) and seizure mice (KA) within the ipsilateral (i) and contralateral (c) lateral septal nucleus (LSN). (B) Counts of FjB-positive cells within the amygdala nucleus at the level of the injection (inj) point (AP, −0.96 mm), rostral (ros) and medial (med) levels. (C) Counts of FjB-positive cells in lateral dorsal (LD) and ventrolateral (VL) at the level of injection point, rostral hippocampus and medial hippocampus. Data are mean ± SD for $n = 4$ per group.

positive cells were present in the contralateral amygdala at any level examined in control or KA-injected mice.

Significant FjB staining was detected in all KA-injected mice within the ipsilateral lateral septal nucleus (LSN) at the level of the amygdala injection point (Figs. 3A and 4A). FjB-positive cells were present within the contralateral LSN in 50% of KA-injected mice (Figs. 3A and 4A).

2.5. *FjB staining in thalamus after seizures*

Finally, we examined FjB staining at the level of the injection point and rostral and medial hippocampus, for three thalamic nuclei. Occasional scattered FjB-positive cells were present in some KA-injected mice in the lateral–dorsal thalamus and ventro-lateral thalamus at the level of rostral hippocampus (Figs. 3A and 4C,D). Counts of thalamic FjB-positive cells showed no significant difference between controls and KA-treated mice and no FjB-positive cells were present in KA-injected mice on the contralateral side for any of the thalamic areas at any level.

2.6. *Short latency epileptogenesis following intra-amygdala KA microinjection in mice*

The time of onset of spontaneous, recurrent seizures following damage caused by intra-amygdala KA microinjection in mice has not yet been investigated. To profile epileptogenesis in this model we used implantable EEG telemetry units to record surface EEG in freely-moving mice. Four mice were subject to two weeks of 24 h/day EEG recordings after KA microinjection. Intermittent EEG recordings from vehicle-injected mice did not detect any epileptic seizures (data not shown).

No mice died after intra-amygdala KA microinjection or during long-term recordings, and all mice developed spontaneous seizures (Fig. 5). Epileptic seizures were typically characterized by a brief period of immobility during onset. This was followed by jaw and forelimb clonus and elevated tail (Straub-tail). Epileptic seizures typically progressed to rearing and falling and in more severe events included loss of posture and jumping (up to Racine scale 6).

The first day in which a spontaneous seizure was detected by surface EEG was day 3 after KA microinjection (1 of 4 mice). By day 4 after KA three of four mice had had an epileptic seizure, and all mice had displayed an epileptic seizure by day 5 after KA microinjection (Fig. 5B). From day 4 thereafter, mice averaged 1–4 seizures per day (full range 0–10). The average electrographic seizure duration was 20.2 ± 15.1 s (range 10–90 s, from 142 captured events). Daily spontaneous seizure rates averaged across the group remained relatively consistent over the course of the recording period (Fig. 5B). Spontaneous seizures for individual mice showed variability between days and across the recording period (Fig. 5C).

2.7. *Hippocampal astrogliosis and mossy fiber rearrangement in epileptic mice*

Finally, we examined sections from control mice and epileptic mice killed on day 15 after the two week EEG recordings post-KA, to visualize long-term changes to the hippocampus. NeuN immunostaining confirmed the presence of macroscopic ipsilateral CA3 lesions in epileptic mice (Fig. 6A). These were not present on the contralateral side of epileptic mice (Fig. 6A).

Next, we counter-stained NeuN-labeled sections with antibodies against glial fibrillary acidic protein (GFAP) as a marker of astrogliosis. Examination of GFAP-stained hippocampi in epileptic mice revealed increased staining in the ipsilateral CA3

