Antiepileptic Drug-Induced Neuronal Cell Death in the Immature Brain: Effects of Carbamazepine, Topiramate, and Levetiracetam as Monotherapy versus Polytherapy

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ABSTRACT

The aim of this study was to test the potential neurotoxicity of three antiepileptic drugs (AEDs), carbamazepine (5H-dibenzepine-5-carboxamide), topiramate [2,3,4,5-bis-O-(1-methylethylidylen)-β-D-fructopyranose sulfamate], and levetiracetam [2-[2-oxopyrrolidin-1-yl]butanamide], in the developing rat brain, when given alone or in combinations. The extent of cell death induced by AEDs was measured in several brain regions of rat pups (postnatal day 8) by terminal deoxynucleotidyl transferase dUTP nick-end labeling assay 24 h after drug treatment. Carbamazepine alone did not increase neurodegeneration when given in doses up to 50 mg/kg, but it induced significant cell death at 100 mg/kg. When combined with phenytoin, carbamazepine, 50 but not 25 mg/kg, significantly exacerbated phenytoin-induced cell death. Although topiramate (20–80 mg/kg) alone caused no neurodegeneration, all doses exacerbated phenytoin-induced neurodegeneration. Levetiracetam (250–1000 mg/kg) alone did not induce cell death, nor did it exacerbate phenytoin-induced neurodegeneration. Of the combinations examined, only that of levetiracetam (250 mg/kg) with carbamazepine (50 mg/kg) did not induce neurodegeneration. Our data underscore the importance of evaluating the safety of combinations of AEDs given during development and not merely extrapolating from the effects of exposure to single drugs. Although carbamazepine and topiramate alone did not induce neuronal death, both drugs exacerbated phenytoin-induced cell death. In contrast, because cotreatment with levetiracetam and carbamazepine did not enhance cell death in the developing brain, it may be possible to avoid proapoptotic effects, even in polytherapy, by choosing appropriate drugs. The latter drugs, as monotherapy or in combination, may be promising candidates for the treatment of women during pregnancy and for preterm and neonatal infants.

Exposure to antiepileptic drugs (AEDs) during a critical period in brain development causes long-term detrimental effects on cognitive and behavioral outcomes (Lauer et al., 1987; Glauser, 2004; Wolansky and Azcurra, 2005; Motamedi and Meador, 2006). One mechanism underlying adverse outcomes of AED exposure may be neuronal apoptosis during late gestation and the perinatal period. Experimental data indicate that even brief exposure to therapeutic levels of AEDs such as valproate, phenobarbital, or phenytoin induces neuronal apoptosis in the rat brain during the first 2 postnatal weeks, with a peak effect at postnatal day (PD)7 (Bittigau et al., 2002). Of particular clinical relevance is the observation that when two AEDs are combined, cell death may be substantial even when each drug is given in a dose subthreshold for causing cell death by itself (Bittigau et al., 2002). Drug combinations are problematic clinically, especially during pregnancy and early childhood; polytherapy causes more adverse effects on cognition compared with monotherapy (Adab et al., 2004; Motamedi and Meador, 2006). Because AEDs are often given in combination, it is crucial to determine whether certain drug combinations minimize or avoid developmental proapoptotic actions.

The early postnatal period in the rat when AEDs exert proapoptotic activity coincides with the brain “growth spurt” period (Bittigau et al., 2002). This period of rapid synaptogenesis and normal developmental apoptosis corresponds with the 3rd trimester of pregnancy through early infancy in...
humans (Bittigau et al., 2002). Therefore, proapoptotic effects of AEDs may affect not only term or preterm infants with epilepsy but normal children exposed to AEDs in utero or through breast feeding. The need for women with epilepsy to take AEDs during pregnancy makes it critical to identify AEDs (and AED combinations) with minimal risks for the developing brain. Therefore, we evaluated carbamazepine, a traditional AED, and two newer AEDs, topiramate and levetiracetam, for effects on neuronal death in rat pups when given alone and in combination with phenytoin.

