Hippocampal Neuropathology of Domoic Acid–Induced Epilepsy in California Sea Lions (*Zalophus californianus*)

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ABSTRACT

California sea lions (*Zalophus californianus*) are abundant human-sized carnivores with large gyrencephalic brains. They develop epilepsy after experiencing status epilepticus when naturally exposed to domoic acid. We tested whether sea lions previously exposed to DA (chronic DA sea lions) display hippocampal neuropathology similar to that of human patients with temporal lobe epilepsy. Hippocampi were obtained from control and chronic DA sea lions. Stereology was used to estimate numbers of Nissl-stained neurons per hippocampus in the granule cell layer, hilus, and pyramidal cell layer of CA3, CA2, and CA1 subfields. Adjacent sections were processed for somatostatin immunoreactivity or Timm-stained, and the extent of mossy fiber sprouting was measured stereologically. Chronic DA sea lions displayed hippocampal neuron loss in patterns and extents similar but not identical to those reported previously for human patients with temporal lobe epilepsy. Similar to human patients, hippocampal sclerosis in sea lions was unilateral in 79% of cases, mossy fiber sprouting was a common neuropathological abnormality, and somatostatin-immunoreactive axons were exuberant in the dentate gyrus despite loss of immunopositive hilar neurons. Thus, hippocampal neuropathology of chronic DA sea lions is similar to that of human patients with temporal lobe epilepsy. J. Comp. Neurol. 522:1691–1706, 2014.

INDEXING TERMS: hippocampal sclerosis; mossy fiber sprouting; somatostatin; stereology; dentate gyrus

Temporal lobe epilepsy is common in adult humans (Engel et al., 1997). Typically, patients have a history of a precipitating brain insult (French et al., 1993). Anticonvulsant drugs fail to control seizures in many cases (Kwan et al., 2011). With the exception of surgical resection of temporal lobe tissue, current therapies only suppress seizures and do not cure the epileptic condition. Ideally, temporal lobe epilepsy would be prevented by administering treatments following a brain insult. No such anti-epileptogenic treatment exists currently. A variety of rodent models of temporal lobe epilepsy are available to investigate mechanisms of temporal lobe epilepsy and develop new treatments (Buckmaster, 2004), but differences in the brains of rodents versus humans may contribute to the poor success rate of translating treatments for neurological disorders to human patients (Kola and Landis, 2004). There is a paucity of large animal models of temporal lobe epilepsy. In dogs, for example, spontaneous epilepsy is common (Potschka et al., 2013), but temporal lobe epilepsy is rare (Buckmaster et al., 2002b; Kuwabara et al., 2010). Non-human primate models of temporal lobe epilepsy have been developed (Ribak et al., 1998; Gunderson et al., 1999) or attempted (Perez-Mendes, 2005), but they involve ethical questions and practical limitations. California sea lions (*Zalophus californianus*) offer a possible alternative.

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Sea lions are abundant along the west coast of the United States. They are large carnivores with gyrencephalic brains approximately one-fourth the size of human brains (Bininda-Emonds, 2000; Montie et al., 2010). Sea lions develop epilepsy after consuming domoic acid (DA), the amnesic shellfish poisoning toxin identified in a Montreal area incident when over 100 humans became ill after eating tainted mussels (Perl et al., 1990). Natural DA exposure also affects other species including birds (Work et al., 1993), otters (Kreuder et al., 2003), dolphins (Torres de la Riva et al., 2009), and whales (Doucette et al., 2012). During seasonal blooms, DA-producing *Pseudo-nitzschia* algae are consumed and concentrated by grazing planktivorous fish, such as anchovies, which then are eaten by sea lions that absorb the toxin through the gut, develop neurological signs including status epilepticus, and become stranded on beaches where they may be observed and reported to The Marine Mammal Center in Sausalito, California, which rescues stranded animals for rehabilitation and eventual release (Scholin et al., 2000). Some sea lions that survive initial DA toxicosis appear to recover following treatment, but many develop spontaneous recurrent seizures, fail to thrive upon release, and restrand in poor condition requiring euthanasia (Goldstein et al., 2008).

It seems likely that DA-exposed sea lions develop temporal lobe epilepsy. DA is a potent ligand of kainate-type glutamate receptors (Debonnel et al., 1989a,b; Stewart et al., 1990; Tasker et al., 1991) and to a lesser degree AMPA receptors (Larm et al., 1997). DA generates unusually long-lasting (non-desensitizing) channel activation (Zhang et al., 2008) and strongly excites hippocampal neurons (Zaczek and Coyle, 1982; Sari and Kerr, 2011), some of which express high levels of kainate receptors (Künig et al., 1995). At high doses, DA was found to cause hippocampal damage in laboratory animals (Sutherland et al., 1990; Tryphonas and Iverson, 1990; Strain and Tasker, 1991). Human patients who died during the Montreal DA incident (Teitelbaum et al., 1990) and a survivor who later developed temporal lobe epilepsy displayed hippocampal neuron loss (Cendes et al., 1995). Sea lions that died after DA exposure displayed histological evidence of excitotoxicity in the hippocampus (Silvagni et al., 2005). Magnetic resonance imaging (MRI) reveals hippocampal atrophy in sea lions that recover from DA toxicosis (Goldstein et al., 2008). However, hippocampal neuron loss in DA-exposed sea lions has not been quantified and compared with data from human patients. Furthermore, tissue from DA-exposed sea lions has not been evaluated for epilepsy-related synaptic reorganization. The present study addressed whether sea lions naturally exposed to DA display patterns and extents of hippocampal neuron loss and synaptic reorganization similar to that reported previously for human patients with temporal lobe epilepsy.

MATERIALS AND METHODS

Subjects were sea lions (*Zalophus californianus*) that stranded along the central California coast in 2010 and were admitted to The Marine Mammal Center in Sausalito, California for rehabilitation but did not respond to treatment and were euthanized due to poor clinical prognosis. Stranded sea lions were collected under a Letter of Authorization from the National Marine Fisheries Service to The Marine Mammal Center. Sex and age determination was based on published criteria (Greig et al., 2005): pup (0–1 years), yearling (1–2 years), juvenile male (2–4 years), subadult male (4–8 years), juvenile or subadult female (2–5 years), adult male (8+ years), and adult female (5+ years). Control subjects (44% female) were pups ($n = 3$), yearlings ($n = 1$), juveniles ($n = 1$), subadults ($n = 2$), or adults ($n = 2$) that were euthanized because of pneumonia, malnutrition, cancer, or severe shark bite wounds. Subjects known or suspected of previous exposure to DA were identified by previously described criteria (Goldstein et al., 2008; Thomas et al., 2010), which included intermittent seizures (at least 2 weeks apart and/or at least 2 weeks following admission to The Marine Mammal Center), unusual behaviors, stranding individually (not in clusters during blooms of *Pseudo-nitzschia* algae, like acute DA-exposed animals), and/or hippocampal atrophy evident by MRI.

These "chronic DA" animals were yearling ($n = 1$), juvenile ($n = 2$), and subadult ($n = 3$), but mostly adults ($n = 7$) and mostly females (71%). DA toxicosis is common in adult females, probably because their normal migration patterns increase the likelihood of exposure (Gulland et al., 2002; Goldstein et al., 2008). Five chronic DA sea lions were admitted in status epilepticus, three of which had DA in their feces or urine. Detection of DA in bodily fluids is not always possible because plasma half-time is short (Truelove and Iverson, 1994). Four of the five sea lions that were admitted in status epilepticus were observed to have later spontaneous seizures. Another sea lion was included in the chronic DA group because of intermittent seizures. Six others were included because of hippocampal atrophy, two of which also were observed to have spontaneous seizures. Two sea lions were not initially suspected of being chronic DA animals, were not tested for DA exposure or hippocampal atrophy, and were euthanized within 9 days of admission, but were added to the chronic DA group because they displayed

hippocampal sclerosis histologically. The minimum duration between possible or known DA exposure and the first observed spontaneous convulsive seizure was 26 ± 7 days (mean $\pm$ SEM). The minimum duration between possible or known DA exposure and euthanasia was 52 ± 14 days.