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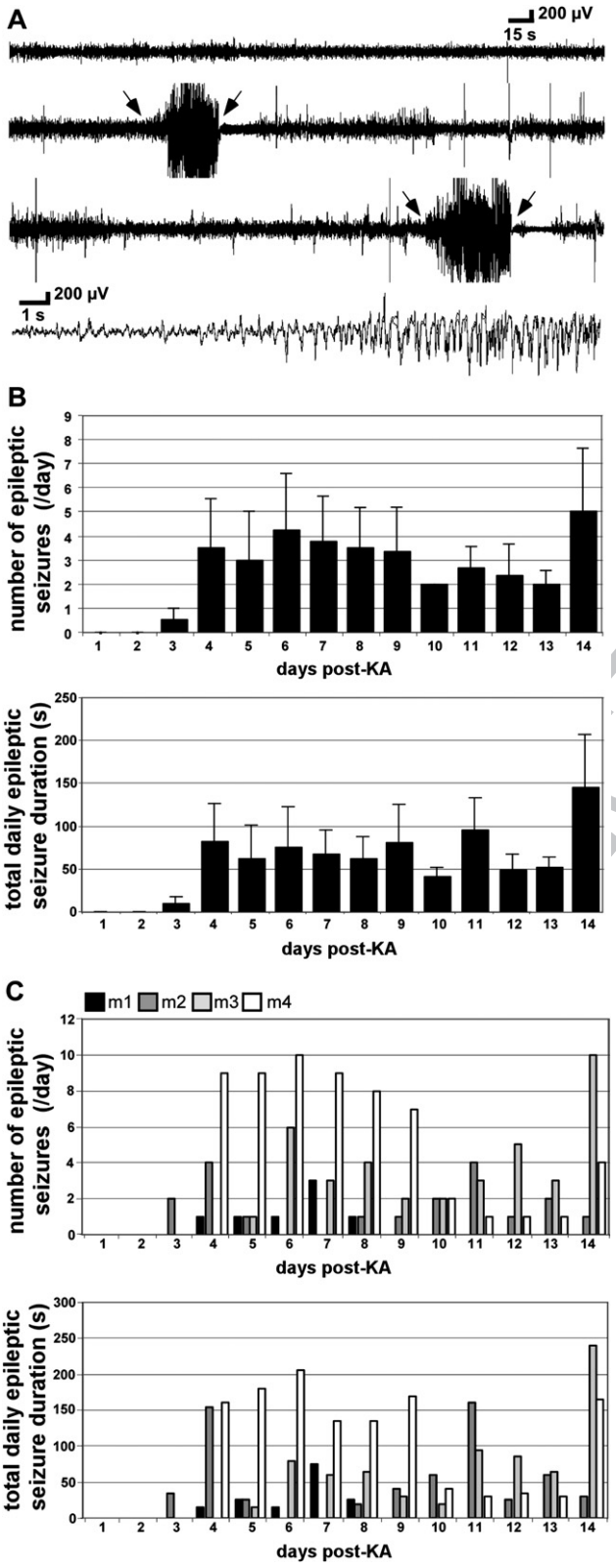
region of epileptic mice, and the presence of thickened astrocyte processes (Fig. 6B).

We also stained sections from epileptic mice for FjB to examine ongoing degenerative changes (Fig. 6C). Hippocampal FjB staining was largely negative, although one epileptic mouse had a small cluster of FjB-positive cells within ipsilateral CA3 (data not shown) (Fig. 6C). A small number of FjB-positive cells (range 7–58) were also present in ipsilateral cortex in sections from epileptic mice (data not shown).

Last, we undertook immunostaining for neuropeptide Y (NPY) on hippocampal sections from epileptic mice, as a surrogate marker of mossy fiber axon rearrangement (Borges et al., 2003). Compared to control, there were prominent increases in NPY immunoreactivity in ipsilateral hippocampus, including mossy fibers throughout the suprapyramidal region of CA3 and inner molecular layer of the dentate gyrus (Fig. 6D). While asymmetric, increased NPY immunostaining in the mossy fiber pathway was also apparent in the contralateral hippocampus (Fig. 6D).



