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Phenobarbital and Midazolam Increase Neonatal Seizure-Associated Neuronal Injury

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Status epilepticus is common in neonates and infants, and is associated with neuronal injury and adverse developmental outcomes. γ -Aminobutyric acidergic (GABAergic) drugs, the standard treatment for neonatal seizures, can have excitatory effects in the neonatal brain, which may worsen the seizures and their effects. Using a recently developed model of status epilepticus in postnatal day 7 rat pups that results in widespread neuronal injury, we found that the GABA_A agonists phenobarbital and midazolam significantly increased status epilepticus-associated neuronal injury in various brain regions. Our results suggest that more research is needed into the possible deleterious effects of GABAergic drugs on neonatal seizures and on excitotoxic neuronal injury in the immature brain.

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In the neonatal rodent brain, γ -aminobutyric acidergic (GABAergic) drugs, which are the standard treatment for neonatal seizures, can have excitatory effects, which might aggravate seizures and their long-term consequences.^{1–4} In clinical use and in some experimental models, GABAergic drugs appear to stop behavioral seizures,⁵ but adverse effects might be hard to detect if they occur in a subpopulation of immature neurons that have little behavioral expression at that age.⁶ We recently developed a model of status epilepticus (SE) in postnatal day 7 (P7) rat pups that resulted in high survival rates and widespread neuronal injury⁷ and for the first time offered us the opportunity to test the effect of GABAergic drugs on SE-associated neuronal injury. We found that both phenobarbital and midazolam treatment of SE increased acute neuronal injury in several brain regions, raising questions about the safety of their clinical use.

Materials and Methods

Male and female Sprague-Dawley albino rats (Charles River Laboratories, San Diego, CA; $n = 66$) were used at P7 with the day of birth considered as day 0, as previously reported.⁷ Lithium chloride (5mEq/kg) was administered intraperitoneally

(i.p.) at P6, and the next day SE was induced with subcutaneous (s.c.) pilocarpine hydrochloride (320mg/kg) together with scopolamine methyl chloride (1mg/kg, i.p.) to block the peripheral effect of pilocarpine. All experiments were conducted with the approval of and in accordance with the regulations of the Institutional Animal Care and Use Committee of West Los Angeles VA Medical Center, and in accordance with the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

In preliminary experiments, we found that high doses of midazolam (6mg/kg, but not 3mg/kg) and phenobarbital (25mg/kg, but not 10mg/kg) induce apoptosis in control (no-seizure) pups. Ten minutes after the development of stage 3 seizures, some pups were treated with midazolam (3mg/kg, i.p., SE+Mz group, $n = 36$) and others with phenobarbital (10mg/kg, i.p., SE+Ph group, $n = 26$). Some pups (SE group, $n = 30$) were kept untreated as SE controls and only received saline (i.p.), whereas other pups receiving only midazolam (3mg/kg, i.p., Mz group, $n = 6$) or phenobarbital (10mg/kg, i.p., Ph group, $n = 8$) were kept as no-seizure controls for injury comparison. An untreated, no-seizure group was also included (sham, $n = 5$). All animals were rehydrated with saline approximately 4 hours after SE (10% of body weight, s.c.) and sacrificed 24 hours after SE onset.

Preparation of tissue for histology and active caspase-3 (caspase-3a) immunohistochemistry was performed as previously reported.⁷ The quantification of neuronal injury using Fluoro-Jade B (FJB) staining was performed by manual counting by an observer blinded to the animal condition using a grid to reduce the possibility of overcounting. Three adjacent coronal sections per animal were taken for each brain region at the following anatomical locations: caudate putamen, septal nuclei, nucleus accumbens (bregma + 1.0mm),^{8–10} globus pallidus (bregma – 1.0mm), dorsal hippocampus, parietal cortex, piriform cortex, thalamus, hypothalamus, amygdala (bregma – 2mm), and ventral hippocampus, lateral entorhinal cortex, substantia nigra (bregma – 4.2mm). Counting of the entire brain structure was performed using a Leica (Wetzlar,

