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contract research in the neurosciences

Assessment of anti-seizure properties of two proprietary compounds in the electrical kindling model of epilepsy

Date

This study was conducted under the terms of a Laboratory Services Agreement between NeuroDetective International and client.

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Introduction

Partial (focal) seizures are the most common type of seizures in adults (Engel, 1998). While anticonvulsant drugs (ACDs) remain the primary treatment for reducing seizure frequency and severity, at least 30% of those afflicted have seizures that are resistant to treatment with the currently available drugs (Löscher and Schmidt, 1994). Thus, the identification of new ACDs remains an obvious and important avenue for therapeutic advancement. Since Merritt and Putnam discovered phenytoin in 1938, the identification of ACDs has depended, and will likely continue to depend, on animal models for screening. Electrical kindling provides an excellent *in vivo* model for determining the basic mechanisms underlying the genesis and progression of partial seizures as well as for the screening of potential ACDs (Teskey, 2001). In particular, the kindled limbic focus appears to provide the only validated animal model of partial seizures (Albright and Burnham, 1980).

The effects of anticonvulsant drugs can be categorized into those that elevate threshold and those that inhibit propagation. In the kindling model, AD threshold serves as an index of a test compound's effect on seizure threshold, whereas duration of AD and the duration and manifestation of clonic motor seizures serve as indices of compound effects on seizure propagation. Thus, we measured the effect of the two test compounds on afterdischarge threshold (ADT), afterdischarge duration (ADD), and seizure severity (SS). Since the intensity of stimulation appears to influence phenytoin's anticonvulsant effect (Rundfeldt et al., 1990; Morimoto et al., 1997), we measured ADD and SS in response to threshold kindling stimulation – the most ecologically valid method of electrically inducing a seizure. In addition, since it has been reported that kindling can change the anticonvulsant properties of a drug (Löscher et al.,1998a), we measured seizure threshold and propagation at three phases of kindling (naïve, partial and full).

This study employed electrical kindling of the rat hippocampus, to assess anti-seizure properties of the two test compounds, compound A and compound B. The hippocampus was chosen as the kindling site because it is the most common seizure focus in people, and it is the structure with the lowest seizure threshold in mammals. Anticonvulsant efficacy was evaluated by measurements of afterdischarge threshold (ADT), afterdischarge duration (ADD), and behavioral seizure severity (SS). ADD and SS were measured in response to threshold stimulation. Observations of clinical effects (behavioral toxicity) were also recorded. Comparisons between the test compounds, a positive control (phenytoin), and vehicle were carried out. Phenytoin served as a positive control because it is one of the most extensively studied ACDs and remains a first-line drug of choice for the treatment of partial epilepsy (Graves and Ramsay, 1996; Tunnicliff, 1996).

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Materials and Methods

Subjects

       Sixty-seven male Long-Evans hooded rats weighing 300-350 g and housed individually served as subjects. Food and water were available *ad libitum*. The rats were maintained on a 12 hr light/ 12 hr dark cycle with lights on at 08:00. All testing was done during the light phase.

Test Compounds and Drugs

       Test compounds (compound A and compound B) at high and low dosages (as defined by client) were freshly prepared according to instructions provided by client. All drugs were infused through jugular catheter at rates according to instructions also provided by client. See Appendix for further details.

       Vehicle and Phenytoin were infused at the same volume as compound A at its high dose.

       Testing commenced 30 minutes post injection. A positive control, phenytoin, was used (also administered iv), as was a vehicle-only control. Rats in the phenytoin and vehicle groups were tested 30 minutes post-injection. Thus, there were a total of 6 groups in this study (2 test compounds [two doses each] plus positive control [phenytoin], plus vehicle), with 8 animals in each group. Extra animals were used because of experimental attrition (head cap loss, jugular catheter failure and electrode failure) as well as mortality due to compound A (see Results).

      General Design
1) Surgery all rats (jugular catheter and chronic electrode implantation)
2) 1 week rest
3) Dose animals iv, wait interval, behavioral test
4) Determine ADT in all animals
5) Kindle at suprathreshold levels twice a day until seizure stages 2 and/or 3.
6) Dose animals iv, wait interval, behavioral test and determine ADT in all animals.
7) Kindle until stage 5 seizures
8) Dose animals iv, wait interval, behavioral test and determine ADT in all animals.
9) Euthanize, and perfuse all animals
10) Slice brains and determine electrode placement.

