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Susceptibility to seizure-induced injury and acquired microencephaly following intraventricular injection of saporin-conjugated 192 IgG in developing rat brain

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Abstract

To study the role of neurotrophin-responsive neurons in brain growth and developmental resistance to seizure-induced injury, we infused saporin-conjugated 192-IgG (192 IgG-saporin), a monoclonal antibody directed at the P75 neurotrophin receptors ($p75^{NTR}$), into the ventricles of postnatal day 8 (P8) rat pups. 7–10 days after immunotoxin treatment, loss of $p75^{NTR}$ immunoreactivity was associated with depletion of basal forebrain cholinergic projection to the neocortex and hippocampus. Kainic acid (KA)-induced seizures on P15 resulted in hippocampal neuronal injury in the majority of toxin-treated animals (13/16), but only rarely in saline-injected controls (2/25) ($P < 0.001$). In addition, widespread cerebral atrophy and a significant decrease in brain weight with preserved body weight were observed. Volumetric analysis of the hippocampal hilar region revealed a 2-fold reduction in perikaryal size and a 1.7-fold increase in cell packing density after 192 IgG-saporin injection. These observations indicate that neurotrophin-responsive neurons including basal forebrain magnocellular cholinergic neurons may be critical for normal brain growth and play a protective role in preventing excitotoxic neuronal injury during development.

Keywords: $p75^{NTR}$; Neurotrophins; Microcephaly; Epilepsy; Neonatal; Kainate

Introduction

Complex partial seizures of temporal lobe origin are the most common focal seizures and mesial temporal lobe epilepsy (MTLE) is the most common medically intractable epilepsy syndrome (Engel, 1996). Kainic acid (KA)-induced seizures have been studied as a model of MTLE for over two decades, as limbic seizures produced by systemic injections of KA result in a distinctive pattern of neurodegeneration in the hippocampus that resembles human hippocampal sclerosis (Ben-Ari, 1985; Nadler et al., 1978; Schwob et al., 1980). Moreover, KA-induced status epilepticus is followed, after an apparent latent period, by the development of chronic, recurrent, spontaneous seizures in adult rats (Ben-Ari and Cossart, 2000; Hellier et al., 1998; Zhang et al., 2002). In immature rats prior to postnatal age 21 (P21), however, KA injection causes neither cell death nor chronic spontaneous seizures (Albala et al., 1984; Stafstrom et al., 1992). The mechanism of resistance of immature animals to seizure-induced injury is not well understood, but may in part be due to the abundance of neurotrophic growth factors (Liu et al., 1997; Tandon et al., 1999).

Neurotrophin activity is mediated by both high and low affinity receptors. Whereas high-affinity Trk receptors mediate trophic responses, the role of the low-affinity neurotrophic factor receptor ($p75^{NTR}$) appears to depend on the cellular context. $p75^{NTR}$ can promote survival by interacting with TrkA (Holtzman et al., 1992; Kaplan and Miller, 2000). Alternatively, in some systems, $p75^{NTR}$

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appears to promote death through an interaction with the ceramide signal transduction pathway (Casaccia-Bonnefil et al., 1996) or in conjunction with the co-receptor sortilin (Nykjaer et al., 2004). $P75^{NTR}$ also may inhibit nerve growth by binding to Nogo receptor (Kaplan and Miller, 2003; McKerracher and Winton, 2002). Although $p75^{NTR}$ is present in both fore- and hind-brain, and is widely expressed during early postnatal development (Fusco et al., 1991; Koh and Higgins, 1991; Yan and Johnson, 1988), much attention has focused on its role in basal forebrain magnocellular neurons where $p75^{NTR+}$ neurons are exclusively cholinergic. Developing cholinergic neurons of the basal forebrain express TrkA and TrkB receptors as well as $p75^{NTR}$ (Anderson et al., 1995; Holtzman et al., 1992; Koh and Higgins, 1991; Koliatsos et al., 1994; Li et al., 1995), and respond to NGF and BDNF by increasing ChAT activity both in vivo (Gnahn et al., 1983; Mobley et al., 1986; Morse et al., 1993) and in vitro (Alderson et al., 1990; Friedman et al., 1995; Hefti et al., 1985). More importantly, NGF and BDNF expressions appear to be regulated by cholinergic activity (Boatell et al., 1992; Ferencz et al., 1997; Lindefors et al., 1992; Rossner et al., 1997).

During the first two postnatal weeks in developing rodents, basal forebrain cholinergic neurons undergo marked hypertrophy and extensive neurite outgrowth (Koh and Loy, 1989; Sofroniew et al., 1987). These phenomena correlate with the peak of NGF mRNA and protein expression (Auburger et al., 1987) and a period of explosive synaptogenesis in their target areas (Fiala et al., 1998). The first 2 weeks of life also represent a period of relative resistance to seizure-induced neuronal injury, although animals at this developmental stage manifest increased seizure susceptibility (Nitecka et al., 1984). Resistance to seizure-induced injury in the immature brain remains unexplained. To study the role of trophic factor-responsive neurons in seizure-induced injury in immature rats, we treated P8 rat pups with the potent highly selective immunotoxin, 192 IgG-saporin, which kills $p75^{NTR}$ neurons, and then examined the anatomic effects of seizures induced by KA on P15, an age at which KA-induced seizures typically cause no detectable neuronal injury or death.

