1990

Expression Of The Proto-oncogene C-fos Following Electrical Kindling In The Rat

G Campbell Teskey

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EXPRESSION OF THE PROTO-ONCOGENE C-*FOS* FOLLOWING ELECTRICAL KINDLING IN THE RAT

by

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Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
November, 1989

ISBN 0-315-55282-4

ABSTRACT

Kindling is an example of permanent change in brain function that results from repeated elicitation of epileptiform neural activity. The molecular/genetic mechanisms by which kindling is produced and maintained are just beginning to be addressed. Recently two proto-oncogenes, c-*fos* and c-Ha-*ras*, have been identified as possible mediators of intracellular signal transduction and may mediate the kindling process. Additionally, c-*fos*, has been proposed as the gene responsible for turning on molecular events that might underlie the long-term neural changes that occur during kindling. The objective of the present study was to: i) further investigate the enhancement of c-*fos* and c-Ha-*ras* levels following kindled seizures and ii) investigate the role of c-*fos* in the plastic changes underlying kindling.

In this study, male hooded rats, were electrically kindled and the resulting c-*fos* and c-Ha-*ras* gene expression was quantified using Northern blot analysis. Initially the time course of c-*fos* and c-*ras* expression after a stage 5 kindled seizure in the amygdala-pyriform was assessed. This was followed by measuring c-*fos* levels in different neural tissues, those that kindle (hippocampus, and neocortex) and those that do not (cerebellum). The accumulation of c-*fos* at different kindling stages on both the stimulated and contralateral hemispheres was determined. Different forms of

electrical stimulation, kindling and non-kindling, were delivered to the amygdala and c-*fos* accumulation was determined. An assessment of the level of c-*fos* due to individual variation in afterdischarge (AD) duration was performed. The reinduction of c-*fos* was examined by delivery of a second kindling stimulation to a group of kindled animals. Finally the effect of pentylenetetrazol (PTZ) injection, which either did or did not produce a convulsion, on c-*fos* accumulation was determined.

The results indicated that: i) c-*fos* was constitutively expressed in forebrain and cerebellum, ii) basal levels of c-*fos* were equivalent in naive and in fully kindled rats that has been seizure free for 3 weeks, iii) an amygdala-pyriform kindled seizure massively and transiently increased c-*fos* levels, iv) kindled seizure enhancement of c-*fos* was observed throughout forebrain and cerebellum, v) enhancement of AD duration corresponded to enhanced c-*fos* levels, vi) enhanced c-*fos* levels were observed in the amygdala-pyriform contralateral to the kindled site, and the enhancement did not depend on the occurrence of AD in the contralateral amygdala-pyriform, vii) electrical stimulations not resulting in AD increased c-*fos* levels, viii) c-*fos* levels were increased by control stimulations, ix) c-*fos* induction was partially refractory, x) PTZ caused c-*fos* induction independent of a motor convulsion, and xi) kindled seizures did not alter the

expression of c-Ha-*ras*.

The most parsimonious explanation that accounts for the observations made in this study is that c-*fos* was expressed simply as a consequence of neural activity and not exclusively due to the specific neural activity (afterdischarge) required for kindling. This does not preclude a role for c-*fos* in the long-term response to external stimuli, but it does suggest that c-*fos* is not the crucial "master switch" in turning on a molecular program that might underlie kindling.

ACKNOWLEDGEMENTS

I would like to thank Peter Cain for his infectious enthusiasm, unfailing encouragement, and critical commentaries. More than just a supervisor he acted as a confidant, "sounding board", and role model. I am grateful.

Special thanks to Burr Atkinson for the generous use of his facilities as well as his judicious employment of the carrot and stick.

Invaluable technical assistance and moral support were altruistically provided by many, including; Francis Boon, Eric Hargreaves, Zoe Dennison, Norman Yu, Jeff Tomlinson, Ing Goping, Karen Walden, Ling Lau, and Roger Frappier.

James and Colleen Dobbin, David Carey, Dwight Stewart and Francis and Kathy Boon provided the much appreciated distractions and friendship necessary for thesis survival.

I have cached away the sage advice and philosophies imparted by Peter Ossenkopp, Martin Kavaliers, Frank Prato, and Case Vanderwolf.

Finally, to Tannis, who's unfaltering love and strength allowed me to complete this document. Thank you.

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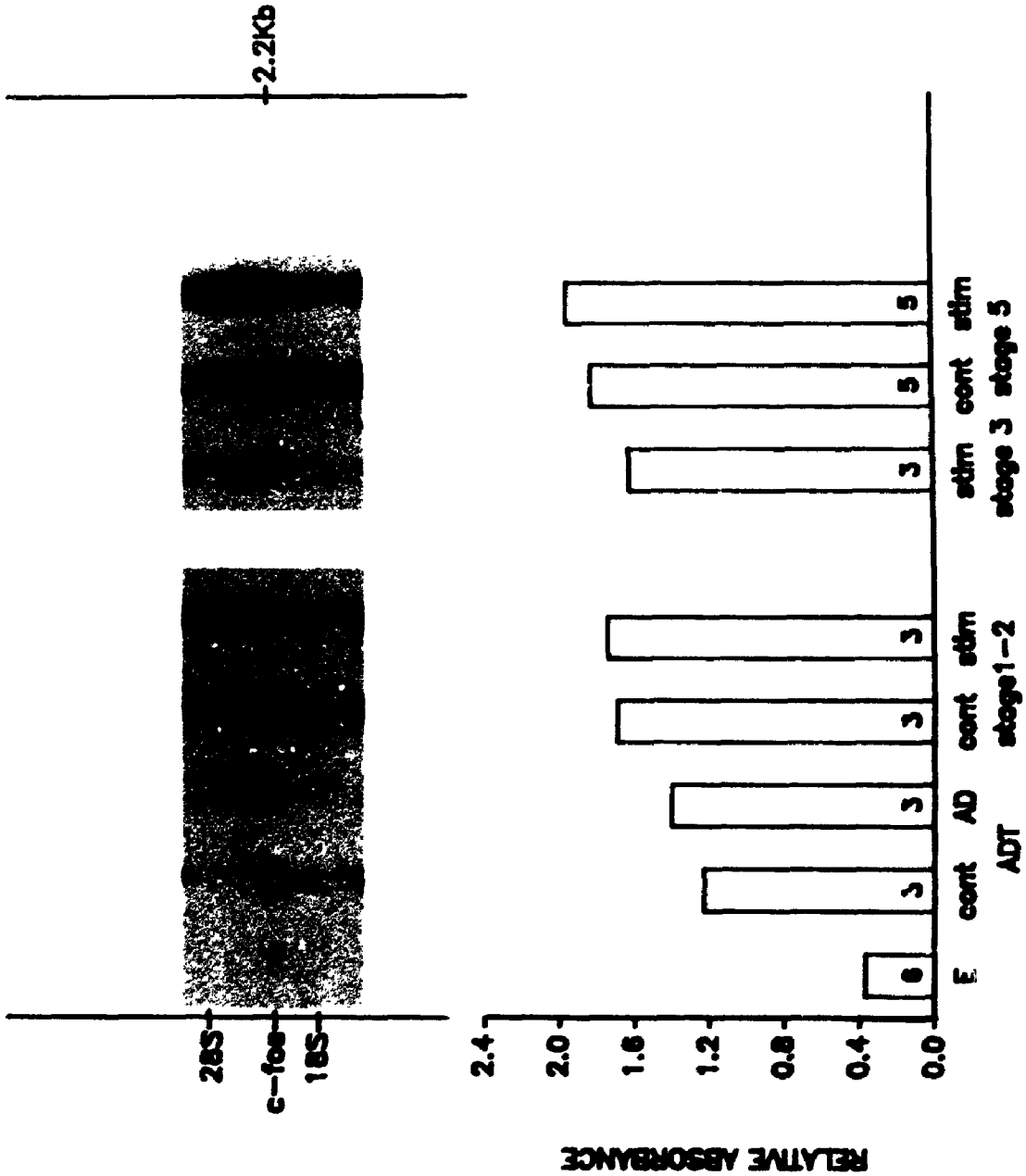

2.2Kb
28S
c-fos
18S
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
6
3
3
3
3
3
5
5
E cont AD cont stim cont stim
ADT
stage1–2
stage 3 stage 5

LTP	long-term potentiation
MOPS	3-[N-Morpholino]propane-sulfonic acid
mRNA	messenger ribonucleic acid
ms	millisecond
myc	avian myelocytomatosis (AMV) oncogene
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PC12	pheochromocytoma cell line
PKC	protein kinase C
PTZ	pentylenetetrazol
ras^{Ha}	Harvey murine sarcoma (Ha-MuSV) oncogene
SD	spreading depression
SDS	sodium dodecyl sulfate
sis	simian sarcoma oncogene
src	Rous sarcoma virus (RSV) oncogene
SSC	0.15M NaCl and 0.015M trisodium citrate
V	volts
yes	Yamaguchi 73 avian sarcoma virus (Y73-ASV)

PART 1

INTRODUCTION

In 1969, Graham Goddard and colleagues described an animal model for neuronal plastic change which they termed "kindling" (Goddard *et al.*, 1969). Initially, Goddard was interested in the effects of amygdaloid electrical stimulation on avoidance conditioning in rats. He observed that repeated stimulation caused his rats to develop seizures. Although many others had observed this somewhat irritating confound, Goddard believed that the brain was changing in response to the invariant stimulation, and that this change could provide a useful neural model for learning and memory as well as for focal epilepsy. Goddard coined the term "kindling" in analogy to starting a fire with an initially ineffective stimulus. Thus, kindling refers to the progressive development of epileptiform activity and associated behavioural convulsions in response to the repeated application of initially subconvulsant, relatively low-intensity, electrical stimulation of the brain. Michael Corcoran, in his 1988 review (p.82), provided an excellent summary of kindling with the following statement.

> Kindling comprises a cascade of physiological, biochemical, and presumably morphological phenomena, some of which we may be starting to understand. These events represent a pathological distortion of the mechanisms of synaptic plasticity

> that participate in other forms of sensitization in the nervous system. The very fact that they are so exaggerated may mean that it will be easier eventually to identify and characterize them than it will be with more elusive, but less aberrant, forms of sensitization. Although pathological in itself, kindling may open a window on the normal functioning of the nervous system.

Kindling is particularly well suited for study because unlike other models, precise control can be maintained over the experimental variables. Behavioural and electrographic development is gradual enough to permit manipulation at various phases of the process. Results are easier to interpret because kindling does not require the use of exogenous substances such as alumina cream, penicillin, or kainic acid, and the choice of kindling site permits experimental investigation of particular foci or neurotransmitter pathways. Kindling provides us with an animal model which allows precise control over the neural changes and thus an excellent opportunity to explore the molecular/genetic basis of neuronal plastic changes.

KINDLING:

The kindling phenomenon usually involves the chronic implantation of electrodes, using stereotaxic techniques, and the repeated administration of a 1 second train of 60 Hz biphasic square waves at a regular interval (1-48 hrs). The electrical stimulation, if sufficiently strong, evokes a self-

sustaining epileptiform response, termed afterdischarge (AD), that can be recorded in the electroencephalogram (EEG). The AD consists of a series of "spikes" with amplitudes of 0.5-5.0 mV and a frequency of 1-3 Hz. On the first day of a typical kindling study, the minimum electrical current required to evoke AD, the afterdischarge threshold (ADT), is determined. The ADT for electrodes in forebrain regions ranges from 50-200 μA. On subsequent sessions, suprathreshold current (50 μA > threshold) is applied and the resulting AD recorded. Racine (1972a,b) has demonstrated that the spaced and repeated occurrence of AD is both necessary and sufficient for the development of kindling. Furthermore, repeated subthreshold stimulation will lower the ADT but will not contribute to the kindling process itself (Racine, 1972a).

A second standard paradigm for studying kindling has also evolved. This is achieved when intracranial infusions of convulsant chemicals are slowly applied and used to elicit AD. The following discussion and experimentation concentrates on kindling produced by discrete electrical stimulation of the rat brain.

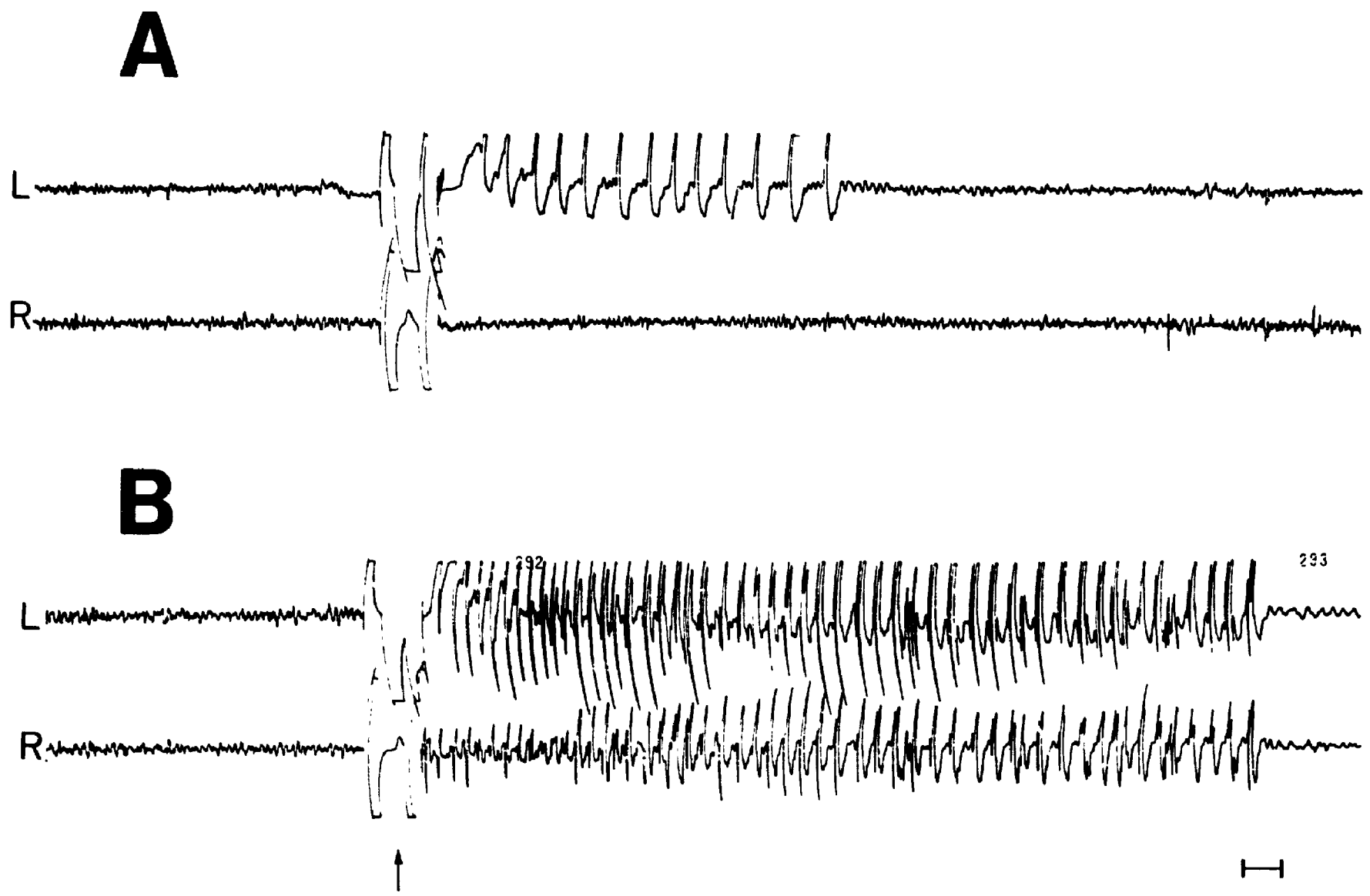
The repeated occurrence of AD results in a strengthening of the AD. On the first day AD duration in the amygdala averages approximately 6 seconds, while following the 11th session, with a daily stimulation interval, the AD duration

in the amygdala averages approximately 50 seconds. The AD duration, frequency, complexity, and propagation to secondary sites all progressively increase (Racine, 1972b), while the amplitude, at the site of stimulation, decreases after a few stimulations and then progressively increases (Teskey *et al.*, 1988). Figure 1 compares the AD on the first and the eleventh sessions.

Behaviorally, the animal progresses through stages of motor seizure severity, culminating in a fully generalized convulsion involving most or all of the skeletal musculature. Racine (1972b) has classified the convulsive behaviour in the rat into five distinct stages: 1) motor arrest accompanied by facial automatisms such as vibrissae twitching, 2) head nodding and chewing, 3) unilateral forelimb clonus, 4) rearing on the hindlimbs with bilateral forelimb clonus, and 5) a seizure characterized by rearing, clonus of both forelimbs, and falling. Although animals are usually considered to be fully kindled after displaying a stage 5 convulsion, continued stimulation for an extended period of time will result in more severe seizures and even spontaneous seizures which can occur in the absence of a triggering pulse of electrical current (Pinel and Rovner, 1978).

Immediately following the AD and convulsive episode a brief (1-60 sec) flattening of the EEG and an absence of behaviour is observed. This state has been termed post-ictal

Figure 1. Bipolar recordings from the left (L) and right (R) amygdala. **A.** The first session. **B.** The 11th session in the same rat, which resulted in a stage 5 convulsion. A few seconds of baseline EEG precedes the arrow (↑) which indicates the stimulation. The calibration (⊢⊣) denotes one second. Stimulation was delivered to the left amygdala only.


A
L
R
B
L
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292
293

depression. Afterwards, the rat's EEG and behaviour seems to be normal with the exception of the occurrence of interictal spikes in the EEG. These are single epileptic spikes which occur more frequently following a seizure and become less frequent with greater temporal separation from the seizure.

The brain changes that result from kindling seem to be very long lasting. Animals kindled to stage 5 and allowed to remain unstimulated for periods up to a year or more have responded to stimulations of the same current intensity with a stage 5 convulsion (Goddard *et al.*, 1969; Wada *et al.*, 1974; Corcoran, 1988). This has given rise to the view that kindling is essentially permanent. However, in amygdala kindled rats, we have found that after an interval of twelve weeks from the last stimulation, there was some falling back to earlier stages, and less electrographic output (Dennison *et al.*, 1989). Nevertheless, there were significant savings in the number of stimulations to return to a stage 5 convulsion, and the passage of time does not return an animal to a naive state.