Catheter Surgical procedures

       All rats were anaesthetized with 58.83 mg/kg ketamine (85%) xylazine (15%) at 1.0 ml/kg injected intramuscularly. An incision over the left shoulder was made and the underlying tissue was blunt dissected. A rubber silastic tube catheter (0.5 mm ID 0.9 mm OD with 2 moveable beads) and rat injection port (Access Technologies, Norfolk Medical, Skokie, IL) was then filled with 0.9% saline solution and sewn into the muscle tissue. Another incision was made over the left jugular, where the underlying tissue was once again blunt dissected. The catheter was then fed from the shoulder subcutaneously to the jugular where it was inserted into the left jugular vein dorsal to the thoracic cavity and secured with silk suture thread. Rats were then placed in a standard stereotaxic apparatus for electrode implantation.

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Electrode Surgical procedures
      Twisted wire bipolar stimulating and recording electrodes was prepared from Teflon-coated stainless steel wires, 178 um in diameter (A-M Systems, Everett, WA). Uninsulated ends of the electrodes were connected to gold-plated male amphenol pins. The two poles of the electrodes were separated by 1.0 mm. Rats were anaesthetized with 58.83 mg/kg ketamine (85%) and xylazine (15%) at 0.5 ml/kg, injected intramuscularly. Lidocaine 2% (Austin, Joliette, QA), a local anesthetic, was administered subcutaneously at the incision site.

      Two bipolar electrodes were chronically implanted according to the stereotaxic coordinates of Swanson (1992). The stimulating electrode was implanted 4.5 mm posterior to Bregma, and 4.5 mm lateral to midline, then slowly inserted ventrally to a depth of 7.0 mm from brain surface, a position which places the stimulating electrode into the CA1 region of the ventral hippocampus. Gold-plated male amphenol pins connected to the electrodes were inserted into a 9-pin McIntyre connector plug, and adhered to the skull with four or five stainless steel screws along with dental cement. One of the stainless steel screws served as the ground reference. Experimental procedures commenced no earlier than seven days post surgical implantation.

      The anticonvulsant efficacy of each drug was evaluated from measurements of afterdischarge threshold (ADT), afterdischarge duration (ADD), and behavioral seizure stage (SS). Afterdischarge thresholds were determined by delivering one set of 25 uA stimulation trains, consisting of balanced biphasic square wave pulses, each 1.0 ms in duration, at 60 Hz for a total duration of 1 s. Failure to elicit a discharge resulted in increasing the current in 25 uA steps, waiting 30s and then re-stimulating until the threshold was surpassed. The lowest intensity of stimulation that induced 4 seconds of AD was arbitrarily defined as threshold (ADT). ADD, a measure of electrographic seizure activity, is the total duration of spikes in the EEG, with an amplitude of at least twice that of baseline EEG and a frequency greater than 1/s. SS was graded according to Racine (1972):
      stage 1, chewing, and/or pronounced salivation, and/or facial automatisms;
      stage 2, head-jerking and/or circling toward side of stimulation;
      stage 3, unilateral forelimb clonus on ipsilateral side;
      stage 4 bilateral forelimb clonus;
      stage 5 bilateral forelimb and hindlimb clonus.

      EEG signals were amplified and filtered at 1 Hz (high pass) and 100 Hz (low pass) with Grass Model 12 EEG amplifiers. On non-drug days rats were stimulated twice daily with current intensities 200 uA above threshold and polygraph records were recorded.

Anticonvulsant assessment
      Comparisons among the test compounds and the positive control group (phenytoin) and vehicle group were made with respect to ADT (seizure susceptibility), ADD and Seizure stage (seizure propagation). Clinical signs were also recorded, especially any signs of sedation or motor impairment.

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Behavioral (Clinical) assessment
  All animals were observed in an open-field box, and their behavior scored on a five-point index of sedation and muscle relaxation (modified from Hönack and Löscher, 1989). Sedation/muscle relaxation is classified as follows:

  0 = normal forward locomotion, no decrease in neck and abdominal muscle tone;
  1 = slightly reduced forward locomotion, slight decrease of muscle tone;
  2 = reduced locomotion with rest periods in between (eyes partly closed), further decrease of muscle tone;
  3 = reduced locomotion with more frequent rest periods, more pronounced decrease in muscle tone;
  4 = no forward locomotion, animal sits quietly with eyes closed, total loss of muscle tone.

