AFTERDISCHARGE THRESHOLD REDUCTION IN THE KINDLING MODEL OF EPILEPSY

by

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ABSTRACT

Afterdischarge threshold (ADT) changes were studied in the amygdala kindling model of epilepsy. Racine (1972a) reported that electrical stimulation lowers the ADT in amygdala-kindled rats, and that the presence of electrodes alone has no effect. More recently, however, Löscher et al. (1993, 1995) have produced data that suggest that ADT drop results from the presence of electrodes alone and that electrical stimulation may have no effect on ADTs. These conflicting views must be reconciled before meaningful research on threshold drop – and agents designed to prevent it – can be attempted.

In Experiment 1, Racine's original finding that electrical stimulation lowered ADTs was replicated. The ADT of the stimulated amygdala was significantly lowered by daily subthreshold stimulation. No change was seen in the ADT of unstimulated control subjects. As in Racine's previous experiments, stimulation was done in male Long Evans rats and begun 4 weeks after electrode implantation. The "Half-Split" procedure was used for determination of ADTs.

In Experiment 2, Racine's procedures (Half-Split ADT determination, male rats) were applied at Löscher's time intervals. In agreement with Löscher, it was found that ADTs were significantly lower in subjects tested 4 weeks after implantation than in subjects tested 1 week after implantation, even without stimulation. This drop in thresholds disappeared in subjects tested at 8 weeks after implantation. Added stimulation did not produce an ADT drop in the 4 week group.

In Experiment 3, Löscher's original experiment was replicated with the addition of an unstimulated time-matched control group. As in Löscher's previous experiments, stimulation was done in female Wistar rats using a standard current intensity (500 μ A, base-to-peak) and begun 1 wk after implantation. The "Ascending Series" procedure was used for threshold determination. Initial ADTs were significantly lower in subjects 4 weeks after implantation, even in the absence of stimulation. This drop in thresholds disappeared in subjects tested at 8 post-operative recovery weeks. Comparison of stimulated subjects to unstimulated time-matched controls, showed clear threshold reduction effects in the 4 and 8 week subjects.

These results make it clear that there are consistent changes in ADT following electrode implantation, and that these are independent of electrical stimulation – as Löscher suggested. When these are taken into account, however, the stimulation-induced changes reported by Racine can be seen. The effects of electrical stimulation on ADTs, therefore, must be assessed by a comparison of stimulated subjects to a group of non-stimulated subjects of similar post-operative recovery time. If this is done, pharmacological testing of agents designed to block ADT drop should produce valid results.

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ABBREVIATIONS

AD	afterdischarge
ADT	afterdischarge threshold
EEG	electroencephalogram
g	gram
hr	hour
Hz	Hertz
kg	kilogram
mA	milliAmpere
mg	milligram
min	minute
mL	millilitre
mm	millimetre
ms	millisecond
s	second
μ A	microAmpere

CHAPTER 1

INTRODUCTION

1.1 EPILEPSY: THE CLINICAL PROBLEM

1.1.1 Definition/Clinical Background

The term “epilepsy” refers to a group of chronic neurological disorders characterized by spontaneous, recurrent seizures (Burnham, 1997). Epilepsy is one of the commonest of the central nervous system (CNS) disorders, occurring in one of every 100 people (Rogawski and Porter, 1990). Although epilepsy may occur at any time during life, onset is often in childhood (Janz, 1997). There is no common etiology for the epilepsies—causes are varied and may include neoplasms, vascular anomalies, scars, stroke, genetic factors, birth trauma, and brain injury (Engel, 1989; Ettinger, 1994). In approximately 70% of the cases, the cause is unknown (Bruton, 1988).

Current pharmacotherapy for epilepsy is aimed at controlling seizure occurrence (Shin and McNamara, 1994), and does not cure the underlying cause(s) of epilepsy. Almost 20-30% of epileptic patients fail to respond to anticonvulsant drugs, however (McNamara, 1996). These patients may be candidates for surgery (Awad et al., 1991; Rasmussen, 1974).

1.1.2 Low Seizure Threshold: The Central Problem in Epilepsy

Although seizures are the hallmark of epilepsy, the essence of epilepsy is not seizures but a chronic low seizure threshold (Burnham, 1997). Every normal brain has the circuitry necessary for seizure production, but most individuals do

not produce spontaneous seizures. The crucial abnormality in epileptic brains is a chronically low seizure threshold that promotes spontaneous seizures. Thus, low seizure threshold may be considered the central issue in epilepsy.

1.1.3 The Development of Low Seizure Threshold

How do low thresholds develop in epileptic patients? Genetics certainly plays a role (Treiman, 1993; Annegers, 1994), as do structural lesions in some patients (Ettinger, 1994). In part, however, low thresholds may result from repeated episodes of neural excitation. During the pre-drug era, it was observed that, as focal seizures recurred, there was an increasing tendency for the interval between attacks to grow shorter, and for minor attacks to lead to major ones (Burnham, 1991). It has also been reported that Jacksonian and post-traumatic seizures in humans often develop progressively more severe clinical manifestations over time (Wada, 1977; Servit and Musil, 1981). The increase seen in seizure frequency presumably relates to a drop in focal thresholds. The increase seen in clinical manifestations may relate to threshold drop outside the focus. Certainly, the initially low thresholds found in epileptic patients tend to drop even further as repeated attacks occur (Morrell, 1973).

1.2 THE KINDLING MODEL OF EPILEPSY

1.2.1 Animal Models

Animal models of epilepsy are used to study the basic mechanisms of seizure disorders. Experimental manipulations can be performed in animals that cannot

be performed in human subjects. For useful reviews of animal models, see Woodbury et al. (1983) and Fisher (1989).

1.2.2 The Kindling Model

The kindling model is an experimental model of focal epilepsy, secondarily generalized (Cain, 1992; Albright, 1983). It involves the repeated application of electrical stimulation to brain tissue, via a chronic indwelling electrode (Goddard et al., 1969). Electrical stimulation is applied at regular intervals, usually once per day. Initially, stimulations evoke only freezing, possibly accompanied by facial movements. With repetition, however, stimulations come to elicit progressively stronger motor responses, culminating in a generalized convulsion. This involves (in the rat) bilateral forelimb clonus, rearing, and falling (Racine, 1972b; see Figure 2-2). Once the susceptibility to fully developed motor convulsions develops, it is permanent (Racine, 1972b). For useful reviews, see Sato et al. (1994) and Cain (1992).

1.2.3 Advantages of the Kindling Model

It has been proposed that the ideal experimental model of epilepsy should allow for all of the following (Wada, 1975): 1) precise experimental control over the location and size of the epileptic lesion; 2) creation of an epileptic lesion without induction of destructive pathology; 3) experiment control over the time course of seizure development; 4) experimenter control of seizure occurrence; and 5) the eventual development of a spontaneous recurrent seizure state.

The kindling model of epilepsy satisfies all of these criteria: 1) in kindling, standard stereotaxic techniques are used for precise implantation of electrodes; the electrode tip is placed where the investigator wants the focus to be located; 2) the focus is created without extensive scarring; 3) the experimenter is in control of the time course of seizure development; 4) in the early stages of kindling, seizures occur as a result of brief trains of electrical stimulation, and do not occur in their absence—convulsions are stimulus-locked and under experimenter control; and 5) if stimulations are continued over an extended period, spontaneous seizures develop, marking kindling as a true epilepsy model (Pinel, 1978).

1.2.4 Basic Phenomena of Kindling

Electroencephalographic (EEG) studies have revealed two different types of functional reorganization which accompany the kindling process: enhanced extrafocal propagation, and focal threshold drop. Both are permanent:

1) Enhanced extrafocal propagation is the phenomenon that presumably underlies the development of motor seizures (Racine, 1972a,b,c). Early stimulations in kindled subjects trigger only local afterdischarges (ADs). With repeated stimulation, however, the ADs propagate farther and farther, and eventually spread to reach motor structures (Racine, 1972a; Burnham, 1976).

