A KCNQ Channel Opener for Experimental Neonatal Seizures and Status Epilepticus

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Objective: Neonatal seizures occur frequently, are often refractory to anticonvulsants, and are associated with considerable morbidity and mortality. Genetic and electrophysiological evidence indicates that KCNQ voltage-gated potassium channels are critical regulators of neonatal brain excitability. This study tests the hypothesis that selective openers of KCNQ channels may be effective for treatment of neonatal seizures.

Methods: We induced seizures in postnatal day 10 rats with either kainic acid or flurothyl. We measured seizure activity using quantified behavioral rating and electrocorticography. We compared the efficacy of flupirtine, a selective KCNQ channel opener, with phenobarbital and diazepam, two drugs in current use for neonatal seizures.

Results: Unlike phenobarbital or diazepam, flupirtine prevented animals from experiencing development of status epilepticus when administered before kainate. In the flurothyl model, phenobarbital and diazepam increased latency to seizure onset, but flupirtine completely prevented seizures throughout the experiment. Flupirtine was also effective in arresting electrographic and behavioral seizures when administered after animals had developed continuous kainate-induced status epilepticus. Flupirtine caused dose-related sedation and suppressed electroencephalographic activity but did not result in respiratory suppression or result in any mortality.

Interpretation: Flupirtine appears more effective than either of two commonly used antiepileptic drugs, phenobarbital and diazepam, in preventing and suppressing seizures in both the kainic acid and flurothyl models of symptomatic neonatal seizures. KCNQ channel openers merit further study as potential treatments for seizures in infants and children.

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Epileptic seizures occur commonly in the first days after birth.[1] Such neonatal seizures are usually symptomatic, arising as a result of developmental abnormalities, in utero injuries, perinatal hypoxia-ischemia, infection, and other causes. Patients experiencing neonatal seizures are at substantial risk for mortality and long-term morbidity, including static encephalopathy, cerebral palsy, and chronic epilepsy.[2,3] The first-line drugs given to neonates, phenobarbital and phenytoin, are effective in less than 50% of cases.[4] Moreover, phenobarbital, benzodiazepines, and phenytoin have been shown to cause widespread neuronal apoptosis when given to young rodents, raising concerns about administration of these drugs in human infants.[5–7] The need for new treatments for neonatal seizures that are both safer and more effective has been widely acknowledged.[1,7,8]

Genetic, physiological, and pharmacological studies call attention to KCNQ potassium channels (also termed Kv7 and M-channels) as potential molecular targets for treatment of neonatal seizures.[9] Loss-of-function mutations in the *KCNQ2* and *KCNQ3* genes cause benign familial neonatal seizures (BFNSs), an uncommon but highly penetrant, dominantly inherited syndrome characterized by seizures that recur frequently over the first weeks of life.[10–12] Experimental inhibition of KCNQ channels in rodents, by pharmacological and genetic methods, dramatically promotes neuronal and network hyperexcitability and seizures in neonatal animals and in slices from neonatal brain; these effects diminish or disappear progressively with maturation.[13–17] KCNQ2 and KCNQ3 are highly expressed on both myelinated and unmyelinated axons, and are strictly colocalized with clusters of voltage-gated sodium channels in the proximal axon (where action potentials initiate) and at the nodes of Ran-

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vier.[14,17–19] It has been proposed that this strategic localization at the exact sites where action potentials arise and are conducted allows KCNQ channels to exert strong control over neuronal firing.[14,17,18]

Selective neuronal KCNQ channel openers have been identified among drugs already in use for humans, including diclofenac (a widely used antiinflammatory agent), flupirtine (a nonopioid analgesic in approved use in Europe), and retigabine (which is currently in phase 3 clinical trials for adult partial epilepsy in the United States and Europe).[20–22] Additional potent and selective KCNQ channel openers have recently been discovered through directed high-throughput screening and have begun to be subjected to preclinical testing for adult epilepsy and pain.[23–25] KCNQ openers increase channel activity significantly above normal levels by speeding opening rates, slowing closing rates, and reducing the membrane depolarization required for activation. Flupirtine and retigabine are close structural analogues (Fig 1). Flupirtine and retigabine have been shown to exhibit neuroprotective activity in several experimental systems, including models of infantile neuronal ceroid lipofuscinosis.[26–28] Remarkably, KCNQ channel opener compounds have not been evaluated as a potential treatment for neonatal seizures or status epilepticus (SE). The human KCNQ2 and KCNQ3 channel loss-of-function phenotype, BFNS, suggests the channels are present, active, and important during the neonatal period. Thus, augmenting KCNQ channel activity is a rational strategy for safe suppression of hyperexcitability during the neonatal period.

In early trials using adult rodent models and adult human patients, flupirtine showed evidence of antiepileptic efficacy.[29] Here, we have tested the utility of flupirtine for treatment of neonatal seizures and SE. We have compared flupirtine with two drugs widely used for neonatal seizures, phenobarbital and diazepam. In two well-established models, using the glutamate receptor agonist kainic acid or the convulsant gas flurothyl, flupirtine appeared more effective than either standard agent in preventing the onset of seizures in rat pups. In the kainic acid model, which produces seizures that evolve into SE lasting hours, flupirtine was more effective than either standard agent in diminishing the severity of electrographic and behavioral seizures. Indeed, flupirtine was effective even when its administration was withheld until after kainate-induced seizure activity had progressed to continuous SE. These findings indicate that KCNQ channel opener therapy for neonatal seizures deserves further investigation.

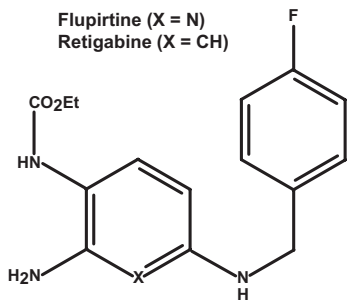


Fig 1. Flupirtine and retigabine are close analogues. X *indicates the position varied in the two compounds; substitutions are as indicated.*

Materials and Methods

Chemicals and Drugs

Kainic acid (Sigma, St. Louis, MO) stocks dissolved in water were stored at −20° C; aliquots were diluted in normal saline solution immediately before use. Flupirtine maleate (Sigma) was freshly dissolved in 1:2 (vol/vol) mixture of dimethylsulfoxide and saline solution on the day of use. Solutions of diazepam (Hospira, Lake Forest, IL) and phenobarbital (Baxter, Deerfield, IL) for injection were purchased from the pharmacy of the Hospital of the University of Pennsylvania and, where needed, serially diluted in either dimethylsulfoxide/saline 3:7 (vol/vol, diazepam) or saline (phenobarbital). All drugs were administered by intraperitoneal (IP) injection. All experiments were performed in parallel on littermates randomly assigned to receive vehicle or one or more therapeutic drugs, in equal volumes.

