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Deletion of *puma* protects hippocampal neurons in a model of severe *status epilepticus*

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Abstract

Prolonged seizures (*status epilepticus*) can activate apoptosis-associated signaling pathways. The extent to which such pathways contribute to cell death might depend on the insult intensity, whereby the programmed or apoptotic cell death component is reduced when seizures are more severe or protracted. We recently showed that mice lacking the pro-apoptotic Bcl-2 homology domain 3-only protein Puma (Bbc3) were potently protected against damage caused by *status epilepticus*. In the present study we examined whether Puma deficiency was protective when the seizure episode was more severe. Intra-amygdala microinjection of 1 µg kainic acid (KA) into C57BL/6 mice triggered *status epilepticus* that lasted about twice as long as with 0.3 µg KA prior to lorazepam termination. Hippocampal damage was also significantly greater in the higher-dose group. Over 80 % of degenerating neurons after seizures were positive for DNA fragmentation assessed by terminal deoxynucleotidyl dUTP nick end labeling (TUNEL). Microscopic analysis of neuronal nuclear morphology in TUNEL-positive cells revealed the proportion displaying large rounded clumps of condensed chromatin was ~50 % lower in the high-dose versus low-dose KA group. Nevertheless, compared to heterozygous and wild-type mice subject to *status epilepticus* by high-dose KA, neuronal death was reduced by ~50 % in the hippocampus of Puma-deficient mice. These data suggest aspects of the apoptotic component of seizure-induced neuronal death are insult duration- or severity-dependent. Moreover, they provide further genetic evidence that seizure-induced neuronal death is preventable by targeting so-called apoptosis-associated signaling pathways and Puma loss likely disrupts caspase-independent or non-apoptotic seizure-induced neuronal death.

Keywords

Apoptosis; Bcl-2; Caspase; Epilepsy; Hippocampal sclerosis; Necrosis

A prolonged seizure (*status epilepticus*) or repeated brief seizures over time can cause neuronal death in both animal models and humans (Meldrum, 1993, Wasterlain et al., 1993, Fujikawa et al., 2000a). Seizure-induced neuronal death is mainly triggered by prolonged over-activation of glutamate receptors which results in loss of cellular ion homeostasis, generation of free

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radicals, edema and rupture of various cell membranes, and necrosis (Choi, 1988, Meldrum, 1991, Fujikawa, 2005). However, coordinated (programmed) cell death pathways underlie a major component of the seizure-induced neuronal death process, in as much as there is an ordered cascade of signaling events which progress in a predictable manner that are amenable to pharmacologic or genetic disruption (Liou et al., 2003, Henshall and Murphy, 2008). A molecular signature of apoptosis-associated signaling pathways is found within the hippocampus and other brain regions in several experimental models of seizure-induced neuronal death, and also in resected human temporal lobe material from patients with refractory epilepsy. For example, induction of Bcl-2 family proteins, release of apoptogenic molecules from mitochondria, activation of caspases and DNA fragmentation (for review see (Engel and Henshall, 2009)). Caspase-independent apoptosis signaling pathways (Cheung et al., 2005, Heo et al., 2006, Zhao et al., 2010) and non-apoptotic proteases such as calpains (Araujo et al., 2008, Wang et al., 2008), also contribute to excitotoxic neuronal death. Apoptosis-associated genes such as *bcl-2* have potent effects not only against caspase-dependent apoptosis but also caspase-independent apoptosis and necrotic cell death (Zhong et al., 1993, Kane et al., 1995, Susin et al., 1999, Single et al., 2001).

The extent of involvement of programmed or apoptotic signaling may be dictated by the severity of the insult, whereby milder insults produce more apoptotic cell death (Ankarcrona et al., 1995, Bonfoco et al., 1995, Ward et al., 2007). Studies of *status epilepticus* have also suggested that when seizure development is delayed, the apoptotic molecular signature is more pronounced (Kondratyev and Gale, 2004). Our group has shown that focal-onset *status epilepticus* in mice induced by intra-amygdala kainic acid (KA) results in nuclear morphology consistent with an apoptotic or programmed form of cell death in ~30 % of neurons positive for DNA fragmentation (Shinoda et al., 2004a). Various biochemical features are also present in this model, including p53 involvement, release of cytochrome *c* and caspase activation (Murphy et al., 2007, Engel et al., 2010). Increasing the dose of KA in this model produces greater cumulative seizure time and a larger CA3-hilar lesion (Hatazaki et al., 2007, Tanaka et al., 2010). It is unknown whether this affects the relative contribution of apoptosis-associated processes.