The neurotoxic profile of carbamazepine, one of the most frequently used AEDs during pregnancy and childhood, has not been reported. Clinical data indicate that exposure to carbamazepine monotherapy in utero is without significant detrimental cognitive effects compared with other AEDs (Meador, 2004). If adverse cognitive effects are related to AED-induced cell death in the developing brain, then carbamazepine may be less likely than other traditional AEDs to induce neurodevelopmental apoptosis. To test this hypothesis, we studied carbamazepine alone and in combination with phenytoin for effects on neuronal death in the neonatal rat.

Among new generation AEDs examined for neurotoxicity in neonatal rats, topiramate and levetiracetam hold promise for minimizing the risk of neuronal death (Glier et al., 2004; Manthey et al., 2005). However, neither drug has been examined for effects on neuronal death when given in combination with other AEDs. This is of particular concern because topiramate and levetiracetam are indicated as add-on medication and are frequently combined with traditional AEDs. Therefore, we examined topiramate and levetiracetam, alone and in combination with phenytoin, for effects on neuronal death in the neonatal rat model.

The dose of phenytoin selected for our studies (50 mg/kg) is close to that raising the threshold for spike wave-type after-discharge in rat pups (Krssek et al., 1998) and near the ED50 in a rat kindling model of complex partial seizures (Renfrey et al., 1989). The range of carbamazepine doses selected (25–100 mg/kg) included doses attenuating pentyleneetetrazol-induced tonic seizures in PD7 rats (Kubova and Mares, 1993). Hoogerkamp et al. (1994) found plasma levels within the therapeutic range (phenytoin, 10–40 mg/l; carbamazepine: 4–25 mg/l) with anticonvulsant doses (40 mg/kg each) of phenytoin and carbamazepine in rats.

The doses of levetiracetam and topiramate selected for the present study extended above the therapeutic range. Levetiracetam up to 100 mg/kg did not enhance neuronal death in rat pups when given alone (Manthey et al., 2005). Although the therapeutic range for levetiracetam in children is often 10 to 60 mg/kg/day (Lagae et al., 2005), doses up to 315 mg/kg/day have been used (Koukkari and Guarino, 2004), warranting a more extensive range of doses to estimate a therapeutic index for this drug. Accordingly, we selected higher doses (250–1000 mg/kg) than previously tested. Topiramate, given alone in doses under 50 mg/kg was found not to induce neurodevelopmental apoptosis (Glier et al., 2004); corresponding doses are anticonvulsant in infants (4.8–38.5 mg/kg/day) (Glauser et al., 1999; Ormrod and McClellan, 2001) and in PD7 rats (20 and 40 mg/kg) (Haugvicideva et al., 2000). The doses of topiramate selected for our studies (20–80 mg/kg) fell within the anticonvulsant range previously examined by Glier et al. (2004).

Materials and Methods

Animals. Male and female Sprague-Dawley rat pups (Harlan, Indianapolis, IN) between PD7 and PD8 were used. Pups were maintained with their dam in a temperature-controlled (21°C) room with a 12-h light cycle. All protocols were in compliance with the American Association for Accreditation of Laboratory Animal Care standards and were approved by the Georgetown University Animal Care and Use Committee. Efforts were made to minimize the number of animals used and any discomfort. There was no mortality in this study.

Drug Treatment. Levetiracetam (250, 500, and 1000 mg/kg; Keppra oral solution; UCB Pharma, Smyrna, GA), topiramate (20, 40, and 80 mg/kg; Sigma, St. Louis, MO), sodium valproate (400 mg/kg, sodium 2-propylpentanoate; Sigma), sodium phenobarbital (75 mg/kg, 5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione; Sigma), and MK801 (0.5 mg/kg; Sigma) were diluted and dissolved in saline. Phenytoin (50 mg/kg, sodium diphenylhydantoin, 5,5-diphenylimidazolidine-2,4-dione; Sigma) was dissolved in alkalized saline, pH 9 to 11. Carbamazepine (25, 50, and 100 mg/kg; Sigma) was suspended in saline solution containing 1.0% Tween 80 (Sigma) and sonicated. Propylene glycol was intentionally avoided as a vehicle because it substantially alters brain levels of phenytoin (Morris et al., 1987). Control groups received equivalent volumes of vehicle. The age at which the maximal extent of cell death was induced by AEDs and MK801 was at PD7 (Ikonomidou et al., 1999; Bittigau et al., 2002), reaching a peak at 24 h after drug treatment using TUNEL or silver staining for detection (Ikonomidou et al., 1999; Bittigau et al., 2002; Glier et al., 2004; Manthey et al., 2005). Therefore, in the present study, all injections were given i.p. at PD7, 24 h before sacrifice.