Materials and methods

Experimental design

All experiments were conducted under a protocol approved by the institutional sub-committee on research animal care and conformed to NIH guidelines regarding the use of experimental animals. 13 litters of Sprague–Dawley rats (Charles River Laboratories, Cambridge, MA) (both male and female pups, 5–10 animals in each litter) were used as summarized (Table 1). Because these studies were conducted prior to sexual maturity, animals were not selected by sex. No sexual dimorphism in response to immunolesions as assayed by ChAT activity following neonatal intraventricular 192 IgG has been reported although Ricceri et al. have noted sexual dimorphism in layer-specific loss of cortical thickness (Ricceri et al., 2002). To establish the optimum dosage and volume to achieve consistent cholinergic denervation, 1–4 μg of 192 IgG-saporin in 1–5 μl of PBS was injected into 16 animals and the brains were processed for acetylcholinesterase (AChE) histochemistry on P15. Two micrograms of 192 IgG-saporin given in 1 μl of PBS was well tolerated and consistently caused essentially complete depletion of AChE-positive basal forebrain neurons by P15 ($n = 8$), whereas lower doses produced inconsistent results. We therefore used this dose for all subsequent experiments. For KA experiments, on P8, animals received intraventricular injection of 192 IgG-saporin (Chemicon, Temecula, CA) in phosphate-buffered saline (PBS) ($n = 20$) and their littermates received PBS alone (saline, $n = 30$). Seven control animals received no surgery. Because all animals received intraventricular saporin or saline on P8, while still nursing, and because we were concerned that 192 IgG-saporin treatment could adversely affect competition for maternal attention/nursing, we housed each cohort of 192 IgG-saporin-treated animals together as a group with one dam and each cohort of saline-treated animals as a group with another dam. These animals were drawn from 10 different litters. On P15, pups from 192 IgG-saporin ($n = 16$) or saline ($n = 25$) groups were treated with KA (5 mg/kg in PBS, pH 7.4) or an equal volume of PBS ($n = 9$) by intraperitoneal injection. Animals were sacrificed on P18 by transcardiac perfusion and brains were processed for AChE histochemistry, $p75^{NTR}$ immunocytochemistry, Nissl stain, and in situ nick translation histochemistry to detect DNA fragmentation, a robust positive marker of neuronal injury (Weiss et al., 1996).

Table 1
Summary of experimental groups

Group	P 8 (*n*)	P 15 (*n*)	P 18 (*n*)
saporin-KA	192 IgG-saporin (16)	KA (16)	perfused (16)
saporin-PBS	192 IgG-saporin (4)	PBS (4)	perfused (4)
saline-KA	PBS (25)	KA (25)	perfused (25)
saline-PBS	PBS (5)	PBS (5)	perfused (5)
Dose response	192 IgG-saporin (16)	Sacrificed for AChE histochemistry (8)	
No surgery	No treatment (7)	KA (3) PBS (4)	perfused (7)

Immunotoxin injection

P8 pups (weight 17–20 g) were anesthetized with methoxyflurane for 3 min (min) for incision and creation of a burr hole in the skull. Pups were placed on a preformed mold made with dental cement and immobilized with tapes. Using a 5-μl Hamilton syringe, 1 μl of 192 IgG-saporin (2 μg/μl) or PBS was injected over 1 min into the left lateral

ventricle (AP −1.5 mm, ML +2 mm, and DV −2.5 mm relative to bregma). The syringe was left in place for an additional 60–90 s to allow diffusion of the solution. Pups were kept in an incubator warmed to 36 ± 1°C until full recovery from the surgery (within 1 h), grouped with similarly treated animals, and returned to designated dams.

Behavioral observation and seizure grading

Following KA injection on P15, animals were closely observed and their behavior recorded for 3 h. A seizure severity grade was assigned based on the maximal response achieved on a scale from 0 to IV as follows: 0, no response; I, single limb clonus and scratching; II, multiple limb clonus and staggering gait; III, limb clonus, tonic extension of limbs and falling; IV, continuous grade III seizures for longer than 30 min (status epilepticus).

Detection and quantification of DNA fragmentation by in situ nick translation histochemistry

Animals were sacrificed by pentobarbital overdose (100 mg/kg ip) and transcardiac perfusion with 10 ml of PBS followed by 20 ml of ice-cold 4% paraformaldehyde in 100 mM PBS, pH 7.4. Brains were removed, post-fixed for 24 h, and cryoprotected in 20% sucrose overnight. Each brain was mounted and coronal or horizontal 50-μm-thick sections were cut. Serial coronal sections spanning dorsal hippocampus and serial horizontal sections through the anterior commissure were collected in 6-well plates. Six horizontal sections or 6 coronal sections (every 6th sections, 300 μm apart) from each brain were selected and processed for in situ end labeling (ISEL) nick translation as previously described (Koh et al., 1999). Adjacent sections were processed for AChE histochemistry, $p75^{NTR}$ immunocytochemistry, and Nissl stain. For quantification of DNA fragmentation, three sections (6 hippocampal sections per brain) from 16 192 IgG-saporin- and 25 saline-treated animals were examined. Images were captured digitally at 10× magnification, converted to gray scale, and areas of ISEL-positive cells were highlighted at a threshold set at constant level (120) for all specimens using an image analysis system, MetaMorph (v. 6.1, Universal Imaging Corp.). An average percent of area above the threshold was calculated per animal and comparison was made between all 192 IgG-sapoin-KA and saline-KA animals.