We recently reported that mice deficient in *puma* (*bbc3*), a potent pro-apoptotic Bcl-2 family protein, were protected against seizure-induced neuronal death in the lower-dose KA model (Engel et al., 2010). In the present study we investigated whether the higher-dose KA model produces a different apoptotic contribution, and the extent to which Puma deficiency may or may not be protective under those conditions.

Experimental Procedures

Mouse model of focal-onset status epilepticus

All animal procedures were performed 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 (Engel et al., 2010). Adult male C57BL/6 mice (20-25 g) (Harlan, UK) and *puma*^{+/+, +/-, -/-} mice on a C57BL/6 background (Villunger et al., 2003) were used. Briefly, 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 craniotomies were performed. Mice were affixed with three recording electrodes (Bilaney Consultants Ltd, Sevenoaks, UK) to record surface electroencephalogram (EEG). Electrodes were placed above

dorsal hippocampus and a third over frontal cortex. A guide cannula was affixed over the dura (coordinates from Bregma: AP = -0.94; L = -2.85 mm) (Paxinos and Franklin, 2001) and the entire skull assembly fixed in place with dental cement. Anesthesia was discontinued, EEG recordings were commenced using a Grass Comet XL lab-based EEG (Medivent Ltd, Lucan, Ireland), and then a 31-gauge internal cannula (Plastic Ones Inc.) was inserted into the lumen of the guide to inject KA (Ocean Produce International, Nova Scotia, Canada) [0.3 µg or 1.0 µg in 0.2 µl of vehicle; phosphate-buffered saline (PBS), pH adjusted to 7.4] into the amygdala. The EEG was recorded until intravenous lorazepam (6 mg/kg) administration at 40 min and then recorded 1 h thereafter. Seizure time was calculated from the EEG as the duration of high amplitude and high frequency discharges (HAHFDs) during the 40 min experimental period, as described (Shinoda et al., 2004a).

Histopathology and immunohistochemistry

Mice were euthanized 72 h after anticonvulsant and brains were flash-frozen whole in 2-methylbutane at -30°C and sectioned in the coronal plane at 12 µm. Sections were defrosted, air-dried and post-fixed in 10 % formalin for 30 min, followed by washes in PBS. For assessment of overall (total) neuronal death, sections were stained using Fluoro-Jade B (FJB) (Millipore, Cork, Ireland), as described (Tanaka et al., 2010). For immunohistochemistry, sections were permeabilized with 0.3 % Triton X-100, blocked in 5 % goat serum for 1 h and then incubated overnight at 4°C with antibodies against NeuN (1:400, Chemicon, Hampshire, UK). Sections were washed again in PBS and incubated in goat anti-mouse AlexaFluor 568 (Bio Sciences Ltd, Dun Laoghaire, Ireland). DNA fragmentation was detected using a Fluorescein-based terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit according to manufacturer's instructions (Promega, Madison, WI) (Engel et al., 2010). Sections were mounted with medium containing 4, 6 diamidino-2-phenylindole (Vector Laboratories Ltd, Peterborough, UK) to visualize nuclei. Imaging was performed using a Nikon 2000s epifluorescence microscope with a Hamamatsu Orca 285 camera (Micro-optica, Dublin, Ireland). Cell counts were performed under 40× or 60× magnification and were the average of two adjacent sections assessed by an observer blinded to experimental treatment. NeuN or TUNEL-stained cells were counted along the entire CA3 subfield from border of CA3a with CA2 through to the hilar region, according to standard demarcations. For assessment of TUNEL nuclear morphology, 90-210 cells were examined per slide on sections from mice from each dose group ($n = 3-4$ each). Assignment of apoptosis-like or non-apoptotic features was according to the presence or absence of rounded or spherical clumps (< 5 per cell) within TUNEL-positive nuclei of cells in the CA3 subfield.