Immediately after euthanasia by barbiturate overdose, hippocampi were isolated from brains and placed in 0.37% sodium sulfide for 30 minutes before transfer to 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C for 2 days. After equilibration in 30% sucrose in 0.1 M PB, hippocampi were frozen and sectioned (40 μm) from the septal pole to the temporal pole. Beginning at a random point near the septal pole, a 1-in-72 series of sections was mounted on slides and Nissl-stained with 0.25% thionin (10–14 sections/hippocampus). Adjacent series of sections were processed for somatostatin immunoreactivity and Timm staining.

An investigator blinded to subject group counted Nissl-stained neurons in the granule cell layer, hilus, and pyramidal cell layer of CA3, CA2, and CA1. The hilus was defined by its border with the granule cell layer and straight lines drawn from the tips of the granule cell layer to the proximal end of the CA3 pyramidal cell layer (Fig. 1A). The border between the CA3 and CA2 pyramidal cell layer was determined by the distal end of granule cell axons, labeled black in the stratum lucidum of CA3 in adjacent Timm-stained sections. The transition from CA2 to CA1 was identified by dispersion of the pyramidal cell layer. The border between CA1 and the subiculum was identified by the point at which superficial CA1 pyramidal cells ceased being contiguous. Isolated hippocampi did not include the subiculum in its entirety, so subicular neurons were not counted. Numbers of neurons per hippocampus were estimated by using the optical fractionator method (West et al., 1991). Dissector height was total section thickness. For other sampling parameters, see the summary in Table 2, which shows mean coefficients of error much smaller than coefficients of variation, indicating sufficient sampling within hippocampi.

Granule cell layer thickness was measured in control sea lions and other mammals. Nissl-stained sections (30–40 μm thick) of hippocampi from previous studies were available from macaque monkeys (Austin and Buckmaster, 2004), squirrel monkeys (Lyons et al., 2010), dogs (Buckmaster et al., 2002b), rats (Thind et al., 2010), and mice (Buckmaster and Lew, 2011). From control animals of each species a hippocampal section from the mid-septotemporal level was selected, and the height of the granule cell layer was measured at a representative straight length of the internal part of the granule cell layer.

Sections were processed for somatostatin immunocytochemistry by using an established protocol (Buckmaster et al., 2002b). Briefly, sections were incubated in polyclonal rabbit antiserum to somatostatin for 40 hours at 4°C (Table 1). The somatostatin antiserum had been tested with a radioimmunoassay by the manufacturer and was reported to cross-react with somatostatin-14, −28, −25, and [Des-Ala^{1}]-somatostatin, but not [D-Trp^{8}]-somatostatin, prosomatostatin-32, somatostatin analog RC-160, somatostatin analog (CTOP-NH_2), substance P, neuropeptide Y (NPY; porcine), vasoactive intestinal protein (VIP), insulin (human), or glucagon (human). The antiserum stains the appropriate pattern of cellular morphology and distribution in rats (Buckmaster and Dudek, 1997), dogs (Buckmaster et al., 2002a), monkeys (Austin and Buckmaster, 2004), and wild-type mice but not somatostatin knockout mice (Buckmaster et al., 2002a). Between extensive rinses, sections were exposed to a biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase. Immunopositive structures were visualized by diaminobenzidine reaction product. Sections from control and chronic DA sea lions were processed together in the same solutions.

Timm staining was developed in slide-mounted sections for 75 minutes in 120 ml 50% gum arabic, 20 ml 2 M citrate buffer, 60 ml 0.5 M hydroquinone, and 1 ml 19% silver nitrate. After rinsing, Timm-stained sections were exposed to 5% sodium thiosulfate for 4 minutes before dehydration and coverslipping with DPX. Mossy fiber sprouting was evaluated by an investigator blinded to subject group using an established protocol (Buckmaster and Lew, 2011). Briefly, NIH ImageJ was used to measure the percentage of the area of the granule cell layer plus the molecular layer that displayed black Timm staining in a 1-in-72 series of sections. The granule cell layer plus the molecular layer was outlined, and area measurements were used to calculate the percentage that was stained black. Timm-positive areas were selected by adjusting with a darkness threshold tool.

Only brightness and contrast were adjusted in digital images.

RESULTS

The hippocampus of control sea lions (Fig. 1A) contained over 6 million neurons (Table 2). Sea lions appeared to have a relatively small proportion of neurons in the granule cell layer compared with other mammals. To test what might be contributing to this difference, the height of the granule cell layer was measured in control sea lions and from the comparable