Acetylcholinesterase histochemistry and $p75^{NTR}$ Immunocytochemistry

To assess cholinergic denervation, AChE histochemistry was performed as previously described (Hedreen et al., 1985) 7 days (dose response study) and 10 days (KA study) after 192 IgG-saporin treatment. We also used a mouse monoclonal antibody against $p75^{NTR}$ (192 IgG, Oncogene Science) as another specific and sensitive marker of central cholinergic neurons, and to further assess the effect of 192 IgG-saporin treatment on non-cholinergic $p75^{NTR}$-bearing cells and fibers on day 10. Horizontal sections from saline- and 192 IgG-saporin-treated animals were processed to compare $p75^{NTR}$ immunoreactivity in the forebrain, thalamus, diencephalon, cerebellum, and brain stem. Six sections from each brain adjacent to those used for in situ nick translation were processed free-floating using the ABC-3,3′-diamino-benzidine method with nickel (II) sulfate intensification, as previously described (Koh and Loy, 1989). Briefly, sections were incubated in 0.1% Triton X-100 in PBS, blocked with normal horse serum, and then incubated with primary antibody 192 IgG (2 μg/ml overnight) followed by biotinylated secondary anti-mouse antibody (rat adsorbed, Vector Laboratories, Burlingame, CA). Endogenous peroxidases were blocked with 0.3% H_2O_2 prior to incubation with avidin-biotin-peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA).

Volumetric analysis of cell size and cell packing density

Eighteen 50-μm-thick Nissl-stained horizontal sections from the level of anterior commissure containing ventral hippocampus, amygdala, and entorhinal cortex, and 300 μm ventral and dorsal to that section, were selected from 3 192 IgG-saporin and 3 saline-injected animals (a subset from saporin-KA and saline-KA, Table 1) and the cell size of a total of 1710 hilar neurons from 36 hippocampal sections was measured. The initial section for each animal was selected randomly, while the sixth section above and below was selected by protocol for analysis to provide an unbiased sample. Neurons within the hippocampal hilar region were chosen for the volumetric analysis because the area is easily defined and neurons are well dispersed (Fig. 5). We used the nucleolus in the sections to determine the plane of focus and for cell counts. Only cells with a distinct nucleolus and generous cytoplasmic staining characteristic of neurons were included in the analysis. Quantitative analysis of neuronal size and cell packing density (number of cells per unit volume) was made bilaterally in 15 equally sized boxes of 12,000 μm^3 within the hilus, spanning 180,000 μm^3 per each brain. An American Optical no. 146 ocular reticule was used at a final magnification of 1000×, and the analysis was done using a three-dimensional cell counting system (Debassio et al., 1996; Williams and Rakic, 1988).

Statistics

The *z* test with Yates correction (SigmaStat v. 3.0 SPSS Inc.) was used to compare differences in proportion of animals that sustained neuronal injury after KA-induced seizures at P15. Quantification of DNA fragmentation was compared using Student's *t* test. The non-parametric Mann–Whitney test was used to compare packing density and neuron size. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison was performed to com-

pare body weights and brain weights among experimental groups (GraphPad Prism v. 4.0, GraphPad Software Inc.). Values are expressed as mean ± standard error of means (SEM). Significance was defined as $P < 0.05$ for all tests.

Results

Immunotoxin-induced cholinergic depletion

Within 10 days after a unilateral intraventricular injection of 2 μg of 192 IgG-saporin, we found nearly complete loss of $p75^{NTR}$-bearing basal forebrain cholinergic neurons and their afferent projections to hippocampus and neocortex (Fig. 1). Medial septum and vertical and horizontal limbs of the diagonal band of Broca were completely devoid of 192-IgG-positive neurons. Only a few scattered immunoreactive neurons remained (2–6 cells per section) within the most ventral part of nucleus basalis magnocellularis. AChE staining confirmed the loss of basal forebrain cholinergic cell bodies and processes, and showed the relative sparing of striatal cholinergic interneurons (Fig. 1B, insert). Indeed, basal forebrain cholinergic neurons appear to be specifically targeted by 192 IgG-saporin. 7–10 days after toxin treatment, no detectable difference in $p75^{NTR}$ immunoreactivity was found between saline- and 192 IgG-saporin-injected animals in the glomerular layer of olfactory bulb, subependymal cells of the lateral ventricle, cerebellar Purkinje cells, principal and spinal trigeminal nuclei and tract, nucleus tractus solitarius, ventral coclear nucleus, prepositus hypoglossal nucleus, area postrema, mesencephalic trigeminal nucleus, blood vessels, and meninges.