Statistical analysis

Data are presented as means ± S.E.M. Data were analyzed using analysis of variance with *post hoc* Fisher's PLSD test or for two group comparison Student's t-test (StatView software; SAS Institute, Inc., Cary, NC). Significance was accepted at $p < 0.05$.

Results

KA dose-dependency of seizures

We recently reported that seizure duration was related to KA dose during *status epilepticus* induced by intra-amygdala KA in SJL mice (Tanaka et al., 2010). Here, we began by examining the relationship between seizure damage and seizure duration for the 0.3 and 1 µg KA doses in C57BL/6 mice. *Status epilepticus* induced by 0.3 µg KA mainly caused damage to the ipsilateral CA3a subfield of the hippocampus, as assessed by TUNEL staining of cells containing fragmented DNA (Fig. 1A, B). TUNEL-positive cells were also occasionally found in sectors CA3b and CA3c, but rarely in the hilus (Fig. 1A, B). In contrast, TUNEL staining in mice injected with 1 µg KA was typically more extensive in CA3a and extended into CA3b

and CA3c subfields (Fig. 1A). TUNEL-positive hilar neurons were also present in mice given 1 μ g KA (Fig. 1A, B). Counts of TUNEL-positive cells revealed a significant difference between the doses in both CA3 and hilar subfields (Fig. 1B).

To investigate the cause of the additional damage in the higher dose group we examined EEG records. Total seizure time was significantly longer in mice subject to *status epilepticus* induced by 1 μ g KA compared to 0.3 μ g-injected mice (Fig. 2A). Further analysis of the EEG indicated that HAHFDs (i.e. polyspike seizure discharges) lasted longer in the higher dose group (Fig. 2A). There was also a strong trend to earlier seizure onset in 1 μ g KA-injected mice, although this did not reach statistical significance (data not shown). Representative EEG traces from both groups are shown in Fig. 2B.

Majority of degenerating neurons following status epilepticus are positive for DNA fragmentation

To assess the proportion of dying neurons which were positive for DNA fragmentation we stained tissue sections from 0.3 μ g KA-injected mice for FJB to label all irreversibly damaged neurons, and adjacent sections for TUNEL (Note, double-staining of FJB and TUNEL on the same sections was not technically feasible). Hippocampal FJB counts in the CA3 subfield were higher than TUNEL counts in all mice studied. The proportion of FJB-stained degenerating neurons which were TUNEL positive was 83.5 % (range 62.5 – 92 %, $n = 7$ mice).

Reduced apoptosis-like nuclear morphology following more prolonged status epilepticus

To explore whether the balance between apoptosis-like and non-apoptotic or necrotic cell death differed between the seizure models, we examined the morphology of TUNEL-positive nuclei. TUNEL-positive nuclei were considered not to have apoptosis-like morphology if fragmented nuclear material was predominantly evenly dispersed or the TUNEL-positive nucleus had a “bean-shaped” appearance (Fig. 3A). In contrast, apoptosis-like morphology included nuclei displaying one or more large spherical clumps within the TUNEL-positive nucleus (Fig. 3B). Mice subjected to *status epilepticus* induced by 1 μ g KA had a significantly lower proportion of TUNEL-positive nuclei displaying apoptosis-like features compared to 0.3 μ g KA mice (Fig. 3C). However, raw counts of TUNEL-positive CA3 cells with apoptosis-like features was very similar between models (average total of 34 and 31 cells per CA3 subfield for 0.3 μ g vs. 1 μ g, respectively).

Neuroprotection in Puma-deficient mice subjected to 1 μ g KA-induced status epilepticus

Genetic ablation of *bim* or *puma*, members of the pro-apoptotic BH3-only proteins, confers neuroprotection against *status epilepticus* (Engel et al., 2010, Murphy et al., 2010). These studies were performed with the injection of 0.3 μ g KA. Since Puma deficiency provided a stronger neuroprotection than Bim deficiency we reasoned that Puma deficiency might also protect against a more necrotic insult. To explore this, we compared 1 μ g KA-induced seizure damage in wild-type mice to *puma*^{+/-} and *puma*^{-/-} mice.