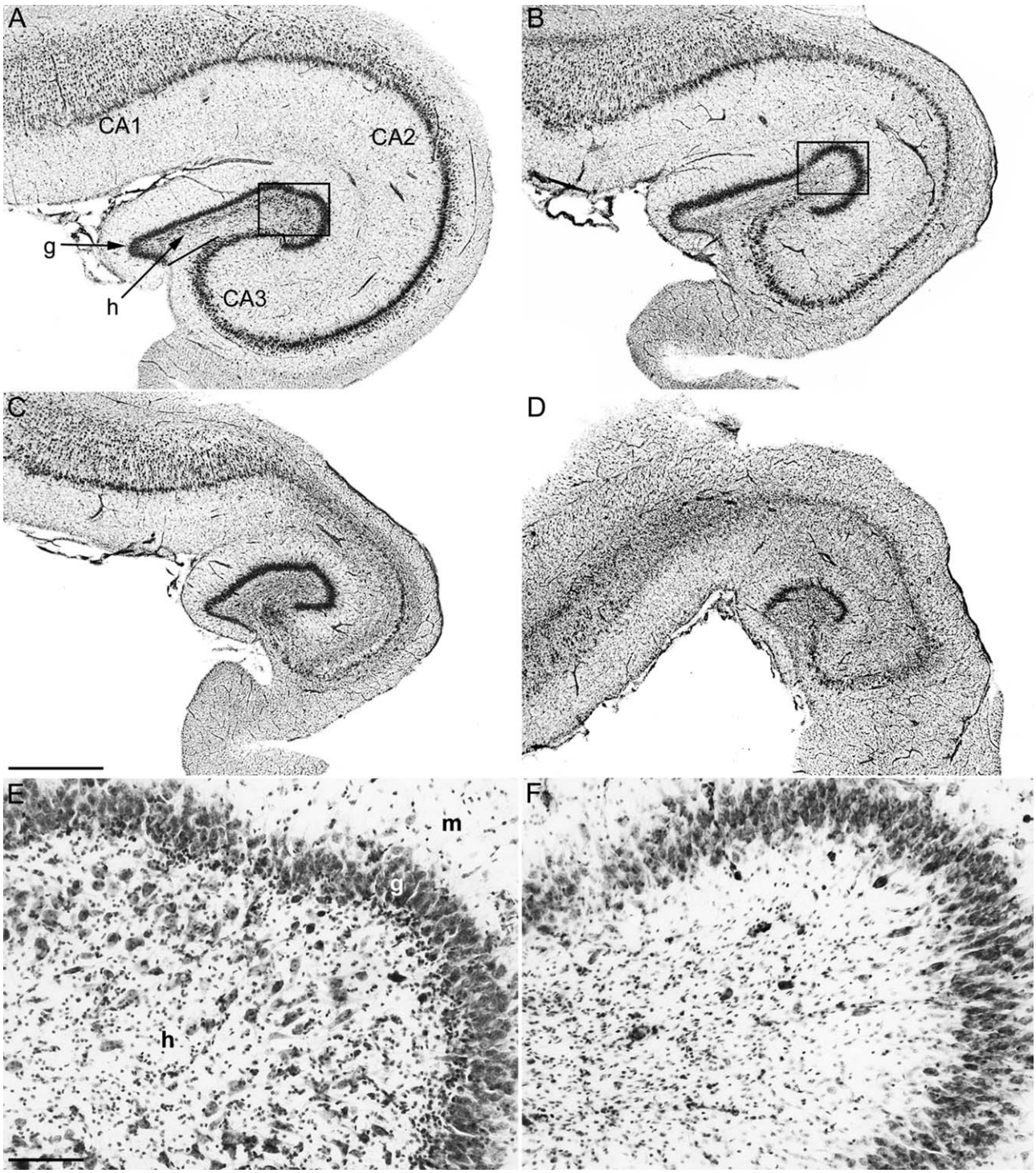

Figure 1. Nissl-stained hippocampi from a control (A) and chronic domoic acid (DA) sea lions (B–D). All sections from similar septotemporal levels. **A:** Lines indicate border between the hilus (h) and CA3 field. g, granule cell. **B–D:** Increasing levels of neuron loss in three chronic DA sea lions. All were admitted in status epilepticus with DA toxicity and were euthanized 22–53 days later. **E,F:** Higher magnification views of hilar regions indicated by rectangles in A and B, respectively. Scale bar = 1 mm in C (applies to A–D); 100 μm in E (applies to E,F).

part of hippocampi in other mammals, including macaque monkeys, squirrel monkeys, dogs, rats, and mice (Fig. 2). The average height of the granule cell layer in control sea lions (56 ± 2 μm [mean ± SEM]) was less than that of all other species, and only 69–71% of macaques (79 ± 4 μm, $P = 0.002$, analysis of variance

[ANOVA] with the Holm–Sidak method), squirrel monkeys (80 ± 6 μm, $P = 0.019$), and dogs (81 ± 7 μm, $P = 0.009$). Average height of the granule cell layer in mice (57 ± 4 μm) was significantly less than that of macaques and dogs ($P < 0.02$). Average height of the granule cell layer in rats (73 ± 4 μm) was not significantly different from that of any other species. In addition to a thinner layer, granule cells of control sea lions appeared less densely packed. These findings suggest that sea lions have proportionally fewer granule cells than other mammals in part because the granule cell layer is thinner.

All chronic DA sea lions displayed significant hippocampal neuron loss, but to varying degrees (Fig. 1B–D). To compare neuron loss across hippocampal subfields, averages were calculated from "affected" hippocampi, which had neuron numbers in any subfield at least 2 standard deviations below average control values. When this criterion was used, significant neuron loss was evident in the left hippocampus of four chronic DA sea lions, the right hippocampus of seven, and bilaterally in three. Although right-sided neuron loss was more frequent, the difference was not significant ($P > 0.05$, χ^2 test). Compared with controls, average neuron loss in affected hippocampi of chronic DA sea lions was substantial in all hippocampal subfields (Table 2), most severe in the hilus (20% of controls, $P < 0.05$, ANOVA on ranks with Dunn's method), least severe in the granule cell layer (54%, not significantly different) and CA1 pyramidal cell layer (55%, $P < 0.05$), and intermediate in the CA2 (35%, $P < 0.05$) and CA3 pyramidal cell layers (32%, $P < 0.05$) (Fig. 3A).

Neuron loss in chronic DA sea lions was most variable in the granule cell layer. The coefficient of variation for granule cell layer neuron counts in affected hippocampi from chronic DA sea lions was 0.798. Some affected hippocampi in chronic DA sea lions displayed severe granule cell loss with values only approximately 20% of controls, whereas others displayed no granule cell loss. In other subregions of the hippocampus, neuron loss was more consistent. When granule cell loss occurred, it appeared as thinning of the layer and sometimes gaps. Bilayers of granule cells, as reported in some cases of human temporal lobe epilepsy (Houser, 1990; Thom et al., 2002; Blümcke et al., 2009), were not observed in chronic DA sea lions.

All of the male chronic DA sea lions were young (one yearling, two juveniles, and one subadult), whereas females were mostly older (two subadults and eight adults). In the granule cell layer, hilus, and CA3 field, neuron loss was less severe in the younger males compared with the older females (Table 3). Affected hippocampi in male chronic DA sea lions had an almost normal average number of granule cells (95% of controls), whereas females had only 39% of controls (male vs. female, $P = 0.02$, t test). The average number of

TABLE 1.

Primary Antibody Used in This Study

Antigen	Immunogen	Manufacturer	Dilution
Somatostatin-14	Synthetic somatostatin-14	Peninsula Laboratories (Belmont, CA), rabbit polyclonal, #IHC 8001	1:4,000

TABLE 2.

Parameters and Results of Stereological Analysis of Hippocampal Neuron Numbers in Control and Chronic DA Sea Lions Known or Suspected of Previous Exposure to Domoic Acid

	Granule cell layer	Hilus	CA3	CA2	CA1
Counting grid	200 × 200 μm	200 × 200 μm	400 × 400 μm	200 × 200 μm	500 × 500 μm
Counting frame	20 × 20 μm	50 × 50 μm	50 × 50 μm	50 × 50 μm	50 × 50 μm
Cells counted/hippocampus (mean ± SEM)	260 ± 16	207 ± 16	231 ± 15	227 ± 15	283 ± 14
Coefficient of variation	0.44	0.56	0.48	0.36	0.49
Mean coefficient of error[1]	0.06	0.10	0.08	0.10	0.06
Neurons/hippocampus of control sea lions ($n = 9$ animals, 18 hippocampi)(mean ± SEM)	2,120,000 ± 350,000	333,000 ± 14,000	1,350,000 ± 170,000	332,000 ± 98,000	2,310,000 ± 300,000
Neurons/hippocampus of chronic DA sea lions ($n = 14$ animals, 17 affected hippocampi)(mean ± SEM)	1,150,000 ± 250,000[2]	68,100 ± 9,400[3]	433,000 ± 51,000[3]	117,000 ± 14,000[3]	1,260,000 ± 120,000[3]
Neurons/hippocampus of chronic DA sea lions ($n = 11$ animals, 11 unaffected hippocampi)(mean ± SEM)	2,470,000 ± 140,000	349,000 ± 12,000	1,570,000 ± 60,000	386,000 ± 27,000	2,670,000 ± 110,000

[1]Calculated according to West et al. (1991).
[2]Only different from unaffected hippocampi, $P < 0.05$, ANOVA on ranks with Dunn's method.
[3]Significantly different from controls and unaffected hippocampi in chronic DA sea lions.