Kainate seizure-induced neuronal injury

KA on P15 resulted in seizures in all animals injected. There was no significant difference in seizure severity among the control (no surgery, $n = 3$, Grade III), PBS (Grade II, 31%; Grade III; 51%; Grade IV, 18%) or 192 IgG-saporin (Grade II, 23%; Grade III, 62%; Grade IV, 15%) injected animals. We examined seizure-induced neuronal injury within the hippocampus using in situ detection of DNA fragmentation. In 13/16 (81%) animals pretreated with 192 IgG-saporin that caused near complete cholinergic denervation, we found significant injury most consistently in CA3 pyramidal cells, and to a lesser extent in CA4 and CA1 pyramidal subfields (Fig. 2). By contrast, in 2/25 (8%) animals injected with intraventricular saline, we found hippocampal neurons manifesting DNA fragmentation (z test, $P < 0.001$), consistent with numerous reports that immature brain is relatively resistant to KA seizure-induced injury (Albala et al., 1984; Koh et al., 1999; Nitecka et al., 1984; Stafstrom et al., 1993). Quantification of DNA fragmentation on six hippocampal sections from each brain using the MetaMorph image analysis system confirmed that significant seizure-induced neuronal injury occurs following 192 IgG-saporin treatment (unpaired Student's t test, $P < 0.0001$) (Fig. 3). Lateral septal nuclei (5/16) and reticular thalamic nuclei (8/16) also showed DNA fragmentation after seizure in 192 IgG-saporin-treated brains, while amygdala, entorhinal cortex, and ventromedial thalamus, areas vulnerable to seizure-induced injury in adults (Weiss et al., 1996), show no evidence of injury.

Hippocampal injury spanned the septo-temporal axis of hippocampus and was symmetric in 7/13 animals. When

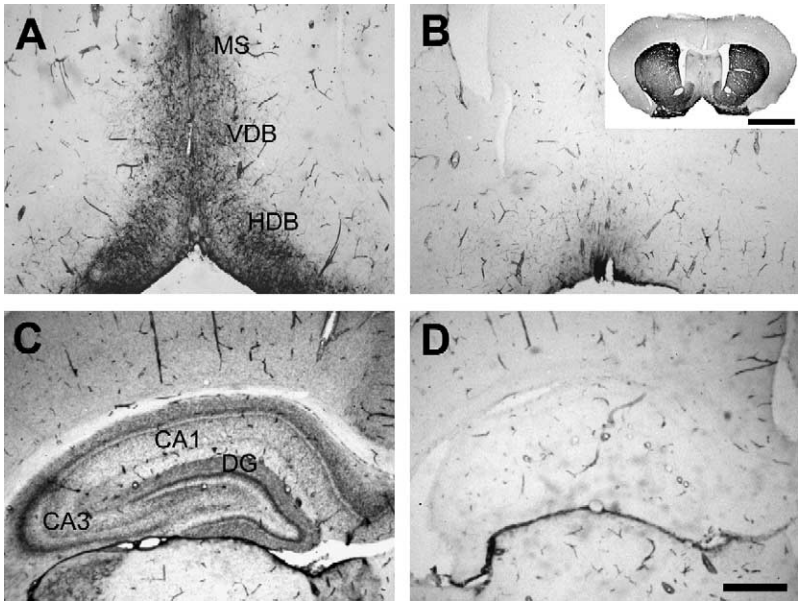

Fig. 1. Loss of $p75^{NTR}$-immunoreactive basal forebrain neurons and their afferent projections following intraventricular injection of 192 IgG-saporin. (A and C) P18 saline-injected control. DG: dentate gyrus. (B and D) Animal treated with 192 IgG-saporin. The medial septal nucleus (MS), vertical (VDB), and horizontal diagonal band of Broca (HDB) (A) and hippocampus (C) are completely devoid of staining. Scale bar = 100 μm. Insert in B: AChE histochemistry showing intact striatal cholinergic neurons. Scale bar = 1 cm.