Seizure durations were not significantly different between the three genotypes (data not shown). Counts of TUNEL-positive cells 72 h after *status epilepticus* within the CA3 subfield of the hippocampus revealed ~50 % less in *puma*^{-/-} mice compared to wild-type and *puma*^{+/-} mice (Fig. 4A, B). To support these data we compared neuronal survival at 72 h between the different genotypes by assessing numbers of NeuN-stained CA3 cells displaying normal neuronal morphology. Counts of surviving CA3 neurons were significantly higher in *puma*^{-/-} mice when compared to wild-type and *puma*^{+/-} mice (Fig. 4C, D). Representative immunofluorescence microscopy images from TUNEL- and NeuN-stained tissue sections are shown in Figures 4B and 4D. Typically, wild-type mice displayed extensive loss of CA3

neurons throughout the subfield. In contrast, Puma-deficient mice had fewer dying cells in the CA3a and CA3b/c regions.

Discussion

In the present study we found that increasing *status epilepticus* duration led to increased seizure damage and a lower percentage of cells displaying apoptosis-like nuclear TUNEL-positive morphology. Puma deficiency nevertheless conferred strong protection against seizure-induced neuronal death *in vivo*. These data provide genetic evidence that Puma influences seizure-induced neuronal death and suggest this likely extends beyond caspase-dependent apoptosis to caspase-independent apoptotic or non-apoptotic pathways.

The present study explored the relationship between seizure duration, hippocampal damage and mode of cell death in a mouse model of *status epilepticus*. This remains an important issue since it has the potential to influence how to protect brain against seizure damage (Meldrum, 2002, Henshall and Murphy, 2008). Here, we attributed the mode of cell death as apoptosis on the basis of nuclear morphology in TUNEL-positive cells. Previously we used the term programmed cell death positive/negative (Shinoda et al., 2004a) but the criteria here were the same. Over 80 % of dying neurons were TUNEL-positive in our study and nuclear morphology consistent with apoptosis-like cell death was evident in a subpopulation of these cells, in agreement with our previous reports (Araki et al., 2002, Shinoda et al., 2004a, Murphy et al., 2007). Occasional cells also display TUNEL-positive material dispersed throughout the soma in this model (Murphy et al., 2007), probably the result of nuclear membrane breakdown during apoptosis (Taylor et al., 2008). Chromatin masses have been reported to be TUNEL-positive during physiological apoptosis in the developing rat brain (Ishimaru et al., 1999) and we found here and previously that rounded nuclear clumps were invariably TUNEL-positive (Araki et al., 2002, Shinoda et al., 2004a, Murphy et al., 2007). This contrasts reports that heavily condensed chromatin in apoptotic cells remains TUNEL-negative (Barth et al., 2002, Fujikawa et al., 2010). Whether this represents a failure of the TUNEL stain to penetrate, different stages of an essentially similar process, or a substantive and important difference in the form of cell death, is not certain. While we did not use electron microscopy in the present study to determine morphology, multiple independent markers of apoptosis are present in the model including caspase cleavage, cytochrome *c* release and modulation by pharmacologic and genetic disruption of specific pathways (Araki et al., 2002, Murphy et al., 2007, Engel et al., 2010, Murphy et al., 2010). The TUNEL-positive cells not showing apoptosis-like nuclear morphology in our study presumably represent DNA fragmentation arising from non-caspase-dependent signaling (e.g. apoptosis-inducing factor, AIF), or non-apoptotic cell death pathways (Dong et al., 1997, Cheung et al., 2005, Kroemer and Martin, 2005, Taylor et al., 2008, Zhao et al., 2010). The remaining ~20 % of TUNEL-negative dead neurons presumably represent necrotic cells in which programmed pathways were minimally activated or absent (Fujikawa, 2005, Fujikawa et al., 2010).

Little doubt remains that activation of ordered cell death signaling cascades make a major contribution to seizure-induced neuronal death (Liou et al., 2003, Engel and Henshall, 2009). The relative contribution of caspase-dependent versus -independent pathways or non-apoptotic pathways is less understood and seems strongly model-, species- and development-stage dependent (Fujikawa, 2005, Engel and Henshall, 2009). The present study explored whether a more severe seizure insult generates a less “apoptotic” profile of cell death. This concept has support from several studies (Ankarcrona et al., 1995, Bonfoco et al., 1995, Du et al., 1996, Portera-Cailliau et al., 1997, Ward et al., 2007) and has previously been examined in the setting of seizure-induced neuronal death where it was affirmed (Kondratyev and Gale, 2004) but also rejected (Fujikawa et al., 2010). Our data show that when *status epilepticus* was more severe there was a reduction in the relative proportion of dying cells displaying apoptosis-like nuclear