Kindling has been demonstrated in all animal species in which it has been attempted, from frog to baboon (McNamara, 1986) and it occurs in most brain areas including neocortex, basal forebrain, limbic structures, and brain stem (Cain, 1977; Racine, 1978; Wada, 1982; Burnham, 1985). The only major brain structure that is known not to kindle is the

cerebellum (Maiti and Snider, 1975).

Kindling is not the result of a pathology caused by electrode implantation, poisoning by metallic ions, edema, or gliosis (Goddard *et al.*, 1969). This has been shown by experiments in which electrodes have been implanted and a period of time has been allowed to pass. If damage is involved in kindling, one would predict a more rapid rate of kindling in this group, than in a group kindled immediately after recovery from surgery. However, there is no difference between the rate of kindling in the two groups. Furthermore, it has been demonstrated that there is no tissue damage in kindled animals that does not also occur in implanted unkindled controls (Goddard and Douglas, 1975), and there is no damage to synaptic regions at the site of stimulation (Racine and Zaide, 1978). The seizure development effects of the kindling treatment are relatively independent of stimulus intensity, again suggesting that tissue damage at the tip of the electrode is not a cause of the seizure development (Racine, 1972b). Thus, kindling represents a robust modification of brain function, but what is its relevance to the normal functioning of the nervous system?

Since its discovery twenty years ago, kindling is considered to be the best available model of secondary generalized temporal lobe epilepsy (Racine, 1978; Wada, 1978; Goddard, 1983; McNamara, 1986) and many have suggested that

kindling may be an expression of neuronal plasticity that modifies synaptic function in a manner similar to that which occurs in learning and memory (Goddard and Douglas, 1975; Morrell and Toledo-Morrell, 1986).

The epilepsies are a family of neurological disorders that have in common a transient, recurrent, self-sustained interruption of normal brain function and a simultaneous hypersynchronous activation of a large population of neurons in one focal area or generally throughout the brain. Kindling provides a model for epilepsy of the focal kind. In many ways kindling provides the best model system because the animal seizure mimics human seizure behaviour and has a similar EEG pattern. Following either a kindled seizure in animals or a human ictal (seizure) event, interictal spikes are observed in the EEG. Kindling and human focal seizures also share similarities in a close correspondence of pharmacology. Anticonvulsant drugs used as a treatment for the human condition will dampen the kindled seizure and retard the development of kindling.

Kindling-like effects may even contribute to human epileptogenesis (McNamara, 1984). In humans, a focus arising from one temporal lobe may progress so that both temporal lobes eventually trigger the seizures. This phenomenon is known as the development of a mirror focus. During kindling, progression to secondary sites is observed, with the secondary sites becoming independent generators of AD and not simply

reactive to the primary site. A single case in humans demonstrates that repeated periodic electrical stimulation is sufficient to cause the development of motor seizures (Sramka *et al.*, 1977). Electrodes were implanted in an individual's left thalamus to ameliorate intractable pain. Stimulation lead to a convulsion of the right arm, that progressed to generalized tonic-clonic convulsions and were not due to complications such as abscess or haemorrhage. Thus, kindling provides an excellent and practical model for investigations into some pathological processes that affect the brain. In a broader perspective, the understanding of kindling would provide valuable insights into normal brain function.

What then are the similarities between kindling and learning and memory? I should begin by clarifying what is meant when kindling is referred to a model of learning. To begin, most of the processes typically associated with learning, such as "reinforcement", "pairing of stimuli", or involvement of "attentional mechanisms", are either not present or not identified during kindling. However, if we restrict our interest to the "unit physical change" that underlies "engram formation", or the physical trace, then kindling may have some utility. Probably the most compelling evidence that kindling and engram formation may be related, is that kindling involves a permanent change in brain function in adult animals. Fully kindled animals when left for long periods of time, relative to their life spans, display a

seizure when restimulated. Secondly, forebrain structures, which are regarded to be more directly involved in the processes of engram formation than brainstem sites, kindle more rapidly (Goddard *et al*, 1969; Racine *et al*, 1972). Thirdly, analogously with learning, intermittent trials result in more rapid acquisition then massed trials. I should point out that there are many arguments for not viewing kindling as a model of learning and memory, (for review see Racine and Zaide, 1978). Most neuroscientists probably view a relationship between epilepsy and learning as unconvincing. Perhaps kindling is best viewed as a model for neural plastic change (Morrell and Toledo-Morrell, 1986; Corcoran, 1988).

Tsukamari (1981) defined plasticity as any persistent change in the functional properties of single neurons or neuronal aggregates. Kindling results in a permanent functional change that does not seem to be a consequence of pathology (Morrell and Toledo-Morrell, 1986), and is therefore considered to be a good model of neural plasticity (Racine, 1978; Corcoran, 1988). The alterations in brain that accompany kindling appear to be based on a mechanism that is expressed at the cellular level. Evidence for neural plasticity comes from four general observations i) progressive changes in AD and behaviour during kindling ii) occurrence of transfer, iii) enhancement of evoked potentials and iv) increase in the bursting properties of neurons. Evidence supporting these statements will be reviewed in the following

section.

There are progressive increases in AD in the primary site as well as the appearance of AD at anatomically related secondary sites. Initially, regions with strong anatomical connections to the primary kindled focus support stronger ADs that are synchronous with the evoked AD, than areas synaptically further away. However, during the later stages of kindling independent AD is recorded from secondary sites in response to stimulation of the primary kindling site. This implies that the secondary sites have themselves become kindled.

Transfer is observed when fewer ADs are required to evoke fully generalized seizures in secondary sites (e.g. the contralateral amygdala) following kindling of the primary site (Goddard *et al.*, 1969; Racine, 1972b; Burnham, 1981; Cain, 1986). This facilitation between sites suggests that transsynaptic neural changes occur as a result of kindling the original brain site. Furthermore, transfer is not eliminated by destruction of the primary site prior to stimulating the secondary site (Racine, 1972b), which indicates that transfer does not operate simply by activating the kindled primary site through stimulation of the secondary site.

Evoked potentials are electrical responses recorded in target sites that are evoked by the application of electrical

pulses to neuroanatomically related regions. Evoked potentials that were recorded following kindling in areas neuroanatomically related to the kindling site but separated by at least one synapse showed an enhancement in amplitude when compared to evoked potentials elicited prior to kindling (Racine *et al.*, 1972; Racine *et al.*, 1976). In addition, Racine *et al.*, (1983) found no change in potentials evoked by stimulation of sites other than the primary focus. This suggested that the changes seen in transmission are specific to the pathways directly involved in seizure development. Therefore, the changes seen as a result of kindling involve alterations in normal transsynaptic conduction, and not merely changes specific to epileptiform activity.

The possibility that neurons themselves change and fire in an "epileptiform burst" mode has been examined using intracellular recordings. Racine *et al.*, (1986) has shown that the response to intracellularly applied depolarizing current was often stronger in cells from kindled slices. Cells from kindled ventral hippocampal slices often responded at the onset of the depolarizing pulse with a burst-like discharge of action potentials, while synaptic input remained normal (Racine *et al.*, 1986). The idea that cells may develop abnormal response properties as a result of kindling is further supported by the work of McIntyre and Wong (1986), who observed large changes in the strength of epileptiform discharges in the isolated amygdala/pyriform slice

preparation. Thus, high-frequency activation alters some neurons such that single pulses and other treatments can evoke a stronger bursting response in kindled neurons relative to controls (Racine *et al*, 1986; McIntyre and Wong, 1986; King *et al.*, 1985).

The neuronal plastic changes discussed above are thought to be based on, at least in part, a form of neural growth. Evidence for new growth comes from two main findings i) changes in synaptic terminals, ii) kindling retardation by protein synthesis inhibitors.

Goddard and Douglas (1975) observed electron micrographs of kindled tissue and found that synaptic terminals were increased in size, although their experimental design did not allow them to conclude that kindling increased terminal size. There have been reported increases in the size or numbers of dendritic spine contacts following electrical stimulation (Rutledge *et al.*, 1974; Van Harreveld and Fifkova, 1975; Sutula *et al.*, 1988) as well as changes in synaptic morphology, the ratio of perforated to nonperforated synapses, following kindling (Geinisman *et al.*, 1988). Racine and Zaide (1978) reported a significant increase in terminal size but not number of terminals. However, Racine (1978) failed to replicate the observation of an increase in terminal size in a later study. Thus, although the exact nature of morphological change during kindling remains uncertain, it

seems likely that some form of change occurs.

It is generally accepted that alterations in synaptic connectivity that permanently facilitate neural transmission are likely to be dependent, at least in part, on neural growth (McIlwain, 1977). Since growth processes require the synthesis of new proteins, a treatment that disrupts protein synthesis, and therefore growth processes, may alter kindling. This possibility was approached by studies using cycloheximide (CHX) as a protein synthesis inhibitor. Although epileptiform AD was inhibited in both frog and rabbit it was not clear whether CHX blocked the development or the expression of the seizures (Morrell *et al.*, 1976; Ogata, 1977). Cain *et al.* (1980), using anisomycin (ANI) as a protein synthesis inhibitor, which is essentially free of anticonvulsant effects, found that ANI-treated mice showed little or no tendency to develop kindled convulsions despite being stimulated 14 times. ANI was also found to reduce seizure duration in mice. Rats that received ANI pretreatment displayed a significantly reduced development of kindling (Jonec and Wasterlain, 1979). This latter finding has been replicated (Teskey, unpublished).

In my study, two groups of animals were used. One half hour before receiving the kindling stimulation rats received intraperitoneal injection of either 60 mg in 2.5 ml/kg ANI or 2.5 ml/kg physiological saline. One half hour after ANI injection protein synthetic inhibition is maximal. Thus, at

the time of stimulation, protein synthesis was at a minimum. Animals that received saline treatment kindled to a stage 5 seizure in an average of 12.5 sessions, while animals that received ANI did not reach a stage 5 seizure. After 20 sessions, ANI-treated animals were having on average a behavioural seizure of stage 2-3. In a cross-over design, those animals that received saline and were fully kindled now received ANI. These animals, following ANI injection and stimulation, displayed a stage 5 seizure that was undistinguishable from a seizure preceded by saline injection. In the other group, those animals that h ' received ANI and 20 stimulations were then administered sa..ne. These animals under the saline condition required an average of 11 sessions to reach a stage 5 seizure. From this study it can be concluded that ANI inhibited the development of kindling but did not affect the expression of the seizures.

In summary, the evidence presented suggests that the process of kindling involves long-term changes in transsynaptic connections and the intrinsic hyperexcitability of neurons. Furthermore, these changes are probably dependant, in part, on neural growth processes. Such growth processes would be expected to be dependant on cellular mechanisms for growth and would therefore require altered expression of cellular genetic information. Thus, we would expect a change, either an increase or decrease, in the synthesis of specific mRNAs and their proteins that are

directly involved in effecting a transition to the kindled state. Given this, there is a need to identify specific mRNAs and polypeptides that are related to the neural plastic changes underlying the kindling phenomenon. Fortunately, the discovery of oncogenes and their involvement in growth processes may provide some good candidates for mRNAs and proteins involved in kindling.

ONCOGENES:

Oncogenes are genes with potential properties for the induction of neoplastic transformation in either natural or experimental conditions. Most oncogenes have been isolated from acute transforming retroviruses, which act as oncogene transducers. Oncogenes were discovered by the unexpected presence of avian tumour virus RNA and proteins in uninfected chicken embryo cells (Leong *et al.*, 1972; Hayward and Hanafusa, 1973; Chen *et al.*, 1974). Furthermore, it was demonstrated that the cells of uninfected chickens contain homologous genome nucleotide sequences to the transforming gene of avian sarcoma viruses (Stehelin *et al.*, 1976). Similarly, cellular DNA and RNA sequences, homologous to acute transforming retroviruses, were found in rat tumours as well as in normal rat tissues (Anderson and Robbins, 1976, and Scolnick *et al.*, 1976). This showed that the cellular genome harboured, in apparently

latent form, a gene which could assume malignant roles when properly activated.

For clarity, proteins encoded by oncogenes are specified by the short form, e.g. FOS. The gene is abbreviated and either italicized or underlined, as in *fos*. Viral alleles carry the designation v as in v-*fos*, while cellular alleles carry the designation c as in c-*fos*.

Today, cellular oncogenes have been found in all vertebrate species studied, as well as in invertebrates. Oncogenes seem to be present and expressed in all multicellular animals including sponges, and at least some are present in yeast, archaebacteria and plants (Shilo, 1984; Zabulionis *et al.*, 1988). The widespread distribution of oncogenes in nature suggests that their expression has essential biological function, most probably growth, since their oncogenic counterparts induce abnormal growth.

At least 18 cellular oncogenes and perhaps 12 more carried by various DNA tumour viruses are known. But how do these 30 oncogenes exert their mechanism of action? It is known that oncogene proteins elicit only exaggerated versions of the phenotypes produced by their normal cellular counterparts. Thus it seems reasonable that the control of growth of a cell must be controlled by either: i) intercellular signals (growth factors), ii) cell surface

receptors, or iii) the proteins responsible for transducing growth signals from the receptors to critical intracellular targets.

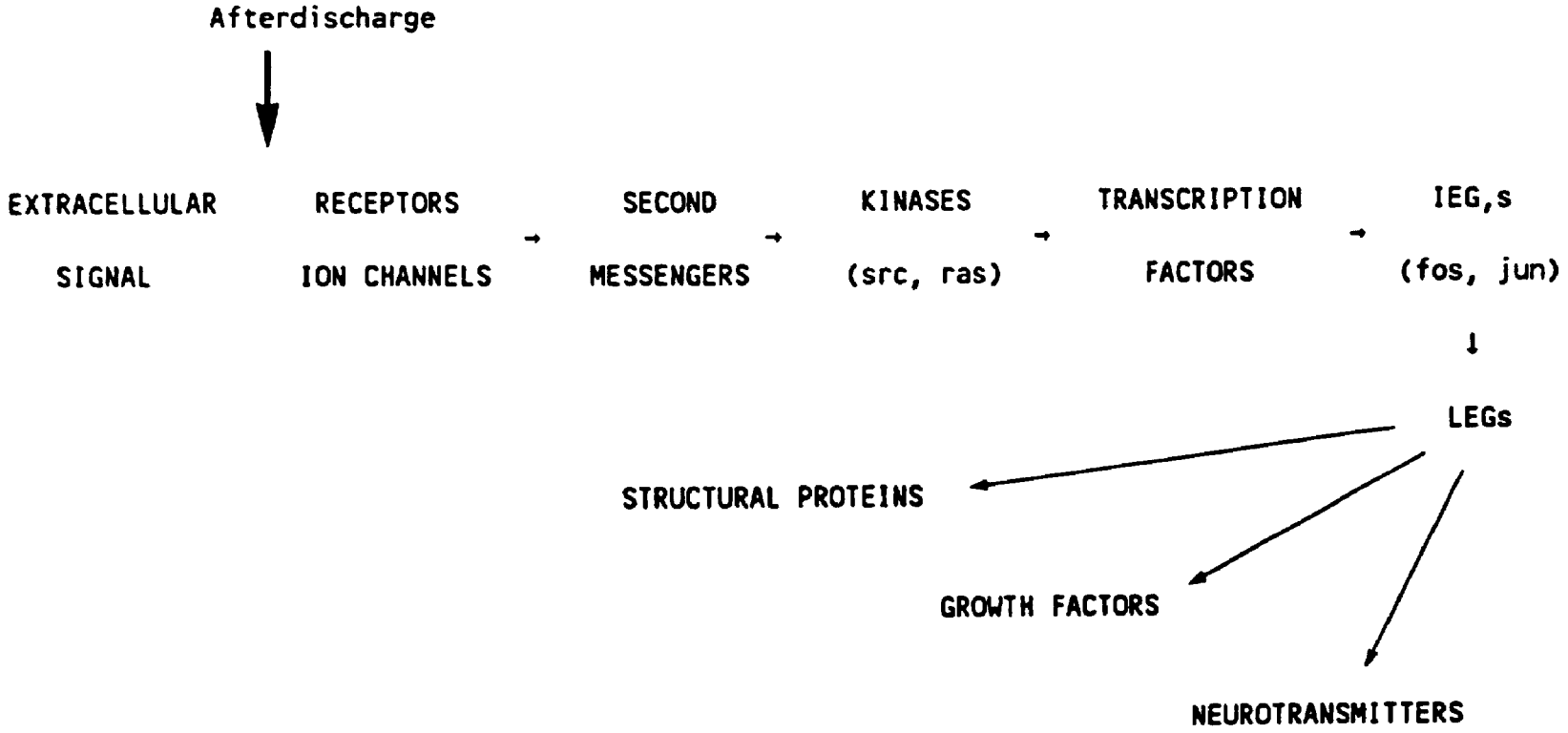
Recent evidence suggests that there are oncogenic proteins of all three types. The identification of oncogenes whose products clearly fit into the first two categories came from comparison of the predicted amino acid sequences of the oncogenic proteins with those of purified growth factors or growth factor receptors. The product of v-*sis* oncogene of simian sarcoma virus shows a strong homology with the partial sequence of human platelet-derived growth factor (Waterfield *et al.*, 1983; Doolittle *et al.*, 1983; Devare *et al.*, 1983). Another connection between oncogenes and growth control emerged from the comparison of the sequence of several tryptic peptides derived from epidermal growth factor (EGF) receptor. The EGF receptor, purified from the human tumour cell line A431, showed an almost perfect match with the predicted product of the v-*erb*-B oncogene of avian erythroblastosis virus (Jansen *et al.*, 1983, and Gray *et al.*, 1983; Sherr *et al.*, 1985). Some preliminary evidence suggests that the protein products of either v-*fms* or c-*neu* may also act as growth receptors (Anderson *et al.*, 1984; Manger *et al.*, 1984; Schecter *et al.*, 1984).

The third type of oncogene product is one that acts at a postreceptor site in a cellular growth control pathway. There are several candidates for this type of mediation. The

src family of oncogenes, including the *yes*, *fgr*, *fps/fes*, *ros* and *abl* oncogenes, all encode proteins with protein-tyrosine kinase activity. These proteins, which act as enzymes, might phosphorylate proteins which are normally targets for the growth factor-activated receptor protein-tyrosine kinases and thus provide the intracellular growth stimulus. Recent evidence implicates the *ras* proteins in signal transduction from cell surface receptors by regulating adenylate cyclase and hence cAMP levels. There is also a series of oncogenes whose products are nuclear; namely *jun*, *myc*, *myb*, and *fos* genes. The nuclear location of their products suggests that they might play a role in regulating the expression of other genes needed in the growth response (Cochran *et al.*, 1984).