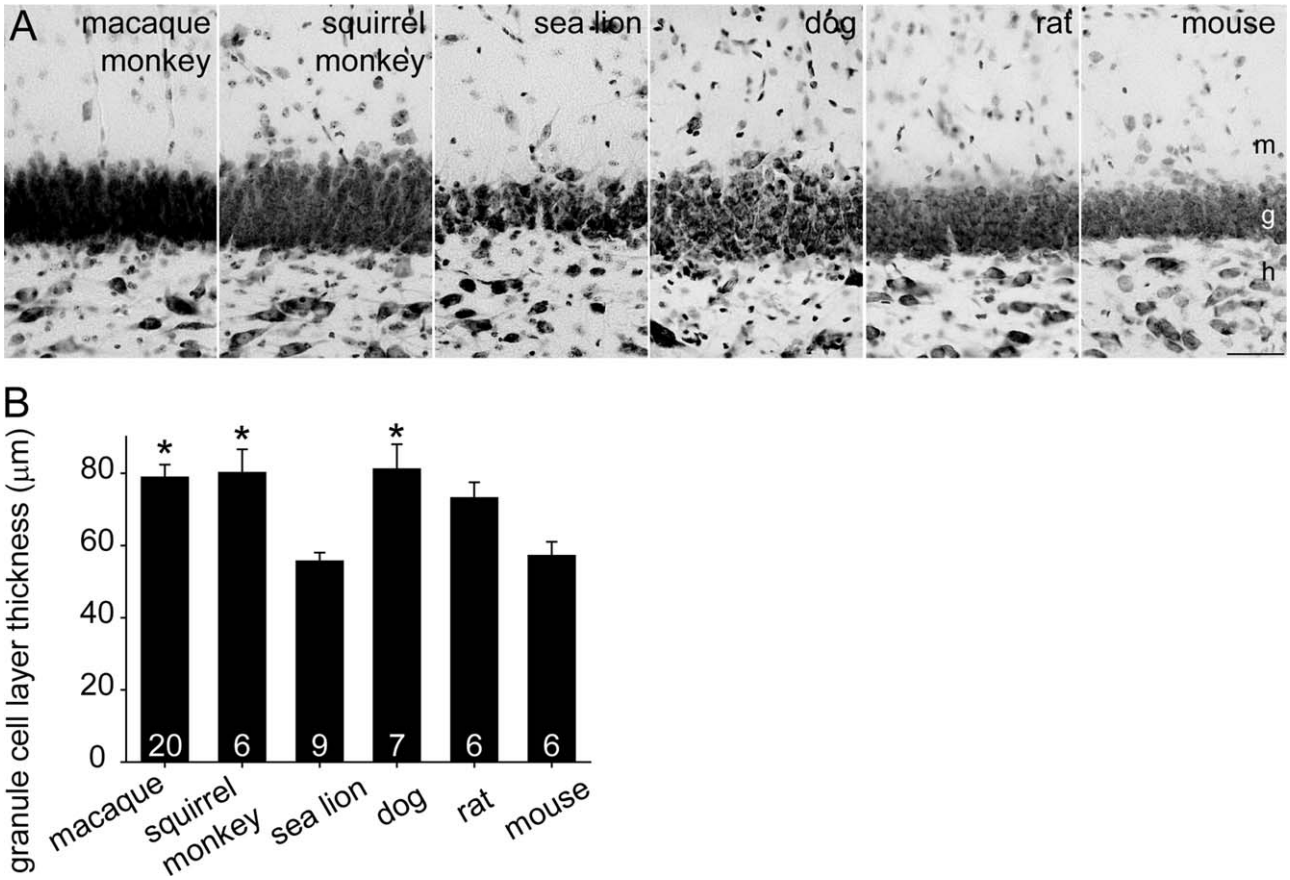

Figure 2. **A**: Dentate gyrus granule cell layer thickness in different mammals. All images and measurements obtained from a straight region of the internal part of the granule cell layer (g) at the mid-septotemporal level of the hippocampus. h, hilus; m, molecular layer. **B:** Granule cell layer thickness values (mean ± SEM). Number of animals indicated at base of bars. *Average granule cell layer thickness was greater in macaques, squirrel monkeys, and dogs compared with sea lions ($P < 0.02$, ANOVA with Holm–Sidak method). Scale bar = 50 μm in A.

hilar neurons was 31% of controls in males, whereas it was only 16% in females ($P = 0.01$). The average number of neurons in CA3 was 44% of controls in males, whereas it was only 27% in females ($P = 0.04$).

In control sea lions, granule cells, hilar neurons, and CA2 pyramidal cells were distributed relatively evenly in sections along the septotemporal axis (Fig. 4A). CA1 pyramidal cells were least numerous at the septal pole and most numerous at the extreme end of the temporal pole. CA3 pyramidal cells were increasingly more numerous in the temporal third of the hippocampus. Neuron loss in affected hippocampi of chronic DA sea lions occurred relatively evenly along the septotemporal axis in all regions (Fig. 4C–F) except in the granule cell layer (Fig. 4B). In the granule cell layer of chronic DA sea lions, neuron numbers were 81% of controls in the septal half of the hippocampus but only 48% of controls in the temporal third.

To test whether the extent and pattern of hippocampal neuron loss in chronic DA sea lions was similar to that in human patients with temporal lobe epilepsy, results were compiled from previously published studies of human tissue that had been obtained after temporal lobectomy (Babb et al., 1989; Kim et al., 1990; Sass et al., 1990; Lee et al., 1995; Mathern et al., 1995, 1996, 1997; Bahh et al., 1999; de Lanerolle et al., 2003; Blümcke et al., 2007) or autopsy (Thom et al., 2005). Substantial neuron loss was evident in all hippocampal subfields of patients with temporal lobe epilepsy and chronic DA sea lions compared with controls (Fig. 3B). In sea lions neuron loss was more severe in the hilus, CA3, and CA2 subfields compared with humans. In humans neuron loss was more severe in CA1. Sea lions and humans displayed similar levels of granule cell loss.

Neuron loss was unilateral in 11/14 chronic DA sea lions (Fig. 5A,B). Similar to human patients with temporal lobe epilepsy (Thom et al., 2005), in chronic DA sea lions, neurons were spared in hippocampi contralateral to affected hippocampi (excluding bilateral cases).

Average numbers of neurons in each subfield of contralateral hippocampi of chronic DA sea lions were 105–117% of control values (Table 2) and not significantly different from controls but significantly higher than affected hippocampi ($P < 0.05$ ANOVA on ranks with Dunn's method).

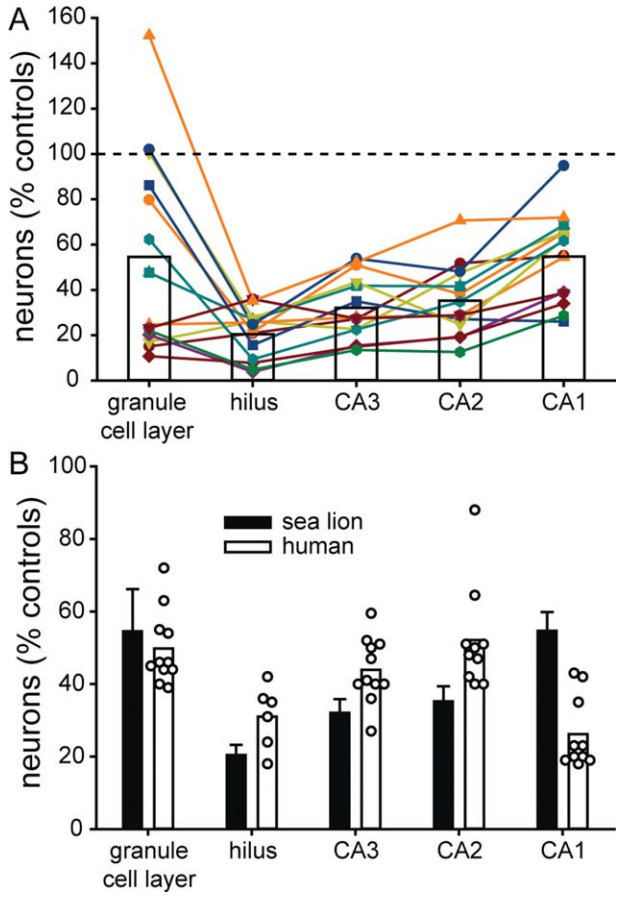

Figure 3. Neuron loss in hippocampal subregions. A: Neurons per affected hippocampus in chronic domoic acid (DA) sea lions ($n = 14$ animals, 17 hippocampi) normalized to average control values ($n =$ nine animals, 18 hippocampi). Each line-scatter plot is from an individual chronic DA sea lion. Bars indicate averages. **B:** Neurons per affected hippocampus for chronic DA sea lions (mean ± SEM) and neuron densities reported in previously published studies for patients with temporal lobe epilepsy (Babb et al., 1989; Kim et al., 1990; Sass et al., 1990; Lee et al., 1995; Mathern et al., 1995, 1996, 1997; Bahh et al., 1999; de Lanerolle et al., 2003; Thom et al., 2005; Blümcke et al., 2007). Symbols indicate results from individual studies. Bars indicate averages.