For testing drug combinations, phenytoin was selected because 1) it consistently induces neuronal cell death with minimal behavioral impairment, and 2) its metabolism is not significantly affected by the drugs of interest in the present study (Morris et al., 1987; Swiader et al., 2000; Siadidiya et al., 2002; Foutain et al., 2007). The following drug combinations were tested: levetiracetam (500 mg/kg) + phenytoin (50 mg/kg) or MK801 (0.5 mg/kg), topiramate (20, 40, and 80 mg/kg) + phenytoin (50 mg/kg), carbamazepine (25 and 50 mg/kg) + phenytoin (50 mg/kg), and levetiracetam (250 and 500 mg/kg) + carbamazepine (50 mg/kg). The first drug was given 2 h before the second drug, and animals were sacrificed 24 h after the second drug treatment.

Tissue Preparation. Brains were removed, quickly frozen in isopentane, and stored at −80°C. Coronal cryostat sections (20 μm) throughout the entire brain were examined for cell death using TUNEL and Fluoro-Jade B (see below).

TUNEL Assay. To measure apoptotic cell death, TUNEL staining was performed using the Apoptag peroxidase in situ apoptosis detection kit (Chemicon International Inc., Temecula, CA) according to the manufacturer’s recommendations. For quantification of cell death, photomicrographs (10×) of three sequential sections at 200-μm intervals were taken within each of several brain regions of each animal. TUNEL-positive cells within a 1.00-mm2 area were counted by an observer blind to the treatment conditions. Data are presented as mean ± S.E.M. per tissue section.

Fluoro-Jade B Staining. For detecting neuronal degeneration, Fluoro-Jade B staining was performed according to the procedures described previously (Schmued and Hopkins, 2000).

Statistics. Statistical comparisons were performed by one-way analysis of variance followed by post hoc Tukey test with p < 0.05 as the criterion for significance.

Results

Regional Cell Death, Acute Behavior, and Brain and Body Weight. To validate and calibrate our measurements, we replicated previous reports of cell death after phenytoin,
valproate, phenobarbital, and MK801 in several brain areas of the rat pups (for summary, see Table 1). As previously reported (Ikonomidou et al., 1999; Bittigau et al., 2002), the most vulnerable areas were located within thalamus and striatum. In addition, several cortical regions exhibited AED-induced cell death including retrosplenial, frontal, and parietal cortices. MK801 and phenobarbital induced the most cell death in thalamus, striatum, and cortical areas; valproate induced marked cell death in thalamus and cortex but not in striatum; and the effect of phenytoin was largely limited to thalamus and striatum.

A subset of drugs affected the behavior and growth of the pups. At the doses used, MK801, phenobarbital, and valproate caused sedation and a reduction both in brain weight (approximately 5–9%) and in body weight gain (approximately 15% less than controls). These treatments tended to increase the brain/body weight ratio, indicating possible malnutrition (Table 2). Phenytoin did not cause sedation and had the least impact on body weight gain compared with the other previously tested AEDs. Accordingly, we selected phenytoin for experiments involving drug combinations. Carbamazepine (25 mg/kg), levetiracetam, and topiramate, the focus drugs for the present study, caused little or no sedation or effects on body or brain weight. Carbamazepine (50 mg/kg) induced mild sedation but did not retard body growth.