3. Discussion

In vivo models of prolonged seizures have been valuable tools for advancing our understanding of the cell and molecular pathogenesis of human MTLE (Morimoto et al., 2004). A majority of hippocampi obtained from patients with temporal lobe epilepsy display neuropathologic changes that comprise preferential unilateral neuron loss within the endfolium and CA1, with relative sparing of CA2. Additional features include (astro)gliosis, dentate granule cell layer dispersion and mossy fibre rearrangement (Najm et al., 2006). Limitations exist with some animal models in terms of reflecting this pathology. Systemic administration of pilocarpine or KA produces bilateral damage that is often extensive in the cortex, while hippocampal damage, where present, may be the result of secondary processes such as ischemic hemorrhage (Fariello et al., 1989; Sloviter, 2005; Sloviter et al., 2007). Commonly used mouse strains also lack characteristic seizure damage in hippocampus after systemically-delivered convulsants (Schauwecker and Steward, 1997). Focal elicitation approaches such as electrical stimulation of the perforant path appear to more faithfully model the unilateral and hippocampal-predominant neuropathology of human MTLE (Harvey and Sloviter, 2005; Kienzler et al., 2006; Sloviter, 1983; Sloviter et al., 2007). An alternative approach has been to elicit seizures by intra-amygdala KA microinjection. Originally described in rats (Ben-Ari et al., 1980), we adapted this model to mice (Araki et al., 2002; Shinoda et al., 2004a). While independent groups have subsequently employed the model in mice (Kasugai et al., 2007; Li et al., 2008), the present study provides important additional characterization. First, our experiments show damage in CA3 spans the full rostro-caudal extent of the ipsilateral hippocampus and was similar at each level examined.

Fig. 5 – Temporal course of epilepsy emergence after intra-amygdala KA microinjection using radiotelemetry EEG in mice. (A) Representative EEG traces showing baseline EEG and examples of typical epileptic seizures captured by EEG telemetry (between arrows). Bottom trace shows typical electrographic seizure onset. (B) Graphs represent (top) average number of epileptic seizures per day, and (bottom) cumulative average duration of epileptic seizures per day. Data are mean ± SD for $n = 4$ per group. (C) Graphs showing daily seizure number and cumulative seizure time for the individual recorded mice (m).