2) The thresholds for induction of AD, or “afterdischarge thresholds” (ADTs) undergo a progressive reduction as kindling proceeds (Racine, 1972a). This drop in threshold does not correlate with motor seizure development

(Racine, 1972a), although it may be related to the eventual development of spontaneous attacks (Pinel, 1978).

In the next section ADT reduction will be discussed in detail since it is the principal focus of the present project.

1.3 AFTERDISCHARGE THRESHOLDS AND AFTERDISCHARGE THRESHOLD DROP IN THE KINDLING MODEL

An important feature of the kindling model is that ADTs can be easily measured via the implanted electrodes. In this respect, kindling differs from other focal models (e.g. cobalt) where threshold measures are seldom done.

1.3.1 ADT in Kindled Subjects

When thresholds have been measured in kindled subjects, it has been found that some limbic structures have lower initial ADTs than others. Limbic structures, for instance, generally have lower thresholds than the neocortex (Racine, 1972a; Racine, 1975; Burnham, 1978; for review, see Burnham, in press).

In addition, as mentioned above, ADTs of some brain sites in the kindling preparation are lowered as a result of electrical stimulation (Racine, 1972a). Unlike motor seizure development, ADT reduction seems to relate to stimulation per se, and does not depend on the occurrence of ADs (Racine, 1972a).

ADT reductions are greater in limbic structures than in the cortex, and greater in some limbic structures than in others (for review, see Burnham, in press). In the kindled amygdala, ADT reduction occurs mostly during the first 20

days of stimulation, and they may be permanently lowered by as much as 40-60% by daily 1 s trains of stimulation (Racine, 1972a). In contrast, in the hippocampus only a 25% drop is seen (Racine, 1972a). During the course of neocortical (suprasylvian gyrus) kindling in cats, however, Sanei et al. (1992) reported no changes in ADT.

Surprisingly, threshold reduction is—at least in part—a local phenomenon. Threshold reduction in one brain site does not necessarily lead to ADT reduction in other sites. This was initially reported by Racine (1972a), who found in rats that ADT reduction in the amygdala had no significant effects on ADTs in the contralateral amygdala, septal area, or hippocampus. Sanei et al. (1991), however, have recently reported that in cats, ADT reduction in the amygdala or the hippocampus causes a significant drop in the unstimulated pyriform cortex. Thus, threshold drop outside the stimulated focus may occur, but it seems to be a highly restricted phenomenon.

Reports of drug effects on focal thresholds are inconsistent. Albright (1983) demonstrated that anticonvulsants effective against tonic-clonic attacks raise focal thresholds. Wise and Chinerman (1974) reported that phenobarbital raised focal thresholds, but diazepam did not. Racine et al. (1975) agreed that diazepam was ineffective, and noted that phenytoin was ineffective as well. In contrast, Ehle (1980) and McNamara et al. (1989) reported phenytoin did raise focal thresholds. Albertson et al. found that diazepam and carbamazepine was effective on the amygdala threshold (1983, 1984).

Studies in which stimulation intensity was set at threshold plus a known increment (e.g., 20%), offer an indirect measurement of drug/threshold effects (Burnham, 1991). These have suggested that most of the clinical anticonvulsants raise thresholds in both the amygdala and cortex (Albright and Burnham, 1980; Wada et al., 1976).

1.3.2 Sub-threshold and Supra-threshold Stimulation

Pinel et al. (1976) investigated the role of current intensity in lowering kindled seizure thresholds. It was reported that initial ADTs were lowered by “low-intensity” stimulations, but transiently raised by “high-intensity” stimulations. ADTs from subjects who received “high-intensity” stimulation returned to baseline (initial) threshold levels after a 7 day rest period, suggesting that the stimulation had produced no threshold drop. This finding does not seem to agree with Racine’s original report (1972a), in which it was shown that “supra-threshold” electrical stimulation permanently lowered ADTs in the amygdala, although it was not as effective as sub-threshold stimulation. Insights gained from this thesis address this apparent incongruity (General Discussion).

1.3.3 Procedures for Measuring ADTs in Kindled Subjects

Traditionally, kindled ADTs have been determined by one of two methods: the Ascending Series technique (Pinel et al., 1976), or the Half-Split method (Racine, 1972a). These methods involve different procedures:

1) The Ascending Series technique involves administering an ascending series of current intensities, once per minute, until an AD is elicited. This stimulation intensity is then deemed the “ADT”. The advantage of the Ascending Series technique is that the ADT for a subject is determined within one session. This is especially important in studies that involve acute drug effects on focal thresholds.

2) In contrast, the Half-Split method involves administering a standard initial current intensity, and adjusting the subsequent stimulation intensity depending on the outcome: if an AD is elicited upon stimulation, the current intensity is halved and applied 48 hours later; if an AD is not elicited upon stimulation, the current intensity is doubled and applied 48 hours later. This procedure is repeated until the difference between the AD-eliciting current intensity and the previous non-effective level is no more than 20% of successful current. This stimulation intensity is then deemed the “ADT”. The Half-Split procedure may require more than 1 week for a single ADT determination, as opposed to 1 d with the Ascending Series technique. Hence it is not appropriate for the study of the acute effects of drugs on focal thresholds.

It has been suggested that these two techniques may give different results. Swartzwelder and Mobray (1980) directly compared the Ascending Series ADT determination method against the Half-Split determination method in the dorsal hippocampus. In Phase I of their experiment, ADTs were first assessed using the Ascending Series technique, and then, 48 hours later, reassessed using the Half-Split method. Forty-five days later, during Phase II of

the experiment, the groups crossed-over and were reassessed using both methods, using the Half-Split determination method first, and then, 48 hours later, using the Ascending Series technique. The Ascending Series method produced higher ADTs which were less stable over time than were those produced using the Half-Split method. Also, threshold level ADs produced using the Ascending Series technique were of longer duration in Phase II of the experiment than in Phase 1. These changes in AD duration were not seen with the Half-Split method. Thus, Swartzwelder and Mobray (1980) suggest that the Ascending Series technique may yield more variable data. They contend that the repetitive low-level stimulation used in the Ascending Series technique may render the stimulated area to be refractory to stimulation, thus elevating the ADT.

Certainly the Ascending Series technique can produce elevated thresholds if short interstimulus intervals or intertest intervals are used. Freeman and Jarvis (1981) investigated the role of interstimulus intervals in assessing ADTs with the Ascending Series technique. It was found that an inter-stimulus interval of 30 s yielded higher ADTs and motor seizure thresholds than the other time intervals tested (1, 3, or 5 min). The role of the inter-test interval was also evaluated in the same report. Using the Ascending Series technique of ADT determination, it was found that an inter-test interval of 24 hr yielded progressively higher ADTs over five tests, whereas stable ADTs were produced when the inter-test interval was 48 hr.

In the present work, Racine's Half-Split technique—with a 48 hr inter-test interval—was used in Experiments 1 and 2. The Ascending Series Technique was used only in Experiment 3, which directly replicated Löscher's work.

1.3.4 Attempts to Block ADT Reduction in Kindled Subjects

The study of drug effects on focal threshold and focal threshold reduction in kindling addresses a central problem of epilepsy. The potential to arrest or slow threshold drop might translate into knowledge about the progressive nature of epilepsy—such as the events that lead up to late-onset post-traumatic epilepsy—and could lead to novel therapeutic approaches.

As yet, few explicit studies of drug effects on ADT reduction have been done. Most of these have been pharmacological studies. So far, all reports have been negative: no drugs have been found that have been able to block ADT drop. Wise and Chinerman (1974), for instance, reported that neither phenobarbital nor diazepam blocked threshold drop in the amygdala. Ehle (1980) found phenytoin to be ineffective in stopping threshold drop as well.

The study of drug effects on threshold drop is an important and under-researched field. Before relevant studies can proceed, however, an important paradox in the literature must be resolved.