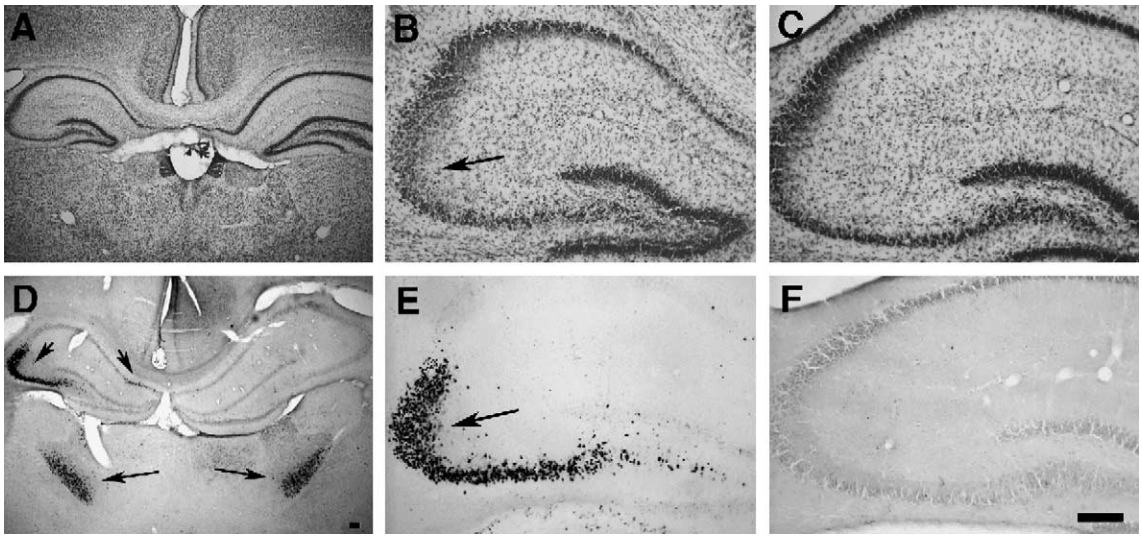

Fig. 2. KA seizure-induced neuronal injury after cholinergic denervation in immature brain. A–C, Nissl stain; D–F, in situ nick translation histochemistry. (A and D) Low magnification coronal section through the dorsal hippocampus of 192 IgG-saporin-treated animal 3 days after KA seizures. Note the DNA fragmentation within the hippocampus (short arrows) on the side that is smaller and in the reticular thalamic nuclei bilaterally (long arrows). Scale bar = 100 μm. (B and E) High magnification coronal section through dorsal hippocampus of animal treated with 192 IgG-saporin on P8 and KA on P15, and sacrificed on P18. Neuronal injury is seen in the CA3 (arrow). (C and F) High magnification coronal section through the dorsal hippocampus of saline-KA animal treated with saline on P8 and KA on P15. No DNA fragmentation occurs after KA. Scale bar (B–F) = 200 μm.

injury was asymmetric, it was worse on the side that demonstrated maximal atrophy (Fig. 2A). Even when hippocampal injury was asymmetric, thalamic and septal injury was bilateral (Fig. 2D). In four animals treated with 192 IgG-saporin that did not experience seizures (192 IgG-saporin on P8, and PBS on P15, see Table 1), no injury–except along the needle tract at the dorsal most hippocampus in one–was apparent.

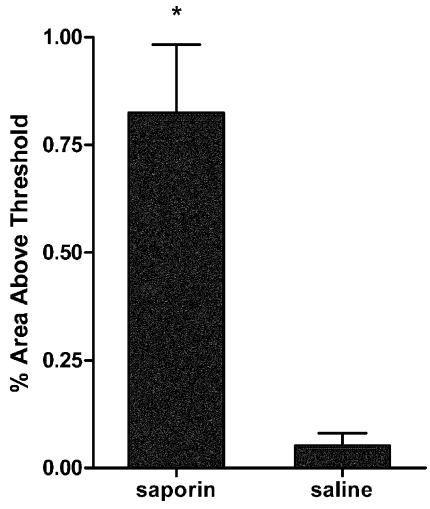

Fig. 3. Quantification of hippocampal DNA fragmentation. An average percent area above threshold (threshold area containing stained cells/total hippocampal area captured at 10× magnification) was calculated using MetaMorph image analysis system from 16, 192 IgG-saporin and 25 saline brains 3 days after KA injection at P15. There is a significant increase in seizure-induced DNA fragmentation following 192 IgG-saporin treatment (*$P < 0.0001$ Student's t test).

Effect of intraventricular 192 IgG-saporin on developing brain

Early in the course of these studies, we were surprised to note that on gross inspection brains from 192 IgG-saporin-treated animals were smaller than brains from either PBS treated or no surgery controls at the time of sacrifice 10 days after the injection (Fig. 4). We therefore measured brain and body weights on all subsequent experimental animals (a subset of the entire set of experimental animals). While body weights did not differ significantly between groups (no surgery: 44.9 g ± 2.0 (n = 7); saline: 40.3 g ± 2.5 (n = 10); 192 IgG-saporin: 38.6 g ± 2.7 (n = 7), P = 0.26, one-way ANOVA), brain weights of 192 IgG-saporin-treated animals were significantly lower than controls (P = 0.0061, one-way ANOVA with Bonferroni's adjustment for multiple comparisons). Pairwise analysis demonstrated significant differences in brain weight between 192 IgG-saporin (0.87 g ± 0.03) and no surgery (0.99 g ± 0.02, $P < 0.01$), and between 192 IgG-saporin and saline (0.97 ± 0.02, $P < 0.05$) (Fig. 4B). KA treatment on P15 had no effect on the body or the brain weight on P18 (body weight: KA 40.4 g ± 1.3 (n = 11) vs. saline 42.4 g ± 2.5 (n = 13), P = 0.25; brain weight: KA 0.97 g ± 0.02 vs. saline 0.93 ± 0.03, P = 0.14). In all 192 IgG-saporin-treated animals (with or without KA, $n > 20$), cortical neurons appeared more densely packed, suggesting reduced neuronal size or loss of neuropil. Similarly, hippocampal neurons also appeared small and more compact. To further characterize these observations, we prepared Nissl-stained horizontal sections for volumetric analysis from a subset of animals treated after we initially recognized a difference in brain size between