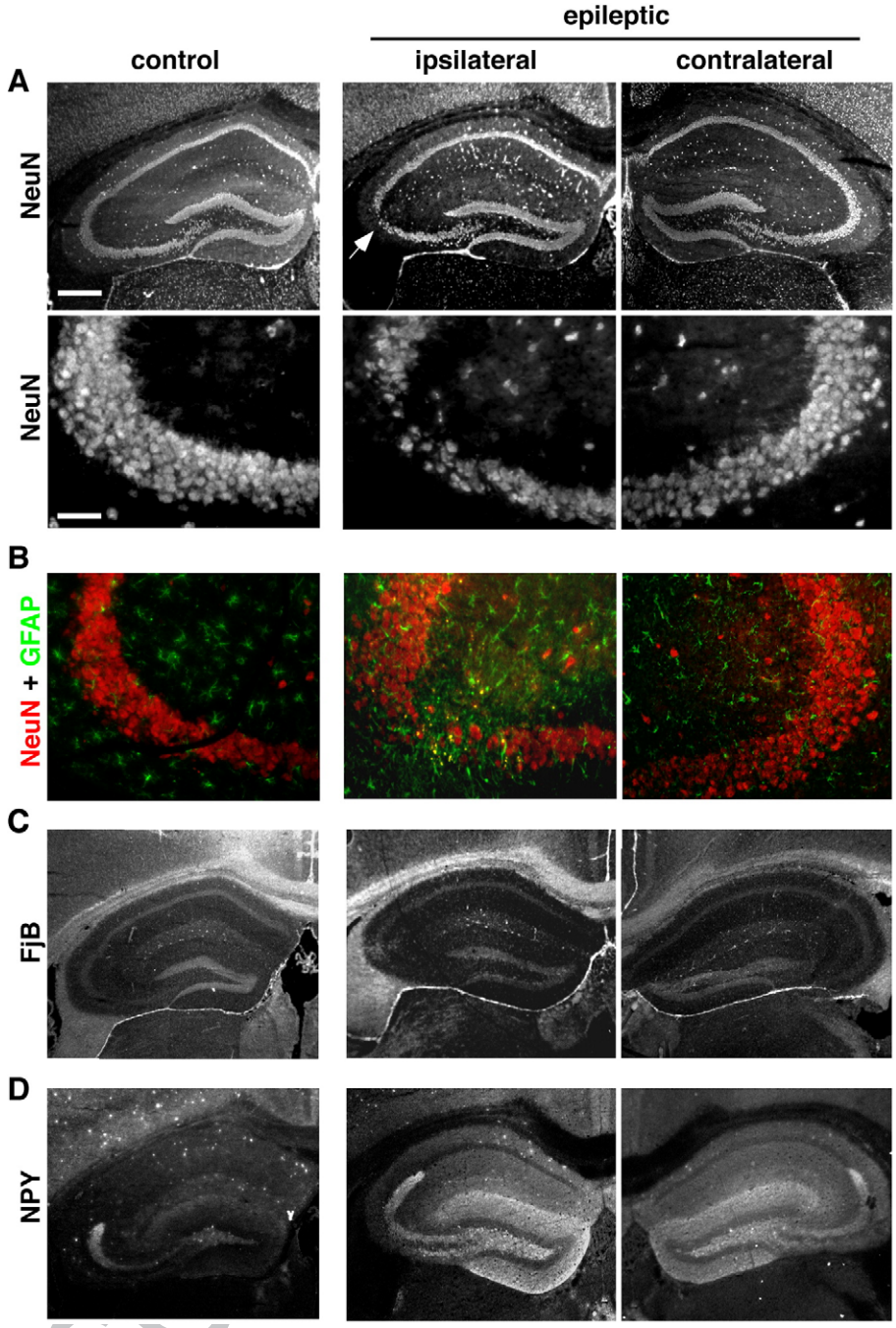


Fig. 6 – Chronic hippocampal changes in epileptic mice. Representative photomicrographs of hippocampal changes in the ipsilateral and contralateral hippocampi of epileptic mice. (A) Photomicrographs showing (top) 4× and (bottom) 20× lens field views of NeuN immunostaining in hippocampi from control and epileptic mice at day 15. Note the presence of a lesion in ipsilateral but not contralateral CA3 of epileptic mice (arrow). (B) Representative NeuN/GFAP double immunostaining (20× lens) in control and epileptic mice. Note elevated GFAP staining within the lesioned region of the ipsilateral CA3. (C,D) Representative photomicrographs (4× lens) showing FjB and neuropeptide Y (NPY) in control and epileptic mice. Note enhanced NPY staining in epileptic mice but lack of FjB staining. Scale bar in top left panel in A, C 500 μm; bottom left in A, 100 μm.

This supports the injury as seizure-related, rather than a result of diffusion of KA from the injection site. Indeed, the extent of the CA3 lesion can be further restricted by administering lorazepam 30 min after intra-amygdala KA microinjection in this model (Li et al., 2008). The CA2 subfield was not damaged by intra-amygdala KA-induced seizures, in agreement with the resistance reported previously using this model (Araki et al., 2002; Kasugai et al., 2007; Li et al., 2008; Shinoda et al., 2004a). Damage to CA1 is often present in human MTLE and we find as before (Araki et al., 2002; Shinoda et al., 2004a), that CA1 injury, though not strongly induced, is nonetheless present in mice after intra-amygdala KA microinjection, particularly in temporal

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hippocampus. Since human MTLE pathology is mainly unilateral and affects the hilar, CA3 and CA1 hippocampal subfields, the present study supports the relevance of the modeling approach and species used.

Cortical neuronal injury has not previously been investigated following intra-amygdala KA microinjection in mice. The present study shows only small numbers of ipsilateral cortical neurons are damaged in this model. This matches similar findings in rats with this model (Henshall et al., 2002), and supports the clinical relevance of the model since cortical neuron loss in human MTLE is typically limited or absent (Bothwell et al., 2001; Dawodu and Thom, 2005; Doherty et al., 2003). The model does not appear to evoke significant damage to the thalamus, which is an area vulnerable in some models (Kubova et al., 2001) and in patients after status epilepticus (Fujikawa et al., 2000). Experimental SE often causes amygdala injury (Nissinen et al., 2000; Tuunanen et al., 1999), and this was seen in the present study, although injury at the rostral extent likely resulted from mechanical (cannula) or direct excitotoxic effects of KA. A limitation of the model lies with achieving complete seizure cessation after intra-amygdala KA microinjection. However, some control is possible through varying anticonvulsant administration time (Araki et al., 2002; Li et al., 2008; Shinoda et al., 2004a). An additional advantage of the present model is its adaptability for use in genetically-modified mice, where it has recently been employed to characterize the anti-apoptotic role of *bcl-w* (Murphy et al., 2007) and epileptogenic effects of adenosine kinase (Li et al., 2008).