1.4 LÖSCHER VERSUS RACINE: PARADOXICAL FINDINGS

An important anomaly involving threshold reduction in the kindling model exists.

In Racine's (1972a) original study of threshold reduction, the effect of daily electrical stimulation on the ADT in the amygdala was assessed (see Figure 1-1). Stimulation was set at 80% of the determined ADT. For a detailed

Figure 1-1. ADT Drop in the Amygdala (Reproduced from Racine, 1972a)

description of this procedure, see Procedure for ADT Reduction, General Methods. It began 4 weeks after electrode implantation and was administered for 60 days. Male, Long Evans rats were used as subjects. The ADT of the stimulated amygdala was significantly lowered—as compared to unstimulated controls—by the threshold reduction paradigm. Thresholds were retested later, after a 6-week rest period (no stimulation), to measure the permanence of the ADT reduction. A significant, though slight, increase was found, but ADTs were still significantly lower than the original thresholds. These data suggest that ADT

reduction depends on stimulation, and does not relate to the presence of electrodes per se.

In contrast, Löscher et al. (1995) studied 4 groups of subjects at different times after electrode implantation (1 wk, 2 wks, 4 wks, or 8 wks). Female, Wistar rats were used as subjects.

Figure 1-2. Effects of Stimulation on ADT (reproduced from Löscher et al., 1995)

Significant difference of pre- or post-kindling ADT to the data determined after 1 week of post-surgical delay is indicated by asterisk (P at least <0.05). Within groups, the post-kindling ADT was significantly ($P < 0.01$) lower than pre-kindling ADT in groups with 1, 2 and 8 weeks but not the group with 4 weeks post-surgical delay.

They found that pre-kindling ADTs at 2 and 4 weeks post-implantation were significantly lower than the pre-kindling ADT 1 week after implantation. Pre-kindling ADTs returned to 1-week levels in the 8 week post-implantation group

(see Figure1-2). This suggests that ADTs drop without stimulation, simply due to the presence of the electrodes. When the groups were subsequently kindled, the effect of electrical stimulation of the amygdala was not observable in subjects that began stimulation 4 weeks after electrode implantation—the time when un-stimulated threshold drop was maximal. (ADTs in this study were compared only to original values: there was no unstimulated control group.) These data seem to imply that threshold drop may all relate to electrode implantation, since there was no stimulation induced drop in the 4 week group.

These data directly disagree with the original report on threshold reduction by Racine (1972a), which showed threshold reduction in subjects where stimulation began 4 weeks after implantation.

In an earlier study, Löscher et al. (1993) had also reported a significant difference in the un-stimulated seizure threshold of rats determined at either 1 week or 4 weeks after implantation of an electrode into the amygdala. Subjects with 4 weeks post-implantation time yielded an initial (pre-kindling) ADT significantly lower than subjects with 1 week post-implantation. Thus, Löscher has found an un-stimulated drop in two different experiments. Löscher et al. (1995) suggest—based on previous observation of iron deposits induced by electrode implantation and the epileptic effect of iron in cortical and limbic regions—that their results are due to deposition of iron from hemoglobin destruction in local microhemorrhages caused by the implantation. It is not clear how this explanation would relate to their 8-week data, where ADTs has risen again, and a stimulation effect was seen.

It is important that the nature of ADT reduction be solved before further study of ADT changes can be done. If threshold reduction relates simply to the implantation and presence of electrodes, it may have little relevance to the clinical problems of epilepsy, and it is unlikely that anticonvulsant drugs could affect it.

1.5 PREVIOUS WORK DONE ON THE EFFECT OF ELECTRODE IMPLANTATION

Is it possible that electrode implantation alone can cause changes in ADT? Many electrophysiological studies require stereotaxic penetration of the brain for stimulation and recording of responses. The effects of the small lesions resulting from electrode implantation are generally considered minimal. Even so, the effect of electrode implantation, *per se*, has been shown in several reports to induce histological (Robinson et al, 1975), behavioural (e.g. Boast et al., 1976); Hertz et al., 1974), and neurochemical (e.g. Benattia et al., 1992; Ashton et al., 1980) changes.

Robinson et al. (1975), for instance, found enzyme changes, mainly confined to a narrow area of tissue damage surrounding the electrode tract. In this report, numerous changes in enzymes were found as a result of trauma. Enzymes controlling tissue respiration showed early and rapid changes, increasing in hyperactive, swollen glial cells and vascular endothelium, and decreasing in nerve cells and neuropile. Acid phosphatase activity also increased rapidly in glial cells; other phosphate-releasing enzymes increased more gradually with time. A turning point in these changes occurred between 25 and 40 days, followed by a reversion to more normal levels at 60 days.

Ashton et al. (1980) observed in frontal cortex that [³H]spiperone binding—a measure of serotonergic receptors—was 30% lower in amygdala-implanted (non-stimulated) rats than in non-operated age-matched controls.

The effect of electrode implantation has been observed behaviourally as well. Boast et al. (1976) demonstrated a defect in learning processes in mice caused merely by the implantation of either stainless-steel or platinum/iridium electrodes into the dentate gyrus. This change was correlated with significant hemorrhagic vascular damage, and the deposition of iron around electrode tracts. Since iron deposits were found around the tracts of both stainless-steel and platinum/iridium electrodes, the iron originated from hemoglobin destruction, and was deposited by the extravasation of blood during electrode induced microhemorrhages. Furthermore, the visualization of the vascular tree of the hippocampus using india ink demonstrated vascular damage which was closely related to the appearance of iron. Dahl and Ursin (1969) also correlated the accumulation of ferrous ions in the region of the electrode tract with behavioural alterations.

Similarly, the duration of electrode implantation has been reported to affect the rate of kindling. Blackwood et al. (1982) made a comparison between the rate of kindling in subjects that had been implanted with electrodes either at 1 week or 4 weeks before daily stimulation was started. It was found, using stainless-steel or platinum/iridium electrodes, that the rate of kindling was significantly enhanced after the longer implantation time. Using Teflon-coated stainless steel electrodes, Löscher et al. (1995) also found an enhanced rate of

kindling in rats when stimulation was started 4 and 8 weeks after electrode implantation versus rats that began stimulation 1 week after electrode implantation.

Löscher et al. (1993) have reported long-lasting changes in amino acid levels of rat brain regions as a result of prolonged intra-amygdala electrode implantation. Electrode-implanted groups differed significantly from non-implanted naïve rats in several regions, including the olfactory bulb, amygdala, pyriform cortex, and hypothalamus. The greatest difference was an increase of glycine levels suggesting that electrode implantation led to alterations in glutamatergic neurotransmission.

1.6 OBJECTIVES OF THE PRESENT STUDY

1.6.1 General Objective

The general objective of the present study is to clarify the respective roles of electrode implantation and stimulation in threshold drop in the kindling model, so that meaningful studies on the pharmacological modification of threshold and threshold drop can be conducted.

1.6.2 Specific Objectives

1. Experiment 1 To determine whether the results of Racine (1972a) could be replicated. Stimulation was done in male Long-Evans rats, starting 4 weeks after electrode implantation. The 'Half-Split' technique will be used for

threshold determinations, and Racine's threshold reduction paradigm was used to drop ADTs.

2. Experiment 2 a) To determine whether the variations in initial ADTs reported by Löscher would be seen if Racine's technique (as above) was applied at different times after implantation. b) To assess the effect of kindling stimulation, if any, on the above groups.

2. Experiment 3 To determine whether the results of Löscher et al. (1995) could be: a) replicated, and b) extended—by adding 4 groups of unstimulated (but handled) subjects. These unstimulated controls—which were missing in Löscher's original experiments—give an indication of what ADT would be in stimulated subjects at the time of their final ADT determination if they had not undergone the ADT reduction procedure. As in Löscher et al. (1995), stimulation was done in female Wistar rats, starting at either 1, 2, 4, or 8 weeks after electrode implantation. The 'Ascending Series' technique was used for threshold determinations.