TUNEL-positive morphology. Critically, however, absolute numbers of such cells remained about the same. If we assume caspase-dependent signaling is important for apoptosis-like morphology (Kuida et al., 1998, Kroemer and Martin, 2005, Taylor et al., 2008), which is not certain (Joza et al., 2001), then extending seizure severity or duration does not appear to alter this component. Instead, only the non-caspase-dependent apoptotic or non-programmed necrotic component is enhanced. Kondratyev *et al.* reported that caspase-dependent apoptosis signaling increased when *status epilepticus* was slower to develop, but since that study only investigated molecular pathways we cannot be certain of whether the morphology component changed or simply the biochemical signature (Kondratyev and Gale, 2004). Likewise, we cannot directly compare our data to Fujikawa and coworkers since they found neither mild nor severe seizures produced any apoptotic morphology or biochemical signature (Fujikawa et al., 2010). Indeed, little or no TUNEL staining was reported in their study, nor previously using either pilocarpine or systemic kainate models (Fujikawa et al., 2000b, Fujikawa et al., 2007). This differs from findings by many other groups including ours, where TUNEL staining is widespread (Faherty et al., 1999, Shinoda et al., 2004a, Heo et al., 2006, Mikati et al., 2008). However, the conclusions drawn by Fujikawa *et al.* are apt given their data and it remains difficult to reconcile that substantive differences would exist in the mechanism of seizure-induced neuronal death between models. Debate over which animal model best represents the pathology of human temporal lobe epilepsy continues (Sloviter, 2008). Notably, altered Bcl-2 family proteins, caspase processing and DNA fragmentation are all evident in resected material from patients with temporal lobe epilepsy (Shinoda et al., 2004b, Schindler et al., 2006, Yamamoto et al., 2006, Narkilahti et al., 2007, Yang et al., 2008). A human case of extensive apoptosis as a result of *status epilepticus* has also been documented (Cherian et al., 2009).

Puma is a potent activator of the mitochondrial cell death pathway via neutralization of anti-apoptotic Bcl-2 family proteins and possibly direct Bax-activating effects, leading to mitochondrial outer membrane permeabilization, caspase activation and apoptosis (Villunger et al., 2003, Cartron et al., 2004, Willis et al., 2007). However, caspase-independent apoptosis such as mediated by AIF is also regulated by Bcl-2 and certain BH3-only proteins (Susin et al., 1999, Culmsee et al., 2005, Liou et al., 2005, Landshamer et al., 2008). It is possible that Puma can influence AIF-induced cell death (Wang et al., 2007). Moreover, Bcl-2 has also been shown to protect against necrotic cell death as mitochondrial permeabilization also leads to mitochondrial dysfunction (Zhong et al., 1993, Kane et al., 1995, Single et al., 2001). We previously reported that seizure-induced neuronal death was reduced by over 70 % in Puma-deficient mice (Engel et al., 2010). The present study demonstrated that seizure-induced neuronal death was again strongly inhibited in Puma-deficient mice despite a reduced overall apoptosis component in this model. Protection was however less than when compared to our previous study (Engel et al., 2010). Although the influence of Puma was reduced, our data provide evidence that Puma influences both caspase-dependent and caspase-independent apoptosis and perhaps has effects on non-apoptotic pathways or necrosis, e.g. by protecting mitochondrial integrity (Kroemer and Martin, 2005). Last, it is noteworthy that in human epileptic brain tissue, cells displaying active caspase-3 are negative for nuclear AIF and *vice versa* (Schindler et al., 2006), implying autonomous apoptosis-related signaling pathways can be activated in neighboring cells subject to the same insult.

While the mechanism by which Puma mediates its effects in the present study is unknown, Puma induction after seizures is chiefly p53-dependent (Engel et al., 2010). Beyond the therapeutic potential for neuroprotection in *status epilepticus*, the wider value of targeting Puma in neurologic disorders is uncertain. Despite induction following cerebral ischemia, Puma appears to be dispensable for neuronal death in *in vivo* mouse models of focal cerebral ischemia (Kuroki et al., 2009). Puma is also not required for neuronal death following intra-hippocampal *N*-methyl-D-aspartate injection (Concannon et al., 2007).