While the molecular mechanisms underlying signal transduction in the nervous system have been studied extensively, much remains to be discovered. My intention here is to draw attention to the path-like interconnection of oncogene action that might modulate gene activity and, potentially, subsequent neural plastic change. A speculative intracellular signal transduction pathway is depicted in Figure 2. Following a stimulation that produces an AD, receptors and/or ion channels are activated leading to an influx in ions, most notably calcium. Calcium itself, or in combination with other second messengers, such as inositol triphosphate, activates protein kinases (SRC, RAS). The kinases in turn may phosphorylate transcription factors which

Figure 2. A speculative intracellular signal transduction pathway.


Afterdischarge
EXTRACELLULAR SIGNAL
RECEPTORS ION CHANNELS
SECOND MESSENGERS
KINASES (src, ras)
TRANSCRIPTION FACTORS
IEG,s (fos, jun)
LEGs
STRUCTURAL PROTEINS
GROWTH FACTORS
NEUROTRANSMITTERS

may activate immediate early genes (IEGs). At this point IEGs (FOS, JUN) could up-regulate certain genes and down-regulate others (including themselves). This can lead either to an accumulation, or loss, of late effector gene (LEGs) proteins. These LEGs could be the proteins that are involved directly, or as regulators, in the signal transduction pathway. The LEGs could also be growth factors and neurotransmitters thought to effect intercellular communication, as well a structural proteins involved in neuronal morphology. The signal transduction pathway provides a model to account for the production of long-term changes in cell metabolism and morphology following cell activation by extracellular signals (Greenberg and Ziff, 1984). Since individual oncogenes influence the activity state of the pathway, and thereby the plastic (growth) properties of mammalian neurons, the exploration of their regulation and expression following kindling is needed.

From the intracellular signal transduction pathway it is obvious that *fos* (and probably *jun*) serve as a primary target of signal transduction; *fos* then transforms the incoming signal into a change of gene expression. The induction of *fos* is rapid, large with respect to it's preinduction levels, and transient (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Lau and Nathans, 1987). Furthermore, *fos* is transcriptionally activated, a protein synthesis-independent mechanism, and under the control of constitutively expressed transactivating

proteins that bind to DNA and regulate gene transcription (Curran and Morgan, 1987). Thus, *fos* is thought to turn on a developmental program by activation of LEGs, and for this reason *fos* has been termed the "master switch" (Marx, 1987). The FOS protein, which shows DNA-binding activity, can activate or repress the protein synthesis-dependent and more durable LEGs (Robertson, 1988), which would be responsible for growth changes.

Fos:

The c-*fos* gene is the normal cellular counterpart of the viral oncogene v-*fos* which has been identified as the bone transforming gene of the FBJ-murine osteosarcoma virus (Curran *et al.*, 1983; Verma, 1988). The cellular gene encodes for the FOS protein which undergoes extensive post-translational modifications and exists as a nuclear phosphoprotein in forms 55-72 kDa that exhibit both non-specific and sequence specific DNA binding properties (Curran *et al.*, 1984; Adamson *et al.*, 1985; Sambucetti and Curran, 1986; Curran *et al.* 1985). Recently, it has been observed that the c-*fos* gene was rapidly and transiently expressed in many tissues in response to growth factor stimulation (Greenberg and Ziff, 1984; Muller *et al.*, 1984; Cochran *et al.*, 1984; Kruijer *et al.*, 1984; Greenberg *et al.*, 1985; Curran and Morgan, 1985; Kruijer *et al.*, 1985). These results suggested a non-mitogenic role for c-*fos* in cellular differentiation and growth.

In vitro, c-*fos* mRNA and FOS rapidly increased in nondividing neuronally-differentiated pheochromocytoma (PC12) cells after depolarization with K^+, stimulation with cholinergic agonists, or nerve growth factor applications (Kruijer *et al.*, 1984; Greenberg *et al.*, 1986; Morgan and Curran, 1986; Milbrandt, 1986). Application of phorbol esters, which activate protein kinase C (PKC), also induce c-*fos* transcription in PC12 cells (Morgan and Curran, 1986). It should be noted that in PC12 cells, agents that induce c-*fos* fall into two categories (Curran and Morgan, 1986): 1) induction by growth factors and phorbol esters gives rise to a FOS protein that undergoes extensive post-translational modification and 2) induction by depolarization yields a FOS protein that undergoes less extensive post-translational modification. In primary cultures of rat cerebellar granule cells, administration of PKC activators as well as glutamate, NMDA, and quisqualate, enhanced c-*fos* mRNA expression (Szekely *et al.*, 1987). The induction of c-*fos* by excitatory amino acids can be blocked by NMDA-receptor antagonists and high $[Mg^{2+}]$.

Normally the c-*fos* mRNA is present at low levels in rodent brain, particularly neocortex (except layer 1), caudate-putamen (but not globus pallidus), septum, amygdala, hippocampus, dentate gyrus, pyriform cortex, cingulate cortex, entorhinal cortex, and some thalamic and hypothalamic areas (Dragunow *et al.*, 1987; Dragunow and Robertson, 1988; Gubits *et*

al., 1988; Morgan *et al.*, 1987). The location of FOS protein has been identified immunohistochemically in the nuclei of neurons (Hunt *et al.*, 1987; Dragunow and Robertson, 1988). In vivo, the expression of c-*fos* mRNA increased transiently after generalized seizures induced by Metrazole (pentylenetetrazol) injection (Dragunow and Robertson, 1987b and Morgan *et al.*, 1987) and by a unilateral, focal electrolytic lesion of the dentate gyrus hilus (White and Gall, 1987). Both of these procedures cause recurrent seizures. Increases in c-*fos*-like protein have been observed following kainic acid-induced seizures (Le Gal La Salle, 1988 and Popovici *et al.*, 1988) as well as deoxypridoxine-induced seizures (Mizuno *et al.*, 1989). Dragunow and Robertson (1987a) found that a single stimulation to the dorsal hippocampus, which was sufficient to produce AD, caused rapid and transient increases in FOS protein within the nuclei of CA1, CA3, CA4, and dentate gyrus. Similarly, in amygdala kindled rats there was a massive bilateral accumulation of FOS in the cerebral, pyriform, and entorhinal cortices, amygdala and hippocampus (Dragunow *et al.*, 1988). FOS can also be induced by electrically stimulating rat hindlimb motor/sensory neocortex without, presumably, eliciting a seizure (Sagar *et al.*, 1988).

A number of other stimuli have been shown to induce c-*fos* mRNA and protein accumulation. These include ischaemia, stab wounds leading to spreading depression, handling, water deprivation, caffeine, beta-adrenergic receptor agonists,

morphine, and estrogens (for review see Dragunow *et al.*, in press). Thus, c-*fos* levels may be elevated by any stimulus that activates neurons and for this reason Sagar *et al.* (1988) have suggested that FOS accumulation might be used as a high resolution metabolic marker for tracing polysynaptic pathways. Therefore, there may not be a specific link between c-*fos* and plasticity. However, other researchers have suggested that *fos* induction, which follows seizures, may be involved in the long-term adaptive response to convulsions and contributes to neuronal plasticity.

While c-*fos* has been induced following stimuli that are assumed to produce either plastic or non-plastic changes in neural activity, no published reports of induction of c-*fos* after kindled "plastic" seizures with appropriate "non-plastic" stimulation controls are available. The present study attempted to include the appropriate control groups. An experiment addressing the role of c-*fos* as a "master switch" in the plastic changes underlying kindling, would be required to show that c-*fos*: i) was induced only by kindling stimulation and not by stimulation which does not give rise to the kindled state. ii) was not induced simply as a consequence of a non-kindling seizure.

Ras:

The Harvey and Kirsten sarcoma viruses suggest a mechanism by which normal ras proteins may affect cellular

biochemical systems. The three c-*ras* genes encode normal p21 proteins. The p21 proteins have sites for the very tight binding of guanine nucleotides, especially GDP and GTP. This feature is highly significant because GTP/GDP binding is characteristic of a key regulatory protein in the cell's response pathway to external signals that activate cAMP synthesis (Wakelam *et al.*, 1986; Fleischman *et al.*, 1986; Wolfman and Macara, 1987). In mammalian cells membrane-association of ras proteins is essential for their function (Willumsen *et al.*, 1989). The present study also examined the possibility of altered c-Ha-*ras* levels following kindled seizures.

PRESENT RESEARCH:

In order to further explore the enhancement of c-*fos* levels following kindled seizures and investigate the role of c-*fos* in the plastic changes underlying kindling, the following experiments were designed and performed. Northern blot analysis was used because this technique allows for the precise determination of changes in gene expression and for the quantification (relative to an internal control) of the gene expression. See Appendix I for more information on the rationale and technique of Northern blots.

A complete time course of c-*fos* and c-Ha-*ras* mRNA expression in amygdala kindled animals was determined.

Northern blots were made for the amygdala-pyriform region, and comparisons were made between groups of kindled rats (2 stage 5 seizures) that were left for 3 weeks, electrically stimulated and then sacrificed at specified periods (0.25, 1, 2, 3, 5, 8, 24, 72, 148 hours) following the elicited stage 5 seizure. On each blot a lane of sample from both an implanted but unstimulated and a kindled but not restimulated group of rats were included as controls.

At a time of elevated c-*fos* levels (determined above) several structures were examined. These included the stimulated amygdala-pyriform, ipsilateral hippocampus, contralateral amygdala-pyriform, contralateral hippocampus, neocortex, and cerebellum. Comparisons were made with implanted controls. The amygdala-pyriform was chosen because these structures are thought to be a primary area responsible for the generation of chronic epileptiform discharge and have been shown to be critical for normal basal forebrain kindling (Kairiss *et al.*, 1984; Racine *et al.*, 1988). The hippocampus and neocortex were chosen because of their close synaptic connectivity to the amygdala-pyriform, ability to kindle and because they express c-*fos* at low basal levels. The cerebellum was chosen because it does not kindle, and the expression of c-*fos* has been inconsistently localized in the adult cerebellum (Dragunow and Robertson, 1988; Gubits *et al.*, 1988; Sagar *et al.*, 1988).

A comparison of c-*fos* induction in both the stimulated and contralateral amygdala-pyriform regions over the course of kindling was examined following ADT determination, stage 1-2, stage 3 and stage 5. This experiment was performed to investigate the distribution of c-*fos* in relation to the propagation of AD and progression of kindling.

Various controls for electrical stimulation were run to compare the c-*fos* levels relative to a single AD and stage 5 seizure. Some animals received electrode implantation but no current and handling. One group of animals received an equivalent amount of kindling current, but in a form that did not evoke AD. These were termed coulombic controls. Another group received regular kindling stimulation but at subthreshold (for AD) levels. When AD is elicited, changes in the potentiating effects on the response to single test pulses of constant intensity in the affected circuits are also observed (Racine *et al.*, 1972; Cain, 1989). In order to dissociate the effects of AD from the effects of such potentiation, a group of rats received current which resulted in potentiation, but not AD (long-term potentiation (LTP) control). In order to control for the motor seizure itself, a group of rats experienced a single electroconvulsive shock treatment. Since the kindling that results from low-frequency stimulation occurs more rapidly and strongly than normal kindling (Cain and Corcoran, 1981), a group of animals received low frequency stimulation to determine if a greater

amount of c-*fos* could be induced.

In order to determine the effect of varying lengths of AD on c-*fos* accumulation, and to assess the variation of c-*fos* induction, individual animals showing different AD durations were examined.

Kindling stimulations can also be given on several sessions in one day. I gave one group of rats a second stimulation to investigate the refractoriness of c-*fos* induction.

Finally, I investigated the effect of Metrazole (PTZ) injection, which could either produce or not produce a convulsion, on c-*fos* accumulation.

This study was unique because it: i) investigated c-*fos* expression using Northern blot analysis, a technique more sensitive and quantifiable than immunohistochemical methods, ii) investigated plastic changes due to kindling in the kindling "hot" area, the amygdala-pyriform, iii) had controls for electrical stimulation and plasticity, and iv) explored the changes in c-*fos* induction over the course of kindling.

PART 2

GENERAL METHOD

Animals:

Male hooded rats weighing 300-450 g and housed individually served as subjects. Food (Purina Lab Chow) and water were available ad libitum. Subjects were maintained on a 12 hour light-12 hour dark cycle, in which the lights came on at 8 a.m. and turned off at 8 p.m.

Surgery:

Animals were administered 15 mg/Kg atropine methyl nitrate to block bronchiolar constriction and then anaesthetized with 65 mg/Kg pentobarital (Somnotol). While under deep anaesthesia they received implantation of two bipolar electrodes using conventional stereotaxic techniques. The electrodes were constructed of insulated twisted Teflon-insulated Nichrome wire 127 μm in diameter and were stereotaxically aimed at the basolateral amygdala (AP: -1.0 mm; L: 5.0 mm; V: -8.0 mm). Bregma was used as the zero point and the tilt of the animal's head adjusted with the incisor bar to a 5 degree incline. The ventral (V) coordinates were from the skull surface.

The ends of the electrodes were soldered to male Amphenol pins, which were inserted into a miniature connector that was attached to the skull with stainless steel jeweller's screws

and dental acrylic. One male Amphenol pin was soldered to a ground wire of uncoated stainless steel that was attached to a jeweller's screw embedded in the skull.

Kindling Apparatus:

The animals were placed individually into a Faraday cage and attached, via a commutator, to leads connecting the animal's electrodes to the stimulating and recording equipment. The electroencephalogram (EEG) was recorded on a Grass Model 7D polygraph and the stimulation was provided by a Grass Model S88 constant current stimulator connected to a timer.

Kindling Stimulation:

One week after recovery from surgery, the rats were connected to the polygraph and electrical stimulator for determination of the afterdischarge threshold (ADT). An initial current of 50 μA base-to-peak was applied, which was subsequently raised in small steps (25 μA), allowing 30 seconds between stimulations, until an AD was observed on the polygraph. The ADT was defined as the weakest current that would evoke an AD of 4 seconds or longer. The current consisted of biphasic square wave pulses, each 1.0 ms in duration, at 60 Hz and a total duration of 1 second. Kindling stimulations were administered once daily at a stimulation level 50 μA above threshold, to a maximum of 250 μA. Daily stimulations were continued until the desired behavioural

endpoint was achieved. Animals that displayed poor AD, thresholds greater than 225 μA, or slow behavioural and electrographic progression were dropped from the experiments.

In order to provide a reference group to which all experimental manipulations could be compared, six animals had electrodes implanted, as described above, but did not receive any electrical stimulation.

Experiment #1; Time Course Determinations:

Thirty-seven subjects were stimulated once every 24 hours until they reached two stage 5 seizures (Racine, 1972b). They were then left for a three week period before a final stimulation. In order to compare basal gene expression in kindled animals four rats did not receive the final stimulation. Following the last stimulation and resulting stage 5 seizure, designated rats were left for specified time periods (0.25, 1, 2, 3, 5, 8, 24, 72, 164 hours) and then sacrificed. Animals were assigned to the time groups such that each group had an equivalent average duration of AD (50-60 sec). Time course determinations were made for the stimulated amygdala-pyriform regions as well as the contralateral amygdala-pyriform regions.

Experiment #2; Tissue Comparisons:

Five subjects were stimulated once every 24 hours until they reached two stage 5 seizures (Racine, 1972b). They were then left for a three week period before a final stimulation. Following the last stimulation and resulting stage 5 seizure, rats were left for 1 hour and then sacrificed. Comparisons were made between the level of c-*fos* expression in stimulated amygdala-pyriform, contralateral amygdala-pyriform, ipsilateral hippocampus, contralateral hippocampus, neocortex, and cerebellum. Unstimulated brain tissues, from 6 implanted control animals were also compared to assess basal c-*fos* levels.

Experiment #3; Behavioural Stage Comparisons:

Three groups of animals were kindled to either a stage 5, 3, or 1-2 and left unstimulated for a period of 3 weeks then restimulated. Three animals in the stage 1-2 group received a fixed number (4) of stimulations and then left unstimulated for the three week period. A fourth group of 3 animals had only one stimulation session. This group had AD evoked for the first time during the determination of their ADT. This group had AD evoked only on one side. Following a 1 hour delay after AD was evoked, all the animals were sacrificed. Comparisons were made between the stimulated

amygdala-pyriform and contralateral amygdala-pyriform regions.

Experiment #4; Stimulation and Seizure Controls:

No Afterdischarge

Two animals received kindling stimulation but at a subthreshold level that did not evoke AD. These animals received 200 μA, 60 Hz for 1 second.

Coulombic Controls

One group of four animals received the same number of coulombs as the regularly kindled animals but it was delivered so as not to induce AD. These animals received 200 μA, 1 Hz for 60 seconds.

LTP

Three animals were implanted under electrophysiological control with a stimulating electrode in the basolateral nucleus of the amygdala (AP: -1.0 mm; L: 5.0 mm; V: -8.0 mm) and the recording electrode implanted in the lateral entorhinal cortex (AP: -5.8 mm; L: 6.0 mm; V: -6.5 mm). Readings were taken in millimetres with bregma as the zero point and the tilt of the animal's head adjusted with the incisor bar at a 5 degree incline. The ventral (V) coordinates are from skull surface.

Input/Output (I/O) curves were obtained by applying single diphasic rectangular pulses to the amygdala and recording the evoked responses in the entorhinal cortex. Responses were obtained using an Apple II+ microcomputer and an ISAAC Cyborg model 91A analog to digital data acquisition unit analysis system. The pulses were 0.1 ms in duration with an interval of 0.1 ms between them, and they were delivered at a constant rate of 0.2/s. Intensities were varied between the threshold level (400 μA) for triggering an evoked response and the maximum intensity (1400 μA) beyond which no further increments in amplitude were produced. All test pulses and high frequency electrical stimulation were applied only when the rats were completely immobile to avoid the possible confounding effect of movement on the amplitude of the evoked responses (Buzsaki *et al.*, 1981). Animals were potentiated with 1400 μA, a current that had produced the maximal response during the initial I/O test. Potentiating stimulation consisted of 20 trains of 8 pulses per train at 400 Hz, with each train separated by 20 seconds. While potentiating trains were applied an electrographic tracing was taken to insure that no epileptogenic activity was induced. Immediately after the potentiation stimulation was applied and for the next 60 minutes, test pulses were delivered and the degree of potentiation measured.