  Muscle tone was evaluated by palpation and observation. The rats were lifted out of their cage and tilted slightly backwards to observe the exertion made to keep their head up. The amount of leg movement and torso twisting was also observed.

Histology
  Following all experimentation, the rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with saline and formalin. The brains were removed and fixed in 10% formalin with sucrose. Frozen coronal sections 40 um thick were taken and stained with thionin to verify electrode placements. All data reported in graphs and that underwent statistical analysis came from rats with electrodes confirmed to be in their hippocampus (CA1).

Statistical analysis
  Between-subjects analysis of variance was used to determine main effects for ADT and ADD data. Post-hoc Fischer's LSD tests were used to compare ADT and ADD among all groups receiving compounds and vehicle. Statistical analysis of behavioral toxicity and SS were by non-parametric signed rank test. All tests were performed two-sided and $P<0.05$ was considered significant.

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Results

Mortality

Group	Number of rats initially assigned per group	Deaths	Final n per group
Vehicle	9	1	8
Comp A low	12	6	6
Comp A high	9	4	5
Comp B low	9	1	8
Comp B high	8	0	8
Phenytoin	8	0	8

We observed a significant group effect ($F(3, 51) = 4.45$, $p < 0.0075$) on mortality. The groups of rats receiving compound A , at both high and low doses, had significantly ($p < 0.05$) more deaths (less survival) than all other groups, including vehicle.

Behavioral toxicity

There was an overall non-significant (Chi-Square = 8.38, df = 5, p = 0.136) group effect on behavioral toxicity in the naïve condition. Similarly, there were non-significant groups effects on behavioral toxicity in the partially kindled condition (Chi-Square = 7.2, df = 5, p = 0.21) and the fully kindled condition (Chi-Square = 9.08, df = 5, p = 0.106).

All groups experienced some level of behavioral toxicity that we determined was due to the infusion itself. Saline infusions to 3 rats also resulted in equivalent levels of behavioral impairment as in the vehicle and drug treated groups.

Afterdischarge threshold (ADT)

In naïve rats there was a significant group effect ($F(5,37) = 4.14$, $p < 0.004$) in the ADT measure, with post-hoc tests showing that this overall significance was due largely to the compound A (high dose) group having significantly ($p < 0.01$) reduced ADT (i.e. they were more susceptible to seizure than any other group except compound A low dose).

In partially kindled rats there was also a significant overall group effect ($F(5,37) = 2.49$, $p < 0.049$) in ADT, with post-hoc tests showing that this effect was due largely to the phenytoin group having significantly ($p<0.05$) raised ADT (i.e. they were less susceptible to seizure) compared to the Vehicle and compound A high dose groups.

In fully kindled rats there was also a significant group effect on ADT ($F(5,37) = 2.79$, $p < 0.031$), with post-hoc tests showing again that the phenytoin treated rats were significantly less susceptible to seizure ($p<0.05$) than the Vehicle and Compound A high dose groups.

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Two animals receiving Compound A (one receiving the low dose, one the high dose) displayed seizure behavior subsequent to infusion of the drug.

Afterdischarge duration (ADD)

There were no group differences in ADD duration in either naïve rats ($F(5,37) = 1.62$, p = 0.18) or partially kindled rats ($F(5,37) = 1.40$, p = 0.25). However in fully kindled rats there was overall effect of treatment ($F(5,37) = 2.86$, p < 0.028), which was largely due to significantly reduced (p < 0.01) ADD duration in the phenytoin group, compared to the Vehicle and high dose groups of both client compounds.

Seizure severity (SS)

There was no statistically significant effect of treatment on seizure severity in the naïve (Chi-Square = 3.87, df = 5, p = 0.57), partially kindled (Chi-Square = 1.12, df = 5, p = 0.95) or fully kindled (Chi-Square = 2.8, df = 5, p = 0.73) rats. Rats that were kindled showed little seizure behavior under vehicle and drug conditions. The most likely explanation for this is that the infusions themselves blocked expression of seizure behavior.

Summary

1) Compound A increased mortality in rats.

2) Neither Compound A nor Compound B produced anti-convulsant efficacy on a measure of seizure threshold (ADT). In fact, compound A at the high dose showed a pro-convulsant effect in naïve rats. Furthermore, two rats displayed afterdischarge and seizure behaviors during compound A infusion.

2) Neither Compound A nor Compound B produced anti-convulsant efficacy on a measure of seizure propagation (ADD).