A latent period between an initial precipitating neurological injury and subsequent emergence of epileptic seizures is characteristic of acquired MTLE (Williams et al., 2007). Latent periods of a month or more are typically reported for experimental SE-induced epilepsy (Morimoto et al., 2004; Pitkanen et al., 2007). However, spontaneous seizure onset has been detected in subgroups of animals within a week of SE using both systemic and focal elicitation approaches (Harvey and Sloviter, 2005; Nissinen et al., 2000; Shibley and Smith, 2002). By undertaking continuous EEG recordings we could detect epileptic seizures within 3–4 days of intra-amygdala KA-induced SE, with all tested mice epileptic by day 5. This short latent period may result from the localized, reproducible damage in this model and the fidelity of continuous EEG monitoring post-KA (Williams et al., 2007). Our data are consistent with awareness that apparently protracted latent periods in some animals or in some models result in part from inter-animal variability, the extent of hippocampal injury after SE or intermittent EEG monitoring (Williams et al., 2007). The short latent period here also implies protracted pathological processes are unlikely responsible for the emergence of spontaneous seizures in the present model. Mossy fiber sprouting has been linked to the formation of epileptic circuits (Dudek et al., 1994; Wuarin and Dudek, 1996) and can precede emergence of spontaneous seizures (Hendriksen et al., 2001). However, spontaneous seizures occur in post-SE animals without mossy fiber sprouting and the frequency of epileptic events may bear little relation to the extent of sprouting (Nissinen et al., 2000, 2001; Williams et al., 2002). While not excluding a role, the early onset of spontaneous seizures in the present model probably undermines a significant contribution. Nevertheless, mossy fiber sprouting, as implied by NPY immunostaining, was apparent in (epileptic) mice 15 days after KA. The present study also identified astrogliosis within the field of primary damage within the hippocampus. This supports work by Li et al. using the same model, which implicated overexpression of adenosine kinase secondary to astrogliosis in CA3 in epileptic seizure generation (Li et al., 2008).

Neuropeptide Y is an endogenous modulator of excitability and potent inhibitor of seizures. Our data show its presence in normal hippocampus and elevation in hippocampus of epileptic mice, in accordance with reports in chronically epileptic rodents (Nissinen et al., 2000; Schwarzer et al., 1995; Vezzani et al., 1994) and studies showing its elevation in the terminals of mossy fibres (Tu et al., 2005; Vezzani et al., 1996). Whether increased hippocampal NPY levels influences epileptic seizures in the present model is unknown, but anticonvulsant and seizure-terminating roles are supported by findings in mice deficient in NPY which display spontaneous seizures and have increased sensitivity to kainic acid, effects reversed by intracerebral NPY administration (Baraban et al., 1997). Transgenic or viral vector-mediated NPY overexpression in rats also reduces seizures, seizure susceptibility and epileptogenesis (Richichi et al., 2004; Vezzani et al., 2002; Woldbye et al., 1997). However, some data are consistent with seizure-promoting actions of NPY (Liu et al., 1999; Vezzani et al., 1994).

The percentage of animals developing epilepsy after SE shows variance according to model and monitoring employed (Williams et al., 2007). High rates (~90%) are reported following electrical amygdala-stimulation (Nissinen et al., 2000; Pitkanen et al., 2005) and all C57Bl/6 mice surviving pilocarpine-induced SE become epileptic (Borges et al., 2003; Shibley and Smith, 2002). In agreement with findings from hippocampal EEG recordings in mice three weeks after intra-amygdala KA microinjection (Li et al., 2008), we found all mice in our study became epileptic. This supports high-reproducibility of epileptogenesis in this model. However, variability in outcome may be a desired characteristic, for instance for identification of surrogate or biomarkers among animals subject to the same initial precipitating injury.

Daily epileptic event rates remain quite constant in models of amygdala-triggered SE (Nissinen et al., 2000), although the proportion of partial versus secondarily generalized seizures may change (Nissinen et al., 2000; Tanaka et al., 1988). Inter-animal differences in daily event number were found in the present model, but when averaged across the group the daily seizure frequency remained quite consistent once epilepsy emerged. Epileptic seizure durations found in our model were on average of shorter duration than those reported after electrical stimulation of the amygdala (Nissinen et al., 2000; Pitkanen et al., 2007). Since damage was bi-lateral in the studies of Nissinen et al., this supports the influence of the extent of damage and regions affected in the epileptic phenotype.