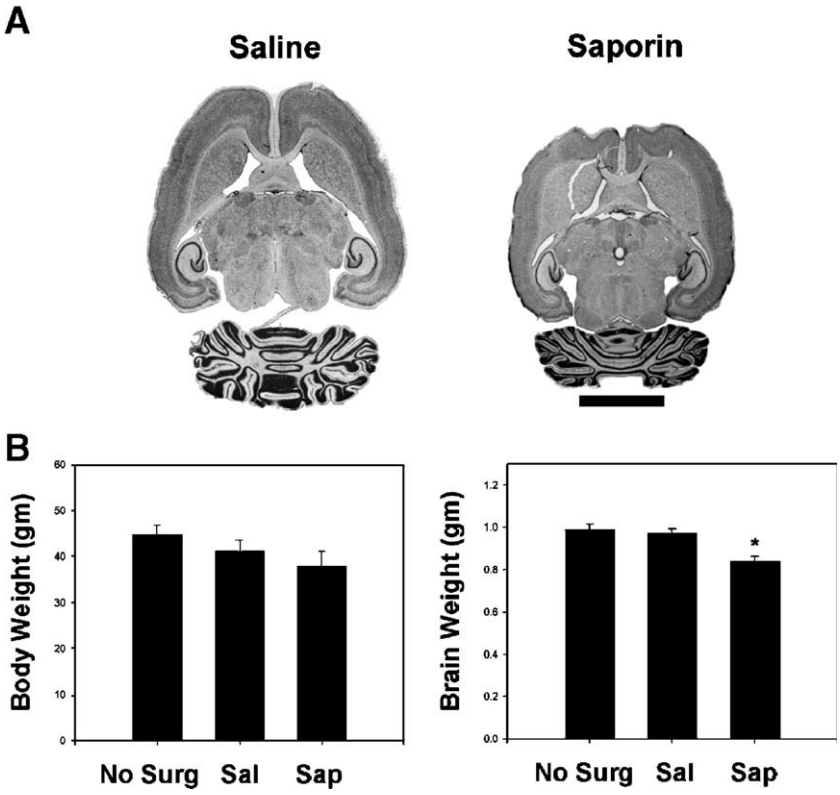

Fig. 4. Microencephaly and decrease in brain weight 10 days after 192 IgG-saporin injections. (A) Low magnification view of Nissl-stained horizontal sections of saline- and 192 IgG-saporin-injected animals. Note the absence of ventriculomegaly or other gross brain abnormality in a 192 IgG-saporin-treated animal, and yet marked brain shrinkage relative to saline control. Scale bar = 1 cm. (B) Reduction in brain weight without a significant loss of body weight after cholinergic denervation (*P = 0.0061, one-way ANOVA). No surg: no surgery control; Sal: saline injected group; Sap: 192 IgG-saporin injected group.

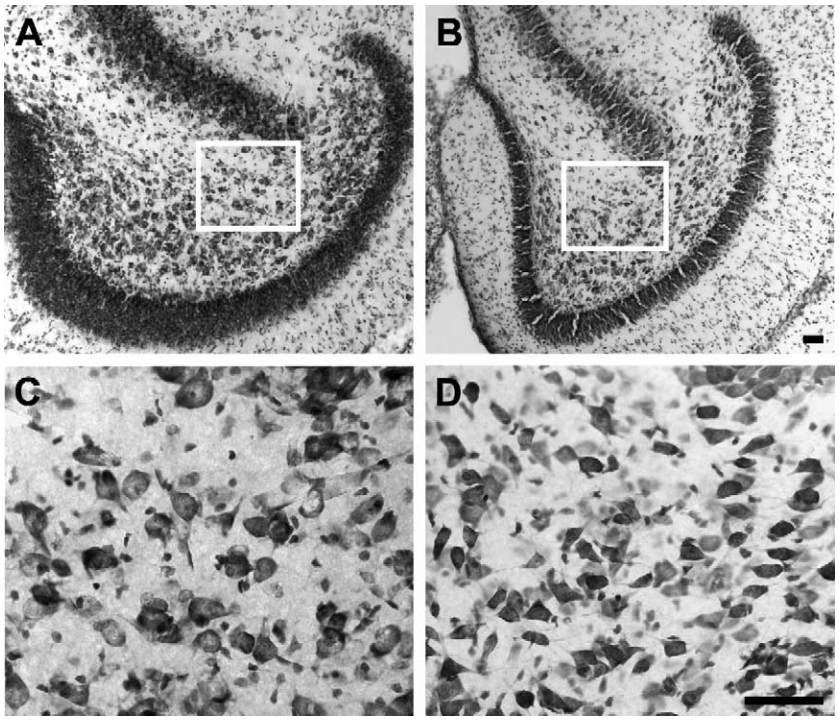

Fig. 5. Decrease in cell size and increase in cell packing density following intraventricular 192 IgG-saporin injection. Nissl-stained sections of a saline (A and C) and a 192 IgG-saporin animal (B and D). C and D are higher magnification views of hilar neurons within the boxed area. Scale bars = 50 μm.

groups. Morphometric measurements of cell size of 1710 hilar neurons and cell packing density spanning 540,000 μm^3 from saline and 192 IgG-saporin brains demonstrated a 2-fold reduction in cell size (saline median 199.7) vs. 192 IgG-saporin (median 95.8) ($n = 3$, $P < 0.05$, Mann–Whitney test) with an associated 1.7-fold increase in cell packing density (neuronal nucleoli/0.1 mm^3) in 192 IgG-saporin-treated animals (saline median 118 vs. 192 IgG-saporin median 186, $n = 3$, $P < 0.05$, Mann–Whitney test) (Fig. 5).