CHAPTER 2

GENERAL METHODS

2.1 EXPERIMENTAL OVERVIEW

The general design used in Experiments 1, 2, and 3 is presented in Figure 2-1. Specific variables related to the three experiments are summarized in Table 2-1.

Table 2-1. Experimental Variables

Experiment	Rat strain/ sex	ADT Threshold Testing	Stimulation parameters for threshold drop	Endpoint criteria	Controls
Expt 1 Racine Replication	Long Evans rat; male	pre- and post- kindling/Half- Split	1.0 s train of biphasic 1ms pulses, 60 Hz	40 days of stimulation	non-kindled (handled)
Expt 2 Racine Time Course	Long Evans rat; male	pre- and post- kindling/Half- Split	1.0 s train of biphasic 1 ms pulses, 60 Hz	40 days of stimulation	
Expt 3 Löscher Replication	Wistar rat; female	pre- and post- kindling/ Ascending Series	1.0 s train of monophasic 1ms pulses, 50 Hz	10 consecutive stage 5 seizures	non-kindled (handled)

2.2 SUBJECTS

Male Long Evans rats (Charles River, La Prairie, Quebec), weighing 250-300 g, and female Wistar rats (Charles River, La Prairie, Quebec), weighing 210-230 g, were used as subjects in the present experiments. All subjects were housed individually, and allowed free access to food and standard laboratory chow. A 12-hr light/12-hr dark cycle was maintained throughout the experiments (lights on at 7 a.m., off at 7 p.m.). After arrival in the vivarium, subjects were allowed at least 1 wk to accommodate before surgery was performed.

Figure 2-1 EXPERIMENTAL OVERVIEW

2.3 SURGICAL PROCEDURES

One week (minimum) after arrival in the animal facility, chronic indwelling electrodes were implanted in all subjects using standard stereotaxic techniques (Skinner, 1971). Subjects were anesthetized for implantation with 60 mg/kg (male dosage) or 30 mg/kg (female dosage) sodium pentobarbital (Somnotol®, MTC pharmaceuticals, Mississauga, Ontario). The head was positioned in a stereotaxic apparatus (DKI 900, David Kopf Instruments, Tujunga, California). The skull was exposed and cleaned, and four jeweler's screws were inserted into the bone. Bipolar electrodes were then lowered into the brain and cemented to the skull with dental acrylic (Nuweld®, L.D. Caulk). The coordinates for electrode placement were as follows:

Table 2-2. Coordinates for Electrode Implantation

Experiment	Site	Coordinates*
Experiment 1 Racine Replication	Right Basolateral Amygdala	AP -1.0 Lat +4.8 Vent -8.5
Experiment 2 Racine-Timecourse Study	Right Basolateral Amygdala	AP -1.0 Lat +4.8 Vent -8.5
Experiment 3 Löscher Replication	Right Basolateral Amygdala	AP -1.9 Lat +4.7 Vent -8.5

*Anterior-posterior and right lateral coordinates are given with bregma (skull landmark where the coronal and sagittal sutures intersect) as a reference point. The ventral reference point is the surface of the dura mater. The upper incisor bar was set at 5 mm above the interaural line in Experiment 1 and 2, and at zero in Experiment 3. Coordinates were derived from publications of Racine (1972a) and Löscher et al. (1995).

All experiments were conducted using commercially available bipolar electrodes (MS 303/1, Plastics One Inc., Roanoke, Virginia). These electrodes consisted of two Polyimide-insulated 0.25 mm diameter stainless steel wires.

Subjects were allowed different post-operative recovery times, depending on the experiment (see Table 2-3, below). They were always handled and accommodated to the test box for at least one week before threshold reduction or kindling commenced.

Table 2-3. Post-operative Recovery Times to Initial ADT Determination

Experiment	Post-operative recovery time
Experiment 1 Racine Replication	4 weeks
Experiment 2 Racine Time Course Study	1 week or 2 weeks or 4 weeks or 8 weeks
Experiment 3 Löscher Replication	1 week or 2 weeks or 4 weeks or 8 weeks

2.4 PROCEDURE FOR ADT DETERMINATION

At an appropriate interval (Table 2-3) after electrode implantation, threshold determination was begun. A Grass Model S-88 stimulator, in series with two PSIU 6 isolation units (Grass Instruments, Quincy, Massachusetts), was

used to generate 1 ms square-wave pulses as indicated in Table 2-4. Intensities and durations differed according to experiment:

Table 2-4. ADT Determination Variables

Experiment	Waveform	Train duration	Method
Experiment 1 Racine Replication	square wave, biphasic	1 sec	Half-Split
Experiment 2 Racine Time Course Study	square wave, biphasic	1 sec	Half-Split
Experiment 3 Löscher Replication	square wave, monophasic	1 sec	Ascending Series

In Experiment 1 and Experiment 2, Racine's Half-Split technique was used to determine ADTs. During threshold determination, testing sessions were conducted every second day (48 hours between sessions). On the first day, a standard current intensity (200 μ A, peak-to-peak) was applied. If the initial current was sufficient to elicit an AD (3 s minimum), it was reduced to half its value and administered during the next testing session. If the initial current was insufficient to elicit an AD, it was doubled in value and applied during the next testing session. This routine was continued until a current intensity was determined such that the difference between the AD-eliciting current and the previous non-AD-eliciting current was no more than 20% of the upper limit. This current intensity was then deemed the "ADT". This procedure exactly duplicates the procedure used by Racine (1972a).

In Experiment 3, the Ascending Series technique was used to determine ADTs. In this procedure, the ADT is determined during a single test session. Current is applied in an ascending series of intensities, once per minute, until an AD is elicited. The series was started with a low standard current intensity (10 μ A, base-to-peak) which was sub-threshold for all subjects. The current intensity was then raised by 20% and applied 1 minute later. This routine was continued until an AD (3 s minimum) was elicited. This current intensity was then deemed the “ADT”. This procedure exactly duplicates the procedure used by Löscher (1995).

Identical procedures were used at the end of each experiment to determine ADTs after threshold reduction.

2.5 PROCEDURE FOR ADT REDUCTION

In Experiments 1 and 2, Racine’s (1972a) procedure for threshold reduction was used. This involved applying daily sub-threshold stimulation to subjects over a course of 40 days. (Forty days were used—instead of Racine’s 60—to shorten the experiment. Racine saw minimal threshold drop during the last 20 days.) On day 1 of stimulation, the stimulation intensity was set at 80% of each experimental subject’s determined initial ADT. This intensity was applied daily until an AD was elicited on two consecutive test days. This stimulation level was then deemed the “new” ADT, and the stimulation intensity was then adjusted to 80% of the “new” ADT. Again, when AD was elicited on two consecutive test days, the stimulation intensity was adjusted as described above, and so on. The

ADT reduction paradigm was carried out daily (5 days/week). Control subjects were placed in the test box and attached to electrodes but received no stimulation. This procedure exactly duplicates the procedure used by Racine (1972a).

In Experiment 3, a standard kindling procedure was used for threshold reduction. This involved applying a supra-threshold standard stimulation intensity (500 μ A, base-to-peak) to experimental subjects until each had 10 consecutive Racine Stage 5 (defined below) seizures. The ADT reduction paradigm was carried out daily (5 days/week). Control subjects were placed in the test box and attached to leads, but received no stimulation. This procedure exactly duplicates the procedure used by Löscher et al. (1995).

2.6 DATA COLLECTION, SCORING, AND ANALYSIS

2.6.1 Electroencephalography

Electroencephalographic (EEG) recording was done during all ADT reduction, kindling, and ADT test trials. A multi-channel electroencephalograph was used (Model 8-10, Grass Instruments, Quincy, Massachusetts). EEG recordings were taken for 10 seconds before each stimulation, and for 20 seconds after the last spike of the AD.