To test whether the frequency of unilateral neuron loss in chronic DA sea lions was similar to that in human patients with temporal lobe epilepsy, results were compiled from previously published human MRI studies (King et al., 1995; Barr et al., 1997; Briellmann et al., 1999) or autopsy findings (Sano and Malamud, 1953; Margerison and Corsellis, 1966; Thom et al., 2005). Unilateral or asymmetric atrophy or hippocampal neuron loss is reported to occur in 63–91% of human patients (81%, average). In the present study 79% of sea lions had unilateral hippocampal neuron loss, which is similar to previous reports that used MRI to detect hippocampal atrophy (Thomas et al., 2010; Montie et al., 2012) or histology to screen for hippocampal damage in chronic DA sea lions (Goldstein et al., 2008) and found asymmetric or unilateral damage in 68–87% of cases (76%, average). These findings suggest that unilateral hippocampal neuropathology is common in human patients with temporal lobe epilepsy and in chronic DA sea lions.

In affected hippocampi of chronic DA sea lions, neuron loss in the hilus most clearly illustrated laterality of lesions (Fig. 5C). In addition to frequently being unilateral, hilar neuron loss consistently was severe, not graded. In affected hippocampi of chronic DA sea lions, hilar neurons were only 4–36% of the average control value. To compare the pattern and extent of hilar neuron loss with another animal model of temporal lobe epilepsy, results from a previous study were plotted that used similar methods to estimate numbers of hilar neurons per hippocampus in control and epileptic kainate-treated rats (Buckmaster and Dudek, 1997). Like chronic DA sea lions, epileptic kainate-treated rats display hilar neuron loss (average 48% of controls). However, in kainate-treated rats, hilar neuron loss is bilateral and graded, not unilateral and "all or none" as in chronic DA sea lions (Fig. 5D).

Immunostaining was used to test whether γ-aminobutyric acid (GABA)ergic interneuron axon sprouting might have occurred in the dentate gyrus of chronic

TABLE 3.

Hippocampal Neuron Numbers in Male and Female Sea Lions Known or Suspected of Previous Exposure to Domoic Acid[1]

	Granule cell layer	Hilus	CA3	CA2	CA1
Male ($n = 4$ animals, 4 hippocampi)	2,000,000 ± 560,000	103,000 ± 9,000	597,000 ± 82,000	143,000 ± 35,000	1,550,000 ± 270,000
Female ($n = 10$ animals, 13 affected hippocampi)	820,000 ± 190,000[2]	54,000 ± 10,000[2]	368,000 ± 52,000[2]	106,000 ± 14,000	1,150,000 ± 120,000

[1]Values represent neurons/hippocampus (mean ± SEM).
[2]$P < 0.05$, t test.

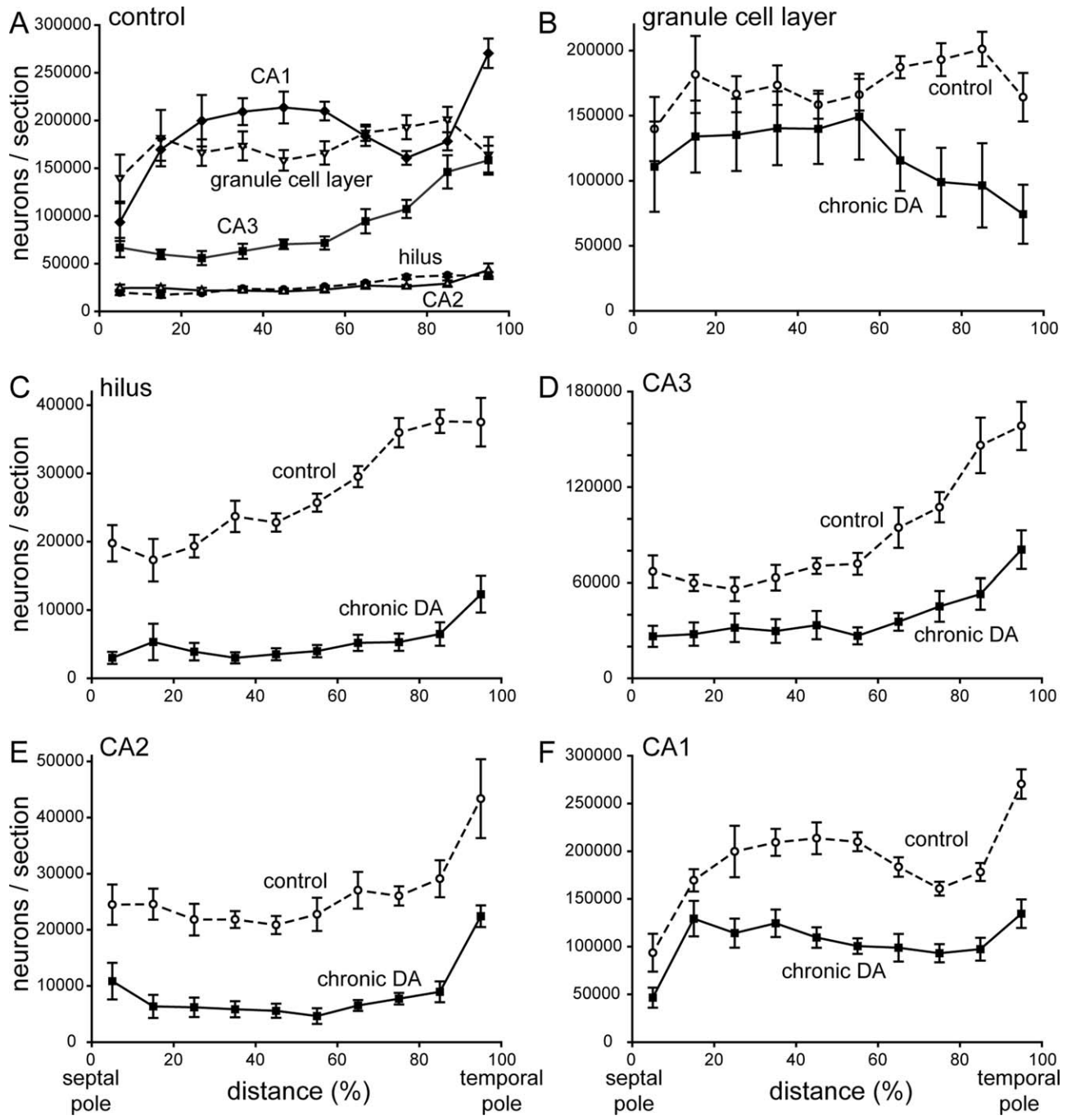

Figure 4. Sea lion hippocampal neurons quantified along the septotemporal axis. **A:** Neurons per section along the septotemporal axis of the hippocampus in controls ($n = 9$ animals, 18 hippocampi). **B–F:** Neurons per section along the septotemporal axis of the hippocampus in the granule cell layer (B), hilus (C), and pyramidal cell layer of CA3 (D), CA2 (E), and CA1 (F) in controls ($n = 9$ animals, 18 hippocampi) and affected hippocampi in chronic domoic acid (DA) sea lions ($n = 14$ animals, 17 hippocampi). Values indicate mean ± SEM.

DA sea lions. Abundant somatostatin-positive hilar neurons were evident in control sea lions but few in affected hippocampi of chronic DA sea lions (Fig. 6). Despite fewer hilar neurons, somatostatin-immunoreactive axons were more evident in affected hippocampi of chronic DA sea lions than in controls.