Status Epilepticus Induction

The Institutional Animal Care and Use Committees of the University of Pennsylvania and Children's Hospital of Philadelphia approved all procedures used. Experimental design emphasized use of a minimal number of animals and minimization of potential discomfort. Animals were warmed under an incandescent lamp during all experimental procedures to maintain body temperature. Pregnant Sprague–Dawley rats were obtained (Charles River laboratory, Kingston, PA) and allowed to give birth. Postnatal day 10 (P10) pups of both sexes were removed from home cages, weighed, placed in an observation cage, given IP injections of kainic acid, and visually monitored for seizure activity by an observer blinded to treatment group. In preliminary experiments, 2mg/kg IP kainic acid consistently induced seizures that progressed to SE in more than 90% of the rat pups within 40 to 50 minutes with a less than 10% mortality rate.

Seizure Induction Using Flurothyl

Flurothyl-induced seizure latencies were measured using procedures previously described.[30,31] P10 rat pups were placed in an air-tight cylindrical plastic chamber (radius, 7.5cm; height, 18cm) within a fume hood. Using a syringe pump (Harvard Apparatus, Holliston, MA), we introduced 50μl/min of the volatile liquid 2,2,2-trifluoroethyl ether (flurothyl; Sigma) into the chamber. Latency to development of forelimb and hind-limb tonic extension was measured by an observer blinded to treatment group.

Electrocorticography

Brain electrical activity was monitored using bilateral screw electrodes placed over the motor cortices.[32] P10 rats were anesthetized with isoflurane. A screw electrode behind the lambda served as ground and reference. To reduce noise contamination, we directed electroencephalographic (EEG) signals to miniature preamplifiers glued with dental adhesive to the skull, powered through the EEG cable. Pups were allowed to recover from surgery and anesthesia for 1 hour. EEG was recorded using a custom-designed 16-channel EEG machine (ALA Scientific Instruments, Westbury, NY), and house-written software was generated using MatLab (MathWorks, Natick, MA). Baseline EEG was recorded for approximately 30 minutes before kainic acid injection. The onset of stage 5 behavioral seizures was well correlated with EEG showing continuous seizure activity (see Results). Antiepileptic drug or vehicle-only control solutions were injected 15 minutes after the onset of stage 5 seizures, determined by both behavioral and electrographic criteria (see later). The EEG was recorded for a minimum of 2 hours after the drug or vehicle treatment, except in uncommon cases where the animal died during SE.

Power and Power Spectrum Analysis

EEG data were digitized at 250Hz. Power calculation was performed and graphically displayed using MatLab software. For analysis of total power, the EEG was filtered using 5Hz high-pass and 60Hz low-pass filters to remove ambient noise, binned in 1-minute intervals, and power within that minute was determined. For spectral analysis, 1-minute periods of EEG data were subjected to a fast Fourier transform algorithm.

Statistical Analysis

Fisher's exact test and Kaplan–Meier and Maltel–Cox log-rank analyses were performed using MedCalc (version 9.5.2.0, www.medcalc.be). To compare the ability of test drugs to suppress EEG power, we first computed the average power within 2-minute time windows (± standard errors) for each treatment condition. Testing for significance of differences in EEG power between groups was performed using a custom-designed Multicomparison for Correlated Repeated Measures test (Javier Echauz, Atlanta, GA), described further in the online supplementary materials. This test accommodates appropriately for multiple simultaneous comparisons and inclusion of partially correlated data acquired during time series using a permutation test.[33] Although this test results in greater estimated p values than classic analysis of variance (ANOVA), it is more appropriate for analysis of our data set because, unlike ANOVA, it is not dependent on assumptions of normality and independence.

Results

Flupirtine, But Not Phenobarbital or Diazepam, Prevents Progression to Continuous Status Epilepticus after Kainic Acid

To model symptomatic human neonatal seizures, we induced seizures in rats at P10, an age established by numerous previous studies as comparable with the human neonatal period.[34] Kainic acid injection caused behavioral seizures that progressively led to SE. A behavioral rating scale, modified from previous work,[35] was used to quantify responses to kainic acid. At stage 1 onset, pups ceased normal exploratory behavior and remained immobile; at stage 2, frequent, sustained hindlimb scratching began; at stage 3, episodes of tonic extension of one side resulted in loss of balance; at stage 4, animals completely lost balance resulting in falling onto their backs, but with recovery; and at stage 5, continuous clonic seizures involving all limbs and persistent loss of righting were present. As illustrated (Fig 2), animals passed predictably through this series of steps, but the rate at which animals progressed showed considerable variability, especially at later stages (ie, stage 5 latency [± standard deviation] was 39.5 ± 12 minutes).

To identify dose–response relations for seizure prevention by flupirtine, phenobarbital, and diazepam, we used a pretreatment protocol. Animals received one of the three antiepileptic drugs, at various doses. After 15 minutes, animals received 2mg/kg kainic acid and were observed to determine whether the drugs delayed or prevented the development of seizure behaviors. In each experimental trial, littermate control animals received vehicle injections, followed by kainic acid. All three drugs showed evidence of sedative effects during the 15-minute wash-in period, manifested as dose-related reduction in spontaneous and stimulation-induced motor activity and hypotonia. At the highest doses tested, all three drugs resulted in essentially complete immobility within 10 minutes, that is, before kainate administration. Because of these dose-related sedative effects, we used kainate-induced progression to seizure stage 5, a robust behavioral end point, for comparison between drug treatment groups (Fig 3). As expected, all control rats (n = 12) given vehicle followed by kainic acid showed signs of seizures, and 91% progressed to stage 5 (SE; see Figs 3A–C). Phenobarbital, at doses up to 100mg/kg, was ineffective in preventing the development of SE (see Fig 3B). The serum concentrations of phenobarbital, 30 minutes after IP administration of 25 and 50mg/kg, were 20.6 ± 2.8 (standard error of the mean, n = 3) and 38.4 ± 2.2μg/ml (standard error of the mean, n = 4), respectively, indicating that the upper clinical therapeutic range was attained. Diazepam pretreatment at doses as high as 16mg/kg was also ineffective at preventing progression of kainic acid–induced seizures to SE (see Fig 3C). Unlike the standard agents, flupirtine suppressed kainic acid–induced seizures in P10 rats potently and dose-dependently. Indeed, none of the young rats that received 50mg/kg flupirtine (n = 7) experienced development of stage 5 seizures (see Fig 3A) within the 2-hour observation period, and all flupirtine-treated animals survived. Despite the small group size of these dose-finding experiments, flupirtine was significantly