2.6.2 Seizure Scoring

Motor seizures, when present, were scored according to Racine's (1972a) seizure stages: Stage 1 (facial clonus); Stage 2 (head nodding); Stage 3 (forelimb clonus); Stage 4 (rearing); Stage 5 (loss of postural control). These stages are illustrated in Figure 2-2.

2.6.3 Histological Verification of Electrode Placements

At the completion of testing, subjects were sacrificed with an overdose of pentobarbital sodium (Somnotol®, M.T.C. Pharmaceuticals) and perfused intracardially with a 0.9% saline solution, followed by 10% formalin. The brains were removed and stored in a 10% formalin solution for a period of at least 3 days. The brains were then frozen at -20 °C, sliced into 30 µm sections (CM3000 Cryostat, Leica, Nussloch), and stained with thionin. Electrode tracts were examined via magnification-projection system (Research Analysis System model 421251, Amersham, Michigan). Only subjects whose electrode placements were correctly positioned were used in data analysis.

2.6.4 Data Analysis

Data were logarithmically transformed to equalize the variance. Comparisons of pairs of means were done using Student's t-tests (Experiment 1) or analysis of variance followed by post-hoc t-tests (Experiments 2 and 3).

Figure 2-2. Seizure stages. A: Stage 1: Mouth clonus; B: Stage 2: Clonic head movements; C: Stage 3: Forelimb clonus; D: Stage 4: Clonic rearing; E: Stage 5: Loss of postural control (falling). F: Post-ictal depression.

CHAPTER 3

EXPERIMENT 1: RACINE REPLICATION

3.1 RATIONALE

The purpose of Experiment 1 was to replicate and confirm Racine's report (1972a) that ADT drops, and that it drops as a result of stimulation, not electrode presence. Male Long Evans rats were used as subjects and no stimulation was given until 4 weeks after electrode implantation. The Half-Split method was then used for ADT determinations and Racine's threshold drop paradigm was used to lower thresholds. These procedures duplicate those used by Racine (1972a), except that threshold reduction was done for 40 rather than 60 days.

3.2 SUBJECTS

Male Long Evans rats served as subjects. Subjects were housed and fed as described in the General Methods. After determination of initial ADTs, subjects were randomly sorted into experimental and control groups.

3.3 PROCEDURE FOR ADT DETERMINATION

Afterdischarge thresholds were determined, according to the Half-Split Method (described in General Methods) twice during the course of the experiment. An initial ADT determination was performed on subjects 4 weeks following electrode implantation. A final ADT determination was performed 1 wk after the 40 day threshold drop regimen was completed.

3.4 PROCEDURE FOR ADT REDUCTION

After their initial ADT determination, experimental subjects began the daily (5 days/week) threshold drop regimen (described in the General Methods). Control subjects were placed in the test box and attached to the leads, but received no stimulation. Stimulation was continued for 40 days, after which subjects were allowed a 7 d rest period with no stimulation before the final determination of ADT.

3.5 RESULTS

The ADT data for stimulated (experimental) and unstimulated (control) subjects are shown in Figure 3-1. As indicated, at the end of the stimulation period, the mean ADT was virtually unchanged in the (unstimulated) control group. The mean ADT in experimental subjects, however, had dropped over 70% ($p < 0.001$).

A curious feature of these data was the fact that initial thresholds were considerably higher in the experimental than in the control subjects. This was a consequence of the random sorting procedure.

The time course of ADT reduction in the experimental subjects is shown in Figure 3-2. ADT reduction occurred fastest during the first 16 days of stimulation, and more slowly from day 16 through day 40. A final ADT test, taken 1 week after the ADT reduction procedure was completed, revealed an ADT value very close to the ADT on day 40.

Figure 3-1. Reduction of afterdischarge threshold as a result daily electrical stimulation using Racine's threshold drop paradigm. Post-operative time to beginning of testing was 4 weeks. Post-operative time to the final ADT test was 14 weeks. Data are shown as means $\pm$ S.E., ($n = 11$, experimentals; $n = 8$, controls). Asterisk indicates significant difference ($p < 0.001$) from Initial ADT.

Figure 3-2. Pattern of afterdischarge threshold (ADT) reduction in the amygdala resulting from daily electrical stimulation using Racine's threshold drop paradigm in experimental and control subjects. Initial and final ADTs are shown as separate points, outside of the curve. Data are shown as means $\pm$ S.E. ($n = 11$, experimentals; $n = 8$, controls).

3.6 DISCUSSION

Experiment 1 was designed to replicate Racine's findings of stimulation-induced reduction in ADT. Racine's findings were fully replicated. ADT dropped over 70% in the stimulated group and rose slightly in the controls. Examination of individual data showed ADT drop in 11/11 of the experimental subjects. In contrast, 5/8 control subjects showed at least a small rise in ADT.

As Racine (1972a) had noted, the ADT reductions in stimulated subjects occurred mostly during the first 2 weeks of stimulation. Mean ADTs then remained virtually unchanged from day 16 to day 40. Examination of individual data, however, revealed that ADT in some subjects continued to drop in small increments, and at a slow rate, during the later stages of stimulation.

The timing of the final ADT tests should be noted for later reference. As mentioned above, initial ADT determination was done 4 weeks after electrode implantation. Since stimulation was done for 40 days (5 days/wk), final ADTs reflect threshold values approximately 14 weeks after electrode implantation.

Experimental and control subjects had different ADTs before the start of stimulation. This difference in initial ADTs was most likely due to two subjects in the experimental group that had very high thresholds. Histological examination of the placements yielded no obvious explanation for these high thresholds. This kind of disparity could be avoided by sorting subjects on the basis of ADTs following the initial determination.

CHAPTER 4

EXPERIMENT 2: RACINE TIME COURSE STUDY

4.1 RATIONALE

Experiment 1 verified that ADTs are lowered by stimulation when stimulation is started 4 weeks after implantation and Racine's techniques are used. Experiment 2 was designed to see whether the changes in pre-kindling threshold, previously reported by Löscher (1995), would also be seen if Racine's techniques were applied. The basic design of Löscher's experiment (1995) was repeated, and stimulation was begun 1, 2, 4, or 8 wks after implantation. Racine's Half-Split and threshold reduction paradigms were used, however, and male Long Evans rats served as subjects.

4.2 SUBJECTS

Male Long Evans rats served as subjects. Subjects were housed and fed as described in the General Methods.

Subjects were randomly sorted into 4 groups with initial ADTs to be determined at 1 week (1 wk group), 2 weeks (2 wk group), 4 weeks (4 wk group), and 8 weeks (8 wk group) after electrode implantation. Electrode implantations were done on the 4 different groups (1, 2, 4, or 8 wk) at 4 different times (8 wk first, 4 wk second, etc.), so that all subjects were the *same age* when their initial ADTs were determined. (Subjects in Löscher et al. (1995) were operated on at

the same time, and tested at different times after implantation (1, 2, 4, or 8 weeks) Thus, ages in Löscher's subjects differed by as much as 7 weeks.)

4.3 PROCEDURE FOR ADT DETERMINATION

The technique used for ADT determination was the Half-Split technique used in Experiment 1 (General Methods). As indicated above, initial ADT determinations were performed on subjects 1 week (1 wk group), 2 weeks (2 wk group), 4 weeks (4 wk group), or 8 weeks (8 wk group) after electrode implantation. Final ADT determinations were performed 1 week after the threshold drop regimen was completed.

4.4 PROCEDURE FOR ADT REDUCTION

After initial ADTs were determined, all subjects received daily sub-threshold stimulations using Racine's threshold reduction paradigm (General Methods) as in Experiment 1. Stimulation was continued for 40 days, after which subjects were allowed a 7 d rest period with no stimulation before the final ADT determination.