**Carbamazepine Alone or in Combination with Phenytoin.** To determine whether carbamazepine enhances cell death in the developing brain, we measured TUNEL-positive cells after carbamazepine (25, 50, and 100 mg/kg) treatment. No significant increase in cell death was observed after 25 or 50 mg/kg; a significant increase in cell death occurred in thalamus after 100 mg/kg (Fig. 1). When combined with phenytoin, carbamazepine (50 but not 25 mg/kg) exacerbated the phenytoin-induced cell death. This combination resulted in more severe cell death in thalamus than that induced by 100 mg/kg carbamazepine alone. Moreover, this combination induced cell death in striatum and frontal cortex, two areas that were not significantly affected by either drug given alone (Fig. 1).

**Effect of Topiramate on Phenytoin-Induced Cell Death.** Topiramate alone (20–80 mg/kg) did not increase cell death compared with controls. However, all doses of topiramate tested in combination with phenytoin (50 mg/kg) significantly exacerbated the cell death in several brain areas, including thalamus and striatum (Fig. 2). This combined effect of topiramate and phenytoin was significant with a topiramate dose of 20 mg/kg, reached a peak with a topiramate dose of 40 mg/kg, and showed no further increase in the presence of 80 mg/kg.

**Effect of Levetiracetam on Neuronal Cell Death Induced by Phenytoin.** Levetiracetam alone (250–1000 mg/kg) did not increase TUNEL-positive cells in any brain region examined compared with controls. Moreover, repeated doses (1000 followed by 500 mg/kg after 8 h) did not increase cell death measured 24 h after the first dose (control versus levetiracetam, 14.6 ± 2.2 versus 24.9 ± 4.6 in ventromedial thalamus; 21.3 ± 2.0 versus 14.7 ± 2.4 in dorsomedial striatum; 10.9 ± 1.9 versus 7.2 ± 1.0 in frontal cortex; units = TUNEL-positive cells per millimeter squared, n = 6). We examined entire coronal sections extending from the frontal cortex to the entorhinal cortex and midbrain, quantifying cell death in those regions in which it was evident (Fig. 3). We also evaluated the tissue using Fluoro-Jade B staining, which detects degeneration selectively associated with neurons. The Fluoro-Jade B staining profiles were consistent with TUNEL assay results (Fig. 3, G–J).

To determine whether levetiracetam can exacerbate phenytoin-induced cell death, rat pups were given both drugs in combination. Even with a relatively high dose of levetiracetam (500 mg/kg), no change in phenytoin-induced cell death was observed (Fig. 3).

**Effects of Levetiracetam Combined with Carbamazepine on Neuronal Cell Death.** The combination of levetiracetam (250 mg/kg) and carbamazepine (50 mg/kg) caused no change in cell death in any of the brain areas examined. However, a higher dose of levetiracetam (500 mg/kg) combined with 50 mg/kg carbamazepine caused an increase in cell death limited to thalamus (Fig. 4).

The fact that 500 mg/kg levetiracetam interacted with carbamazepine to cause cell death in thalamus, whereas the same dose did not enhance phenytoin-induced cell death, suggested that the interactive effect of levetiracetam may depend upon the mechanism of action of the drug with which it is combined. To further explore this, we examined the combination of levetiracetam with the noncompetitive N-methyl-D-aspartate receptor antagonist, MK801 (0.5 mg/kg).

### TABLE 1

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>vm thal</th>
<th>dl thal</th>
<th>dm stri</th>
<th>rsvp ctx</th>
<th>Frontal ctx</th>
<th>Parietal ctx</th>
<th>Rhinal ctx</th>
<th>Hypothalamus</th>
<th>Hippocampus (DG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>50</td>
<td>*</td>
<td>$\sqrt{\cdot}$</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
</tr>
<tr>
<td>Valproate</td>
<td>400</td>
<td>*</td>
<td>$\sqrt{\cdot}$</td>
<td>(\sqrt{\cdot})</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>75</td>
<td>*</td>
<td>*</td>
<td>(\text{\textbackslash}V)</td>
<td>$\sqrt{\cdot}$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
</tr>
<tr>
<td>MK801</td>
<td>0.5</td>
<td>*</td>
<td>$\sqrt{\cdot}$</td>
<td>$\sqrt{\cdot}$</td>
<td>$\sqrt{\cdot}$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>25–50</td>
<td>*</td>
<td>*</td>
<td>(\text{\textbackslash}V)</td>
<td>$\sqrt{\cdot}$</td>
<td>$\sqrt{\cdot}$</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>100</td>
<td>*</td>
<td>*</td>
<td>$\sqrt{\cdot}$</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Topiramate</td>
<td>20–80</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(\text{\textbackslash}V)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>250–1000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

vm thal, ventromedial thalamus; dl thal, dorsolateral thalamus; dm stri, dorsomedial striatum; rsvp ctx, retrosplenial cortex; frontal ctx, frontal cortex; parietal ctx, parietal cortex; rhinal ctx, rhinal cortex; DG, dentate gyrus.