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Germany) $\times 40$ objective and the number of FJB⁺ cells for each of the 3 coronal sections was averaged for each region. To compensate for minor anatomical variations in hippocampus, FJB⁺ cells were expressed per millimeter length of each cell layer. Parietal cortex was counted from the top of the cingulum down to the rhinal fissure. Only cells that had visible nuclei were counted. FJB⁺ cell counts were corrected for cell size by a modified Abercrombie factor,¹¹ in which population cell size was estimated by averaging the diameter of the counted cells' nuclei in the section per neuronal population. Three animals per group were chosen at random, and a total of 70 to 100 profile measurements for experimental groups (SE, SE+Mz, SE+Ph) and 15 to 30 for control groups (sham, Mz only, Ph only) per brain region were used for this average. The average correction factors ranged from 0.73 to 0.79 (Supplementary Table) and were not significantly different among groups, suggesting that differential swelling of the tissue did not occur.

For a subset of SE+Mz and SE+Ph animals, we first stained 3 adjacent sections (bregma -2 mm) for caspase-3a, counted the cells in thalamus, then removed the coverslip, performed FJB staining, and counted the FJB⁺ cells. With these data, we obtained a percentage of FJB⁺ cells that expressed caspase-3a in thalamus.

Experimental groups were analyzed with nonparametric statistical methods: Kruskal–Wallis test followed by Dunn test for multiple comparisons. Statistical significances were considered when $p < 0.05$.

Results

The Course of SE in P7 Pups

The combination of high-dose lithium (5mEq/kg, i.p.) and pilocarpine (320mg/kg, s.c.) induced SE in 66 of 66 pups (100%), an incidence much higher than we previously reported with administering pilocarpine i.p.⁷ Shortly after pilocarpine injection, pups became hyperactive, showing running seizures with vocalization (stage 3). After midazolam or phenobarbital treatment, however, this behavioral component was reduced as the pups became sedated. Sedation was transient and was more severe after midazolam (pups unresponsive) than after phenobarbital (pups moving and responsive). Animal survival at 24 hours post-SE was 64% (30 of 47) in the untreated SE group, 77% (36 of 47) in the SE+Mz group (not significant), and 65% (26 of 40) in the SE+Ph group (not significant). As shown in the Table, neither midazolam nor phenobarbital alone caused a significant increase in neuronal injury compared to sham ($p > 0.05$, Kruskal–Wallis analysis).

Effect of Treatment on Distribution of SE-Associated Neuronal Injury

THALAMUS. Both midazolam and phenobarbital treatment significantly increased SE-associated neuronal

injury in the thalamus (+135%, $p < 0.01$; +136%, $p < 0.001$). Neuronal injury varied among thalamic nuclei, predominating in dorsolateral nuclei in some pups and ventromedial or other nuclei in others (Fig 1).

BASAL GANGLIA. Both midazolam and phenobarbital treatment significantly increased neuronal injury in caudate putamen (+277%, $p < 0.0001$; +205%, $p < 0.01$), globus pallidus (+127%, $p < 0.0001$; +155%, $p < 0.0001$), and substantia nigra (+367%, $p < 0.0001$; +833%, $p < 0.0001$) compared to the untreated SE group (see Fig 1). However, only midazolam treatment significantly increased neuronal injury in the nucleus accumbens (+111%, $p < 0.05$).

HIPPOCAMPUS. Neuronal injury in the stratum pyramidale of dorsal and ventral CA1/subiculum was examined in SE, SE+Mz, and SE+Ph animals at 24 hours after SE induction. As shown in the Table, midazolam treatment significantly increased neuronal injury in dentate gyrus (+150%, $p < 0.05$) and ventral CA1/subiculum (+82%, $p < 0.05$; Fig 2). Interestingly, phenobarbital treatment significantly decreased injury in dorsal CA3 (−60%, $p < 0.05$). The distribution of hippocampal injury was similar to that previously reported.⁷

OTHER LIMBIC REGIONS. Phenobarbital treatment significantly increased neuronal injury in hypothalamus and septal nuclei (+988%, $p < 0.0001$; +293%, $p < 0.0001$), but midazolam increased it only in septal nuclei (+37%, $p < 0.0001$; see Fig 2). As shown in the Table, amygdala neuronal injury was not affected by midazolam or phenobarbital treatment. Injury among amygdaloid nuclei varied and, therefore, the structure was counted as a whole.