Timm staining was used to test whether mossy fiber sprouting occurred in chronic DA sea lions. Control sea lions ($n = 9$ animals, 18 hippocampi) displayed black Timm staining primarily in the hilus of the dentate gyrus and stratum lucidum of CA3 (Fig. 7A,C). Affected hippocampi in chronic DA sea lions ($n = 12$ animals, 15

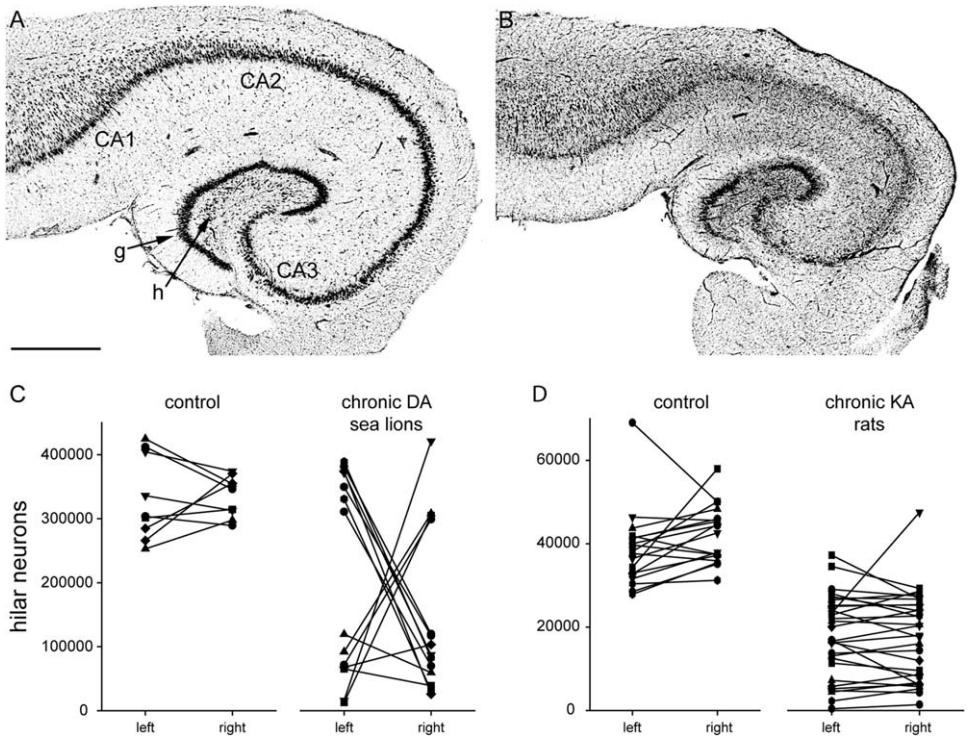

Figure 5. Laterality of hippocampal neuron loss in chronic domoic acid (DA) sea lions. **A,B:** Hippocampi of a chronic DA sea lion admitted in status epilepticus with DA toxicity and euthanized 26 days later. Neuron loss was substantial in the right (B) but not left hippocampus (A). g, granule cell layer; h, hilus. **C:** Hilar neurons per right and left hippocampus in control and chronic DA sea lions. **D:** Hilar neurons per right and left hippocampus in control and chronic kainate (KA)-treated rats reported previously (Buckmaster and Dudek, 1997).

hippocampi, with two animals omitted because of poor Timm staining) displayed an extra band of black Timm staining in the granule cell layer and especially in the inner third of the molecular layer, indicating aberrant mossy fiber sprouting (Fig. 7B,D). The average percentage of the granule cell layer plus the molecular layer that was Timm-positive was abnormally elevated at all septotemporal levels except the extreme end of the septal pole (Fig. 7E). The extent of mossy fiber sprouting was greater in the septal half than in the temporal end of the hippocampus. The difference might be attributable to more severe loss of granule cells in the temporal hippocampus (Fig. 4B). Loss of granule cells would be expected to reduce numbers of mossy fibers and extent of Timm staining. The average amount of black Timm staining in the granule cell layer plus the molecular layer per affected chronic DA sea lion hippocampus was 4.0-fold higher than controls and 2.6-fold higher than contralateral chronic DA sea lion hippocampi ($P < 0.05$, ANOVA on ranks with Dunn's method) (Fig. 7F). Contralateral hippocampi of chronic DA sea lions ($n = 11$ animals, 11 hippocampi) displayed slightly (1.5-fold) but not significantly higher levels of black Timm staining in the granule cell layer plus the molecular layer compared with controls.

DISCUSSION

The main findings of the present study are that after natural exposure to domoic acid (DA), sea lions frequently develop unilateral hippocampal neuron loss, hilar somatostatin-immunoreactive neuron loss but axon sprouting by surviving cells, and mossy fiber sprouting. These neuropathological abnormalities are similar to those of human patients with temporal lobe epilepsy.

Sea lion hippocampal anatomy compared with other species

Qualitatively, the cytoarchitecture of the sea lion hippocampus is similar to that of other mammals, but there are quantitative differences. The hippocampus of control sea lions contained over 6 million neurons, which is over three times more than rats (West et al., 1991) and 17% of humans (West et al., 1994). Compared with other mammals, sea lions have a smaller proportion of neurons in the granule cell layer. In rats (West et al., 1991) and humans (West et al., 1994), granule cells account for 64% and 50% of hippocampal neurons, respectively, but in sea lions only 33%. The difference is specific to the granule cell layer. For example, not surprisingly, sea lions have two to three times

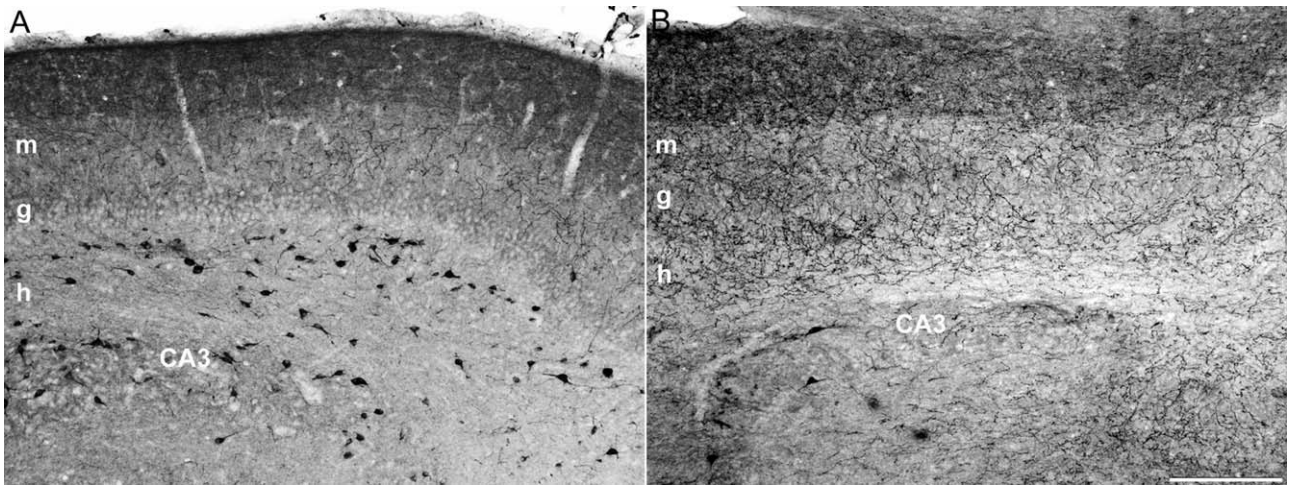

Figure 6. Somatostatin immunoreactivity in sea lion hippocampi. **A,B:** Sections from the mid-septotemporal level of the hippocampus in a control (A) and an epileptic sea lion suspected of previous DA exposure at least 28 days previously (B). Somatostatin-positive cell bodies are abundant in the hilus (h) and proximal CA3 pyramidal cell layer (CA3) in the control but not the chronic DA sea lion. However, somatostatin-positive fibers are abundant in the hilus, granule cell layer (g), and molecular layer (m) of the chronic DA sea lion. Scale bar = 250 μm in B (applies to A,B).

more hilar neurons than smaller carnivores (Mitchell et al., 1999; Buckmaster et al., 2002b) but unexpectedly only a fifth as many granule cells (Amrein and Slomianka, 2010). Average granule cell layer thickness was significantly lower in control sea lions compared with control macaque monkeys, squirrel monkeys, and dogs, all of which have substantially smaller body sizes. Thus, sea lions appear to have fewer granule cells at least in part because of their relatively thin granule cell layer. Granule cell layer thickness is only one of many parameters that can affect the total number of granule cells, and more work is needed to identify the mechanisms underlying this species difference. It is not known whether other marine mammals also have proportionally fewer granule cells, and the functional consequences of fewer granule cells are unclear.