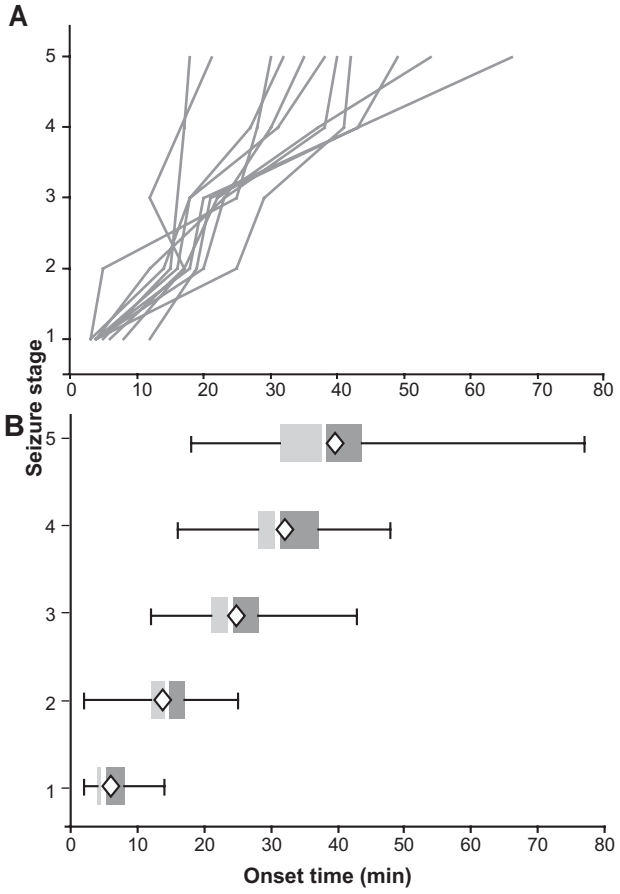


Fig 2. Seizure manifestations develop predictably in postnatal day 10 (P10) rats after kainic acid. Animal behaviors were monitored after injection of 2mg/kg kainic acid intraperitoneally. The time from injection until first onset of behavioral immobility (stage 1), episodes of rapid hind-limb scratching (stage 2), unilateral falling caused by loss of motor control and extensor posturing (stage 3), bilateral loss of motor and postural control with posturing (stage 4), and sustained, irreversible loss of motor control with tonic and tonic-clonic activity (stage 5) were noted. (A) Representative time courses for 11 animals show that animals progressed similarly through seizure stages, but latencies differed. (B) Summary of results for 49 animals. At each seizure stage, diamonds *indicate mean latency,* light *and* dark boxes *indicate second and third quartiles, respectively, and* bars *indicate maximal and minimal latencies observed.*

better than vehicle ($p = 0.004$) or either of the two approved drugs ($p = 0.003$) in prevention of progression to stage 5 seizures (Fisher's exact test).

Flupirtine Is More Effective Than Phenobarbital or Diazepam in Preventing Flurothyl-Induced Seizures

Inhalation of flurothyl vapors is potently convulsant, and has been used as a model of recurrent seizures in neonates and for quantification of seizure susceptibility changes resulting from SE and brain injury.[30,36,37] To assess the efficacy of flupirtine in a second neonatal seizure model, we pretreated P10 rats with high doses of either flupirtine, phenobarbital, or diazepam, then monitored the time between first flurothyl exposure and development of tonic limb extension seizures (Fig 4). Control rats not given any antiepileptic drug devel-

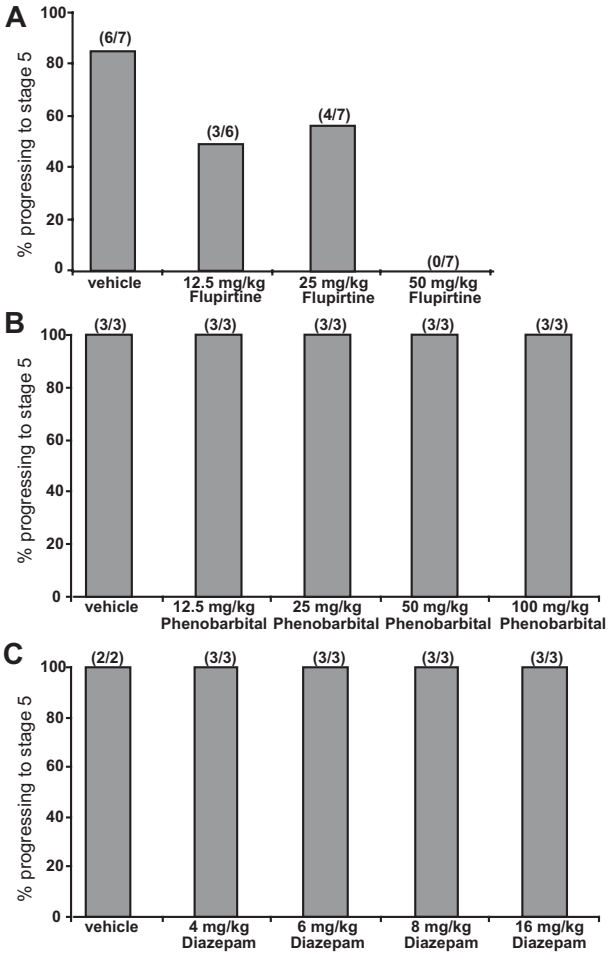


Fig 3. Pretreatment with flupirtine, but not phenobarbital or diazepam, prevents the progression to stage 5 seizures after kainic acid. Animals were injected either with vehicle or the indicated doses of flupirtine, phenobarbital, or diazepam. After 15 minutes, kainic acid was given (2mg/kg intraperitoneally), and animals were monitored for 120 minutes for evidence of behavioral seizures. Flupirtine pretreatment resulted in dose-dependent suppression of behavioral seizures, but phenobarbital and diazepam did not. Numbers above each bar indicate number of animals showing stage 5 seizures (numerator), and number of animals tested at the indicated dose (denominator). Height of bar indicates percentage of animals in each treatment group exhibiting stage 5 seizures (continuous clonic activity of all four limbs with loss of righting). The suppression of progression to stage 5 seizures by all doses of flupirtine pooled was statistically superior to control (p = 0.004) or either diazepam or phenobarbital (p = 0.003). The highest flupirtine dose (50mg/kg) group taken alone was also superior to control (p = 005) or to 50 to 100mg/kg phenobarbital or 8 to 16mg diazepam (p = 0.0005, Fisher's exact test).

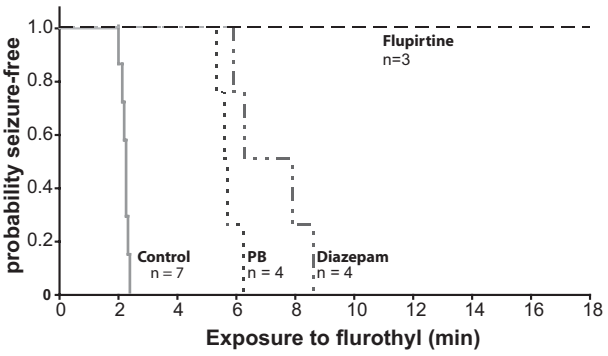


Fig 4. Flupirtine prevents development of flurothyl-induced seizures more effectively than phenobarbital (PB) or diazepam. Comparison of latency with first tonic limb extension seizure in control postnatal day 10 (P10) rats (n = 7) and rats pretreated 15 minutes before flurothyl exposure with phenobarbital (50mg/kg, n = 4), diazepam (8mg/kg, n = 4), or flupirtine (50mg/kg, n = 3). No flupirtine-treated animals experienced development of seizures during 18 minutes of exposure to flurothyl. Phenobarbital and diazepam increased the mean latency but did not fully prevent seizures.

oped tonic limb extension 2.22 ± 0.14 minutes (standard deviation, n = 7) after flurothyl exposure. Phenobarbital and diazepam delayed average latency to 5.67 ± 0.39 (standard deviation, n = 4) and 7.16 ± 1.28 minutes (standard deviation, n = 4), respectively. Rats given flupirtine (n = 3) failed to exhibit tonic limb extension during exposure to flurothyl for 18 minutes, the entire planned duration of the experiment. The observed ranked differences in seizure-free survival were strongly significant ($p < 0.0001$, log-rank test, Kaplan–Meier survival analysis).