NEOCORTEX. Both midazolam and phenobarbital treatment significantly increased neuronal injury in lateral entorhinal cortex (+171%, $p < 0.0001$; +114%, $p < 0.01$), but there was no significant change in piriform cortex. Neuronal injury in parietal cortex was significantly increased by midazolam (+54%, $p < 0.05$), but not phenobarbital. Injury was consistently found along layer 2, although some FJB⁺ cells were spread throughout layer 4 and other cortical layers.

SE Triggers an Active Form of Cell Death

The contribution of caspase-3 to SE-associated neuronal death was assessed by immunohistochemistry using an antibody that recognizes caspase-3a, the active form of that enzyme. As shown previously in this model,⁷ SE resulted in a significant increase in caspase-3a immunoreactivity compared to sham treatment. In a subset of SE+Mz and SE+Ph animals, we found that the percentage of FJB⁺ cells

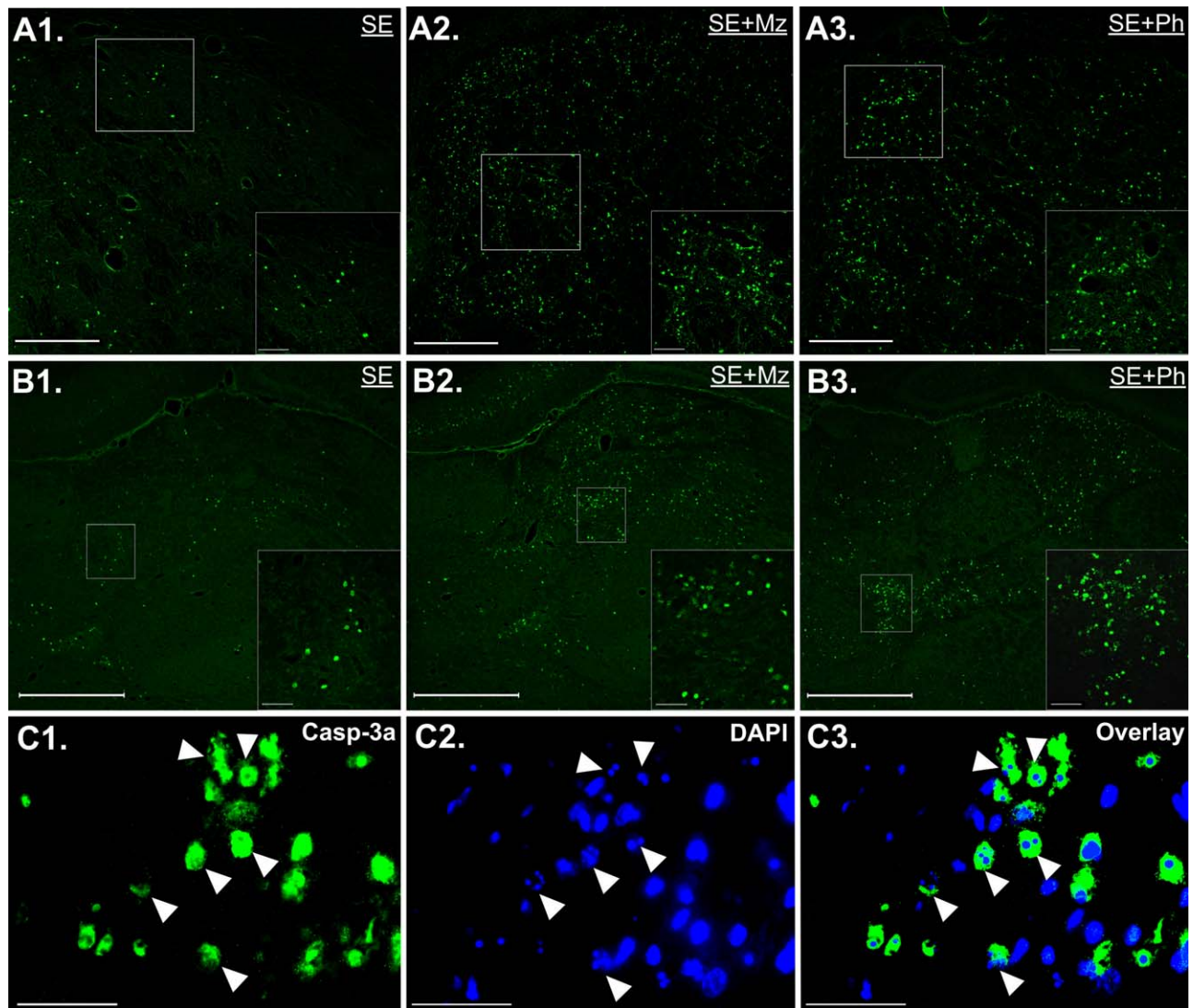


FIGURE 1: Effect of midazolam (Mz) or phenobarbital (Ph) treatment on status epilepticus (SE)-associated neuronal injury in caudate putamen and thalamus, and mechanism of thalamic injury. (A) Images of Fluoro-Jade B (FJB) staining in caudate putamen of (A1) SE, (A2) SE+Mz, and (A3) SE+Ph pups with a higher magnification of the boxed area on the bottom right of each image. SE induces neuronal injury throughout caudate, and this injury is exacerbated following midazolam or phenobarbital treatment by 24 hours post-SE. (B) Images of FJB staining in thalamus of (B1) SE, (B2) SE+Mz, and (B3) SE+Ph pups. Both midazolam and phenobarbital treatment increase FJB cell distribution throughout various thalamic nuclei to approximately the same extent. Scale bars: (A) long bars = 200 μ m and short bars = 20 μ m; (B) long bars = 500 μ m and short bars = 50 μ m. (C) Images of overlay of active caspase-3 (Casp-3a) immunoreactivity (green) and 4,6-diamidino-2-phenylindole (DAPI; blue) staining in thalamus of SE+Mz pups. On the left, high magnification shows distribution of caspase-3a-immunoreactive cells in ventromedial thalamus of an SE+Mz pup; in the middle, high-magnification images show that caspase-3a-immunoreactive cells have fragmented nuclei indicative of neuronal cell death; on the right, this overlay shows that many of these caspase-3a-immunoreactive cells have fragmented nuclei, suggesting a caspase-dependent form of cell death. Scale bars = 50 μ m.

expressing caspase-3a in the thalamus was $79 \pm 10\%$ and $73 \pm 16\%$, respectively. As shown in Figure 1C, these caspase-3a-immunoreactive cells had distinct changes in nuclear morphology, such as fragmented nuclei, suggesting that the neuronal injury resulting from midazolam and phenobarbital treatment is irreversible.

Discussion

This is the first reproducible model showing evidence that GABAergic drugs, a standard treatment for neonatal

status epilepticus, may aggravate SE-associated neuronal injury in some regions of the neonatal rodent brain. We used drug dosages too low to cause apoptosis by themselves, and found that early treatment (10 minutes post-SE induction) with the barbiturate phenobarbital or the benzodiazepine midazolam significantly increased seizure-associated neuronal injury in thalamus, basal ganglia, hypothalamus, septal nuclei, ventral CA1/subiculum, parietal cortex, and entorhinal cortex in P7 rat pups 24 hours after SE induction. Neither treatment significantly