3) Neither Compound A nor Compound B produced anti-convulsant efficacy on a measure of seizure propagation (SS), although this measure was blunted in all groups due to the iv infusion.

4) Neither Compound A nor Compound B produced behavioral toxic effects, but these measures were elevated in all groups as a result of the infusion.

5) The positive control (phenytoin) raised seizure thresholds (ADT) in partially and fully kindled rats as well as reduced seizure propagation (ADD) in fully kindled rats.

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Notes on Phenytoin;

Phenytoin has been reported to exert variable anticonvulsant effects in the kindling model and there has been the suggestion that phenytoin may be the exception to the general observation that anticonvulsant drugs that suppress clinical symptoms also suppress kindled seizures (McNamara et al., 1989; Lothman et al., 1991). It has been reported that phenytoin can have no anticonvulsant effects (Rundfeldt et al., 1990; Rundfeldt and Löscher, 1993; Morimoto et al., 1997), proconvulsant effects (Callaghan and Schwark, 1980; Schmutz et al., 1988; Ebert et al., 1997), anticonvulsant effects at toxic doses (Albright and Burnham, 1980; Ehle, 1980; Mace and Burnham,1987; Morimoto et al., 1997; Otsuki et al., 1998), as well as anticonvulsant effects at non-toxic doses (Albright and Burnham, 1980; McNamara et al., 1989; Löscher et al., 1998b). Factors such as route of administration (McNamara et al., 1989), gender (Ebert et al., 1994), genetic differences (Löscher and Rundfelt, 1991; Ebert and Löscher, 1999), stimulus intensity (Rundfeldt et al., 1990; Morimoto et al., 1997) and amount of kindling (Löscher et al., 1998a) have been touted as being responsible for the lack of concordance in the results.

Studies that have examined the anticonvulsant efficacy of phenytoin using the rat kindling model have yielded variable results. It has been suggested that some of this variation may be accounted for by the fact that, because phenytoin is only water soluble at a pH of more than 10, it may be poorly absorbed following an intraperitoneal injection (McNamara et al., 1989). Our results are in agreement with studies in rats that reported minimal, if any, adverse effects of phenytoin (McNamara et al., 1989; Rundfeldt et al., 1990; Löscher et al., 1998a; Otsuki et al., 1998).

Research using the kindling model in rats has convincingly demonstrated the ability of phenytoin to raise the ADT (Ehle, 1980; Albright, 1983; Rundfeldt et al., 1990; Ebert et al., 1994; Löscher and Rundfeldt, 1991; Löscher et al., 1993; Cramer et al., 1998; Löscher et al., 1998a; Ebert et al., 1997). The results from this study also confirm threshold increase under phenytoin.

Studies using the rat kindling model in which stimulation was delivered at or just above AD threshold reported that phenytoin resulted in a reduction in AD duration (Howe et al., 1980; McNamara et al., 1989; Rundfeldt et al., 1990; Rundfeldt and Löscher, 1993; Voits and Frey, 1994; Standley et al., 1994; Morimoto et al., 1997; Otsuki et al., 1998). However, when stimulation intensities well above threshold were used, phenytoin resulted in either no effect on AD duration (Rundfeldt et al., 1990; Rundfeldt and Löscher, 1993; Morimoto et al., 1997), or an increase in AD duration (Callaghan and Schwark, 1980; Schmutz et al., 1988; Ebert et al., 1997), with one exception reporting reduction in AD duration at stimulation intensities which were likely well above threshold (Albertson et al., 1980). Together, the data from the rat kindling model indicate that phenytoin apparently reduces AD duration (i.e., an anticonvulsant effect) at or near threshold stimulation, but has either no effect or results in an increase in AD duration (i.e., a proconvulsant effect) at stimulation intensities well above threshold. This seems to indicate, at least in this model, that phenytoin most likely raises seizure thresholds but perhaps does not mediate its anticonvulsant action through suppressing seizure propagation. Therefore,

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findings from the rat kindling model indicate that the intensity of stimulation is a critical variable when assessing phenytoin's ability to reduce AD duration.

Studies which have examined the effects of phenytoin on seizure severity in the rat kindling model have also indicated variable effects. Many studies have reported a decrease in seizure severity (Howe et al., 1980; Renfrey et al., 1989; Rundfeldt et al., 1993; Voits and Frey, 1994; Standley et al., 1994); one exception was a study that reported an increase in seizure severity in animals expressing partial seizures (Ebert et al., 1997). Other studies have reported decreased seizure severity, but only at high doses.