Discussion

The main finding of our study is that 7 days after intraventricular injection of 192 IgG-saporin immature rats become vulnerable to KA seizure-induced neuronal injury. Injury occurs in limbic structures, especially the hippocampus, septum, and lateral thalamus in a pattern that is strikingly similar to that seen after seizures in mature rats. There was no difference in maximal seizure severity or morbidity between the treatment groups. Seizures after KA typically last 1–3 h, and we noted no systematic difference in seizure duration between groups, although we cannot rule out the possibility that saporin animals spent more time at higher levels of seizure severity during the post-kainate period. Our finding supports the hypothesis that robust cholinergic input to hippocampus is neuroprotective in the immature brain. We hypothesize that high ambient levels of neurotrophic factors present in immature brain support protective cholinergic input to hippocampus and limbic structures.

Whether cholinergic innervation plays a direct neurotrophic role (Slotkin, 2004), or is neuroprotective indirectly via upregulation of neurotrophic factors such as BDNF and NGF (Boatell et al., 1992; Ferencz et al., 1997; Lindefors et al., 1992; Rossner et al., 1997), or some other mechanism remains to be elucidated. Prenatal choline supplementation, presumably acting to increased release of acetylcholine in offspring (Cermak et al., 1998), has been shown to protect against postnatal neurotoxicity induced by NMDA receptor antagonist (MK801) (Guo-Ross et al., 2002) and prevent seizure-induced memory deficit (Holmes et al., 2002; Yang et al., 2000). Furthermore, in adult rats, intact cholinergic innervation may be neuroprotective against seizure-induced injury and retard epileptogenesis, as 192 IgG-saporin-induced cholinergic deafferentation caused increased seizure-induced death of somatostatin-immunoreactive hippocampal hilar neurons (Jolkkonen et al., 1997), markedly facilitated the development of hippocampal kindling (Ferencz et al., 1998; Kokaia et al., 1996), and increased susceptibility to flurothyl or pentylenetetrazole-induced seizures (Silveira et al., 2000). Similarly, neonatal rats treated with 192 IgG-saporin on P7 to lesion basal forebrain cholinergic neurons showed an increased susceptibility to flurothy-induced seizures on P35 (Silveira et al., 2002). If developmental hyper-innervation of hippocampus is neuroprotective in the immature brain, enhancement of cholinergic neurotransmission may be a novel therapeutic strategy to ameliorate or prevent seizure-induced injury in mature brain (Ferencz et al., 1998; Holmes et al., 2002).

Hippocampal injury was sometimes asymmetric with maximal burden of damage noted on the side of maximal hippocampal atrophy. Interestingly, the anatomic consequences of early life seizures in humans are often asymmetric, even when the seizures are generalized. We were unable to determine whether the side of maximal atrophy and injury was always the side of intracerebral injection in our experiments. Nonetheless, we have considered the possibility of a direct local toxic effect of intraventricular 192 IgG-saporin. Bilateral hippocampal injury in the majority of animals and absence of injury in animals that received 192 IgG-saporin but did not have seizures, however, argue against this interpretation. Another possibility is that in some animals the 192 IgG-saporin injection was intraparenchymal rather than intraventricular. However, bilateral depletion of $p75^{NTR}$ and AChE staining was observed in all 192 IgG-saporin-treated animals, making this possibility seem unlikely. It is possible that a toxic effect of 192 IgG-saporin on hippocampus is potentiated by seizure activity or seizure-associated metabolic changes such as hyperperfusion or relative hypoxemia, and that such an effect is completely independent of 192 IgG-saporin's effect on $p75^{NTR}$ neurons. Still, the absence of 192 IgG-saporin-induced DNA fragmentation in hippocampal neurons in animals that did not experience seizures is most suggestive of the hypothesis that neuronal injury is due to seizures following $p75^{NTR}$ neuronal loss rather than 192 IgG-saporin treatment alone. Further experiments using other stressors such as ischemia or trauma may help address this issue.