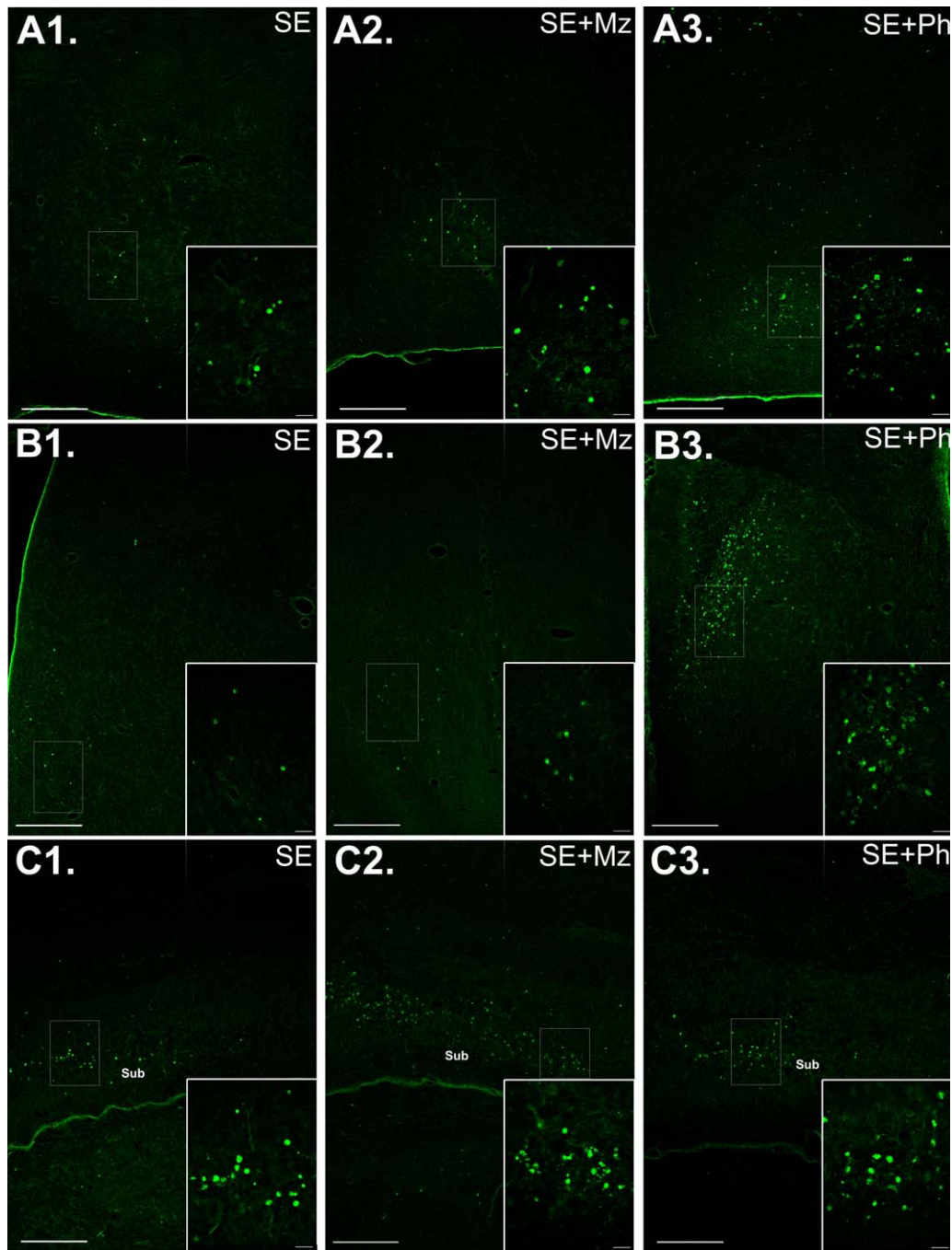


FIGURE 2: Midazolam (Mz) or phenobarbital (Ph) treatment increases status epilepticus (SE)-associated neuronal injury in some limbic regions. (A) Images of Fluoro-Jade B (FJB) staining in hypothalamus of (A1) SE, (A2) SE+Mz, and (A3) SE+Ph pups, with a higher magnification of the boxed area on the bottom right of each image. Phenobarbital treatment significantly increased FJB⁺ cells compared to untreated SE. (B) Images of FJB staining in septal nuclei of (B1) SE, (B2) SE+Mz, and (B3) SE+Ph pups. Both midazolam and (to a greater extent) phenobarbital treatment significantly increase neuronal injury in septum. (C) Images of FJB staining in ventral CA1/subiculum (Sub) of (C1) SE, (C2) SE+Mz, and (C3) SE+Ph postnatal day 7 pups. As shown, SE (C1) induces neuronal injury in ventral CA1/subiculum by 24 hours post-SE and this injury is enhanced following midazolam treatment (C2), although no significant difference is seen after phenobarbital treatment (C3). Scale bars: long bars = 200 μm and short bars = 20 μm . [Color figure can be viewed at wileyonlinelibrary.com]

increased pup survival, discarding the possibility that the increase of neuronal injury is due to the survival of severely injured animals that may otherwise have died. These drugs seemed to stop behavioral seizures, but the possibility exists that they hyperpolarized relatively

mature neurons that have behavioral expression,^{12–14} therefore stopping the behavioral seizures, while depolarizing less mature neurons that have immature connections and little behavioral expression.¹⁵ This might increase neuronal injury in relatively silent areas, and the