Flupirtine Reverses Kainic Acid–Induced Status Epilepticus Rapidly and Persistently

Although pretreatment with antiepileptic drugs is routinely used in preclinical testing of experimental seizure therapies,[38,39] clinical treatment of neonatal seizures is nearly always begun after seizures occur and are diagnosed by clinical criteria, EEG, or both.[1] Arresting an established pattern of recurring seizures may require different mechanisms than preventing the initiation of such seizures. We therefore induced a hyperexcitable state with kainate, allowed seizures to worsen until stage 5 persisted for 15 minutes, and then administered experimental therapeutic agents. Kainic acid injection led to progressive appearance of ictal EEG changes, including predominant fast sharp rhythmic activity, which correlated with gradual worsening in seizure behavior (Fig 5A). Such electrographic and behavioral seizures persisted throughout the recording (approximately 2 hours) in the vehicle-treated animals (see Fig 5A). Flupirtine (50mg/kg) abolished both seizure behavior and associated EEG changes (see Fig 5B). These effects occurred rapidly: suppression of electrographic seizure activity by flupirtine began within the first minute after IP injection, was maximal by 5 to 8 minutes, and was sustained for the entire observation period. Flupirtine resulted in a suppressed background with isolated, large-amplitude, sharp transients (or less frequently, brief runs of sharp transients; see Fig 5B, insets d, e). Phenobarbital (50mg/kg) or diazepam (8mg/kg) also reduced EEG ictal activity and diminished behavioral seizures. Compared with flupirtine-treated animals, the suppression of ictal activity by phenobarbital appeared slightly slower in onset. Furthermore, all phenobarbital-treated animals manifested persistent, though reduced, clonic seizure behavior after treatment, and the EEG showed essentially continuous, fast sharp rhythmic activity after treatment (see Fig 5C). Inhibition of seizure activity by diazepam was rapid in onset, but behavioral seizures that correlated with electrographic activity returned prominently within about 30 minutes after treatment (see Fig 4D).

To allow quantitative comparisons between the drug responses, we determined the electrical power in EEGs of control and therapeutic drug–treated animals. Representative individual EEG power time courses are shown in Figure 6. Kainic acid–induced behavioral seizures and ictal EEG changes were correlated with progressive increases in EEG power, initially greatest at lower frequencies but spreading steadily to higher frequencies (see Figs 6A–D). Kainate-induced increases in EEG power peaked about 30 minutes after clinical and electrographic SE onset and, in control animals, remained high throughout the recording period (see Fig 6A). Inspection of plots of EEG power of individual animals exposed to the therapeutic drugs demonstrated characteristic differences in the responses to the three agents (see Figs 5B–D). Flupirtine caused a rapid and sustained reduction of EEG power to below baseline levels (see Fig 6B). Phenobarbital reduced total power to near baseline levels by 10 to 15 minutes after treatment, but spectral analysis demonstrated persistently increased power in the 15- to 30Hz range. Phenobarbital-treated animals also showed recurrent episodes of more greatly increased power that correlated with the breakthrough seizure activity seen in the EEG and behaviorally (see Fig 5C). Diazepam power analysis demonstrated both an initial favorable drug response and the partial reversal of this response by about 30 minutes after treatment (see Fig 6D). Thus, EEG power plots captured in a quantified form many important features of the electrographic and behavioral responses we observed to the therapeutic drugs.

Figure 7 shows average (± standard error of the mean) EEG power in 2-minute time intervals for groups of control, flupirtine-treated, phenobarbital-treated, and diazepam-treated rats. The three drug-