A relationship between the duration or intensity of SE as the initial precipitating injury and subsequent epileptic phenotype has been demonstrated (Klitgaard et al., 2002; Nissinen et al., 2000; Pitkanen et al., 2005). Moreover, a critical threshold of hilar interneuron loss may be the minimal substrate for formation of epileptic circuits (Zappone and Sloviter, 2004). Taken together, our data are consistent with relatively restricted and unilateral hippocampal neuronal death being sufficient for epileptogenesis.

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Interestingly, mice subject to intra-amygdala KA microinjection that have a lesion restricted to ipsilateral CA3a display hippocampal-only seizures (Li et al., 2008). Monitoring in that study was only during a single day three weeks post-KA and so may have missed some secondarily generalized events. Nevertheless, these data suggest damage in addition to CA3a underlies the expression of seizures we detected on surface EEG. Preventing neuronal death by primary (e.g. glutamate antagonists) or secondary (downstream cell death signaling pathways) neuroprotective agents may therefore be critical to blocking or mitigating epilepsy after SE (Meldrum, 2002). However, results from some studies challenge the efficacy of either class of neuroprotectant as an approach to anti-epileptogenesis (Brandt et al., 2003; Narkilahti et al., 2003).

In conclusion, the present study provides a more complete characterization of the acute hippocampal and extra-hippocampal damage incurred by seizures after intra-amygdala KA microinjection in mice. The pattern of damage reflects that common to human MTLE and the injury elicits epilepsy within a short latent period. Accordingly, intra-amygdala KA microinjection in mice may provide a suitable means for modeling the pathogenesis of MTLE and studying its genetic architecture.

4. Experimental procedures

4.1. *Seizure model*

Animal experiments were carried out in accordance with the principals of the European Communities Council Directive (86/609/EEC) and National Institute of Health's *Guide for the Care and Use of Laboratory Animals*. Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, under license from the Department of Health, Dublin, Ireland.

Studies were performed according to previously described techniques (Murphy et al., 2007; Shinoda et al., 2004a). Mice (C57BL/6 adult male, 20–25 g) were obtained from Harlan, UK. Mice were anesthetized using isoflurane (3–5%) and maintained normothermic by means of a feedback-controlled heat blanket (Harvard Apparatus Ltd, Kent, England). A catheter was inserted into the femoral vein for administration of anticonvulsant. Mice were next placed in a stereotaxic frame and following a midline scalp incision three partial craniectomies were performed and mice were affixed with cortical electrodes (Bilaney Consultants Ltd, Sevenoaks, UK) to record surface EEG. Electrodes were placed above dorsal hippocampus and a third over frontal cortex. EEG was recorded using a Grass Comet digital EEG (Medivent Ltd, Lucan, Ireland). A guide cannula was affixed (coordinates from Bregma: AP=−0.94; L=−2.85 mm) (Franklin and Paxinos, 1997) and the entire skull assembly fixed in place with dental cement. Anesthesia was then discontinued and freely moving mice were placed in a clear Perspex recording chamber. EEG recordings were commenced and after establishing baseline EEG for a few minutes mice were lightly restrained by the experimenter to permit lowering of an injection cannula through the guide cannula to 3.75 mm below the dura for microinjection of KA (Ocean Produce International, Nova Scotia, Canada) (0.3 μg in 0.2 μl phosphate-buffered saline, PBS) into the basolateral amygdala nucleus. Non-seizure control mice received the same volume of intra-amygdala vehicle. Forty minutes following microinjection of KA or vehicle, mice received intravenous lorazepam (6 mg/kg) and the EEG monitored for up to 1 h thereafter. Administration of lorazepam was used to curtail status epilepticus, thereby reducing mortality, morbidity and restricting the extent of damage, as described previously (Shinoda et al., 2004a). Mice were euthanized 24 h following lorazepam and perfused with saline to remove intravascular blood components. Brains were flash-frozen whole in 2-methylbutane at −30 °C and processed for histopathology as described below.