The degree of potentiation was assessed by measuring the amplitude of the population-spike. Population-spike amplitude

was taken to be the length of a vertical line from the maximum height (negativity) of the population-spike to the maximum depth (positivity) of the excitatory post-synaptic potential (EPSP) (Racine and Milgram, 1983 and Racine *et al.*, 1983).

ECS

Saline soaked padded alligator clips were attached to the ears of three naive animals. Resistance, between the ears, was measured. The electrical stimulus, which consisted of a 60 Hz sine wave current, was delivered for a duration of 0.25 seconds. During stimulation the current was measured to determine the voltage that each animal received (155, 200, and 225 V). These animals displayed a fully generalized convulsion with both fore- and hind-limb tonus and clonus.

ADT and Stage 5

The stimulated amygdala-pyriform regions in ADT and stage 5 groups of rats was included in this comparison. The manipulation of these animals has previously been described.

Low Frequency

Three naive animals received low frequency kindling stimulation (Cain and Corcoran, 1981). These animals had bilateral amygdala implants and received 1000 μA, 3 Hz, for either 60, 66, or 75 seconds through one electrode while recording EEG from the contralateral electrode. The animals were stimulated until a stage five convulsion was displayed.

Immediately following observation of the stage five convulsion the current was discontinued and the resulting AD was recorded.

In all cases, after the experimental manipulation, animals were left for one hour in their home cage before they were sacrificed and their amygdala-pyriform regions removed.

Experiment #5; Individual Variation:

Four rats that had been previously kindled to 2 stage 5 convulsions and then left unstimulated for at least two weeks were used. These animals were restimulated and the resulting AD recorded. The analysis of c-*fos* accumulation was performed on each animal individually.

Experiment #6; Second Stimulation:

In this experiment three rats that had previously been kindled to 2 stage 5 convulsions and left for a three week rest, were used. On the day of the experiment these animals received an initial stimulation that evoked AD and a stage 5 convulsion and were then left in their home cage for 2 hours. Following this 2 hour delay the animals received a second stimulation at the same current intensity and the AD was

recorded. One hour following this second stimulation and convulsion the animals were sacrificed. Comparisons were made between the above experimental treatment and the fully kindled animals (Experiment #1) that received only one restimulation and were sacrificed 1, 2, and 3 hours post-convulsion.

Experiment #7; Chemical Convulsion:

Seven animals were injected with either physiological saline 1 ml/kg or 50 mg/kg pentylenetetrazol (PTZ) and observed for 1 hour before decapitation. The PTZ animals were divided into two groups: those observed having severe motor convulsions, including fore- and hind-limb tonus and clonus, and those that exhibited minor convulsive behaviour.

Dissection:

At the specified time period animals were sacrificed by decapitation. The brain was quickly removed from the skull and placed in ice cold phosphate buffered saline (PBS) for two minutes. After the brain cooled the following areas were removed: right amygdala pyriform, left amygdala pyriform, right hippocampus, left hippocampus, neocortex, and cerebellum.

Brain regions were dissected in the following way. The brain was placed ventral side up in the dissecting dish containing ice cold PBS. Coronal slices were made at both the

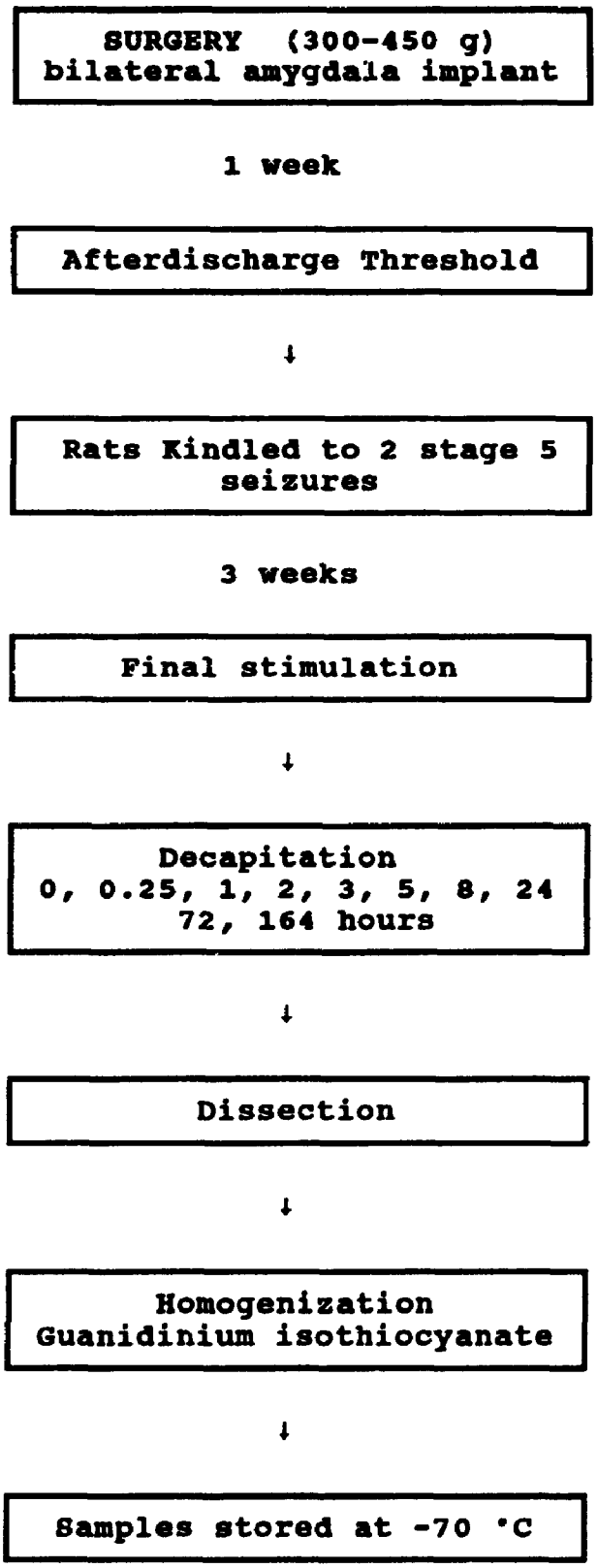
level of the optic chiasm and mammillary bodies. The anterior and posterior tissue was moved aside, leaving the desired portion. All tissue except cerebellum was taken from the remaining block. A horizontal knife cut was made at the rhinal fissure to a depth approximately one quarter the width of the brain. This was followed by a second vertical cut removing the amygdala pyriform area. The neocortex was stripped off with a pair of fine forceps. The hippocampi were then removed with a pair of fine forceps. Finally, the cerebellum was dissected free from the posterior tissue. See Appendix II for anatomical details.

Each brain area was homogenized with 1.0 ml of 4M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium sarcosine, and 0.1 M 2-mercaptoethanol (GIT) per 100 mg tissue. The homogenate was stored in 1.5 ml Eppendorf tubes at -70 °C. See Figure 3 for general methodological flow chart.

RNA Extraction and Electrophoresis:

Total RNA was extracted using the method described by Chomczynski and Sacchi (1987). Following ethanol precipitation and centrifugation the RNA was dissolved in DEP-treated double distilled H_2O, like samples pooled, and stored at -70 °C in 10 μg aliquots. Aliquoted portions of total RNA

Figure 3. Flow chart outlining the general methodological steps in Expt. #1.


SURGERY (300-450 g)
bilateral amygdala implant
1 week
Afterdischarge Threshold
Rats Kindled to 2 stage 5 seizures
3 weeks
Final stimulation
Decapitation
0, 0.25, 1, 2, 3, 5, 8, 24
72, 164 hours
Dissection
Homogenization
Guanidinium isothiocyanate
Samples stored at -70 °C

were removed, lyophilized, and resuspended in RNA sample buffer containing 1 X gel running buffer (1 X = 50 mM MOPS, pH 7.0, 10 mM EDTA), 50% formamide, and 2.2 M formaldehyde. The RNA was denatured in this buffer at 70 °C for 10 minutes, quenched on ice and separated on horizontal 1% agarose-6% formaldehyde gels at 60 V until the dye front (bromophenol blue) was 9.0 cm from the wells (approx. 4 hours). After electrophoresis, gels were stained with 0.5 μg/ml ethidium bromide for 5 minutes in running buffer. The gels were photographed on a shortwave length transilluminator to determine that each lane had equivalent levels of RNA. Gels were then destained in excess running buffer for 20 minutes. The RNA from the gels was transferred with 10 X SSC to Gene Screen *Plus* (NEN, Boston, MA) using a passive transfer procedure for 24-48 hours. Following transfer the filters were baked at 80 °C for 3 hours in a vacuum oven. The gels were stained, and photographed as previously described to confirm a 100% transfer.

Preparation of Oncogene:

The oncogene probes were purchased from Oncor (Gaithersburg, MD) in the form of a DNA insert: v-*fos*, a 1.0 kb Eco R1 fragment from plasmid pfos-1 (van Straaten et al. 1983; Curran *et al.*, 1982) with 80% homology to rat c-*fos* and a GC content of 66% (Van Beveren et al. 1983); v-Ha-*ras*, a 0.7

kb Bam H1 fragment from plasmid Ha-MuSV (Chang *et al.*, 1980). The oncogene inserts (200 ng) were radiolabelled using the Boehringer Mannheim oligolabelling kit and [α ^{32}P]-dCTP (Dupont: Specific Activity of 3000 Ci/mmol) (1 curie = 37 GBq) to a specific activity of ca. 1 X 10^8-10^9 cpm/μg, and 1 X 10^6-10^7 cpm/ml.

Hybridization and Autoradiography:

"Hybrid-Ease" chambers (Hoeffer Scientific Instruments, Mississauga, Ont.) were used for prehybridizations, hybridizations, and washes. Filters were prehybridized in 50% formamide, 10% dextran sulfate, 1% SDS, and 1M NaCl for at least 1 hour at 40 °C. Hybridization was carried out at 40 °C for 20 hours in the prehybridization buffer containing 200 ng of radiolabelled probe. Washings of the filters were in 2.0 X SSC and 1.0% SDS at 50 °C for 30 minutes (for filters probed for c-*fos*). After washing, the damp filters were wrapped in plastic wrap and preflashed X-ray film (Kodak XAR-5) was exposed at -70 °C for 24 and 48 hours. Quantification of autoradiographic bands was performed using an LKB scanning laser densitometer at 695 *nm*. All scans were made with exposures in the linear range of the film and of the densitometer. Baseline absorbency levels were automatically determined for each lane which allowed for comparisons between lanes with minor variations in RNA quantity. Blots that

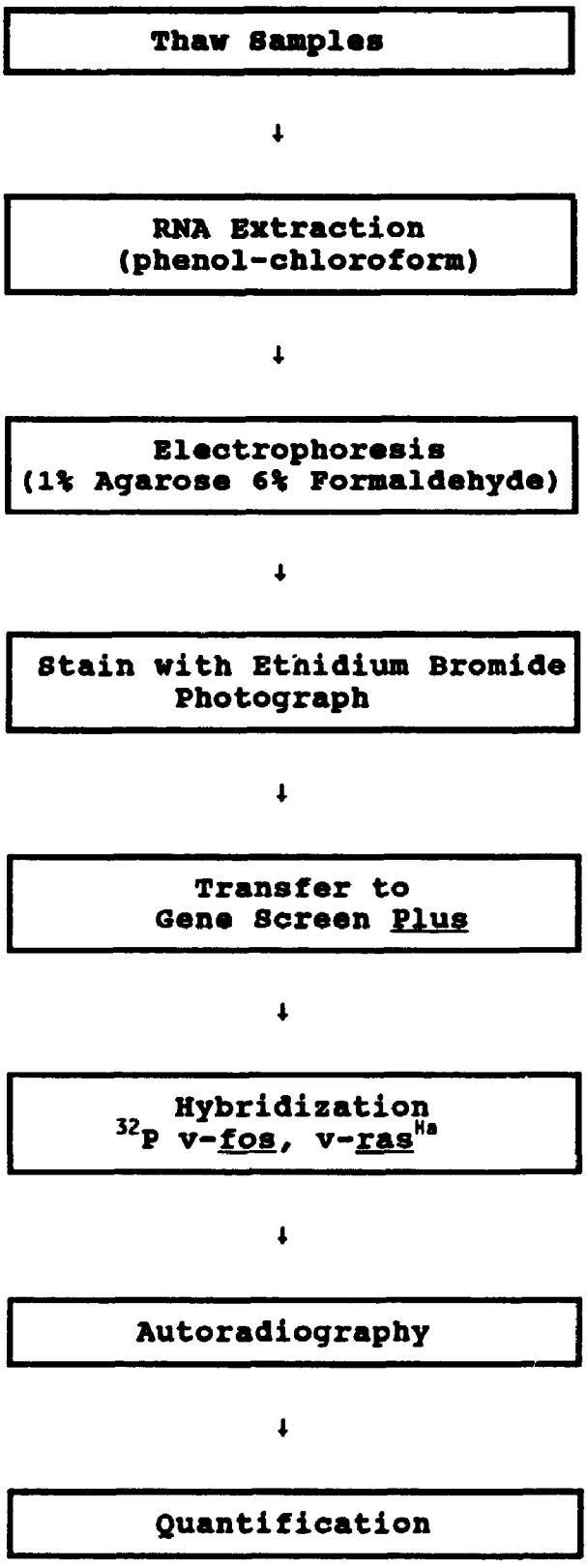
underwent re-hybridization had hybridized probes removed by agitation in boiling 0.1% SSC before re-hybridization. Filters were kept moist between hybridizations and stored at -70 °C. Rehybridization was carried out as on the initial blot. See Figure 4 for methodological flow chart summarizing these procedures.

Statistical Analysis:

In this thesis, RNA from the same experimental treatment group was pooled. Pooling of the RNA was necessary to give sufficient quantities of RNA for repeated analysis. Furthermore, since experimental comparisons can only be made within one Northern blot, this limited the number of lanes that could be compared in one experiment to 14 and therefore required RNA pooling. The pooling of RNA did not allow estimates of individual variation and since inferential statistics require individual variation around mean values, statistical techniques could not be performed. The comparison of lane data within a single Northern blot in the absence of group statistics is a normal procedure with this technique.

Experiment #5 provided an estimate of the variability of c-*fos* induction in the amygdala-pyriform following a stage 5 seizure. Since the variation between animals was low, when the length of AD duration was taken into account, relatively small sample sizes are reasonable.

Figure 4. Flow chart outlining the general molecular methodological steps.


Thaw Samples
RNA Extraction
(phenol-chloroform)
Electrophoresis
(1% Agarose 6% Formaldehyde)
Stain with Ethidium Bromide
Photograph
Transfer to
Gene Screen Plus
Hybridization
32P v-fos, v-ras Ha
Autoradiography
Quantification

PART 3

RESULTS

Experiment #1; Time Course Determinations:

Laser densitometric analysis of Northern blots revealed that fully kindled animals (2 stage five seizures), that were left unstimulated for a 3 week period and electrode-implanted naive animals had equivalent basal levels of c-*fos* (Figs. 5 and 6). Increased c-*fos* accumulation in the stimulated amygdala-pyriform and contralateral amygdala-pyriform was evident by 0.25 hr, maximal by 1 hour, and returned to baseline by 3 hours after a stage five seizure (Figs. 5 and 6). At twenty-four hours post-seizure a slight increase in c-*fos* mRNA was observed in both stimulated amygdala-pyriform and contralateral amygdala-pyriform tissues.

Experiment #2; Tissue Comparisons:

Basal expression of c-*fos* was greatest in amygdala-pyriform (E) and neocortical (C) regions while hippocampal (C) and cerebellar (C) areas had extremely low levels of c-*fos* expression (Fig. 7). One hour following a stage 5 seizure, at the time of maximal c-*fos* accumulation, enhanced c-*fos* levels were observed in the stimulated amygdala-pyriform (stim),

Figure 5. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from pooled stimulated amygdala-pyriform tissue samples. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode-implanted controls, (K) kindled to 2 stage 5 seizures followed by a 3 week rest. Numbers below the histobars (0.25, 1, 2, 3, 5, 8, 24, 72, 164) represent the hours post-stage 5 seizure that the animals were sacrificed; these animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation. Densitometric values for this and all subsequent figures came from one blot for each figure.