$^a$ The majority of cell death was observed in layer II.

$^b$ The majority of cell death was observed in granular layer and polymorph layer of dentate gyrus.
TABLE 2
Brain and body weight at 24 h after drug treatment

Values are expressed as mean ± S.E.M. Δ Body weight = body weight at time of sacrifice minus body weight at time of drug (or vehicle) injection. The n number of vehicle and phenytoin groups was derived from total number of animals across several different experimental sets. In the case of topiramate, levetiracetam, and drug combination groups, only the maximum dose groups have been shown; lower doses induced less change in normal growth.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Δ Body Weight for 24 h</th>
<th>Brain/Body Weight Ratio (Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (n = 36)</td>
<td>0</td>
<td>2.22 ± 0.09</td>
<td>4.79 ± 0.10</td>
</tr>
<tr>
<td>Phenytoin (n = 25)</td>
<td>50</td>
<td>1.02 ± 0.12*</td>
<td>5.04 ± 0.10</td>
</tr>
<tr>
<td>Valproate (n = 8)</td>
<td>400</td>
<td>−0.20 ± 0.43*</td>
<td>5.13 ± 0.17</td>
</tr>
<tr>
<td>Phenobarbital (n = 10)</td>
<td>75</td>
<td>0.48 ± 0.21*</td>
<td>5.30 ± 0.11</td>
</tr>
<tr>
<td>MK801 (n = 10)</td>
<td>0.5</td>
<td>0.03 ± 0.26*</td>
<td>5.21 ± 0.16</td>
</tr>
<tr>
<td>Carbamazepine (n = 14)</td>
<td>50</td>
<td>1.85 ± 0.12</td>
<td>4.57 ± 0.09</td>
</tr>
<tr>
<td>Carbamazepine (n = 10)</td>
<td>100</td>
<td>0.72 ± 0.23*</td>
<td>5.19 ± 0.12</td>
</tr>
<tr>
<td>Topiramate (n = 12)</td>
<td>80</td>
<td>1.62 ± 0.23</td>
<td>5.01 ± 0.30</td>
</tr>
<tr>
<td>Levetiracetam (n = 6)</td>
<td>1000</td>
<td>2.13 ± 0.26</td>
<td>4.50 ± 0.07</td>
</tr>
<tr>
<td>Carbamazepine + phenytoin (n = 10)</td>
<td>50 + 50</td>
<td>0.78 ± 0.21*</td>
<td>4.84 ± 0.09</td>
</tr>
<tr>
<td>Topiramate + phenytoin (n = 8)</td>
<td>80 + 50</td>
<td>0.55 ± 0.18*</td>
<td>4.91 ± 0.33</td>
</tr>
<tr>
<td>Levetiracetam + phenytoin (n = 8)</td>
<td>500 + 50</td>
<td>0.91 ± 0.24*</td>
<td>4.86 ± 0.17</td>
</tr>
<tr>
<td>Levetiracetam + carbamazepine (n = 9)</td>
<td>500 + 50</td>
<td>1.64 ± 0.16</td>
<td>4.38 ± 0.08</td>
</tr>
</tbody>
</table>

* Significantly different from control, p < 0.05.