4.2. *Analysis of spontaneous seizures using EEG telemetry*

A separate group of mice were subject to long-term EEG monitoring to detect the emergence of spontaneous seizures. For these purposes, EEG was recorded using implantable EEG telemetry units (Data Systems International, St. Paul, MN) according to previously reported techniques with modifications (Weiergraber et al., 2005). EEG data were acquired using the Dataquest A.R.T. system with EEG transmitters (Model: F20-EET, DSI) configured to record 2 channel EEG that were affixed over dorsal hippocampi/temporal cortex under anesthesia. Transmitter units were placed in a subcutaneous pocket along the dorsal flank. Following surgery and KA-induced seizures, units were activated and telemetry recordings commenced for 14 days from cages on top of receivers (RPC-1), thus allowing free movement and access to food and water. Continuous EEG data was transferred to a PC via a Data Exchange Matrix (DSI). Mice were euthanized on day 15 and brains processed for histopathology and immunostaining as described above. Telemetry EEG recordings were manually analyzed with epileptic seizures defined according to Pitkanen et al. (Pitkanen et al., 2005) as high frequency (>5 Hz) high amplitude (>2 baseline) polyspike discharges of ≥5 s duration.

4.3. *Behaviour*

Mice were observed during KA-induced seizures and also during spontaneous epileptic seizures and scored according to a modified Racine scale for mice (Borges et al., 2003). Score 0, normal activity; Score 1, arrest and rigid posture or immobility; Score 2, stiffened or extended tail; Score 3, partial body clonus, including forelimb clonus or head bobbing; Score 4, rearing; Score 5, rearing and falling; Score 6 tonic–clonic seizures with loss of posture or jumping.

4.4. *Histopathology*

Brains were sectioned at 12 μm on a Leica cryostat at four stereotaxic levels (AP from Bregma; −0.94 mm (injection point), −1.22 mm (rostral hippocampus), −1.82 mm (medial hippocampus) and −2.92 mm (caudal hippocampus)) according to a mouse stereotaxic atlas (Franklin and Paxinos, 1997). Brain regions studied were ipsilateral and contralateral to injection side as follows: lateral septal nucleus, basolateral amygdala, hippocampal hilus, CA3 and CA1, temporal cortex, entorhinal cortex, perirhinal cortex, piriform cortex, lateral–dorsal thalamus, medio-dorsal thalamus and ventro-lateral thalamus.

Neuronal damage was assessed using the FluoroJade B (FjB) technique. Briefly, sections were air-dried and post-fixed in 10% formalin. Next, sections were immersed in 100% ethanol (3 min),

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followed by immersion in 70% ethanol (1 min) then rinsed in distilled water (1 min). Sections were next transferred to fresh 0.006% potassium permanganate solution for 15 min with gentle shaking. Sections were then rinsed again and transferred to FjB solution (0.001% in 0.1% acetic acid) (Chemicon Europe Ltd, Chandlers Ford, UK). After staining, sections were rinsed again, dried, cleared and mounted in DPX (Sigma-Aldrich). Sections were examined using a Nikon 2000s epifluorescence microscope (Micro-optica, Dublin, Ireland) under Ex/Em wavelengths of 330–380/420 nm (blue), 472/520 nm (green) and 540–580/600–660 nm (red) and imaged using a Hamamatsu Orca 285 camera. Pseudo-colour transforms from monochromatic Hamamatsu Orca 285 images were undertaken using Adobe® Photoshop® 6.0. FjB positive cells were the average of two adjacent sections counted by an observer blinded to experimental treatment (Shinoda et al., 2004a,b).

4.5. *Immunohistochemistry*

Immunohistochemistry was undertaken according to previously described techniques (Murphy et al., 2007). Briefly, fresh-frozen sections (12 μm) were air-dried, post-fixed in 10% formalin and washed in PBS. Next, sections were permeabilized with 0.3% Triton X-100 and blocked in 5% goat serum for 1 h. Sections were then incubated overnight with antibodies against NeuN (Chemicon) or GFAP (Sigma-Aldrich) (both 1:500), or neuropeptide Y (NPY, 1:1000) (Sigma-Aldrich), were washed again in PBS and incubated in goat anti-mouse AlexaFluor 568 (for NeuN and GFAP) and goat anti-rabbit AlexaFluor 488 (for NPY) (Bio Sciences Ltd, Dun Laoghaire, Ireland). Sections were mounted with medium containing 4′,6 diamidino-2-phenylindole (DAPI) (Vector Laboratories Ltd, Peterborough, UK) to visualize nuclei and fluorescence immunostaining examined as described above.

4.6. *Statistical analysis*

Data are presented as mean±SD. Comparison of data was performed using a Mann–Whitney *U* test and significance was accepted at $p<0.05$.

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