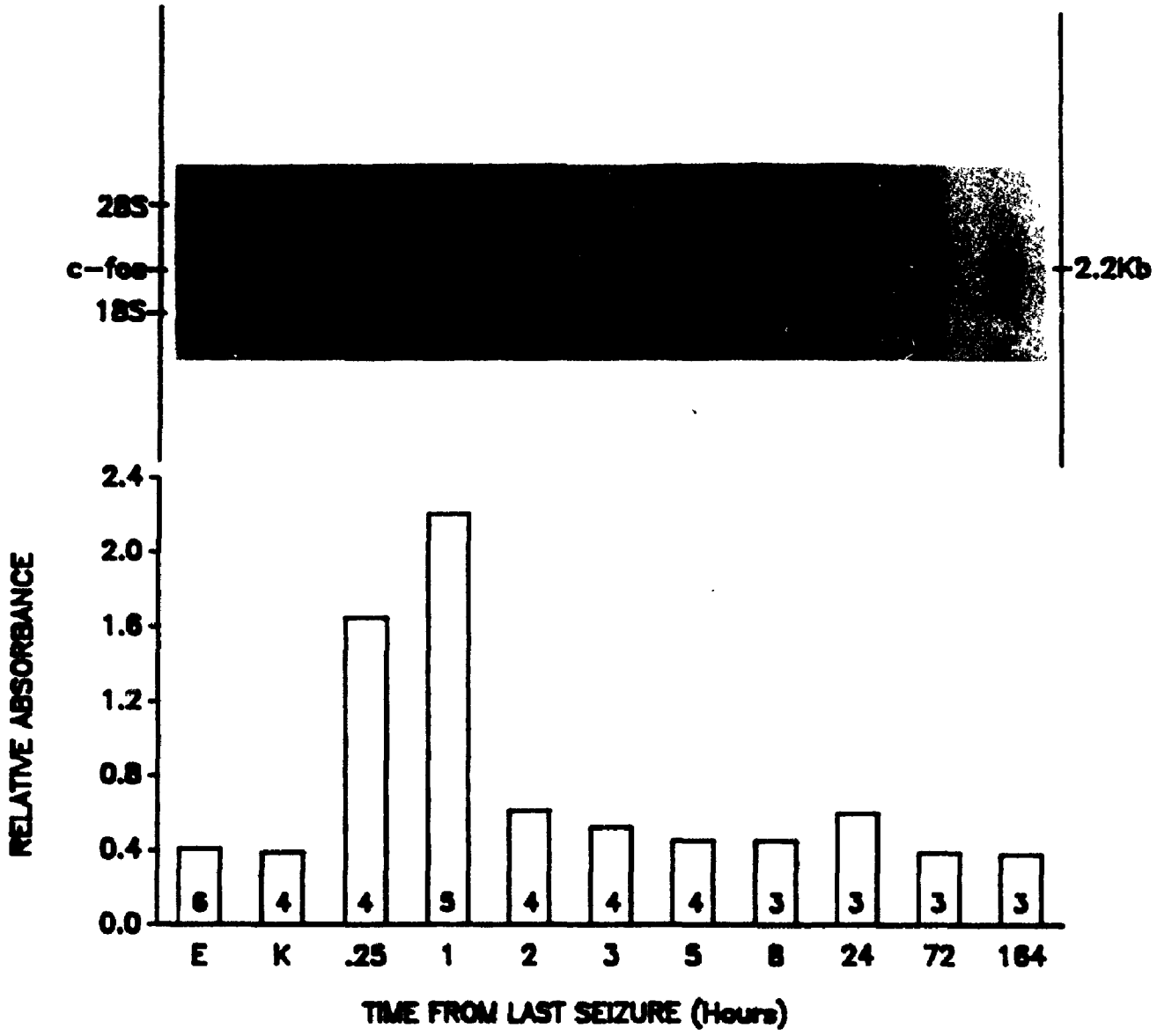

28S
c-fos
18S
2.2Kb
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
6
4
4
5
4
4
4
3
3
3
3
E
K
.25
1
2
3
5
8
24
72
164
TIME FROM LAST SEIZURE (Hours)

Figure 6. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from the pooled contralateral amygdala-pyriform tissue samples. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode-implanted controls, (K) kindled to 2 stage 5 seizures followed by a 3 week rest. Numbers below the histobars (0.25, 1, 2, 3, 5, 8, 24, 72, 164) represent the hours post-stage 5 seizure that the animals were sacrificed; these animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation.

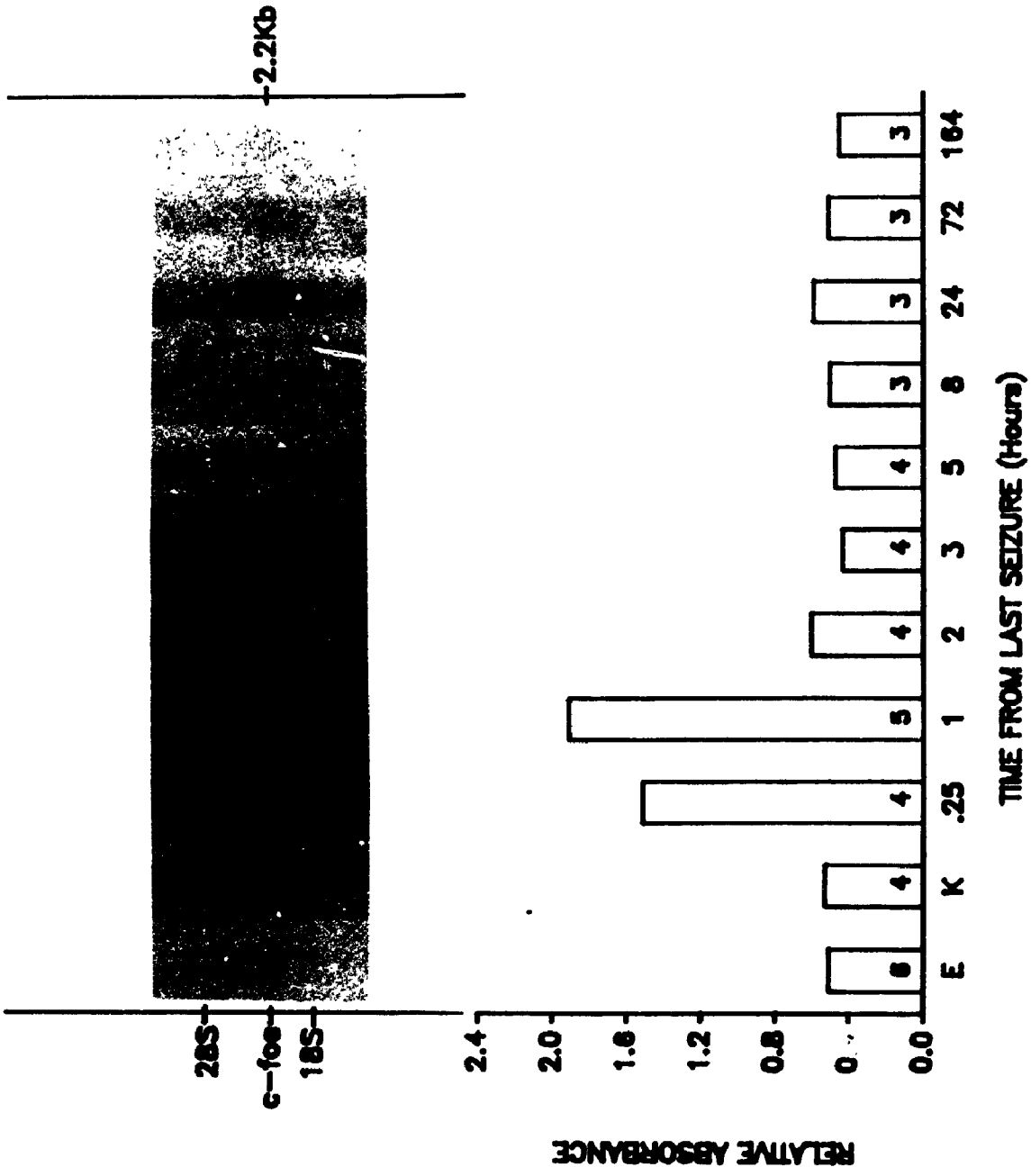
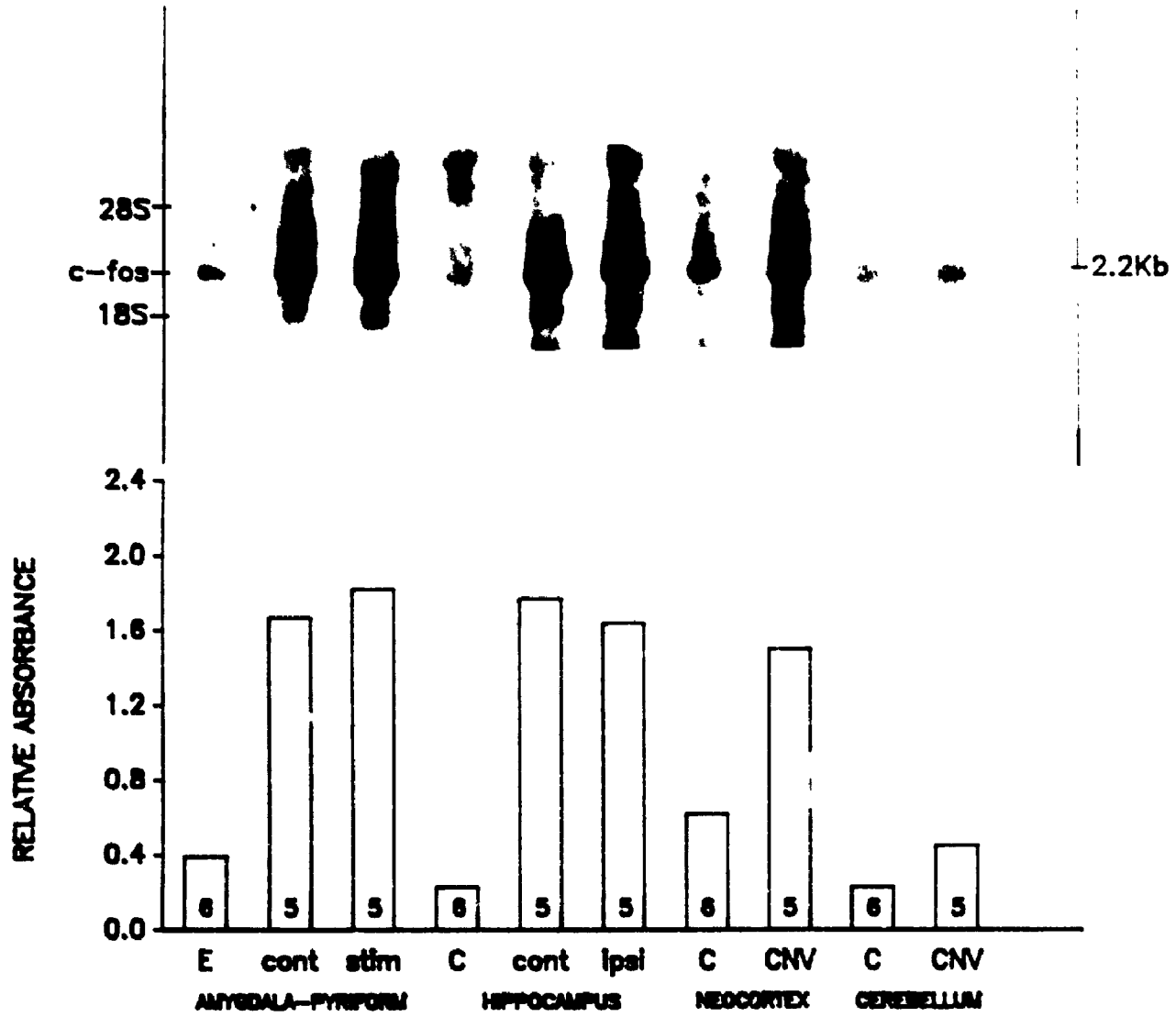

2.2Kb
28S
c-fos
18S
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.0
E
K
.25
1
2
3
5
8
24
72
164
TIME FROM LAST SEIZURE (Hours)

Figure 7. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from amygdala-pyriform, hippocampal, neocortical, and cerebellar tissue samples 1 hour post-stage 5 seizure. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode-implanted controls, (cont) tissue from region contralateral to side of stimulation, (stim) tissue from stimulated site, (ipsi) tissue from region ipsilateral to side of stimulation, (C) control tissue from electrode-implanted animals, and (CNV) tissue from animals experiencing a stage 5 convulsion. All animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation.


28S
c-fos
18S
2.2Kb
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
6
5
5
6
5
5
6
5
6
5
E
cont
stim
C
cont
Ipsi
C
CNV
C
CNV
AMYGDALA-PYRIFORM
HIPPOCAMPUS
NEOCORTEX
CEREBELLUM

contralateral amygdala-pyriform (cont), ipsilateral hippocampus (ipsi), contralateral hippocampus (cont), and neocortex (CNV). Slight enhanced accumulation of c-*fos* was observed in cerebellum (Fig. 7).

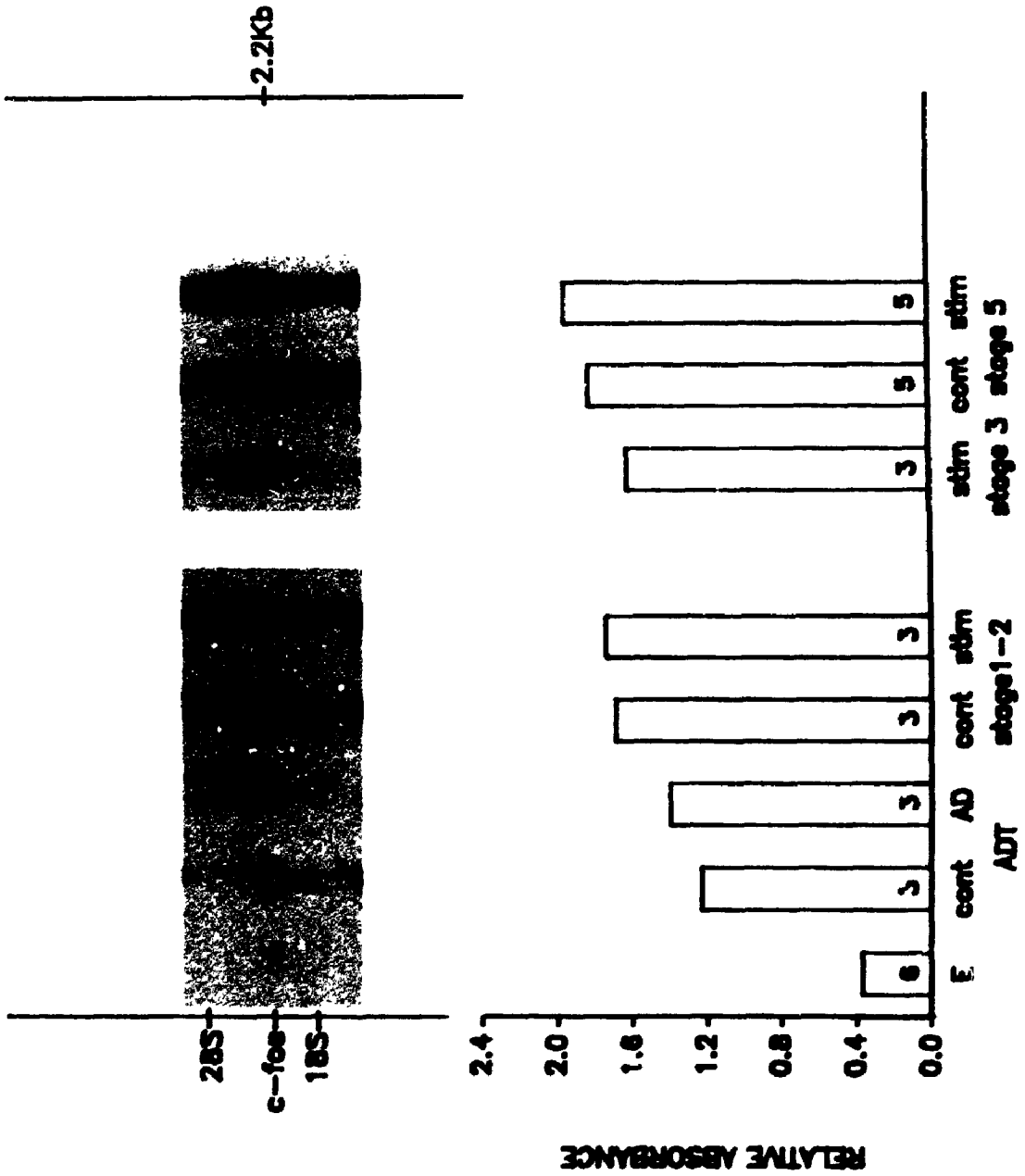
Experiment #3; Behavioural Stage Comparisons:

At one hour following stimulation and AD, in naive (ADT), stage 1-2, stage 3, and stage 5 animals, c-*fos* levels were enhanced in stimulated (stim) and contralateral (cont) amygdala-pyriform regions relative to baseline electrode implanted (E) animals (Fig. 8). In the ADT group, the contralateral (cont) amygdala-pyriform received stimulation but AD was not elicited in that region. The sample for the contralateral stage 3 group was not available.

Experiment #4; Stimulation and Seizure Controls:

The group of animals designated Coulombic Control (CC), that received current equivalent to a kindling stimulation, but in a form that did not evoke AD, and animals that received a subthreshold kindling stimulation that did not evoke AD (NAD), showed a mildly enhanced level of c-*fos* mRNA relative to electrode (E) implanted controls. The animals (LTP) that received high frequency current and showed potentiation (20%,

Figure 8. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from ADT, stage 1-2, stage 3, and stage 5 amygdala-pyriform pooled tissue samples one hour post-stage 5 seizure. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode implanted, (cont) contralateral to side of stimulation, (stim) stimulated site, (AD) tissue from side which showed AD in ADT animals. Stage 5 animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation. Stage 3 animals were previously kindled to a stage 3 seizure followed by a 3 week rest and restimulation. Stage 1-2 animals received 4 stimulation sessions followed by a 3 week rest and restimulation. ADT animals received stimulation on both sides but had AD evoked only on one side. Note: stage 3, contralateral side sample was not available.


2.2Kb
28S
c-fos
18S
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
E cont AD cont stim cont stim
ADT
stage1-2
stage 3 stage 5
6
3
3
3
3
3
5
5
5

15%, and 7% above baseline) but no AD, had enhanced accumulation of c-*fos*. This level of c-*fos* was approximately equivalent to the level induced in the first session (ADT) stimulated amygdala-pyriform region. The amygdala-pyriform c-*fos* levels in stage 5 animals were higher than c-*fos* levels in ADT animals. The single ECS stimulation and resulting seizure caused substantial c-*fos* accumulation. c-*fos* accumulation was substantial in animals that received low frequency stimulation. The highest level of c-*fos* accumulation was observed on the stimulated side of the low frequency stimulated (LFS) animals (Fig. 9).

Experiment #5; Individual Variation:

Generally, in individual rats that had been kindled to stage 5, the longer the duration of AD the greater the c-*fos* accumulation (Fig. 10). In every case the stimulated region showed a greater level of c-*fos* than the contralateral side.

Fxperiment #6; Second Stimulation:

Stage 5 animals that received a second stimulation two hours after an initial stimulation had equivalent AD durations in response to the second stimulation compared to the AD resulting from the initial stimulation. At 1 hour after the second stimulation and resulting AD, c-*fos* accumulation was

Figure 9. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from pooled stimulated amygdala-pyriform tissue samples one hour post treatment. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode implanted, (NAD) stimulation but no AD evoked, (CC) Coulombic control, (LTP) long-term potentiation, (ECS) electroconvulsive shock, (ADT) stimulated tissue from side which showed AD in ADT animals, (SG5) stimulated tissue from stage 5 animals which were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation, (LFS) low frequency stimulated side, and (LFC) low frequency contralateral to side of stimulation.