4.5 RESULTS

The ADT data for subjects with 1, 2, 4, or 8 weeks of post-surgical recovery time before initial ADT determination are shown in Figure 4-1. The solid bars indicate pre-kindling ADTs. As indicated, the pre-kindling ADT was similar in the 1 week and 2 week group. At 4 weeks, it was markedly decreased compared to the ADT at 1 or 2 weeks. After a post-surgical period of 8 weeks,

however, the pre-kindling ADT rose again, and was close to the original (1 week) level.

The stippled bars in Figure 4-1A indicate post-kindling ADTs. Patterns of threshold reduction for each of the groups are illustrated in Figure 4-1B. Comparison of final to initial ADTs shows that they are lower in each case, with the exception of the 4 week post-surgical recovery group. The difference is most evident in the 1 week and 8 week groups. In the 4 week group, the final threshold was higher than the initial threshold. A two-way analysis of variance revealed that neither the effects of post-surgical recovery time (1, 2, 4, 8 wks) or time of threshold test (pre- and post kindling) was significant in themselves. There was, however a significant interaction ($p < 0.05$). Post-hoc unpaired t-tests showed a significant difference ($p < 0.05$) between initial thresholds in the 1- and 4-wk groups. Post-hoc paired t-tests showed significant differences between pre- and post-stimulation ADTs in the 4-wk ($p < 0.05$) group. The difference in the 1- and 8-wk group failed to be significant, perhaps due to variability of the data.

4.6 DISCUSSION

The results of Experiment 2 verify Löscher's previous reports (1995, 1993) that there is a variation in initial ADT with time after electrode implantation. The basic pattern seen was similar to that reported by Löscher, with ADT high at 1 week and 2 weeks, low at 4 weeks and high again at 8 weeks. This was true

even though different subjects and a different technique for ADT determination was used.

The effects of stimulation also resemble those reported by Löscher. In the 1 week and 8 week groups, the reduction of post-stimulation ADT was large (>50%). In the 2 week group, there was a small difference, as the final threshold was only slightly lower than the initial threshold. In the 4 week group, the post-stimulation was higher than the pre-stimulation threshold.

Fig. 4-1A. Pre- and post-kindling ADTs (mean, $\pm$ SE) in 4 groups of rats with 1, 2, 4, or 8 weeks of post-surgical recovery time to kindling. The Half-Split paradigm was used to determine ADTs, and Racine's threshold reduction paradigm. N = 12 (1 wk group); N = 7 (2 wk group); N = 9 (4wk group); and N = 10 (8 wk group). Final ADT determinations were done at the following intervals: 11 weeks (1 wk group); 12 weeks (2 wk group); 14 weeks (4 week group); and 18 weeks (8 wk group).

Downward arrows indicate significant difference between initial and final thresholds ($p < 0.05$, 4 wk group paired t-test; $p < 0.05$, t-test of initial thresholds at 1 wk and 4 wks)

Fig. 4-1B. Reduction of ADT in the amygdala, resulting from daily electrical stimulation using Racine's threshold drop paradigm. Four groups of rats, differing in post-operative recovery period to initial ADT testing, served as subjects:

- 1) 8-wk recovery group (n=10);
- 2) 4-wk recovery group (n=8);
- 3) 2-wk recovery group (n=8);
- 4) 1-wk recovery group (n=12).

Data are shown as means. Final ADTs, determined 1 wk after the last day of stimulation, are shown as solid circles.

A consideration of the variation in pre-stimulation ADT offers a possible explanation for the threshold “rise” seen in the 4 week group post-stimulation—and also for the “variable” effects of stimulation seen in the Löscher paradigm. It must be kept in mind that, in this paradigm, ADT determinations take place many weeks after the initial determinations (see Table 4-1). Final threshold determination for the 4 week group, for instance, began 14 weeks post-implantation. Since initial thresholds are varying, and actually rising from 4 weeks to 8 weeks (and possibly continuing to rise after 8 weeks), the threshold reduction caused by repeated stimulation may be “masked” by time-related threshold rises. Comparing post- and pre-kindling ADTs in the Löscher paradigm confounds the effects of stimulation and time. Because there is a varying baseline, the proper way to show stimulation effects on ADT is to compare stimulated subjects to unstimulated time-matched controls, not to original thresholds. This was done in Experiment 3 (below).

A comparison of the data from the 4 week group in Experiment 2 to Experiment 1 shows an apparent paradox. The subjects and procedures used in Experiment 1 and the 4 week group of Experiment 2 were identical. A clear stimulation effect was seen in Experiment 1, however, but not in the 4 week group in Experiment 2: the final ADT in the 4 week group is actually higher than its original ADT. As mentioned above, the effect of stimulation may have been masked by time-related threshold rises. As final ADTs were measured approximately 14 weeks post-implantation in this group, the ADT would have been significantly higher without stimulation, most likely in the range of the 8

week initial ADT. Thus, in light of the varying baseline, a there most likely was a stimulation effect on ADT. This particular result underlines the fact that valid post-stimulation comparisons can only be made to time-matched, unstimulated controls. This was done in Experiment 3.

Table 4-1. Time of Post-stimulation ADT Determination

(illustrated for the 4 wk group)

Time (in weeks)	Procedure
4	Begin initial ADT determination by Half-Split technique (every other day; ADT determined in 3-4 trials)
5	Start stimulation (threshold reduction paradigm) ↓ (40 days at 5 d/wk = 8 wks)
9	
13	7 day "rest" period
14	Begin finial ADT determination by Half-Split technique (every other day; ADT determined in 3-4 trials)
15	Final ADT is determined

CHAPTER 5

EXPERIMENT 3: LÖSCHER REPLICATION

5.1 RATIONALE

Experiment 2 suggested that post-implantation ADT does vary with time, and that thresholds in stimulated subjects should be compared to thresholds in matched controls, not to the subjects' initial thresholds. If this were done, clear effects of stimulation should be seen even in Löscher's paradigm.

Experiment 3, therefore, exactly replicated Löscher's initial design, except that after determination of initial ADTs, subjects were split into stimulated (experimental) and unstimulated (control) groups—as in Racine's initial design. Using female Wistar rats as subjects, differences in pre-kindling ADTs were assessed at different times after electrode implantation. Subjects were then kindled (experimental) or handled (control), and post-kindling ADTs were later determined. The "Ascending Series" procedure was used for ADT determination, and kindling was done with supra-threshold stimulation. These procedures exactly duplicate the procedures of Löscher et al. (1995).

5.2 SUBJECTS

Female Wistar rats served as subjects. They were obtained and housed as described in the General Methods. Subjects were randomly divided into four large groups, with initial ADTs being determined at 1 week (1 wk group), 2 weeks (2 wk group), 4 weeks (4 wk group), and 8 weeks (8 wk group) after electrode

implantation. These 4 groups were then randomly sub-divided into experimental and control subjects. (See Table 5-1.) Experimental subjects received daily kindling stimulations, as described below. Control subjects were handled and attached to the leads, but not stimulated.

Table 5-1. Grouped Data used for Pre-kindling ADTs

Group	Experimental ADTs	Control ADTs
1 week	484.0 +/- 119.1 (n = 5)	477.5 +/- 131.4 (n = 4)
2 week	372.0 +/- 117.4 (n = 5)	407.8 +/- 104.0 (n = 9)
4 week	368.5 +/- 117.5 (n = 7)	246.6 +/- 84.0 (n = 6)
8 week	440.0 +/- 127.6 (n = 6)	360.0 +/- 84.9 (n = 8)

5.3 PROCEDURE FOR ADT DETERMINATION

Afterdischarge thresholds were determined before and after kindling using the Ascending Series Technique (described in General Methods). Final ADTs (post-kindling ADTs) were determined at least 1 week after the last kindled seizure.

5.4 KINDLING REGIMEN

After initial ADTs had been determined, subjects received daily kindling stimulation (5 days/wk). Subjects were stimulated with monophasic pulses and a

constant current intensity of 500 μ A (base-to-peak) once every day (see Stimulation Parameters, General Methods), until 10 sequential stage 5 (i.e. fully kindled) seizures had been elicited. On each day of the kindling regimen, convulsions (if any) were scored using Racine's (1972a) seizure stages.