TABLE. Distribution of Neuronal Injury among Various Brain Regions in Sham, Mz Only (Mz), Ph Only (Ph), SE, SE+Mz, and SE+Ph Pups

Region	Sham	Mz	Ph	SE	SE+Mz	SE+Ph
Hippocampus						
Dorsal CA1/Sub	0.2 (0.2–0.3)	0.3 (0.2–0.4)	0.2 (0.2–0.3)	8 (4–16)	8 (4–34) ^{c,f}	11 (5–19) ^{b,j}
CA3	0.2 (0.2–0.3)	0.2 (0.1–0.3)	0.2 (0.2–0.4)	5 (2–8)	4 (2–7) ^{b,f}	2 (1–3) ^{b,h,k}
DG	0.1 (0.1–0.2)	0.2 (0.1–0.2)	0.1 (0–0.2)	0.8 (0.4–2)	2 (1–2) ^{d,g,k}	1 (0.4–2) ^{b,i}
Ventral CA1/Sub	0.5 (0.4–0.5)	0.6 (0.4–0.7)	0.3 (0.2–0.6)	11 (7–20)	20 (11–32) ^{d,g,k}	13 (7–20) ^{b,j}
Other limbic regions						
Amygdala	12 (11–15)	11 (9–12)	11 (9–13)	126 (50–221)	140 (63–241) ^{b,g}	118 (61–240) ^{b,j}
Hypothalamus	4 (3–5)	5 (4–6)	5 (3–5)	20 (15–30)	32 (19–76) ^{d,f}	206 (102–300) ^{d,j,n}
Septal nuclei	9 (7–11)	7 (5–8)	6 (6–7)	21 (12–24)	29 (23–101) ^{c,g,n}	81 (60–160) ^{d,j,n}
Thalamus	17 (15–20)	22 (20–25)	17 (12–22)	174 (121–238)	408 (185–782) ^{d,g,l}	410 (279–649) ^{d,j,m}
Cortical regions						
Parietal Ctx	16 (15–18)	19 (18–20)	17 (12–22)	290 (158–366)	434 (227–909) ^{d,g,k}	148 (90–243) ^{a,h}
Piriform Ctx	4 (3–5)	7 (6–8)	6 (4–8)	14 (8–20)	19 (11–37) ^{c,e}	18 (12–27) ^{c,i}
Lateral entorhinal Ctx	6 (3–7)	7 (3–10)	5 (4–6)	12 (5–14)	24 (20–37) ^{c,f,n}	23 (12–41) ^{b,j,m}
Basal ganglia						
Caudate putamen	25 (20–27)	20 (19–25)	23 (21–25)	151 (113–373)	569 (394–918) ^{d,g,n}	460 (183–870) ^{d,j,l}
Nucleus accumbens	8 (7–11)	10 (8–12)	6 (6–8)	118 (93–228)	249 (153–340) ^{d,g,k}	192 (129–366) ^{c,j}
Globus pallidus	4 (2–5)	4 (4–6)	5 (3–6)	11 (7–13)	25 (15–32) ^{d,g,n}	28 (20–32) ^{d,j,n}
Substantia nigra	2 (1–3)	3 (2–4)	2 (0.4–4)	3 (2–6)	14 (10–18) ^{b,e,n}	28 (21–43) ^{b,j,n}