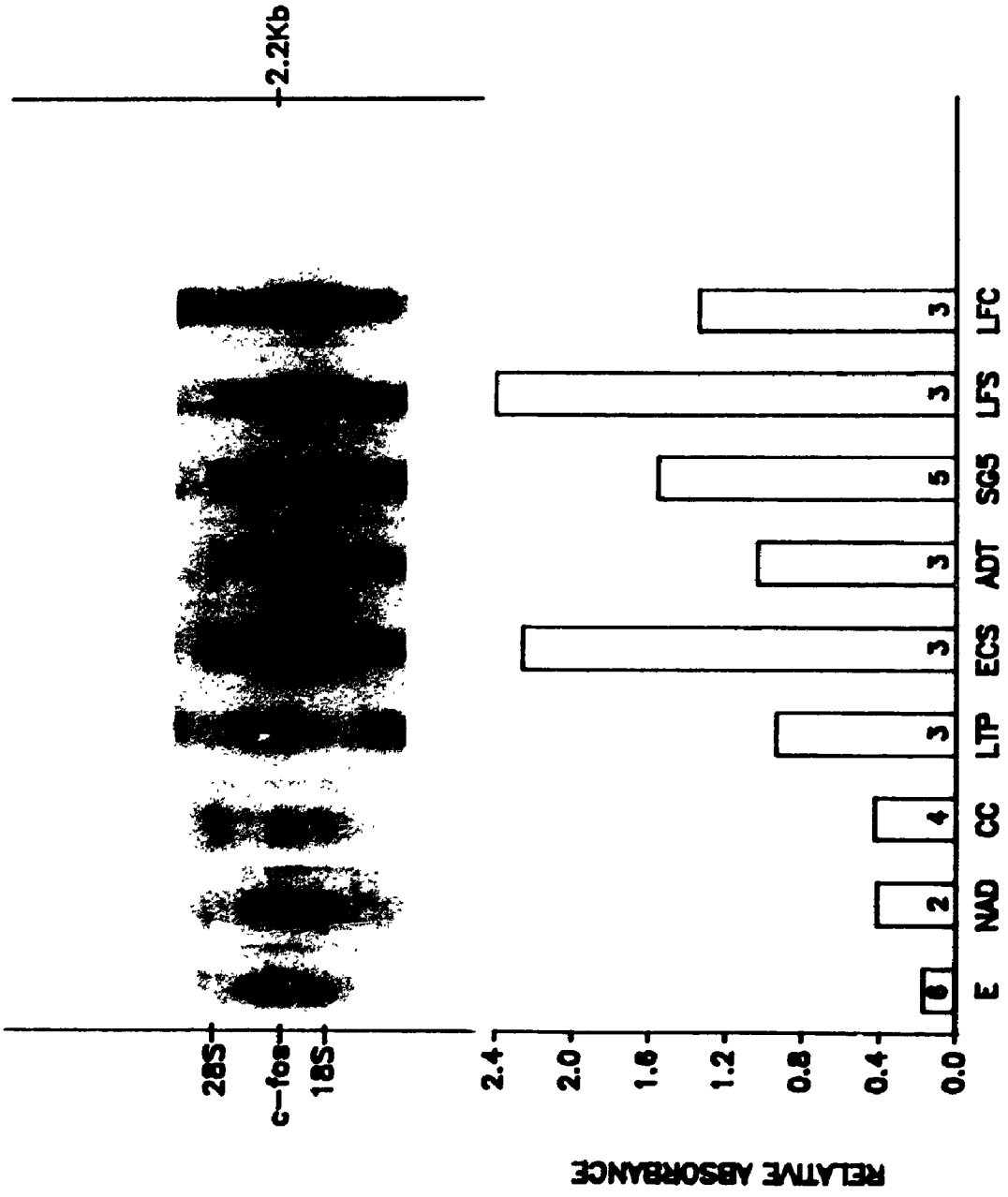
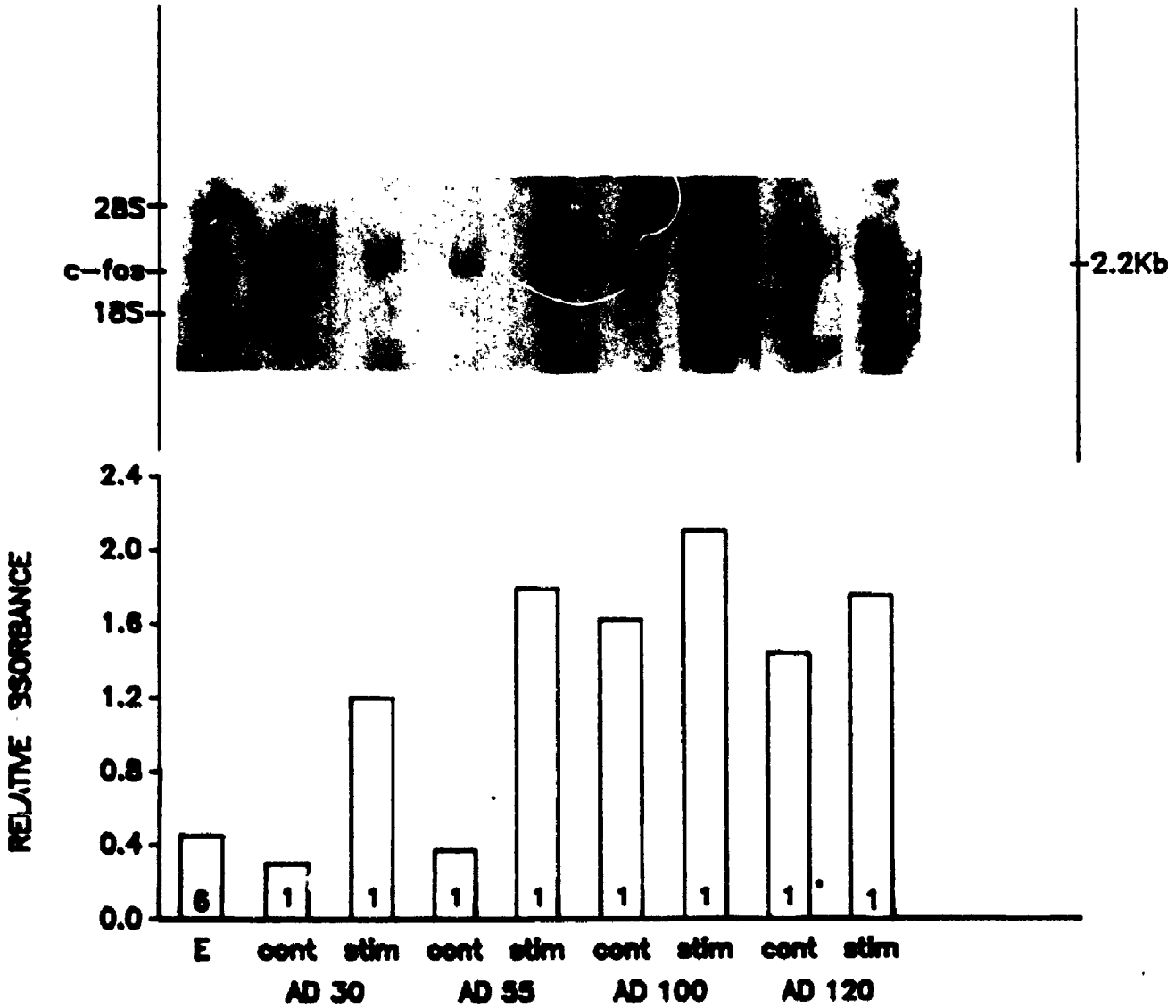

2.2Kb
28S
c-fos
18S
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
E
NAD
CC
LTP
ECS
ADT
SGS
LFS
LFC
6
2
4
3
3
3
5
3
3

Figure 10. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from amygdala-pyriform tissue samples from individual animals one hour post-stage 5 seizure. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate, (AD 30, 55, 100, 120) length of AD from individual animals, (stim) stimulated side, (cont) contralateral to side of stimulation, and (E) electrode implanted. Tissue was obtained from stage 5 animals which were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation.


28S
c-fos
18S
2.2Kb
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
6
1
1
1
1
1
1
1
1
E
cont
stim
cont
stim
cont
stim
cont
stim
AD 30
AD 55
AD 100
AD 120

substantial in both the stimulated (stim) and contralateral (cont) amygdala-pyriform regions. However, these c-*fos* mRNA levels were not quite to the level observed 1 hour after a single stage 5 seizure (Fig. 11).

Experiment #7; Chemical Convulsion:

PTZ injection substantially increased c-*fos* levels relative to a saline injected control (Ctr). PTZ injected animals that displayed a full convulsion (SE) had marginally higher c-*fos* levels than PTZ injected animals that did not display a motor convulsion (Fig. 12).

Ras:

Laser densitometric analysis of Northern blots revealed that in the amygdala-pyriform of both fully kindled animals, seizure free for twenty-one days, and electrode-implanted naive animals had equivalent basal levels of c-Ha-*ras* (Fig. 13). Furthermore, at no time after a stage 5 seizure was there a change in c-Ha-*ras* levels.

Figure 11. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from amygdala-pyriform tissue samples in animals receiving a primary and for some animals second stimulations. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Symbols indicate (E) electrode implanted, and (1, 2, 3) represent the hours post-primary stimulation and stage 5 seizure, that the animals were sacrificed. Animals in the second stimulation group received a second stimulation two hours after the primary stimulation. Symbols for this group indicate (stim) site of stimulation, and (cont) contralateral to side of stimulation. All animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation.

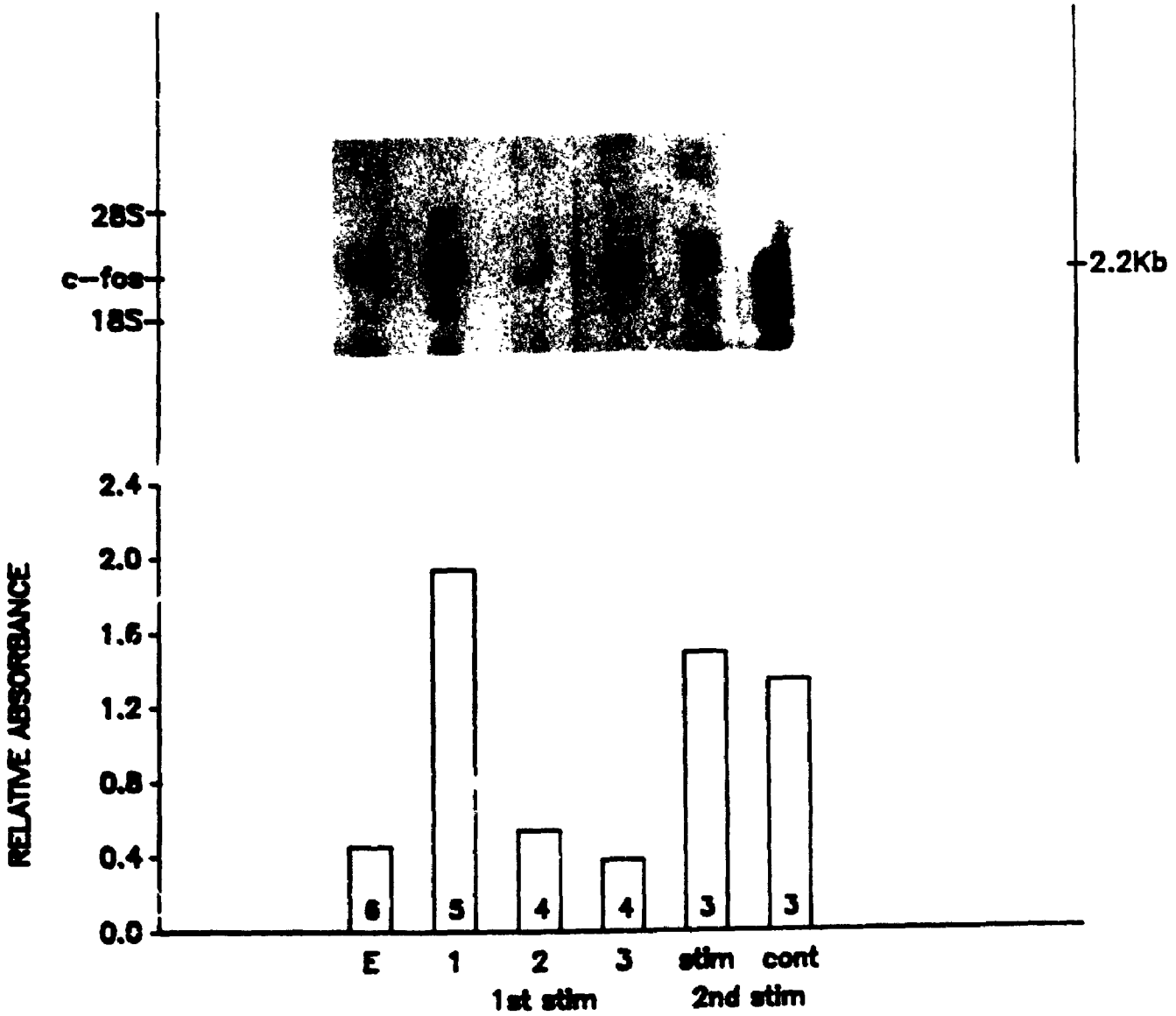

28S
c-fos
18S
2.2Kb
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
6
5
4
4
3
3
E
1
2
3
stim
cont
1st stim
2nd stim

Figure 12. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-*fos* mRNA in total RNA (10 μg/lane) isolated from amygdala-pyriform tissue samples in animals receiving 50 mg/Kg PTZ. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Symbols indicate (Ctr) control, saline (S) injected, (NS) PTZ injected but no seizure, and (SE) PTZ injected with fully generalized motor seizure.

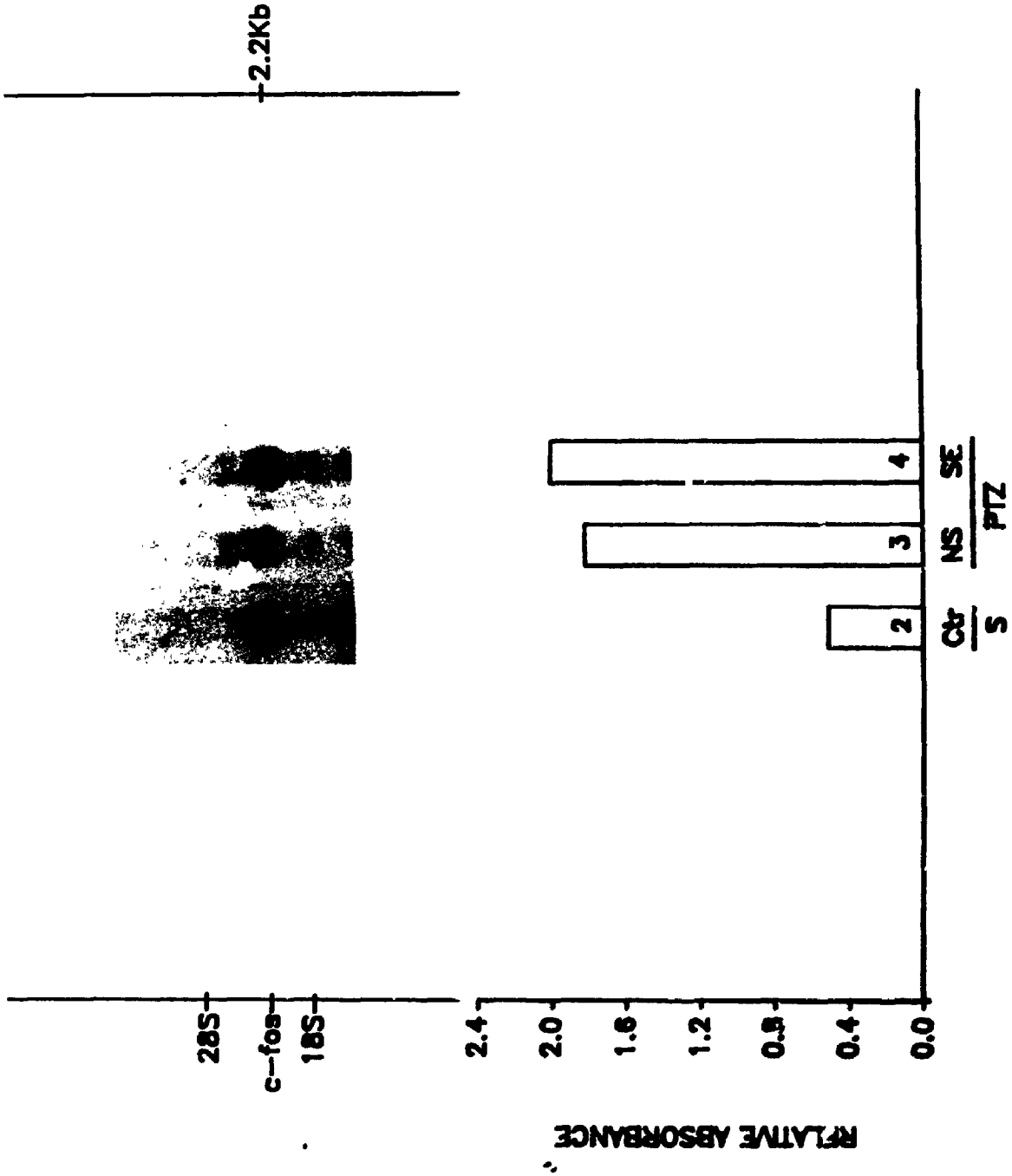
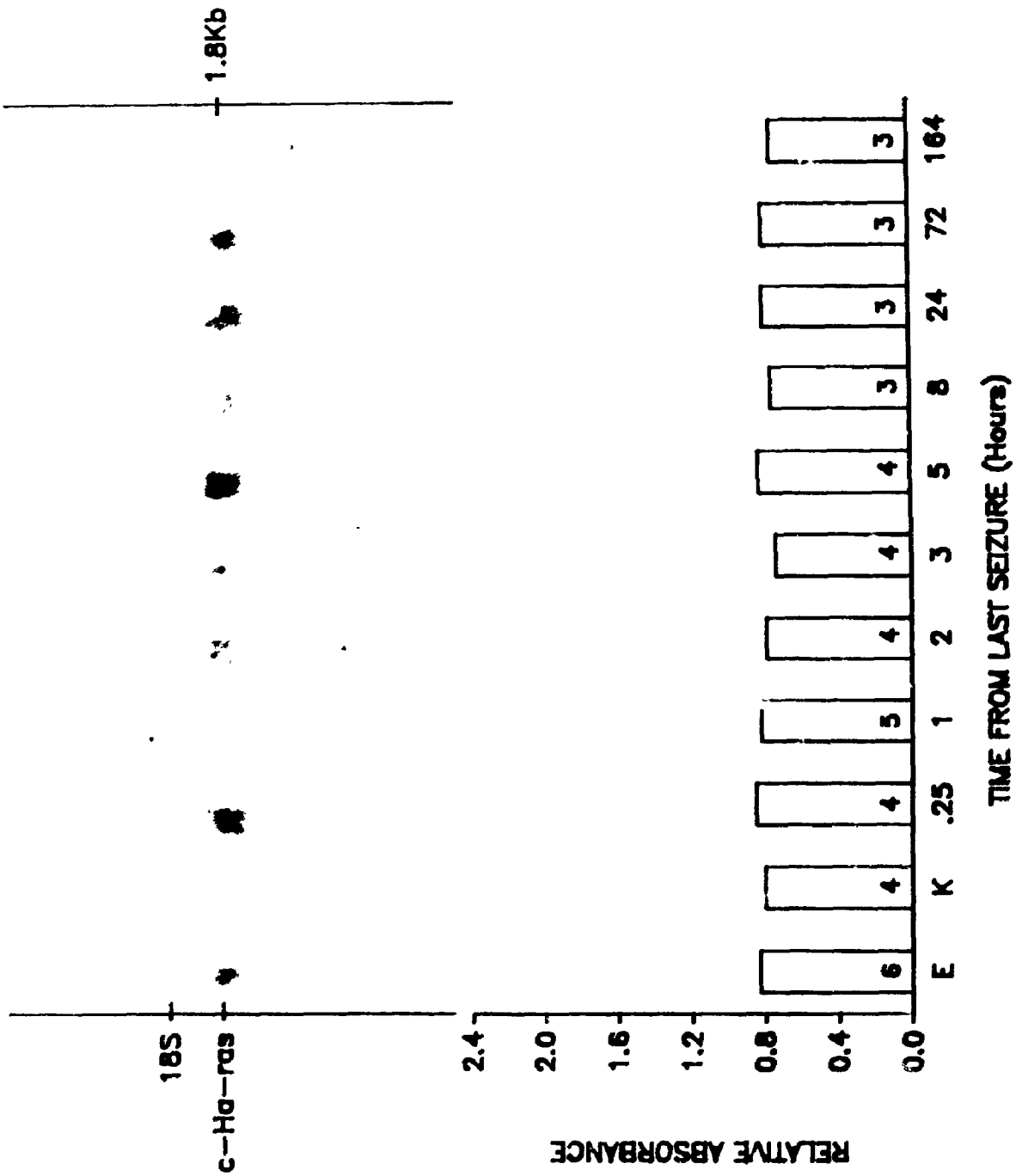