A second previously unreported and unexpected finding is that lesions of $p75^{NTR}$-expressing neurons result in hippocampal neuronal atrophy and apparent microencephaly. Robertson and colleagues found that 192 IgG-saporin injection into the lateral ventricles at P0 and P2 produced long-term depletion of cholinergic neurons while sparing basal forebrain GABAergic neurons and caused a 10% reduction in cortical thickness, decreased dendritic branching, and reduced spine density of neocortical pyramidal neurons (Robertson et al., 1998). Increased density of cortical neurons and a significant reduction in neocortical thickness have also been reported on P7 in rats treated with intraventricular 192 IgG-saporin at P1 and P3 (Ricceri et al., 2002). Using a 10-fold higher dose of 192 IgG-saporin, we observed more marked and diffuse effects on brain growth with preserved somatic growth and weight gain. Previous studies of neonatal 192 IgG-saporin injections at P1, P4, or P7, however, have failed to detect any sign of gross brain atrophy. As in the present study, there was no ventricular enlargement or grossly altered organization or cytoarchitecture (Leanza et al., 1996; Ricceri et al., 1997, 1999). Those

studies used a relatively low dose (0.2–0.4 μg) of 192 IgG-saporin which may have resulted in only partial cholinergic denervation (Waite et al., 1995). It is therefore possible that anatomical and functional compensation by surviving residual cholinergic neurons masked later-life consequences of neonatal cholinergic denervation (Leanza et al., 1996). Alternatively, it is possible that the reduced brain size reported here is a result of a delay in brain development and that at later time points (i.e., adulthood) the size of the brain would be normal. Depletion of the cholinergic system but not of monoaminergic systems results in permanent alterations in cortical morphology, alterations in dendritic branching pattern and spine morphology in sensorimotor cortex, and changes in cortical connectivity of layers IV and V (Bachman et al., 1994; Hohmann and Berger-Sweeney, 1998; Hohmann et al., 1988, 1991). Neonatal 192 IgG-saporin with resultant essentially complete depletion of basal forebrain cholinergic neurons in our study was associated with significant loss of brain weight and concomitantly reduced neuronal size and increased packing density in the hippocampus. At present, we cannot determine whether this finding represents a failure of normal development or an acquired reduction in neuronal size and increase in density from a previously attained level. Nonetheless, together, these results suggest that the cholinergic basal forebrain system may be essential for normal postnatal development of the cortex and hippocampus.

During the early postnatal period, several cell populations and fibers in the cortex, hippocampus, thalamus, brainstem, and cerebellum transiently express $p75^{NTR}$ (Fusco et al., 1991; Koh and Higgins, 1991; Koh and Loy, 1989; Yan and Johnson, 1988). We do not know if 192 IgG-saporin infused into the lateral ventricle of P8 rat pups kills these non-cholinergic $p75^{NTR}$ expressing cells; however, in spite of the dose used, we found no difference in $p75^{NTR}$ immunoreactivity in areas other than basal forebrain in 192 IgG-saporin-treated animals compared to saline controls at the time of sacrifice at P18. In view of the death-promoting role of P75, it is interesting to note that subplate neurons that are destined to disappear during later development transiently express $p75^{NTR}$ and, therefore, even if they were targets of intraventricular 192 IgG-saporin, their loss may produce no developmental consequences. It is therefore possible that apparent widespread atrophy simply represents the loss of non-cholinergic $P75^{NTR}$vcells and their associated projections, but our observation raises the intriguing possibility that acetylcholine, a prototypical neurotransmitter, may have a neurotrophin-like action (Munoz et al., 1999; Slotkin, 2004) during early postnatal development, the lack of which causes a decrease in brain mass and failure of brain growth or shrinkage of brain. Interestingly, two prototypical neurotrophins, BNDF and NGF, have recently been shown to have neurotransmitter-like actions. Application of BDNF to granule cell bodies, for example, evoked a rapid depolarization and action potential firing (Kafitz et al., 1999) associated with a transient increase in calcium concentration (Kovalchuk et al., 2002). Similarly, NGF rapidly and robustly enhanced constitutive acetylcholine release from basal forebrain cultures and this increase was activity dependent and protein synthesis independent (Auld et al., 2001; Nonner et al., 2000).

It is possible that enhanced seizure-induced neuronal injury in immature animals after 192 IgG-saporin pretreatment is secondary to the pervasive developmental effects on brain architecture that we have observed, and not due to a specific defect in cholinergic input created by the lesion. For example, the increased packing density we observed may result in steeper pH or metabolite gradients in regions of excessive neuronal activity, or perhaps increased extracellular glutamate levels due to a smaller volume of distribution (McBain et al., 1990). Additional experiments using microdialysis approaches in combination with dose-response studies of the direct injection of exogenous toxins into 192 IgG-saporin-pretreated brains may clarify this issue.

Disrupted cholinergic basal forebrain innervation has been documented in human developmental disorders including Down's syndrome (McGeer et al., 1985), Rett syndrome (Johnston et al., 1995; Wenk and Hauss-Wegrzyniak, 1999), and fetal alcohol syndrome (Balduini et al., 1994; Costa and Guizzetti, 1999). While acquired microcephaly may be a general response of developing brain to any overwhelming or widespread insults during the early postnatal period as seen in congenital HIV infection (Belman et al., 1988) or perinatal hypoxic–ischemic encephalopathy (Cordes et al., 1994), it is striking that in those developmental conditions associated with cholinergic dysfunction, one of the salient shared features of the disorders is microcephaly (Bauman et al., 1995; Coyle et al., 1986; Wisniewski et al., 1983). These clinical observations together with our findings of decreased brain weight and hippocampal neuronal size as well as loss of developmental resistance to seizure-induced neuronal injury after early postnatal cholinergic denervation suggest that acetylcholine may act as a trophic factor in developing brain.

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