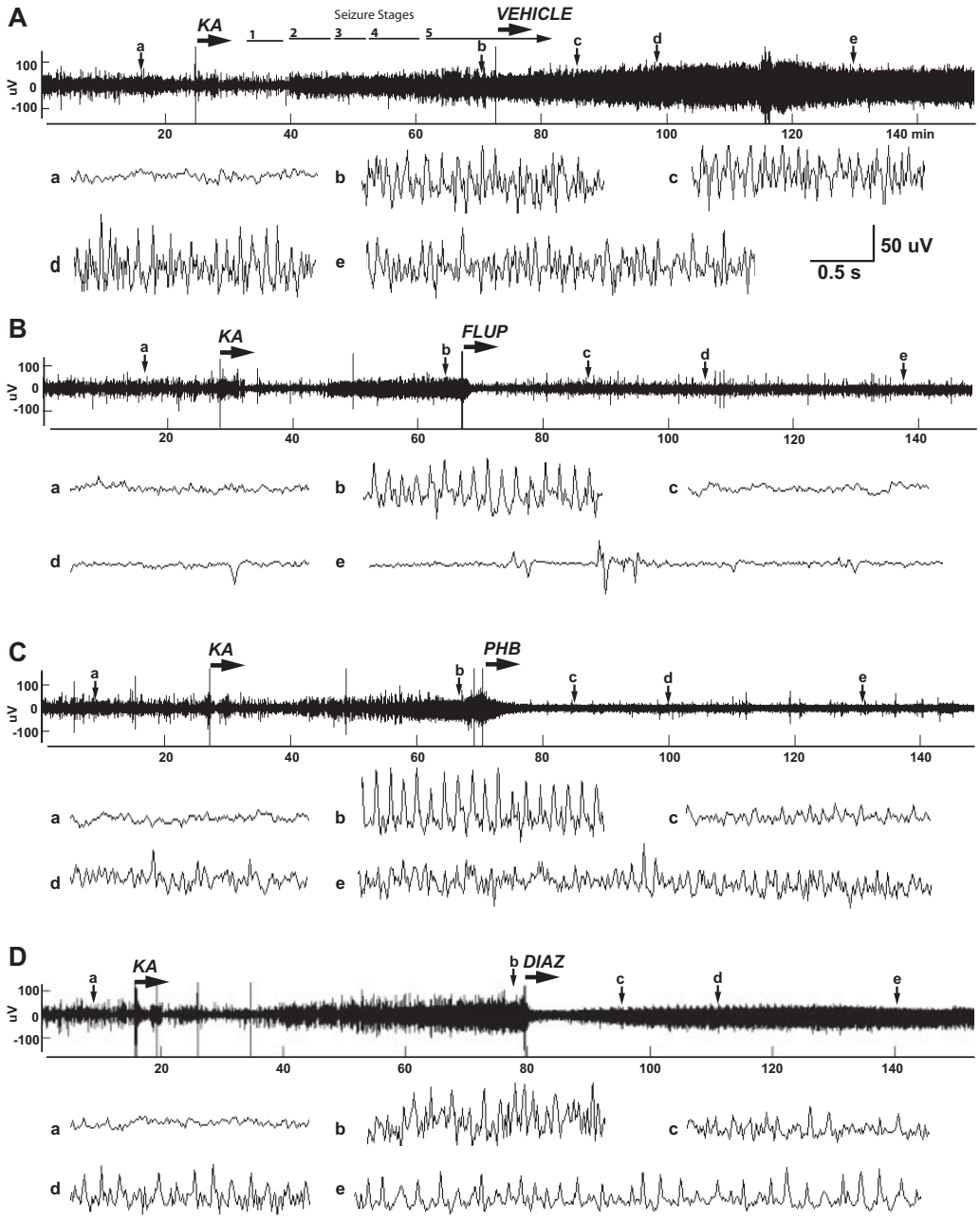


*Fig 5. Flupirtine stops kainic acid (KA) induced electrographic seizure activity more effectively than phenobarbital or diazepam. Rat pups were given KA to elicit seizures. Once stage 5 seizures were observed behaviorally and continuous electrographic seizure activity was seen for 15 minutes, either vehicle (A), 50mg/kg flupirtine (B), 50mg/kg phenobarbital (C), or 8mg/kg diazepam (D) was given. The duration of behavioral seizure stages 1 to 5 are indicated above the EEG trace in A. EEG traces are labeled indicating times of injection of KA and therapeutic drugs (flupirtine [FLUP], phenobarbital [PHB], and diazepam [DIAZ]). Below each continuous EEG trace, EEG excerpts are shown at higher time resolution (*a, *baseline;* b, *during stage 5 seizures before therapeutic drug treatment;* c–e, *15, 30, and 60 minutes, respectively, after therapeutic drug administration). Although all three drugs cause an initial reduction in electrographic seizure activity, the effects of flupirtine appeared more rapid in onset than phenobarbital, and more complete and sustained than those of phenobarbital or diazepam.*

treated groups differed in several respects: onset of the effects of flupirtine appeared more rapid than that of phenobarbital; and the effect of diazepam appeared biphasic, with an initial strong reduction and subsequent increase in average power. We developed a rigorous statistical approach for analyzing whether apparent differences among the four averaged EEG power time courses were statistically significant ($\alpha = 0.05$; see Ma-

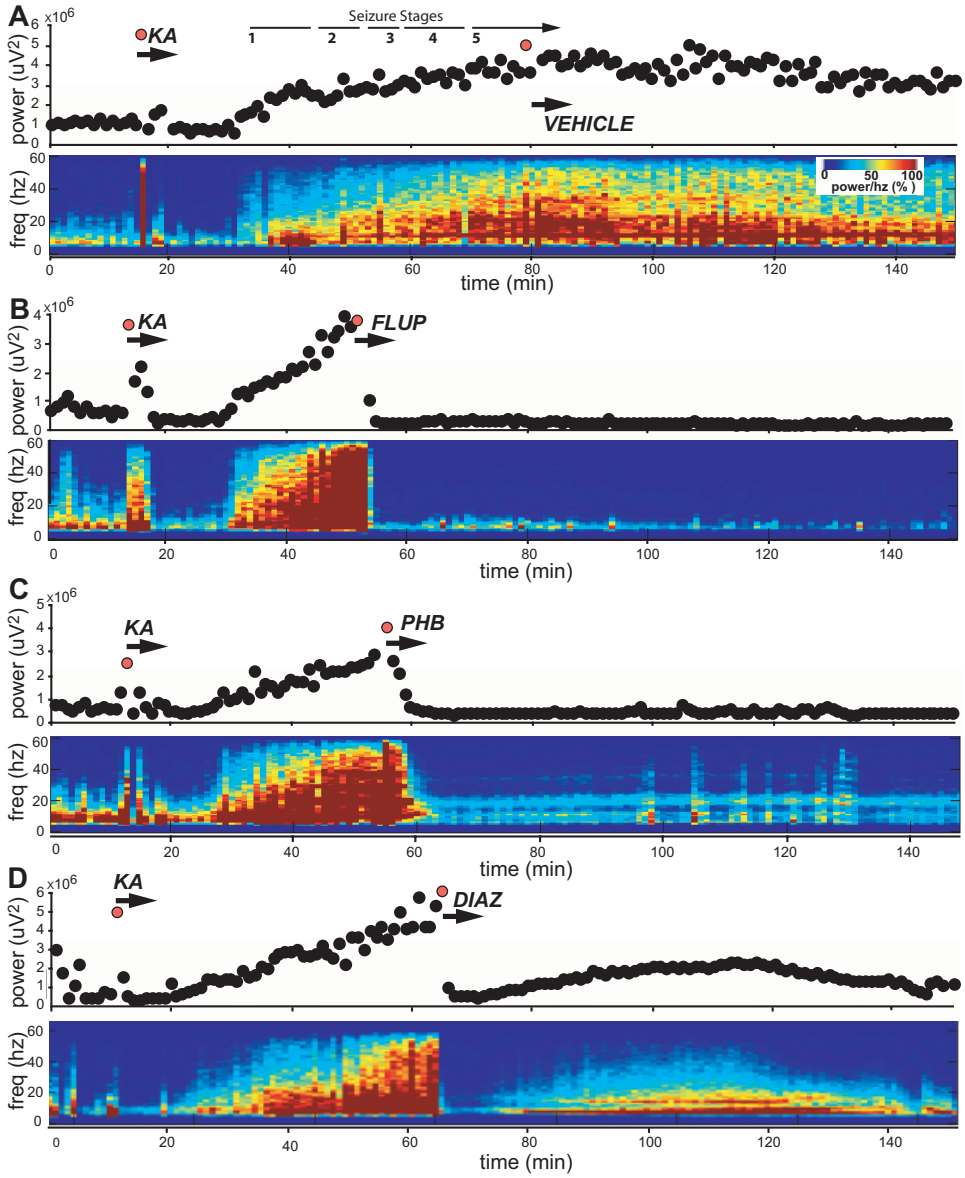
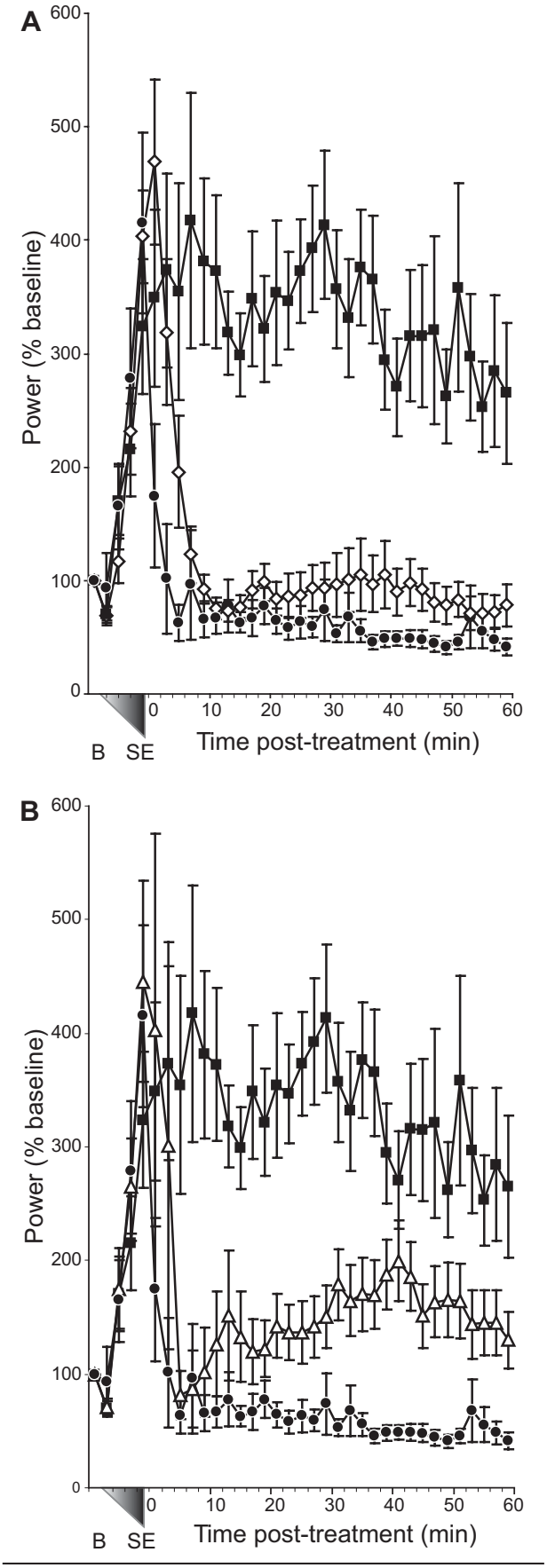