2.2Kb
28S
c-fos
18S
2.4
2.0
1.6
1.2
0.8
0.4
0.0
RELATIVE ABSORBANCE
2
Ctr / S
3
NS / PTZ
4
SE / PTZ

Figure 13. Upper panel: autoradiogram of the northern blot analyses for the accumulation of c-Ha-*ras* mRNA in total RNA (10 μg/lane) isolated from pooled stimulated amygdala-pyriform tissue samples. Lower panel: histobars represent the densitometric values obtained from the samples shown above. The sample size represents the number of pooled animals and is indicated by the number within the histobars. Symbols correspond to histobars in the lower panel and lanes in the upper panel. Letters below the histobars indicate (E) electrode-implanted controls, (K) kindled to 2 stage 5 seizures followed by a 3 week rest. Numbers below the histobars (0.25, 1, 2, 3, 5, 8, 24, 72, 164) represent the hours post-stage 5 seizure that the animals were sacrificed; these animals were previously kindled to 2 stage 5 seizures followed by a 3 week rest and restimulation.


1.8Kb
18S
c-Ha-ras
RELATIVE ABSORBANCE
2.4
2.0
1.6
1.2
0.8
0.4
0.0
E
K
.25
1
2
3
5
8
24
72
164
6
4
4
5
4
4
4
3
3
3
3
TIME FROM LAST SEIZURE (Hours)

PART 4

DISCUSSION

Initially, the levels of c-*fos* and c-Ha-*ras* in naive and fully kindled animals, as well as at various time intervals after a stage 5 seizure, were determined in both stimulated and contralateral amygdala-pyriform regions.

In the amygdala-pyriform region the level of c-Ha-*ras* was equivalent between naive animals, fully kindled animals that were seizure-free for 3 weeks, and at various times after a stage 5 seizure in fully kindled animals. These observations are consistent with previous reports that did not find altered levels of c-Ha-*ras* at any time after a PTZ injection or dentate hilus lesion, both of which led to recurrent convulsions (Morgan *et al.*, 1987; White and Gall, 1987). I conclude that kindled seizures do not alter c-Ha-*ras* expression.

I observed that c-*fos* is expressed constitutively (at low levels), in the amygdala-pyriform region. This observation is in agreement with Gubits *et al.* (1988). Basal expression may reflect the observation that environmental stimuli can induce c-*fos* in neurons (Dragunow and Robertson, 1988), and therefore it may not be possible to measure "true" basal levels of c-*fos* mRNA in brain because it would be impossible to control for all stimuli that may induce its expression.

On the other hand, basal levels of FOS protein have been inconsistently reported. Positive results could be caused by the cross-reactivity of the FOS antibody to FOS itself and other FOS-related nuclear antigens (Franza *et al.*, 1987). The use of an antibody directed against the unique N-terminal region of the FOS protein has resulted in detection of no observable basal levels of FOS in brain or spinal cord (Hunt *et al.*, 1987; Le Gal La Salle, 1988; Popovici *et al.*, 1988). However, due to the greater sensitivity of Northern Blot analysis as compared with immunohistochemical techniques it is probably safe to conclude that c-*fos* is expressed constitutively.

The present results also demonstrate that c-*fos* mRNA is present at equivalent low basal levels in naive and fully kindled animals in the absence of a generalized convulsion during the twenty-one days prior to sacrifice. This result is in agreement with a previous report by Dragunow *et al.* (1988), who showed immunohistochemically that there was no change in basal levels of FOS protein(s) in the brains of amygdala-kindled rats that had been seizure-free for 7 days compared to naive controls. These results lead me to conclude that kindling is not associated with any permanent change in the basal level of c-*fos*.

I observed an enhanced bilateral c-fos accumulation 15 minutes after a stage 5 seizure with maximum accumulation at

1 hour post-seizure. c-*fos* levels returned to basal levels by three hours post-seizure. With respect to the findings at the earliest time (15 min) and maximum time (1 hr.) after induction, these results correspond with the time course following Metrazol- (PTZ) induced convulsions in mice (Morgan *et al.*, 1987). However, c-*fos* levels after a kindled seizure returned to a basal level more rapidly than following PTZ-induced convulsions. This could be expected, since PTZ treatment causes recurrent seizures, and presumably recurrent c-*fos* induction. The time course of c-*fos* accumulation following a kindled seizure is protracted when compared to *in vitro* induction in serum-stimulated fibroblasts and in PC12 or 3T3 cells treated with nerve growth factor (Muller *et al.*, 1984; Greenberg and Ziff, 1984; Curran and Morgan, 1985; Kruijer *et al.*, 1985; Milbrandt, 1986). Perhaps the protracted time course after a kindled seizure reflects further changes in neurons following the AD, such as the re-establishment of pre-AD ionic distributions, that may further induce c-*fos*. Thus, both the nature of the inducing stimulus and the difference between adult neurons *in vivo* and cells lines maintained *in vitro* may account for the protracted c-*fos* accumulation observed after stage 5 seizures.

I also observed a slight increase in c-*fos* levels 24 hours post-stage 5 seizure in both the stimulated and contralateral amygdala-pyriform regions. This increase may simply be due

to variance in basal c-*fos* expression. However, if the increase at 24 hours is real then two different explanations may account for this somewhat surprising observation.

Wasterlain (1974) reported that focal seizures can induce a transient ischemic attack. Jørgensen *et al.*, (1989) found an increase in c-*fos* mRNA in the hippocampus, 24 hours after a 20 minute 4-vessel occlusion which induced cerebral ischemia. Taken together these results suggest that seizure-induced ischemia may give rise to a delayed increase in c-*fos* levels. However, Jørgensen *et al.* (1989) reported that while there were increased c-*fos* levels at 24 hours, the maximal c-*fos* levels occurred at 72 hours in the post-ischemic hippocampus. Although an increase of c-*fos* at 72 hours post-seizure was not observed, in the present study, this could be due to differences in the brain structures examined and severity of the ischemic attack. The seizure-induced ischemia may not have been as severe as the 4 vessel occlusion.

Jørgensen *et al.* (1989) reported that following the 4 vessel occlusion the hippocampal CA1 region showed the highest c-*fos* hybridization. The CA1 region is known to be highly sensitive to ischemia-induced necrosis (Sommer, 1880; Kirino *et al.*, 1984; Pulsinelli *et al.* 1982). Since delayed neuronal death and delayed c-*fos* expression have similar temporal profiles, this suggests that c-*fos* expression might be related to cell

death.

Dragunow and Robertson (1988) also report a prolonged increase in FOS expression. They report that following a cortical injury, caused by a stab wound, increased c-*fos* levels are observed between 1-72 hours in cortical neurons. Although the occurrence of recurrent seizures, caused by the cortical injury, has been put forward as an explanation for the FOS expression, this seems unlikely since induction is confined to the cerebral cortical cells of the wounded hemisphere. A spreading depression (SD), which is known to occur after mechanical damage, may account for the increase in c-*fos* levels. During SD neurons take up Ca^{2+}, which results in a 10-fold decrease in extracellular Ca^{2+} and causes an intense high-frequency burst of action potentials, which is known to induce c-*fos*. Furthermore, SD is usually confined to one hemisphere (Lauritzen, 1987). These speculations for an increase in c-*fos* in neurons do not account for the observation, in the present study, of an increase in c-*fos* levels at 24 and not 72 hours post-seizure. Perhaps the enhanced c-*fos* levels at 24 hours are due to an increase in c-*fos* in non-neural cells.

Although glial cells do not show basal levels of FOS they do show an enhanced FOS level 12-24 hours after injury (Dragunow and Robertson, 1988), at a time that just precedes glial cell division (Ludwin, 1985). This observation is in agreement with the 24 hour temporal delay of enhanced c-*fos*

levels following a stage 5 seizure. Astrocytes regulate the neuronal microenvironment, assist in repair of neural tissue and remove neuronal debris by phagocytosis. It is also known that both mature oligodendroglia and astrocytes will proliferate in response to brain injury (Ludwin, 1985). Furthermore, amygdala kindling results in astrocyte hypertrophy, as detected by stains for glial fibrillary acidic protein, particularly within the amygdala-pyriform region (Racine, personal communication). The increase of FOS in either nerve or glial cells may be related to the regrowth of nerve cell processes or production of nerve axon regeneration factors released from glial cells (Morrison, 1987). It should be pointed cut that Dragunow and Robertson (1987a) did not report FOS in glial cells of the hippocampus after the first and only AD. However, the present study looked for increases in c-*fos* levels in the amygdala-pyriform region after a stage 5 seizure. The differences in the structures studied and especially the much more severe AD induced in the present study may have caused glial activation and increase in c-*fos* levels in glia.

Thus, the two induction times, immediate-early and 24 hour-delayed, may reflect different roles for c-*fos*. The initial c-*fos* enhancement may reflect neuronal activation, while the delayed c-*fos* enhancement may reflect neuronal necrosis, cell death or glial activation.

Comparisons were made between the amount of c-*fos* mRNA in different brain regions in both control animals and at one hour following a stage 5 seizure. In the present study the forebrain, which includes amygdala-pyriform, hippocampus and neocortex, shows basal levels of c-*fos* (Morgan *et al.*, 1987). Although the expression of c-*fos* has been inconsistently localized in the adult cerebellum (Dragunow and Robertson, 1988; Gubits *et al.*, 1988; Sagar *et al.*, 1988) I report very low basal levels.

As previously described, there was a massive accumulation of c-*fos* in both the stimulated and contralateral amygdala-pyriform regions. Massive accumulation of c-*fos* was also observed in the hippocampus both ipsilateral and contralateral to the side of stimulation, and in neocortical tissue. These observations are in agreement with the distribution of FOS protein(s) (Dragunow *et al.*, 1987). These same regions are areas that kindle readily, and this fact had lead others to speculate on a relationship between c-*fos* and the long-term changes in nerve cells that underlie kindling (Dragunow *et al.*, in press). However, the cerebellum also showed an enhanced level of c-*fos* induction following a stage 5 seizure. The cerebellum does not kindle and stimulation of cerebellum leads to an inhibition of convulsive behaviour (Maiti and Snider, 1975). This leads to the conclusion that there is no relationship between the distribution of c-*fos* induction and

readiness to kindle.

The results concerning c-*fos* accumulation over the development of kindling in the amygdala-pyriform region indicated that in general, the amount of c-*fos* increased as the severity of the convulsive behaviour increased. This was probably due to the progressive increase in AD duration, amplitude, frequency, and complexity observed over the course of kindling. The results from comparisons of individual differences in AD duration revealed that generally, as AD duration increased the amount of c-*fos* accumulation increased. The results also showed that there was a fair amount of variation in c-*fos* accumulation in different rats, especially in the hemisphere contralateral to the side of stimulation. Post (personal communication) also reported a surprising variation in the distribution of c-*fos* following electrical kindling. It appears that stronger AD resulted in larger c-*fos* levels.

Interestingly, c-*fos* was induced bilaterally even on the first (ADT) and fifth (Stage 1-2) sessions when AD was localized only in one hemisphere. Even though the amount of c-*fos* accumulation in the contralateral amygdala-pyriform was always somewhat less than the stimulated side, even when AD was not observed in the contralateral amygdala-pyriform, these observations seem to indicate that c-*fos* may be induced as a consequence of neuronal activation and not necessarily as a

result of AD propagation. In apparent agreement with the present results, Dragunow *et al.* (1988), using immunohistochemical techniques, found bilateral hippocampal elevations of FOS after one AD in the hippocampus, and a bilateral amygdala-pyriform elevation of FOS following a stage 5 seizure in amygdala kindled animals. However, only a unilateral elevation of FOS was observed in the stimulated amygdala after a few ADs in amygdala kindled animals (Dragunow *et al.*, 1988). This last observation led Dragunow *et al.* (1988) to the conclude that FOS induction reflects the extent of seizure propagation from the amygdala focus. The results from the present study on c-*fos* distribution do not seem to support this claim.

Thus far my results appeared to indicate no clear relationship between the accumulation of c-*fos* and the presence of AD. In order to further explore the possibility of a non-specific activation of c-*fos*, I examined the effects of different types of electrical stimulation, some of which lead to plastic change and others that do not, on c-*fos* accumulation in the amygdala-pyriform region.

The observations came from "control" animals that received electrical stimulation, which did not give rise to AD, both after "regular" kindling electrical stimulation of the amygdala that was subthreshold and electrical stimulation consisting of the same number of coulombs, as "regular"

kindling stimulation, but given over a longer time period and at a lower frequency. These two types of current delivery modestly increased c-*fos* levels. Thus, electrical stimulation alone that did not produce noticeable changes in EEG, and did not contribute to kindling, was able to increase c-*fos* levels.

High frequency electrical stimulation that caused LTP, but did not produce AD, resulted in c-*fos* levels nearly equivalent to the c-*fos* levels observed after the initial AD in the amygdala-pyriform region. This further suggests that high frequency stimulation and the resulting neuronal activation, but not epileptiform activity, can enhance c-*fos* levels.

In the hippocampus somewhat different observations have been made. Dragunow *et al.* (1989) reported that in chronic awake freely moving rats that received high frequency electrical stimulation to the perforant path, which produced good LTP, elevated c-*fos* and FOS occurred in the dentate granule cells but not in CA1, CA3 or the entorhinal cortex. The production of FOS resulted in a 200% increase, compared with a 1000% increase following AD in the hippocampus. Furthermore, in hippocampus, the degree of FOS production did not correlate with the degree of LTP production. High frequency stimulation that produced LTP at the perforant path-dentate gyrus synapse did not lead to FOS accumulation in granule cells, while under urethane anaesthesia (Bliss *et al.*,

1988) and only stimulation that led to granule cell bursting led to c-*fos* production (Douglas *et al.*, 1988). These observations led Dragunow *et al.* (1989) to conclude that c-*fos* was not involved in LTP production.

Comparisons between amygdala-pyriform and hippocampus may be difficult. The amygdala, which requires relatively high current intensities to induce LTP, also supports brief initial AD durations during kindling that require low current to elicit (Racine, 1972b; Racine *et al.*, 1983). Conversely, in the hippocampus relatively lower currents are required to induce LTP, and long initial AD durations, that require higher currents, are routinely observed during kindling (Cain, 1989). One explanation may account for similar amygdala-pyriform c-*fos* levels following LTP and AD, as well as the different hippocampal c-*fos* levels following LTP and AD. Perhaps the type of electrical stimulation itself, plus the various intensities of neuronal activation induce c-*fos* in a cell-type-specific manner, independent of the plastic changes resulting from the type of activation.

It has been suggested that the degree of FOS production may be critical in distinguishing a long-term change from a transient change (Dragunow *et al.*, in press). In the present study a group of animals received an ECS treatment as an attempt to control for the effects of intense motor activity (convulsion), without the neuronal plastic changes.

Unfortunately, there probably is no perfect convulsion control, since any treatment that leads to cell bursting will probably show some positive transfer to electrical kindling (Cain, 1986). However, a single ECS treatment does not significantly reduce the number of stimulations to reach a stage 5 seizure (Cain, personal communication). In the present study I observed a massive accumulation of c-*fos* in the amygdala-pyriform region following ECS. The level of c-*fos* accumulation was far greater than that observed after both an initial AD and stage 5 convulsion. Furthermore, the duration of convulsion following ECS was much less than the duration of the stage 5 seizure. Thus it appears that activation that results in cell bursting can massively induce c-*fos* without corresponding contributions to the plasticity.