Unilateral lesions after systemic exposure

Unilateral hippocampal neuropathology is common in human patients with temporal lobe epilepsy and in chronic DA sea lions. It is unclear why status epilepticus following systemic exposure to a toxin causes unilateral lesions. In rodents, status epilepticus after exposure to systemic DA (and other convulsants) causes bilateral damage in the hippocampus and other brain regions (Colman et al., 2005). California sea lions are members of the Otariidae family of pinnipeds, who like dolphins can sleep while swimming and display interhemispheric electroencephalographic (EEG) asymmetry during some stages of sleep (Lyamin et al., 2008), suggesting a possible predisposition for hemisphere-specific activity. However, this unusual EEG feature is not necessary for focal damage, because in humans (Fujikawa et al., 2000) and non-human primates (Meldrum et al., 1974), who do not display marked interhemispheric EEG asymmetry during sleep, status epilepticus commonly causes asymmetrical or unilateral hippocampal neuron loss. One possible mechanism for unilateral lesions in sea lions and primates but bilateral lesions in rodents is a difference in hippocampal commissures, which are more developed in rodents than primates (Amaral et al., 1984). Tract-tracing studies have not been reported for sea lions, but cats, another carnivore, have extensive interhemispheric hippocampal connectivity (Jouandet et al., 1985), suggesting that other factors account for unilateral lesions in sea lions.

Human brains contain many more neurons than rodent brains, but the average numbers of synapses received per neuron are roughly similar (DeFelipe et al., 2002), indicating that, on average, neurons in human brains must be less interconnected. One speculative possibility is that generally reduced connectivity in species with large brains (including sea lions) limits seizure spread resulting in more focal seizures and more focal damage. Similar unilateral or asymmetric patterns of hippocampal damage in sea lions (Goldstein et al., 2008; Thomas et al., 2010; Montie et al., 2012), nonhuman primates (Meldrum et al., 1974), and humans (Fujikawa et al., 2000) suggest that common pathophysiological mechanisms cause hippocampal neuron loss in species with large brains that experience status epilepticus. Although temporal lobe epilepsy in humans

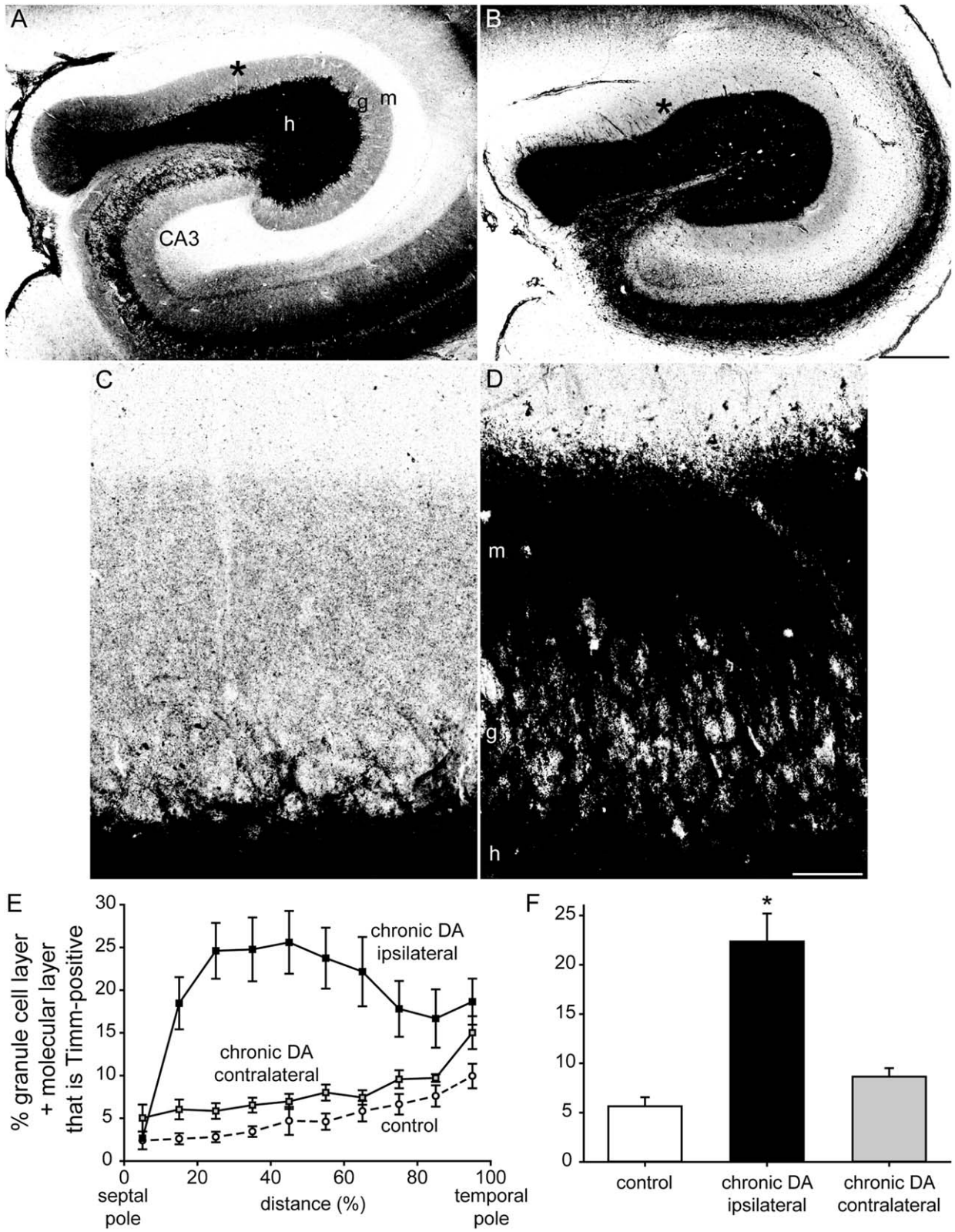

Figure 7. **A–D**: Timm-stained hippocampi from a control (A,C) and a chronic domoic acid (DA) sea lion suspected of DA exposure at least 54 days before euthanasia (B,D). m, molecular layer; g, granule cell layer; h, hilus of the dentate gyrus (A). Asterisks indicate areas shown at higher magnification in C and D. **E,F:** Average percentage of the granule cell layer plus molecular layer that displayed black Timm staining along the septotemporal axis (E) and per hippocampus (F) in control sea lions ($n = 9$ animals, 18 hippocampi) and in affected ($n = 12$ animals, 15 hippocampi) and contralateral hippocampi ($n = 11$ animals, 11 hippocampi) of chronic DA sea lions. Bars indicate SEM. $^*P < 0.05$, ANOVA on ranks with Dunn's method. Scale bar = 500 μm in B (applies to A,B); 50 μm in D (applies to C,D).

is rarely caused by DA-induced status epilepticus (Cendes et al., 1995), the similar pattern of hippocampal damage in sea lions suggests they may be a good model.

Hippocampal neuron loss

Substantial loss of hippocampal neurons is common in human patients with temporal lobe epilepsy and in chronic DA sea lions. As in human patients with temporal lobe epilepsy (Margerison and Corsellis, 1966; de Lanerolle et al., 2003; Swartz et al., 2006; Blümcke et al., 2007), chronic DA sea lions displayed individual variation in the pattern and extent of hippocampal neuron loss. Constraints of most human studies preclude a direct comparison of numbers of neurons for entire hippocampi in humans with temporal lobe epilepsy with that from chronic DA sea lions in the present study. Nevertheless, comparison of neuron density data from previous human tissue studies with neuron number data from sea lion hippocampi revealed average values $\leq$ 55% of controls for every subfield in Ammon's horn and the dentate gyrus.