Fig. 1. Cell death as indicated by TUNEL-positive cells in ventromedial thalamus (A), dorsolateral thalamus (B), dorsomedial striatum (C), and frontal cortex (D) in P8 rat pups treated with vehicle (VEH), carbamazepine (CBZ; 25, 50, and 100 mg/kg i.p.) alone, or the combination of carbamazepine (25, 50 mg/kg) with phenytoin (50 mg/kg i.p.). Values are expressed as mean ± S.E.M. in 1.0 mm² per tissue section (n = 6–14). *, p < 0.05; significantly different from vehicle-treated group. Lower than 50 mg/kg carbamazepine did not induce apoptotic cell death in any of multiple brain regions examined in addition to those shown. In contrast, 100 mg/kg carbamazepine significantly increased cell death in thalamus area. †, p < 0.05; 50 mg/kg carbamazepine significantly exacerbated phenytoin-induced cell death compared with phenytoin alone. ‡, p < 0.05; 50 mg/kg carbamazepine plus 50 mg/kg phenytoin induced significantly more cell death than 100 mg/kg carbamazepine alone. E, area for the cell counting; F to K, photomicrographs of TUNEL-stained section in ventromedial thalamus area. F, vehicle-treated control. G, phenytoin (50 mg/kg). H, carbamazepine (50 mg/kg). I, carbamazepine (100 mg/kg). J, phenytoin (50 mg/kg) + carbamazepine (25 mg/kg). K, phenytoin (50 mg/kg) + carbamazepine (50 mg/kg). Scale, 100 μm.
Levetiracetam up to 500 mg/kg neither exacerbated nor reduced the extent of cell death induced by MK801 (MK801 alone versus MK801/levetiracetam, 104.7 ± 6.6 versus 116.8 ± 10.1 in ventromedial thalamus; 75.3 ± 5.6 versus 77.8 ± 9.3 in dorsolateral thalamus; 74.5 ± 6.9 versus 78.6 ± 10.1 in dorsomedial striatum; 58.7 ± 6.2 versus 54.0 ± 7.1 in frontal cortex; units = TUNEL-positive cells per millimeter squared, $n = 8$).

**Discussion**

Our results demonstrate that compared with most traditional AEDs, carbamazepine, topiramate, and levetiracetam are relatively devoid of proapoptotic actions in the developing brain. However, despite the comparable safety of these drugs when given alone, they differed in their interactions with phenytoin. Although both topiramate and carbamazepine exacerbated phenytoin-induced cell death, levetiracetam did not. Thus, neurodevelopmental toxicity assessments should evaluate drug combinations, especially in view of the prevalence of polytherapy in seizure management during pregnancy (Meador, 2004). At the same time, our data suggest that the relatively low threshold for the proapoptotic action of phenytoin (20 mg/kg) (Bittigau et al., 2002) must be associated with a mechanism not shared with carbamazepine. To the extent that carbamazepine and phenytoin share common mechanisms of seizure control (e.g., actions at voltage-gated sodium channels), the proapoptotic effect may be unrelated to these therapeutic mechanisms. Further support for distinct mechanisms of carbamazepine and phenytoin is the observation that a very high dose (500 mg/kg) of levetiracetam selectively enhanced carbamazepine-induced cell death in thalamus but had no effect on phenytoin-induced cell death.

When combined with phenytoin, however, a therapeutic dose of carbamazepine (50 mg/kg) exacerbated the phenytoin-induced cell death, more than tripling the neurotoxic effect in thalamus. This is likely to reflect pharmacodynamic inter-
actions because carbamazepine does not alter phenytoin levels acutely (Morris et al., 1987). This combination caused more widespread cell death than achieved with 100 mg/kg carbamazepine alone, involving striatum and frontal cortex, regions exhibiting no significant cell death following phenytoin or carbamazepine alone. In this case, the damage caused by combining two different drugs in moderate doses was more severe and pervasive than that caused by a higher dose of a single agent. This reinforces previous reports questioning the benefit of combining these drugs (Morris et al., 1987).

Topiramate alone did not induce neurodegeneration, even in a dose of 80 mg/kg; this is in contrast to Wistar rats in which this dose was proapoptotic (Glier et al., 2004). Interestingly, chronic treatment with 80 mg/kg topiramate in Sprague-Dawley rat pups did not cause long-term histological or behavioral changes (Cha et al., 2002). However, as with carbamazepine, topiramate significantly exacerbated phenytoin-induced cell death; as little as 20 mg/kg topiramate provoked significant cell death in regions unaffected by phenytoin alone. In this case, moderate doses of two drugs in combination produced toxicity not obtained with a 4-fold higher dose of one drug. This is probably a pharmacodynamic interaction because topiramate does not acutely alter levels of phenytoin, although it can potentiate its anticonvulsant action (Swiader et al., 2000).