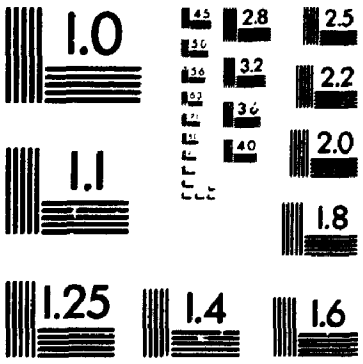
Although a single ECS treatment does not significantly contribute to the rate of kindling, low frequency (LF) stimulation, if given in the proper manner, can result in strong kindling in one session (Cain and Corcoran, 1981). I observed greatly enhanced c-*fos* levels following low frequency stimulation, the resulting AD and stage five seizure. These results provide more evidence for a dissociation between c-*fos* induction and plasticity.

I observed a dissociation between a fully generalized motor seizure and c-*fos* induction. PTZ-injection was able to induce massive accumulations of c-*fos* regardless of the

approximately 30% of c-*fos* levels when compared to animals receiving only the first injection. Percent levels of c-*fos* decreased even more if the second injection was delayed to 4 hours. The larger second c-*fos* levels in the present study may reflect two major differences that exist between the two studies.

As mentioned previously, PTZ-induced seizures are severe, recurrent and long lasting, compared to amygdala-pyriform kindling. It is possible that the second PTZ injection, while inducing a severe convulsion, did not produce a convulsion equivalent to the first convulsion, and thus resulted in a lower second c-*fos* accumulation. On the other hand, PTZ might have activated a larger population of forebrain cells during the primary stimulation leaving fewer cells to take part in the convulsions following the secondary injection. Thus, in the kindling study, a population of cells that did not participate in the initial AD might have been involved in the second AD.

One role of FOS is to form a complex with other nuclear proteins and then bind to the promoter regions of DNA that are responsible for the transcription of c-*fos* itself, as well as other genes (Sassone-Corsi *et al.*, 1988). The FOS protein, in combination with other nuclear extracts (i.e. JUN), form the AP-1 complex that binds to DNA and may serve this autoregulatory, or negative feedback function (Sambucetti and



occurrence of an intense motor convulsion. This seems to indicate that the presence of generalized motor convulsions do not correlate with c-*fos* induction.

Many kindling researchers use a procedure that involves multiple kindling stimulations within each 24-hour period. Although this procedure results in reaching a stage 5 seizure in a shorter amount of time, it does require the delivery of more current, sessions and ADs. To determine if c-*fos* expression is refractory, which one would predict if the role of FOS is that of a negative feedback mechanism (Marx, 1987), I evoked a second stage 5 seizure 2 hours after evoking a first stage 5 seizure. The second seizure came at a time when c-*fos* levels are near basal level and the FOS protein is highly abundant (Dragunow, 1988). At one hour following the second stimulation and resulting stage 5 seizure the animals were sacrificed and c-*fos* accumulation determined relative to animals receiving primary stimulation. I found that when animals were sacrificed after a 2nd stage 5 seizure, c-*fos* levels were approximately 80% of those measured after a single stage 5 seizure in the stimulated amygdala-pyriform. However, the AD was of equivalent duration, complexity, and frequency when primary and secondary ADs were compared.

Morgan *et al.* (1987) performed a similar experiment except they used PTZ-induced seizures in mice. They found c-*fos* levels after a second injection (after 2 hours) to be

approximately 30% of c-*fos* levels when compared to animals receiving only the first injection. Percent levels of c-*fos* decreased even more if the second injection was delayed to 4 hours. The larger second c-*fos* levels in the present study may reflect two major differences that exist between the two studies.

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Curran, 1986; Renz *et al.*., 1987; Sassone-Corsi *et al.*, 1988). However, my results suggest that kindling stimulation can reinduce c-*fos* transcription at a time when the FOS protein is highly abundant. Dynamic changes in the levels and composition of transcription factors are known to occur after seizure (Sonnenberg *et al.*, 1989). Perhaps these alterations provide a mechanism that allows reinduction of c-*fos* during high FOS levels. Other possibilities include: 1) the necessary post-translational modifications of FOS have not occurred at the time of restimulation, 2) FOS is not the critical factor in autoregu. ation.

Taken together the results from the present study suggest a poor correlation between the production of c-*fos* and AD, which is required for kindling. In view of the results from this study the most parsimonious explanation seems to be that electrical stimulation itself plus various types of neuronal activation induce c-*fos*. The occurrence of AD, which is required for kindling (Racine, 1972b), was not the only form of neural activation that induced c-*fos*. Furthermore, morphine injection, which induces c-*fos* in the caudate nucleus of rats, hyperpolarizes rather that depolarizes caudate neurons (Chang *et al.*, 1988). Perhaps changes in synaptic potentials and not cell bursting is sufficient to induce c-*fos*.

This may not mean that c-*fos* is not important in the long-

term adaptive response to seizures. Rather, the non-specificity of c-*fos* induction suggests that c-*fos* may not be the crucial "molecular switch" which turns on the kindling program.

However, there is a possibility that different stimuli are transduced by separate biochemical pathways and this could give rise to different functional forms of FOS. Previous observations suggest that different stimuli induce different post-translational modifications of the FOS protein and these different post-translational modification could affect the function of FOS. In PC12 cells depolarization-induced c-*fos* expression was both calcium and calmodulin dependent (Greenberg *et al.*, 1986; Morgan and Curran, 1986), while NGF-induction of c-*fos* was not and may involve a separate biochemical pathway (Curran and Morgan, 1986). Furthermore, depolarization and growth factor applications to PC12 cells lead to different post-translational modifications of FOS, with fewer post-translational modifications occurring after depolarization (Morgan and Curran, 1986). The possibility of different post-translational modifications of FOS occurring after different types, plastic vs non-plastic, of stimuli needs to be addressed experimentally.

How do we account for the permanence of kindling? It would first appear that a permanent change in genetic expression is required to maintain the long-term alterations

thought to underlie kindling. Permanence probably does not reside at the level of IEG since they are transient. Perhaps a permanent alteration in transcription and/or translation rates of existing proteins or the synthesis of new "kindling proteins" is critical to kindling (Dragunow *et al*, in press). These proteins may be for enzymes, growth factors, ion channels, or structural proteins that may cause permanent biochemical and/or morphological changes in the brain that give rise to the kindled state.

The permanent functional change of the brain after kindling is thought to result from a permanent morphological or biochemical change in neurons. However this permanent functional and morphological alteration may not require a permanent change in gene expression. A transient change in gene expression, and not a permanent change in gene expression, may account for the "permanence" observed after kindling. In a normal functioning cell "house-keeping" genes and proteins are present. These proteins are responsible for maintaining the day to day functioning of the cell. After an AD has been elicited a molecular program may be turned on that creates a new structure or increases an existing structure (e.g. forms a new synapse) of the cell. After the morphological change has taken place the molecular program is turned off and the normal house-keeping genes continue to upkeep this new structure. A small morphological alteration could provide a significant functional change in the cell

without providing a significant, or detectable, change in normal housekeeping gene expression. On this level there might not be any difference between a naive and kindled animal.

CLINICAL RELEVANCE:

At this time the relevance of c-*fos* production to clinical practice is unclear. However, FOS has been found in humans after routine neurosurgical treatment of epilepsy (Dragunow *et al.*, submitted). The production of c-*fos* has also been observed following brain injury (Dragunow and Robertson, 1988). The results from the present study suggest that the delayed (24 hr) c-*fos* production in response to seizure may be related to neural cell death or gliosis.

FUTURE RESEARCH:

In order to better understand the altered cellular processes that occur as a result of kindling the search for genes and proteins involved in long-term adaptive responses must continue. In an attempt to limit the search among the enormous number of genes contained in each cell to only those involved in kindling we should search for those genes that have certain attributes that make them likely candidates for involvement in kindling. Thus, it would be prudent to search for a limited number of genes which possess some or all of the following characteristics.

Look for genes critically involved in crucial life processes. Oncogenes which were recently thought only to be involved in cell division, differentiation and neoplasia have been shown to be rapidly and transiently induced in adult neuron following massive cell discharges. Genes that are expressed constitutively in neurons most probably play an important role in neural functioning. Other DNA binding proteins such as c-*jun*, should be investigated to determine if they are coordinately expressed with c-*fos*. Since kindling is most probably based on some physical change in neurons we should look for structural genes such as actin, and tubulin. Seizures can be considered a form of stress. Thus investigating genes induced as a consequence of stress may prove beneficial. One possibility is the heat-shock genes (e.g., HSP70).

Appendix I The Northern Blot Technique

The Northern blot technique is used to determine the size of a specific mRNA molecule and the amount of that molecule in a test sample. The total RNA from cells is first extracted, purified and quantitated. Extraction is accomplished by homogenizing the cells in a solution that will aid in the liberation of the cell contents and prevent the degradation of RNA by endogenous RNAases. Purification of the RNA is most commonly achieved by a phenol-chloroform extraction procedure which, at a low pH, will allow a phase separation of the RNA from DNA and protein. In order to determine the relative purity and quantity of RNA an aliquot of sample is taken and a spectrum analysis is performed. RNA is read at 260 nm, while protein is read at 280 nm, and an acceptable purity reading is a 260:280 ratio of approximately 1.8 (Maniatis *et al.*, 1982). The relative quantity of RNA is equivalent to 4 times the 260 reading.

The RNA molecules are then denatured by mixing them with a denaturing agent (e.g., formaldehyde) that breaks the hydrogen bonds between base pairs, and thus ensures that the RNA is an unfolded and linear form. A fixed amount of total sample RNA is separated into its specific sizes, by gel electrophoresis. Gel electrophoresis is carried out by pouring a liquid (e.g. agarose) into a mould. As the gel solidifies it forms interconnected pores, or channels, whose

size depends on the concentration of the agarose. The RNA samples to be separated are then loaded into individual wells at one end of the gel and an electric current is passed through the gel. Migration of RNA occurs due to the movement of the phosphate groups caused by the electric current. Larger RNA molecules pass more slowly through the gel matrix than smaller RNA molecules.

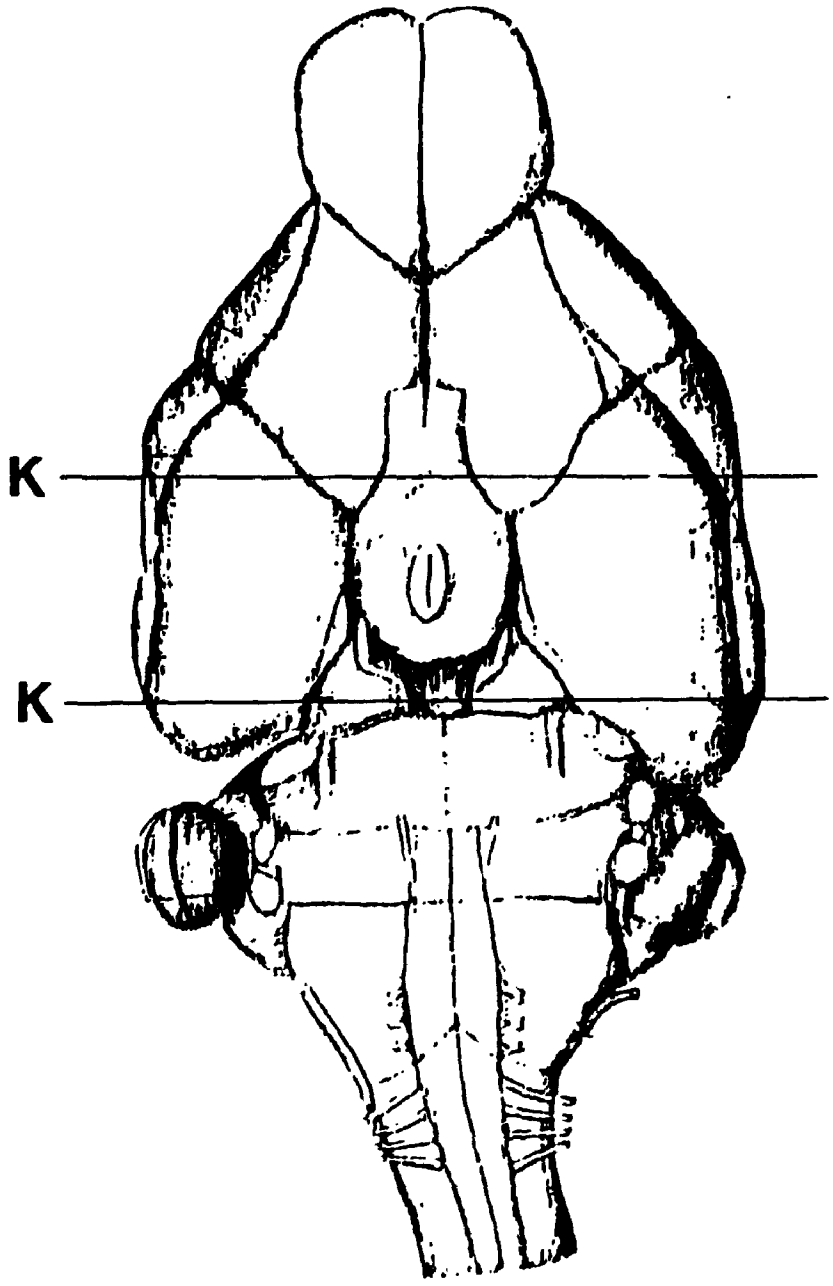
The size-fractionated RNA is then transferred from the gel matrix to a hybridization filter. Hybridization involves bringing together a purified, radioactively labelled DNA probe that is complementary to the mRNA molecule of interest with the filter-bound RNA under conditions that favour specific binding of the probe to the specific mRNA. Finally, an autoradiograph shows the position of the mRNA in the gel, and the intensity of the signal shows the amount of mRNA. Thus, Northern blot analysis can be used to compare the amounts of a specific mRNA (e.g., c-*fos*) generated in cells under different conditions (e.g., seizures).

The Northern blot technique is so extremely sensitive that it detects fragments of RNA complementary to a purified nucleic acid sequence at a level of 1 part in 10^6. This makes the technique highly reliable when comparing lanes, with identical amounts of RNA, on the same blot. However, separate blots that are not probed in the same hybridization solution can not be reliably compared.

Appendix II

View of the ventral aspect of rat brain. Two coronal slices are illustrated (K), one at the level of the optic chiasm and the second at the level of the mamilliary bodies. All tissue for experimentation, except cerebellum, was taken from this block of tissue.

Ventral Aspect of Rat Brain

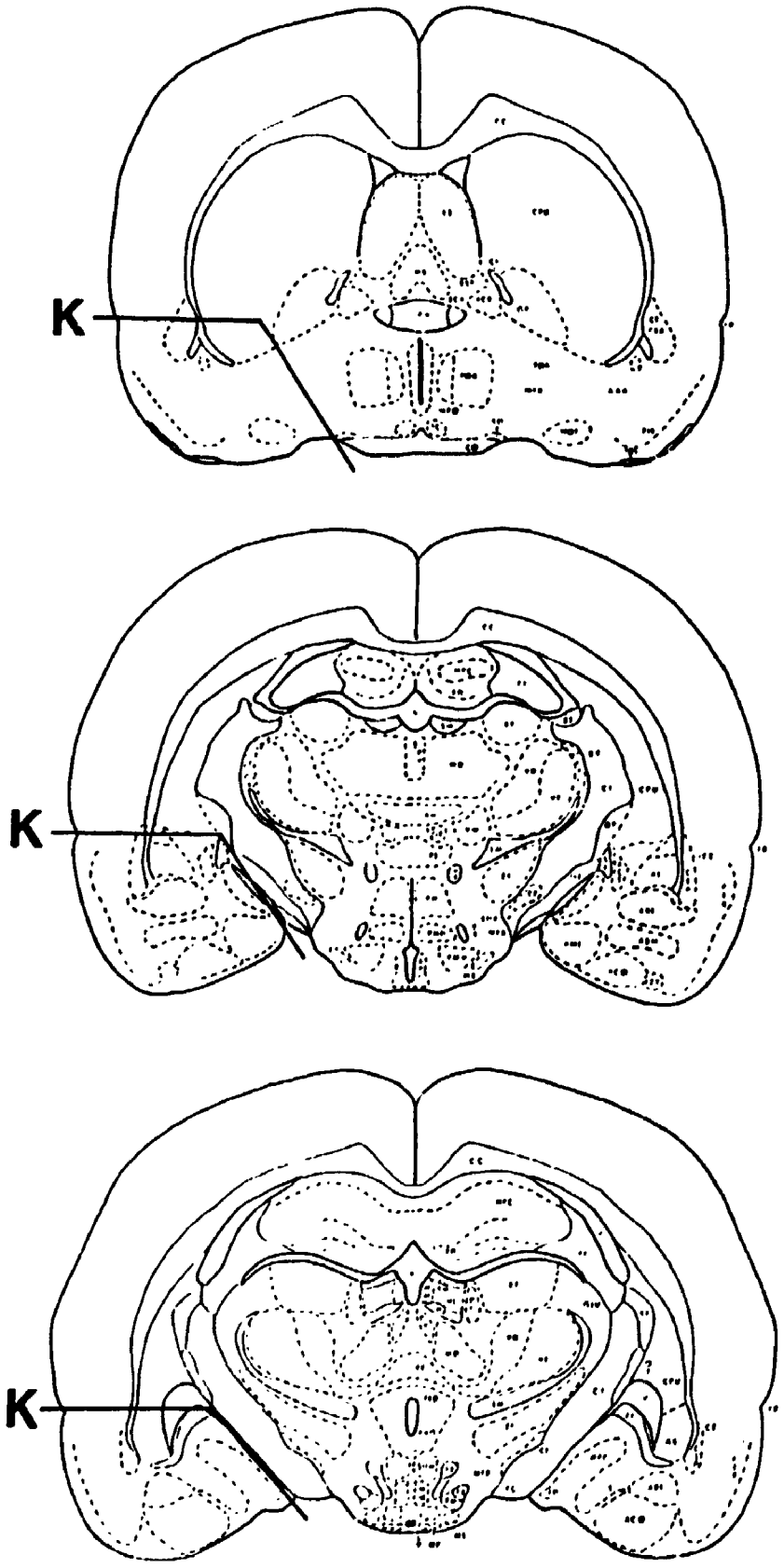


modified from Zeman and Innes, 1963

Appendix II

View of three coronal sections of rat brain. The anterior section represents the section at the level of optic chiasm. The electrode section represents the section at the level of electrode implantation. The posterior section represents the section at the level of mamilliary bodies. A knife cut (K) was made at the rhinal fissure to a depth approximately one quarter the width of the brain followed by a second cut, as shown, that removed the amygdala-pyriform area.

Coronal Sections of Rat Brain



from Pellegrino, Pellegrino and Cushman, 1979

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