5.5 RESULTS

The data from Experiment 3 are shown in Figure 5-1. The first two bars at each time point in Figure 5-1 indicate pre-kindling. As indicated, at 2 weeks and 4 weeks post-implantation, pre-handling ADTs were decreased as compared to the ADT at 1 week. However, after a post-surgical period of 8 weeks, pre-kindling ADTs rose again, and were close to the original (1 week) level.

The striped bars in Figure 5-1 indicate post-kindling ADTs for the experimental (kindled) subjects. Comparison of kindled ADTs to pre-kindling ADTs show that they are lower in each case. The open bars in Figure 5-1 illustrate the final ADTs in subjects that received handling but no kindling. These are the "time-matched" controls that indicate where baseline would be without stimulation. Comparison of post-handling ADT's to pre-handling ADT's show the post-handling ADT's to be lower in the 1- and 2 wk groups, but higher in the 4- and 8-wk groups. Comparison between post-kindling ADTs and 'post-handling' ADTs shows little difference in the 1- and 2-week groups, but a large difference in the 4 and 8 week subjects.

Three-way analysis of variance showed an overall main difference in the 'pre' and 'post' ADT's ($p < 0.05$). Post hoc paired t-tests showed two of the pre-post differences to be significant, the pre-post differences in the kindling group at

1 wk and 8 wks ($p < 0.05$). Post-hoc unpaired t-tests showed significant differences between the post-kindled and post-handled group to be significant in both the 4- and 8-wk groups ($p < 0.05$).

5.6 DISCUSSION

Experiment 3 was designed to replicate Löscher's initial design, except that after determination of initial ADTs, subjects were split into stimulated (experimental) and unstimulated (control) groups. This change was made so that valid post-stimulation comparisons could be made. As indicated, pre-kindling ADTs at 4 weeks after electrode implantation were lower than pre-kindling ADT in the 1 week post-implantation group. At 8 weeks, however, the pre-kindling ADT rose again, and was close to the original (1 week) level. This trend replicates the pre-kindling ADT pattern reported by Löscher et al. (1995) and also the pattern seen in Experiment 2. This effect, therefore, has been now reproduced several times.

A consistent drop in post-kindling ADTs was seen, which was greatest in subjects with 1 week and 8 weeks post-surgical delay to kindling, and less pronounced in subjects in the 2 week and 4 week groups. This approximates the drop in post-kindling ADTs seen by Löscher, except that the drop in the 4 week group was more pronounced in the present experiment. Effects in subjects started at 4 weeks continue to be very variable.

When ADTs in stimulated and unstimulated (handled) subjects were compared, no significant effect was seen in the 1 week or 2 week groups, but

there was a large and significant effect of stimulation in the 4 and 8 week groups. This suggests that stimulation has a relatively limited effect on ADTs if it is started shortly after implantation: Apparent kindling effects in these subjects simply reflect the passage of time. When stimulation is begun at 4 weeks or later, however, clearcut stimulation-related drops in ADT can be seen.

It should be noted that the comparison of post-kindling ADTs to post-handling ADTs gives a clearer indication of stimulation effects, than the comparison of pre- and post-kindling ADTs. The relatively large pre/post difference in the 1 week group turns out to be almost entirely independent of stimulation.

Fig. 5-1. Pre- and post-kindling ADTs (mean $\pm$ SE) in 4 groups of female Wistar rats with 1, 2, 4, or 8 weeks of post-surgical recovery time to testing, respectively. For pre-kindling data, all subjects were used to yield mean ADTs. $n = 9$ (1 week); $n = 14$ (2 weeks); $n = 13$ (4 weeks); and $n = 14$ (8 weeks). Post-kindling ADTs were determined 5 weeks (1 wk group), 6 weeks (2 wk group), 8 weeks (4 wk group), and 12 weeks (8 wk group) after electrode implantation. For post-kindling means, $n = 5$ (1 wk group); $n = 5$ (2 wk group); $n = 7$ (4 wk group); and $n = 6$ (8 wk group); For 'Handled' means, $n = 4$ (1 wk group); $n = 9$ (2 wk group); $n = 6$ (4 wk group); and $n = 8$ (8 wk group). Asterisks (*) indicate significant difference between post-kindling and post-handling ADTs between groups ($p < 0.05$) at 4 and 8 wk group; and significant difference between pre-kindling and post-kindling ADTs between groups ($p < 0.05$) in 1 and 8 wk group.

CHAPTER 6

GENERAL DISCUSSION

In the present work, ADT changes were studied in the amygdala kindling model of epilepsy in order to reconcile the conflicting findings of Racine (1972a) and Löscher (Löscher et al., 1993, 1995). More specifically, the present study set out to clarify the respective contributions of electrode implantation and stimulation to threshold drop in the kindling model. This was necessary so that meaningful studies on the pharmacological modification of threshold and threshold drop could be conducted in the future.

6.1 EXPERIMENT 1

Experiment 1—which replicated Racine's (1972a) study—used male Long Evans rats and Racine's Half-Split and threshold reduction paradigms. Initial ADTs were determined 4 weeks after electrode implantation and final ADTs were determined after 40 days of stimulation (experimentals) or handling (controls). A large drop in stimulated subjects was found (~70%) after 40 days. ADTs in control subjects, on the other hand, were comparable at 4 and 14 weeks after electrode implantation. These results are in good agreement with Racine's original report on ADT reduction (1972a). They verify that—at this time period—threshold does drop with stimulation, and does not drop without it.

Racine's observation that threshold drop occurs mostly during the first 2 weeks of stimulation was also verified. Eighty percent of the total drop occurred during the first 2 weeks. Examination of individual records, however, indicated

that ADT drops did occur in some subjects even during the later days of stimulation. These subjects tended to plateau around 16 days and to remain stable for many days. They would then drop to a lower threshold. Several days later, this slight drop would occur again. These late drops—which occurred in 67% of subjects—account for the slight downward shift seen in the late phases of the group curve. These “late” changes in AD threshold - if they also take place in the threshold for non-triggered events - may relate to the spontaneous seizures which are seen in the kindling preparation after several hundred stimulations (Pinel and Rovner, 1978). Kindling-induced changes are progressive, and do not stop with the first stage 5 seizure. These “late” ADT changes emphasize the need for more studies involving long-term kindling.

It should be noted that the early stages of threshold drop may take place even faster than indicated by Racine’s standard threshold drop paradigm. During the first 4 days, when intensity was dropped by 20%, it was often still supra-threshold. If intensity were dropped in larger increments (e.g., 40%) a truer estimation of the speed of threshold drop would be obtained.

6.2 EXPERIMENT 2

Experiment 2 set out to: 1) determine whether the variations in initial ADTs reported by Löscher would be seen if Racine’s techniques were applied at different times after implantation (1, 2, 4, or 8 weeks) and, 2) examine the effect of Racine’s threshold reduction paradigm on the above groups. Initial ADT determinations revealed that at 4 weeks after implantation, threshold was

markedly lower than at 1 or 2 weeks after implantation. At a post-surgical period of 8 weeks, however, initial thresholds rose again, and were close to the ADT found 1 week after electrode implantation. These results are in good agreement with the reports of Löscher et al. (1993, 1995) regarding the effect of post-surgical recovery time on initial ADTs. This phenomenon has now been shown twice in Löscher's studies (1993, 1995) and twice in the present studies (Experiment 2 and Experiment 3). The occurrence of this phenomenon in Experiment 2 shows that the effect is very robust, since it occurs in different strains and sexes of rat, and with different ADT measurement paradigms.

The post-stimulation ADT—as compared to pre-stimulation ADTs—was clearly reduced in the 1 and 8 weeks group. In the 2 weeks group, the post-kindling ADT was only slightly lower than the original threshold. In the 4 week group, however, the post-kindling ADT was significantly higher than its original threshold. These data seem to suggest that stimulation at 4 weeks raised, rather than lowered, ADT. This directly contradicts the results of Experiment 1.