Our data indicate that levetiracetam alone, even in doses above therapeutic, does not induce cell death in the developing rat brain, nor did it add to, or synergize with, other drugs. Thus, the therapeutic index of levetiracetam in this context is greater than 5-fold and possibly as high as 10-fold. Moreover, even a high dose of 500 mg/kg levetiracetam did not exacerbate the cell death induced by phenytoin or MK801. Thus, this is the first AED identified as devoid of a cell death-promoting action when administered in combination with phenytoin.

Our observations that carbamazepine is unique among traditional AEDs for its lack of proapoptotic actions in the therapeutic dose range and that levetiracetam is unique in its lack of potentiation of phenytoin-induced cell death prompted us to examine the combination of these drugs. The combination of carbamazepine and levetiracetam, even in high doses (50 and 250 mg/kg, respectively), did not cause cell death in any brain area examined. Thus, this combination minimizes the risk for enhancing developmental apoptosis, at the same time that it may enhance therapeutic efficacy and allow a reduction in the dose of carbamazepine (Sisodiya et al., 2002; Fountain et al., 2007).

Our data suggest that sedation is dissociable from the induction of cell death in the immature brain. Although 50 mg/kg carbamazepine induced sedation, it did not induce cell death.
Fig. 4. Cell death as indicated by TUNEL-positive cells in ventromedial thalamus (A), dorsolateral thalamus (B), dorsomedial striatum (C), and frontal cortex (D) in PD8 rat pups treated with VEH or the combination of levetiracetam (250 and 500 mg/kg) with carbamazepine (50 mg/kg i.p.). Values are expressed as mean ± S.E.M. in 1.0 mm^2 per tissue section (n = 6–9). The combination of 250 mg/kg levetiracetam with 50 mg/kg carbamazepine did not cause any cell death in all brain regions examined. However, the combination of 500 mg/kg levetiracetam with 50 mg/kg carbamazepine slightly but significantly increased cell death in the ventromedial thalamus area, although doses of both drugs are subthreshold for causing cell death when given alone. *p < 0.05; significantly different from vehicle-treated group. †, p < 0.05; levetiracetam (500 mg/kg) significantly increased cell death compared with carbamazepine (50 mg/kg) alone. Photomicrographs of TUNEL-stained section in ventromedial thalamus area (E and F). E, carbamazepine (50 mg/kg) + levetiracetam (250 mg/kg). F, carbamazepine (50 mg/kg) + levetiracetam (500 mg/kg). Scale, 100 μm.

dearth, whereas 50 mg/kg phenytoin did not affect behavior but induced cell death. Topiramate markedly exacerbated phenytoin-induced cell death, but the combination was nontoxic. Likewise, previous studies found valproate to cause cell death at a dose (50 mg/kg) below that required for either anticonvulsant or behavioral effects (Polasek et al., 1996; Bittigau et al., 2002). Because nutritional and maternal deprivation in the absence of drug treatment was not sufficient to cause neuronal death in rat pups (Olney et al., 2004), the neuronal death is not merely a secondary consequence of drug-induced sedation or malnutrition.

Our results indicate that a proapoptotic action in the neonatal brain is not common to all antiepileptic medications, nor is the mechanism coupled to the antiepileptic mechanism of action of the proapoptotic AEDs. Furthermore, the threshold for potentiating the effect of phenytoin is not predictably related to the threshold for inducing cell death when given alone, as revealed by the comparison between carbamazepine and topiramate. Finally, the extent to which a drug potentiates the effect of phenytoin varies considerably across drugs and brain areas (Fig. 5), raising the possibility that the mechanisms may be cell population-specific.