Fig 6. Kainate-induced increases in electroencephalographic (EEG) power parallel clinical seizure activity and are reversed by flupirtine, phenobarbital, or diazepam. (A) Control, (B) flupirtine (FLUP), (C) phenobarbital (PHB), and (D) diazepam (DIAZ). EEG data were analyzed for total power and power at various frequencies (see Materials and Methods). For each experimental condition, the top panel shows total EEG power within 1-minute intervals before and after the administration of kainic acid and the indicated therapeutic drug (or vehicle). Time points where EEG artifacts because of handling for injection are shown (red-filled circles). The bottom panels show power per minute within 1Hz frequency bins, calculated by Fourier transformation of the EEG and displayed in color according to the scale bar (inset in A). For each experimental condition, kainic acid (KA) treatment results in progressive increase in total power; in spectral plots, this is first detected at lower frequencies and later at higher frequencies. Note the rapid onset and sustained reduction in power after flupirtine administration. Phenobarbital shows a persistent band (light blue) of increased power at 15 to 30Hz. Diazepam is effective at power suppression in the first 20 minutes of treatment, but power increases prominently at later time points.

terials and Methods and supplementary material). We performed a global analysis (in which the entire time courses were compared) and also assessed the significance of differences detected at individual 2-minute time points after therapeutic drug treatment (see Supplementary Fig S1). The global analysis showed that each of the three therapeutic drugs significantly reduced EEG power compared with controls ($p < 0.025$). Although average EEG power after flupirtine treatment was less than after phenobarbital in 29 of 30 time bins (mean EEG power after flupirtine/mean after phenobarbital = 0.57), these differences did not



achieve statistical significance in the global analysis of the entire time course ($p = 0.3318$; see Discussion). However, EEG power after flupirtine was significantly less than after phenobarbital during the first 4 minutes of treatment ($p < 0.05$; see Supplementary Fig S1), supporting the assertion that flupirtine has a faster therapeutic onset than phenobarbital. In the entire time-course analysis, power after flupirtine treatment was significantly lower than after diazepam (see Fig 6B; $p = 0.0357$), but phenobarbital and diazepam did not differ significantly ($p = 0.3006$). Analysis of individual time windows showed that EEG power after diazepam was significantly less than control during the first 40 minutes after treatment but not during the period 40 to 60 minutes after treatment (see Supplementary Fig S1). This quantitative analysis of our grouped results supports the conclusion that, unlike either diazepam or phenobarbital, suppression of kainate-induced seizures by flupirtine is both rapid in onset and sustained.

Discussion

To assess the utility of flupirtine for neonatal seizures, we have used two animal models of induced seizures that have been extensively used in previous neonatal seizure studies. Either flurothyl or kainic acid robustly induced seizures in rats at P10, an age thought comparable in developmental maturity to human neonates. When give before seizure induction by kainic acid, flupirtine was significantly more effective than either diazepam or phenobarbital in preventing progression to SE (see Fig 3). Flupirtine was also significantly more effective than either approved drug in preventing seizure induction by flurothyl (see Fig 4). Even when administered after kainic acid seizures that were allowed

*Fig 7. Averaged kainate-induced electroencephalographic (EEG) power increases are reversed by flupirtine, phenobarbital, and diazepam. Mean power time courses for control animals (*squares; *n = 5) and those treated with diazepam (n = 8), phenobarbital (*diamonds; *n = 11), or flupirtine (*circles; *n = 8) are shown (± standard error of the mean). Power was sampled either before kainate injection (B, baseline), after kainate (at 25, 50, and 75% of the time interval between injection and status epilepticus [SE] onset, and 5 minutes after SE onset), or at the indicated time intervals after injection of the treatment drug or vehicle (control). (A) EEG power after kainate-induced SE is significantly reduced by 50mg/ml phenobarbital (*p = *0.0006) or 50mg/ml flupirtine (*p = *0.0001). Although 29 of 30 individual flupirtine mean measurements are lower than phenobarbital, results are not statistically different (*p = *0.3318). (B) EEG power after kainate-induced SE is reduced by 8mg/ml diazepam (*p = *0.0242), with a biphasic time course (identical control and flupirtine data to A). Diazepam appears effective transiently after injection, but flupirtine power is significantly less (*p = *0.0357 for entire time course).*

to progress to a state of continuous SE, flupirtine was effective, by behavioral measures, qualitative EEG, and several tests of EEG power (see Figs 5–7). In this post-treatment paradigm, suppression of total EEG power by flupirtine and high-dose phenobarbital (50mg/kg, yielding a serum level of approximately 40μg/ml) did not differ significantly, but the effect of phenobarbital treatment was significantly delayed in onset and was associated with persistent, continuous, high-frequency EEG activity and brief behavioral seizures that were not seen after flupirtine.

This study confirms results of Dzhala and colleagues'[38] earlier study that showed that phenobarbital pretreatment was relatively ineffective for prevention of kainic acid–induced seizures in neonatal rodents and extends the findings to a second agent, diazepam. Dzhala and colleagues'[38] used analysis of EEG power to compare the ability of phenobarbital and the transporter blocking agent, bumetanide, to suppress kainate-induced seizures in neonatal rats. Although our approach was similar in many respects, there are some salient differences. In this study, phenobarbital was used at a greater dose (50 vs 25mg/kg), and trial drugs were given after seizures had progressed to SE (rather than before kainate injection). We used a test of significance that imposed several statistical power-weakening corrections (see Materials and Methods), whereas the previous study used a less stringent, ANOVA-based approach. Indeed, pairwise comparison of flupirtine and phenobarbital treatment using ANOVA and data from Figure 7 yields strong numerical support for superior power reduction by flupirtine ($p = 0.0033$). Because of these methodological differences, and the limitations of power measurement as a marker of more important clinical efficacy end points, drawing conclusions about the relative merits of bumetanide and flupirtine must await additional studies. Currently, it is clear that flupirtine is strongly anticonvulsant in P10 rats.