Neuronal injury median values are shown, with interquartile range in parentheses. Hippocampal regions show FJB⁺ cells/mm, whereas values in other regions represent the total number of FJB⁺ cells per field (see Materials and Methods). The result of statistical analysis of each treatment group (SE+Mz vs sham, Mz, and SE, or SE+Ph vs sham, Ph, and SE) is indicated by the following footnotes:

^a*p* < 0.05 versus sham.

^b*p* < 0.01 versus sham.

^c*p* < 0.001 versus sham.

^d*p* < 0.0001 versus sham.

^e*p* < 0.01 versus Mz.

^f*p* < 0.001 versus Mz.

^g*p* < 0.0001 versus Mz.

^h*p* < 0.01 versus Ph.

ⁱ*p* < 0.001 versus Ph.

^j*p* < 0.0001 versus Ph.

^k*p* < 0.05 versus SE.

^l*p* < 0.01 versus SE.

^m*p* < 0.001 versus SE.

ⁿ*p* < 0.0001 versus SE.

Ctx = cortex; DG = dentate gyrus; FJB = Fluoro-Jade B; Mz = midazolam; Ph = phenobarbital; SE = status epilepticus; Sub = subiculum.

putative cognitive or behavioral sequelae of that injury might not be expressed until much later in development (weeks in rats, possibly years in humans). In mice, flurothyl-induced seizures beginning at P7 result in long-term deficits in social behavior, social interaction, and

learning¹⁶ and exposure to phenobarbital at P6 results in acute and long-term changes to cerebral cortex.¹⁷ In rats, exposure to phenobarbital at P7 results in increased anxietylike behavior and deficits in long-term learning and memory.¹⁸ In a mouse model of inflammation-induced

SE, midazolam treatment induced an abnormal increase in hyperactivity by the chronic phase,¹⁹ indicating that benzodiazepine treatment may have increased the behavioral sequelae of seizures, but SE occurred at P15, when GABA is no longer excitatory in most cells.

Midazolam allosterically enhances chloride flux by binding to the benzodiazepine binding site, between the $\alpha 1$ and the $\gamma 2$ subunits of the GABA_A receptor, and other actions are only seen at higher concentrations. Phenobarbital binds to the barbiturate binding site, but has several other actions at pharmacologically relevant doses.²⁰ Although these two drugs have different mechanisms of action, the finding that both cause widespread increases in SE-associated neuronal injury suggests that this increase may involve GABAergic mechanisms.

The clinical significance of our results is uncertain. They cannot be blindly extrapolated to clinical situations, which deal with a different type of seizures in a much larger brain, but they suggest that more research is needed in animal models as well as in clinical neonatal SE. The possibility that the use of GABAergic drugs for the treatment of neonatal seizures could be deleterious in brain areas that have little behavioral expression at that age raises important questions, because behavioral and clinical expression of the damage could be delayed. Physiological studies of the GABA_A system in rodent neonates provide a potential conceptual framework for that concept, but their clinical significance and their possible developmental effects need to be better understood.

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Author Contributions

All authors were responsible for conception and design of the study. D.T., L.S., and J.N. were responsible for acquisition and analysis of data. D.T. and C.G.W. were responsible for drafting the manuscript or figures. D.T. and L.S. contributed equally to this study.

Potential Conflicts of Interest

Nothing to report.

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