One potential caveat in the present study is that sampling at a single time point may miss key features in the high dose group which develop earlier and disappear, rather than are absent. This is unlikely however, and we have not seen obvious temporal shifts in cell death signaling pathway activation between doses in the model. Of additional note, our studies here establish that seizure duration and seizure damage in C57BL/6 mice are broadly related to KA dose. This supports earlier work in SJL mice that highlighted this relationship in the same model (Tanaka et al., 2010). The higher KA dose appears to induce an earlier onset of electrographic seizures and somewhat longer electrographic discharges (present data and (Tanaka et al., 2010)). While varying seizure time by anticonvulsant administration is common among models and effective in the present model (Henshall et al., 2000, Shinoda et al., 2004a), this KA dose-dependency is a useful property and provides versatility for studying gene or drug manipulations which may increase as well as decrease cell death.

In conclusion, we find that absolute apoptosis-like morphology varies little in relation to *status epilepticus* duration but rather its relative contribution is reduced. We find that absence of pro-apoptotic Puma provides robust protection against seizure-induced neuronal death even where the contribution of apoptosis is effectively lower, implying effects on caspase-independent and non-apoptotic cell death.

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Abbreviations

Bcl-2	B cell lymphoma
BH3	Bcl-2 homology domain
CA	cornu ammonis
EEG	electroencephalogram
FJB	Fluoro-Jade B
KA	kainic acid

NeuN	neuron-specific nuclear protein
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Fig. 1. Influence of KA dose on seizure-damage

(A) Representative photomicrographs showing TUNEL-stained mouse hippocampus 72 h following *status epilepticus* induced by intra-amygdala KA at different doses. Note enhanced cell death in the 1 µg group in each region. Scale bar for CA3a, 60 µm; hilus, 120 µm. (B) Graphs showing TUNEL counts at 72 h for CA3 and the hilus for each group ($n = 3-5$ per group). ** $p < 0.05$ compared to 0.3 µg group.

Fig. 2. Influence of KA dose on seizure time

(A) Semi-quantitative EEG analysis of HAHFDs (high amplitude high frequency discharges) in mice given either 0.3 µg or 1 µg KA demonstrating increased overall seizure time and increased duration of individual HAHFDs at the higher dose ($n = 3-4$ per group). * $p < 0.05$ compared to 0.3 µg group. (B) Representative EEG traces following injection of KA. Note tendency to earlier seizure onset and earlier development of protracted HAHFDs.

Fig. 3. Influence of KA dose on proportion of TUNEL-positive cells displaying apoptosis-like nuclear morphology

(A) Representative photomicrographs (60× lens) of individual TUNEL-positive nuclei displaying non-apoptotic or necrotic features. Bar in A, 7 µm; B, 6 µm. (B) Representative photomicrographs of individual TUNEL-positive nuclei displaying apoptosis-like morphology. (C) Graph showing percentage of TUNEL-positive cells displaying apoptosis-like nuclear features for each KA dose. * $p < 0.05$ compared to 0.3 µg group.

Fig. 4. Neuroprotection in *puma*^{-/-} mice after status epilepticus induced by intra-amygdala microinjection of 1 μ g KA

(A) Graph showing TUNEL counts in ipsilateral CA3 for each genotype 72 h after *status epilepticus* induced by 1 μ g intra-amygdala KA. Note reduced TUNEL in *puma*^{-/-} mice compared to *puma*^{+/-} and wild-type (+/+) mice. (B) Representative photomicrographs showing TUNEL (green) in ipsilateral CA3 subfields from wild-type and *puma*^{-/-} mice at 72 h. Scale bar = 60 μ m. (C) Graph showing NeuN counts in the ipsilateral CA3 for each genotype 72 h after *status epilepticus* induced by 1 μ g intra-amygdala KA. Note higher counts in *puma*^{-/-} mice compared to *puma*^{+/-} and wild-type mice. (D) Representative photomicrographs showing

NeuN (red) immunostaining in ipsilateral CA3 from wild-type and *puma*^{-/-} mice at 72 h. **p* < 0.05 compared to wild-type and *puma*^{+/-} mice (*n* = 4–7 per group).