Although our kainate induction protocol was designed to limit mortality to approximately 10%, it is noteworthy that 8 of 55 control animals (14.5%), 11 of 35 diazepam-treated animals (31.4%), 2 of 48 phenobarbital-treated animals (4.2%), and 0 of 56 flupirtine-treated animals (0%) died within the experimental observation period. Factors underlying these observed mortality rate differences may include, for example, respiratory and hemodynamic effects we have not yet characterized. These may be clinically significant and deserve further study.

Flupirtine has not been approved for use in the United States. However, retigabine is a close structural analogue (see Fig 1) that is currently undergoing phase 3 clinical trial as adjunctive therapy for adult partial epilepsy, after a large stage 2 trial showed tolerability and dose-dependent suppression of seizure frequency.[21] Our findings do not imply that KCNQ openers such as retigabine and flupirtine are more likely to be effective in neonates than in older patients. KCNQ channels continue to be expressed throughout childhood and adulthood.[40–42] Although neonatal brain is unique in its high degree of seizure susceptibility after *blockade* of KCNQ channels,[13–16,43] this finding does not imply progressive diminishment in the experimental or clinical utility of *openers* with age. Evidence has been presented that flupirtine and retigabine are capable of antagonizing glutamate-induced toxicity and enhancing inhibitory synaptic currents,[27,44] in addition to their well-established direct effects as KCNQ openers. Additional studies are required to better specify the mechanisms relevant to the antiepileptic effects of these drugs. As noted earlier, in vivo experiments have raised concerns about potential proapoptotic effects of anticonvulsants currently utilized in neonates.[6–8] Although in vitro results that suggest antiapoptotic and antioxidative effects of flupirtine and retigabine are encouraging,[26,27] it will be important in future studies to directly examine the effects of flupirtine and other KCNQ openers on apoptosis and other aspects of developmental plasticity in the neonatal brain.

This translational investigation was made possible by earlier genetic and basic research.[9] The BFNS syndrome was the first idiopathic epilepsy for which genetic loci were identified.[45–47] Cloning of *KCNQ2* and *KCNQ3,* two potassium-channel genes, at these loci has allowed these novel channels to be studied using in vitro and in vivo methods.[48] Such studies have shown that the channels mutated in BNFS are widely expressed in the central nervous system, are critical regulators of excitability not only in infancy but throughout life, and can be pharmacologically activated by many drugs.[41,49–51] It is somewhat paradoxical that KCNQ2 and KCNQ3 mutations are rare and typically cause a phenotype restricted to the first weeks of life, but KCNQ channels exhibit widespread, lifelong expression and pivotal importance for brain function. This offers an important general lesson for translational neuroscientists: Clinical disease can offer clues allowing the discovery of essential brain proteins and pathways, without fully demonstrating the range of functional activity of these components. This is the case with BFNS and the underlying KCNQ channels.

Neuronal KCNQ channels remain quite poorly understood, and many questions regarding their subunit composition, biological roles, and pharmacology are unanswered. Continued development of KCNQ channel openers with distinctive subunit specificity and potency profiles will be critical for achieving better understanding of channel functions. Flupirtine is a low-potency KCNQ channel opener[22] and is representative of a class of drugs that exert their greatest effects on the KCNQ3 subunit.[52] Currently disclosed KCNQ chan-

nel openers include compounds of greater overall potency (eg, retigabine, various ethyl acrylamides, ICA-27243) and agents that differ from flupirtine in their kinetic mechanisms and selectivity among the neuronal KCNQ subunits (eg, zinc pyrithione).[50] Further in vitro and in vivo testing of novel openers will help clarify differences in biological functions between the KCNQ subunits. In turn, this may allow drug action on these channels to be rationally tailored to maximize therapeutic benefits whereas reducing unwanted side effects for various indications in pediatric and adult patients.

Our studies join recent efforts to translate understanding of the distinctive cellular and molecular features of developing brain into mechanistically novel neonatal seizure therapies. Thus, developmental differences in GABA(A) receptor subunit expression and the transmembrane Cl^- gradient have been found to make synaptic inhibition relatively less effective in the immature compared with the adult brain.[38,53] As noted earlier, the use of bumetanide, which promotes a more negative Cl^- equilibrium potential, and thereby enhances the inhibitory effects of GABAergic neurotransmission, has been investigated to circumvent this.[38,54] In contrast with the functional diminishment of GABAergic inhibition at birth, some excitatory glutamate receptors are overexpressed during early development, making blockade of these receptors another rational treatment approach. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor antagonist NBQX and kainate receptor antagonist topiramate have shown efficacy in the treatment of experimental neonatal seizures,[1,36,39] and they do not cause enhanced apoptosis.[55] Our results further validate the notion that neonatal seizure therapy may be improved through targeting molecules and mechanisms confirmed by laboratory or genetic studies to be of particular importance during early brain development.

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A KCNQ channel opener for experimental neonatal seizures and status epilepticus

Running head: KCNQ potassium channel openers for neonatal seizures

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Supplementary material

Statistical analysis of grouped EEG power data

Here we describe the statistical methods developed for analysis of suppression of EEG power by treatment with phenobarbital, diazepam, or flupirtine, given after the induction of status epilepticus by kainate (representative results shown in Figure 6; average results shown in Figure 7).

Overview of approach: Exact p-values are obtained, often under uniformly most powerful conditions, from careful application of a permutation test.[1] Such an approach requires no assumptions about underlying distributions, other than exchangeability of labels as explained below. Since the total number of possible permutations is astronomical, we made Monte-Carlo simulation estimates of the statistics, generated through use of random shufflings inside a computation loop. The test adopts the spirit of Tukey's honestly significant difference, by studying distribution of extremes, and accounts for multiple comparisons across treatments, repeated measures, correlation structure of the data, and temporal dynamics automatically built into the algorithm. When classical tests such as ANOVA are applicable, our test subsumes them and gives similar

results. For the experiment presented here, however, our test results in higher estimated p-values (making null harder to reject). This is a consequence of provisions for multiplicity, non-normality, and non-orthogonality/dependence, which violate the typical assumptions of classical tests.