Patterns of neuron loss were similar but not identical in human patients with temporal lobe epilepsy and chronic DA sea lions. The hilus was the site of the most severe hippocampal neuron loss in chronic DA sea lions, which is similar to rodent models of temporal lobe epilepsy (Mello et al., 1993; Gorter et al., 2001). In most previous studies of tissue from human patients with temporal lobe epilepsy, the hilus was combined with proximal CA3 for cell counting, probably because the border between the two regions is difficult to clearly visualize in Nissl-stained primate hippocampi (Buckmaster and Amaral, 2001). Some previous studies evaluated the hilus independently and reported that the hippocampal subregion with the most severe neuron loss was the hilus (Sass et al., 1990), CA1 (Mathern et al., 1997; de Lanerolle et al., 2003), or both (Bahh et al., 1999). CA1 cell loss is less severe in sea lions than in humans and least severe in rats (Mello et al., 1993; Gorter et al., 2001). It is unclear why CA1 pyramidal cells are more vulnerable in humans. Hilar neuron loss tends to occur in a unilateral "all or none" pattern in chronic DA sea lions, whereas in rats it is bilateral and graded. In humans, neuron loss frequently is unilateral and neurons in contralateral hippocampi are spared (Thom et al., 2005). Without stereological evaluation of entire human hippocampi, it remains unclear whether hilar neuron loss in human patients is graded as in rodent models or is as consistently severe as in chronic DA sea lions.

It appears that in both chronic DA sea lions and human patients the hippocampus is the site of most severe neuron loss, although other brain regions frequently are affected. Chronic DA sea lions with hippocampal atrophy sometimes display atrophy of the parahippocampal gyrus, which contains the entorhinal cortex (Montie et al., 2012). After DA toxicosis in sea lions, histological lesions occur consistently in the hippocampus and sometimes in extra-hippocampal structures, including the amygdala (Silvagni et al., 2005). Similarly, the entorhinal cortex (Du et al., 1993) and amygdala also are damaged in some human patients with hippocampal sclerosis (Sano and Malamud, 1953; Margerison and Corsellis, 1966).

Hippocampal neuron loss was more severe in older female chronic DA sea lions than younger males. Most DA-exposed sea lions are adult females, which might be attributable to the timing of their congregation at rookeries where frequent blooms of *Pseudo-nitzschia* algae occur (Bejarano et al., 2008; Thomas et al., 2010). Sea lions have a long gestation period, pregnant sea lions are exposed to DA, and DA reaches the fetus, which might be exposed to recirculated toxin for extended periods (Brodie et al., 2006). Thus, it has been proposed that the increasing frequency of younger animals, especially males, with signs of previous DA exposure might be attributable to in utero exposure (Goldstein et al., 2008; Ramsdell and Zabka, 2008). Of the four male chronic DA sea lions in the present study, only one stranded because of acute DA toxicosis, raising the speculative possibility that the other three were exposed in utero. DA-exposure histories of wild sea lions are incomplete. However, if some of the young males in the present study had been exposed during gestation, results of the present study suggest that in utero exposure to DA can cause epilepsy to develop later in life and that hippocampal neuron loss after in utero exposure is less severe than that caused by adult exposure. Similarly, it has been hypothesized that in utero exposure to low levels of DA that do not cause symptoms in adults might cause temporal lobe epilepsy to develop later in developing humans (Stewart, 2010).

Synaptic reorganization

The present study revealed temporal lobe epilepsy-related synaptic reorganization in chronic DA sea lions. Over half of the GABAergic interneurons in the dentate hilus in rats and monkeys express somatostatin (Austin and Buckmaster, 2004). Numbers of somatostatin-immunoreactive hilar neurons are reduced substantially in patients with temporal lobe epilepsy (de Lanerolle et al., 1989), but surviving cells sprout axon collaterals that become even more exuberant than in controls (Mathern et al., 1995). In mouse models of temporal lobe epilepsy, sources of sprouted axons include surviving somatostatin-immunoreactive neurons in the hilus

(Zhang et al., 2009) and CA1 field (Peng et al., 2013). Similarly, chronic DA sea lions displayed loss of hilar somatostatin interneurons but abundant somatostatin-immunoreactive axons in the dentate gyrus, suggesting synaptic reorganization.

Mossy fiber sprouting is a common neuropathological abnormality in patients with temporal lobe epilepsy (Sutula et al., 1989; de Lanerolle et al., 1989; Houser et al., 1990) and in animal models of temporal lobe epilepsy (Buckmaster, 2004). Sprouted mossy fibers synapse predominantly with granule cells, forming a recurrent excitatory circuit in human patients (Babb et al., 1991; Franck et al., 1995; Zhang and Houser 1999) and animal models (Represa et al., 1993; Wenzel et al., 2000; Buckmaster et al., 2002c). Similarly, mossy fiber sprouting was a consistent finding in chronic DA sea lions.

Sea lions as a possible model of human temporal lobe epilepsy

The validity of rodent models generated by systemic treatment with kainic acid and other convulsants is supported by the development of temporal lobe epilepsy in a human (Cendes et al., 1995) and in sea lions after DA intoxication. Sea lions provide opportunities and challenges as a potential animal model of human temporal lobe epilepsy. Advantages include their similar hippocampal neuropathology, human-like size, and availability. On average, The Marine Mammal Center in Sausalito, California admits approximately 70 cases/yr with acute DA toxicosis (Goldstein et al., 2008). Rates of *Pseudo-nitzschia* algal blooms along the coast of California (Sekula-Wood et al., 2011) and cases of DA toxicosis admitted to The Marine Mammal Center appear to be increasing (Greig et al., 2005; Goldstein et al., 2008). Because sea lions are exposed in the wild following ingestion of prey containing varying amounts of DA, doses of DA and exact timing of exposure are unclear, which is an experimental limitation.

Nevertheless, status epilepticus can be recognized, and the presence of DA in feces and urine can be detected. Admitted sea lions with acute DA toxicosis receive anticonvulsive and supportive therapy for days to weeks (Gulland et al., 2002), during which time they also could receive experimental anti-epileptogenic treatments. Upon discharge, continued treatment becomes impossible, and 54% of released status epilepticus survivors ultimately die prematurely or are euthanized (Goldstein et al., 2008). Thus, there is a pressing veterinary need for (and an opportunity to test) anti-epileptogenic treatments that could be administered shortly after precipitating injuries and for a limited period of time. Unlike laboratory animals, sea lions are wild animals protected legally by the Marine Mammal Protection Act. Stranded sea lions cannot be implanted with electrodes for chronic EEG recording or held in captivity for long-term seizure monitoring. Direct measures of epileptogenicity, such as seizure frequency, probably are not feasible. Nevertheless, indirect measures of anti-epileptogenic treatment efficacy can be obtained. One potentially informative parameter is restranding rate, which is measured by identifying individuals from flipper tags they receive before release (Greig et al., 2005). Chronic DA sea lions restrand at a rate of 71% (Goldstein et al., 2008), which is much higher than that of sea lions rehabilitated after stranding for other reasons (~0.5%) (Gulland et al., 2002). Other parameters include movement, migration, and diving patterns that can be monitored from a satellite transmitter glued to the fur, which remains attached for weeks until molting. Chronic DA sea lions exhibit abnormal movement, migration, and diving patterns (Thomas et al., 2010). Thus, experimental anti-epileptogenic treatments could be evaluated in DA-exposed sea lions to test whether restranding rates decrease and whether movement, migration, and diving patterns normalize.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: PSB, IT, FMDG, WVB. Acquisition of data: PSB, XW, IT, FMDG, WVB. Analysis and interpretation of data, drafting of the manuscript, statistical analysis, obtained funding, and study supervision: PSB.

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