Our findings of neurotoxicity with combinations of drugs that alone were devoid of proapoptotic actions indicate that the practice of adding on medications can introduce adverse consequences unanticipated by the safety profile of a single agent. This problem was especially striking with topiramate and carbamazepine. Levetiracetam was the only drug that was relatively devoid of a proapoptotic action even when combined with other antiepileptic drugs.

Our study has focused on one stage of development, corresponding to the late prenatal/neonatal period in humans (Bittigau et al., 2002). Furthermore, within this period, our experiments consistently have utilized a single age, the PD7 to 8 rat pup. This time point was selected because it is maximally sensitive to the proapoptotic action of AEDs (Bittigau et al., 2002). Introduction of AEDs at earlier or later ages has considerably less impact, with PD14 (mid- to late infancy in the human) representing the end of this critical period of vulnerability (Bittigau et al., 2002). From a clinical standpoint, this means that some drug-induced neurotoxicity could be avoided during infancy by delaying the introduction of certain AEDs until after the vulnerable period. Knowing which drugs are most appropriate and safest to use at different stages of development will allow optimal seizure control with minimal impairment of neuronal maturation.

Levetiracetam has many potential advantages for the treatment of epilepsy in early childhood. It is a broad-spectrum AED with high oral bioavailability, low plasma protein binding, linear kinetics, and almost no drug interaction with other agents. Its potential to avoid proapoptotic effects by choosing appropriate doses and drug combinations.

Persistent behavioral and cognitive dysfunction results from acute and chronic exposure to phenytoin (Ogura et al., 2002), alcohol (Ieraci and Herrera, 2006), anesthetic agents (Jevtovic-Todorovic et al., 2003), or N-methyl-D-aspartate receptor antagonists such as MK801 (Harris et al., 2003); these agents promote developmental apoptosis in several species including nonhuman primates (Farber et al., 2005). Thus, drug-induced developmental cell death may contribute to long-term adverse effects, and its prevention may mitigate adverse behavioral outcomes (Ieraci and Herrera, 2006). Conversely, AEDs that do not induce significant developmental neuronal death seem to have negligible behavioral impact. Topiramate does not seem to have long-term deleterious effects on cognition when given chronically to rat pups (Cha...
et al., 2002), whereas carbamazepine monotherapy during pregnancy has been found to be relatively safe in terms of cognitive outcomes in the offspring (Meador, 2004). Moreover, levetiracetam seems to be well tolerated, with relatively little adverse effects (Hovinga, 2001; Coppola et al., 2004). To establish the extent to which excessive drug-induced developmental apoptosis is a risk factor predictive of adverse behavioral outcomes in the clinical setting, additional preclinical and clinical studies are warranted. Our results may have implications that extend to immature neurons in the adult brain because newly generated adult dentate gyrus neurons have been found susceptible to the proapoptotic actions of ethanol (Herrera et al., 2003); it remains to be determined whether these neurons are similarly vulnerable to AEDs and/or to AED combinations.

Although the profile of neurotoxicity in humans is likely to have anatomical and temporal features distinct from that in rodents, the experimental results indicate a need for caution in the use of certain drugs during the perinatal period. At the same time, the induction of excessive neuronal cell death is only one of a host of deleterious actions that AEDs can exert on the developing brain. For example, aberrant synaptogenesis in the absence of cell death can have severe long-term consequences (Holmes et al., 2002). Thus, although AEDs devoid of proapoptotic actions show promise for use in pregnancy and during the early postnatal period, screening for and evaluation of other types of neurotoxicity is needed before concluding that a compound is safe for the immature brain.

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References

Fig. 5. Net increase in TUNEL-positive cells over treatment with phenytoin alone in ventromedial thalamus (A), dorsolateral thalamus (B), dorsomedial striatum (C), and frontal cortex (D) in PDS rat pups treated with carbamazepine (CBZ; 25, 50 mg/kg), levetiracetam (LEV; 500 mg/kg), or topiramate (TPM; 20, 40, 80 mg/kg) combined with phenytoin (50 mg/kg). Both carbamazepine and topiramate exacerbated the cell death induced by phenytoin. In contrast, levetiracetam did not significantly enhance phenytoin-induced cell death. +, p < 0.05; significantly different from phenytoin-only treated group.