Summary of statistical method:
Data set: EEG relative powers data matrix **X** = [35 times x 31 pups], 4 experimental conditions in **X** grouped into [n_1 n_2 n_3 n_4] = [5 10 8 8] columns (pups) assigned to **c**ontrol, **ph**enobarbital, **d**iazepam, and **f**lupirtine respectively

Hypothesis: All conditions equivalent, same group-mean profiles as for control
H_0: c(t) = ph(t) = d(t) = f(t)
where c(t) = ($x_{t,1}$+$x_{t,2}$+$x_{t,3}$+$x_{t,4}$+$x_{t,5}$)/n_1, and similarly for the other groups

Alternatives: One-tailed superiorities
K_1:ph(t)<c(t), K_2:d(t)<c(t), K_3:f(t)<c(t), K_4:f(t)<ph(t), K_5:f(t)<d(t), K_6:ph(t)<d(t)

Assumption: Exchangeability of pup labels under null (no special precondition in any particular group; one common, unknown distribution of EEG relative powers)

Statistics:
(1) Measure of deviation between paired group-mean profiles: Time-dependent Studentized difference in group means, accounting for pup sample size mismatch, arranged in matrix **D** = [35 times x 6 pairings]
= [(ph–c)/s_{12} ⋮ (d–c)/s_{13} ⋮ (f–c)/s_{14} ⋮ (f–ph)/s_{24} ⋮ (f–d)/s_{34} ⋮ (ph–d)/s_{32}],
where s_{12} = pooled standard error for the difference in means obtained from group-pups variability ${\sigma_1}^2$ and ${\sigma_2}^2$, i.e., $\sqrt{\frac{(n_1-1)\sigma_1^2+(n_2-1)\sigma_2^2}{n_1+n_2-2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}$, and similarly for the 5 other group pairs

(2) Time-dependent multicomparison test statistic: Most negative of the 6 measures above at each time, $D_{\min}(t)$ = min{$D_{12}(t)$, $D_{13}(t)$, $D_{14}(t)$, $D_{24}(t)$, $D_{34}(t)$, $D_{32}(t)$}

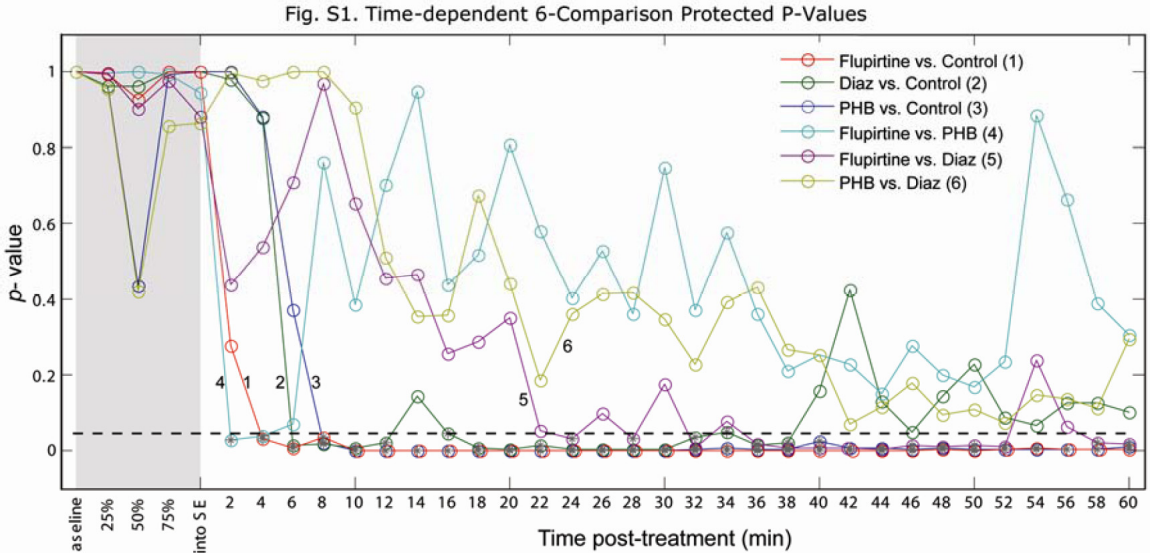
(3) Global multicomparison test statistic: Most negative of the 6 time averages of the measures in (1) above, D_G = min{$\langle D_{12}(t)\rangle$, $\langle D_{13}(t)\rangle$, $\langle D_{14}(t)\rangle$, $\langle D_{24}(t)\rangle$, $\langle D_{34}(t)\rangle$, $\langle D_{32}(t)\rangle$}, where $\langle D_{12}(t)\rangle = \sum_{t=1}^{35} D_{12}(t)/35$, and similarly for the 5 others

Significance test algorithm:
(1) Compute deviations matrix **D** from the experimental observations **X**
(2) Compute the experimental $D_{\min}(t)$ and D_G statistics from **D** (though only its {$D_{12}(t)$, ⋯}, and {$\langle D_{12}(t)\rangle$,⋯} components will be explicitly needed)
(3) Perform a random reassigment of the 31 pups to their labels (shuffle columns of **X**) to obtain a null-hypothetic data matrix replicate **X***
(4) Compute **D*** from **X*** following *exactly* the same steps as done for **X**
(5) Compute $D_{\min}$*(t) and D_G* statistics from **D***

(6) Repeat steps (3) and (4) above M = 20,000 times, thus obtaining empirical distribution of multicomparison test statistics $\{D_{\min}{}^{*(1)}(t), D_{\min}{}^{*(2)}(t), \cdots, D_{\min}{}^{*(M)}(t)\}$ and $\{D_G{}^{*(1)}, D_G{}^{*(2)}, \cdots, D_G{}^{*(M)}\}$
(7) Compute 6 p-value profiles protected for time-dependent inference:
$[\, p_{12}(t) \vdots p_{13}(t) \vdots p_{14}(t) \vdots p_{24}(t) \vdots p_{34}(t) \vdots p_{32}(t) \,]$,
where $p_{12}(t) = \sum_{k=1}^{M} I\left\{D_{\min}^{*(k)}(t) \le D_{12}(t)\right\} / M$, $I\{\cdot\}=1$ if argument true, $=0$ otherwise, and similarly for the 5 other comparisons
(8) Compute 6 p-values protected for global inference: $[\, P_{12}\ P_{13}\ P_{14}\ P_{24}\ P_{34}\ P_{32} \,]$, where $P_{12} = \sum_{k=1}^{M} I\left\{D_G^{*(k)} \le \langle D_{12}(t) \rangle\right\} / M$ and similarly for the 5 other comparisons

Inference: Any time points where p-value profiles ≤ 0.05 in (7) above are remarkable. Any global p-values ≤ 0.05 in (8) above are significant and indicate which of the alternative hypotheses $\{K_1, \cdots, K_6\}$ should be retained.

Results



The approach yields p-values for each of the 6 comparisons at each time epoch during the experiment. In Figure S1, each circle represents a p-value for one two-fold comparison, as indicated in the key (inset). The critical p = 0.05 threshold is indicated as a dashed black line, and time region prior to treatment drug injection is shaded grey. Each circle represents a p-value for a particular time and two-way comparison, calculate as described above and as indicated by the color key. Values lower than 0.05 are indicated by grey asterisks within the circle, and, at time points where red (flupirtine) and blue (phenobarbital) symbols overlap, the red symbol is moved about 0.2 min later in

time so both are visible. This representation provides a detailed account of the significance of difference between treatment groups. Of note, phenobarbital and flupirtine differ significantly at t = 2-4 min, and approaches significance at t = 6 min, supporting the assertion that flupirtine is significantly faster in onset than phenobarbital. The global *p*-values, i.e., for the entire period from 0 to 60 minutes after administration of the therapeutic drug, are shown in Table S1.

Table S1. Global p-values for average EEG power

Comparison	**p-value**
control vs. flupirtine	0.0001*
control vs. phenobarbital	0.0006*
control vs. diazepam	0.0242*
flupirtine vs. phenobarbital	0.3318
flupirtine vs. diazepam	0.0357*
phenobarbital vs. diazepam	0.3006

* indicates $p \leq 0.05$

Reference

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