Consideration of these paradoxical results gave rise to an understanding of the need for unstimulated controls. Since the ADT baseline (initial ADTs) is changing, only comparison with time-matched unstimulated controls can show the true effect of stimulation. This important control group was present in Experiment 3.

6.3 EXPERIMENT 3

Experiment 3 set out to determine whether the results of Löscher et al. (1995) could be: 1) replicated and, 2) extended by adding a group of unstimulated controls. Using the Ascending Series technique of ADT determination, it was demonstrated again that initial ADTs vary with post-surgical recovery time after electrode implantation. As mentioned above, this replicated the trend of ADT changes after electrode implantation reported by Löscher et al. (1995).

More importantly, the presence of 'post-handling' ADTs from control subjects allowed a proper evaluation of the relative effects of stimulation and electrode implantation. These data clearly revealed the effects of stimulation in the 4- and 8-week animals. Unexpectedly, these data also revealed that stimulation has virtually no effect on ADT when stimulation is begun shortly after electrode implantation. Past experiments in which the post-operative recovery time before testing was only 1-2 weeks, Wise and Chinerman (1974) and Ehle (1980) for instance, are invalid. Conceivably, post-stimulation disturbances in the brain inhibit (stimulation induced) ADT drop in the first weeks after surgery (see below).

6.4 NECESSITY FOR AN UNSTIMULATED CONTROL GROUP

The results of Experiment 3 underline the necessity for an unstimulated control group. Without the control group, the ADT reduction in the 1 week group could have been mistaken for a stimulation effect, and the effect of stimulation in

the 4 week group might have been missed. Unstimulated controls should be included in all future ADT studies.

6.5 VARIABILITY IN 4-WEEK DATA

A comparison of data from subjects begun at 4 weeks after electrode implantation in Experiments 1, 2, and 3 reveals very variable results.

Experimental parameters in Experiments 1 and 2 were identical at 4 weeks, yet the pre-kindling threshold was high in Experiment 1 (~350 μ A, peak-to-peak) and low in Experiment 2 (~100 μ A, peak-to-peak). In Experiment 1, post-stimulation ADTs showed a large drop, whereas in Experiment 2, they showed a rise.

Comparing Experiment 2 to Experiment 3, it can be seen that the 4 week pre-kindling ADT in Experiment 2 was about 50% of the 1 wk ADT, whereas in Experiment 3 it was 75%. In Experiment 3, a small decrease in post-kindling ADT is seen. These data suggest that the pre-kindling threshold is very variable at 4 weeks. This may be due to the fact that 4 weeks seems to be around the ADT “turn-around” point—the point at which pre-kindling ADT stops dropping and starts to rise, which would produce an unstable baseline.

Pinel et al. (1976) found that subjects that had been administered “high-intensity” stimulations had post-stimulation ADTs similar to their initial ADTs. A calculation of the timing of the last ADT test, however, revealed that it was taken during this “turn around” period (35 days).

Variability around the 4 week period may also explain a paradoxical finding in Experiment 1. ADTs in the (unstimulated) controls did not rise between

the initial ADT determination (4 weeks) and the final ADT determination (14 weeks). (Similar results were found in Racine, 1972a). The initial ADTs may have been taken when thresholds had not yet dropped lowest in the “trough” — or turn around point—of the natural curve of ADT changes after implantation. Final ADTs in the controls may have been taken after the lowest point of this curve, when ADTs were rising.

Future studies the pharmacological modification of ADT and ADT drop might avoid this dangerous time period.

6.6 EFFECTS OF AGE

The results of Experiment 2 also suggest that the effects of age, or more specifically the age differences between the groups used by Löscher et al. (1995), had little effect on initial ADTs. Unlike the paradigm used by Löscher et al. (1995), all subjects in Experiment 2 were the same age at their initial ADT determination. ADTs have been shown to be higher in the younger rats than in older rats (Moshe et al., 1981; Stark et al., 1986), so that the age differences might conceivably have had an effect. The fact that Experiment 2 replicated the pattern of ADTs reported by Löscher, however suggests that age is not an important variable.

6.7 HOW SHOULD ADT DROP BE MEASURED?

How should ADT drop be measured? The results of the present experiments indicate that a proper evaluation of the effect of stimulation on ADT can be best made by comparing post-stimulation ADTs to ADTs of unstimulated,

time-matched controls of similar age—not to the initial ADT of the subjects in question. Also, the experiment may wish to balance ADTs in experimental and control groups after initial ADT determination, as this will yield less variable results, especially when testing is begun at 4 weeks. It is also crucial to wait at least 2 weeks after implantation to start stimulation, since stimulation effects are not seen at earlier periods. It may be wise to avoid the variable “turn around” period at 4 weeks. It is probably not necessary to stagger surgery times in order to control for age.

6.8 WHY DO UNSTIMULATED THRESHOLDS DROP? POSSIBLE MECHANISMS

Why do unstimulated thresholds drop? As mentioned in the Introduction, electrode implantation has been shown to induce significant histological (Robinson et al., 1975), and neurochemical (Benattia et al., 1992; Aston et al., 1980) changes. These may enhance neuronal excitability, since both Blackwood (1982) and Löscher et al. (1993) report faster kindling rates in subjects with long post-implantation times before kindling. They may also explain threshold drop in un-stimulated subjects.

It is particularly interesting to note the time course of the enzyme changes found by Robinson et al. (1975). Enzymes responsible for cellular respiration showed early and rapid changes, increasing in glial cells and endothelium, and decreasing in nerve cells. Acid phosphatase activity increased rapidly in glial cells, and other phosphate-releasing enzymes increased more rapidly. The turning point for these enzyme changes was very similar to the “turn around”

point for ADTs found in the present experiments: most changes reached their peak at 25–40 days, and returned to baseline levels at 60 days. In light of the parallel, it would not be unreasonable to suspect these enzymes are involved in the ADT changes.

The other effects of electrode implantation—amino acid changes, changes in 5-HT binding, iron deposition, etc.—do not show a similar time course, and are less likely to be involved.

6.9 PROPOSED EXPERIMENTS

Three general lines of experimentation are proposed:

1) Time Course Study: A More Detailed Picture of ADT Changes

The present experiments give insight into baseline (unstimulated) ADT changes up to 8 weeks. Future studies need to investigate ADTs after 8 weeks. They should include more groups, perhaps one group/week after electrode implantation from 1 to 16 weeks. This will give information on the long-term stability (or lack of stability) of ADTs.

In addition, a detailed time course study will give more data on the week 4 “turn around” period, during which the direction of ADT changes is reversing. It would be good to have a better estimate of exactly when “turn around” occurs. It would also be good to know whether it occurs at slightly different times in different groups. (This would require repeated measurements.) The above experiments should also assess the effect of stimulation on ADT at various

periods after electrode implantation. As mentioned above, all experiments on ADT and ADT drop should include unstimulated, time-matched controls.

2) What Causes Un-stimulated ADT Drop?

What processes are occurring, and what changes in the tissue surrounding the electrode tip cause the unstimulated drop in ADT which occurs around 4 weeks? The histological findings (e.g. Robinson et al, 1975) of changes in enzymes around the electrode tract should be confirmed and extended. The importance of these might be tested in experiments which manipulated these systems pharmacologically and monitored effects on ADT.

3) What Causes Stimulated ADT Drop? Can it be Blocked?

The present studies have cleared the way for future studies of stimulated ADT drop. As a starting point strategy, drugs known to retard kindling development could be tested for their ability to block or retard ADT drop. Good candidates would be phenobarbital, valproate, and diazepam, which have good anti-kindling effects (Burnham, 1991; Löscher, 1993). It would also be interesting to test protein synthesis inhibitors, and drugs which affect growth factors.

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