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Fyn tyrosine kinase is required for normal amygdala kindling

Donald P. Cain [a,*], Seth G.N. Grant [b,d], Deborah Saucier [a], Eric L. Hargreaves [a,e], Eric R. Kandel [b,c]

[a] *Department of Psychology, Graduate Program in Neuroscience, University of Western Ontario, London, Ontario N6A 5C2, Canada*
[b] *Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University New York, NY 10032, USA*
[c] *Howard Hughes Medical Institute, 722 West 168th Street, New York, NY 10032, USA*
[d] *Center for Genome Research, The University of Edinburgh, King's Buildings, West Main Road, Edinburgh EH9 3JQ, United Kingdom*
[e] *Department of Psychology, McGill University, Montreal, Quebec H3A 1B1, Canada*

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Abstract

To identify specific genes involved with epileptogenesis kindling was examined in mice carrying mutations engineered by gene targeting. Amygdala kindling was tested in mice with a null-mutation in the Fyn tyrosine kinase gene, a mutation that raises the threshold for the induction of long-term potentiation in the hippocampus. The *fyn* mutants had a normal threshold, duration and stability of epileptiform after-discharge, which is crucial for kindling. Despite the normal after-discharge, *fyn* mutants showed a striking retardation in the rate of kindling. Once the kindled state was established in *fyn* mutants it remained stable. This implicates a Fyn-dependent biochemical pathway in the induction but not the maintenance of normal amygdala kindling. *fyn* is the first gene identified to be required for normal epileptogenesis.

Keywords: Gene targeting; Epileptogenesis

1. Introduction

The identification of genes that influence the genesis of seizures may provide insights into the molecular mechanisms of epilepsy. In addition to gene mapping in seizure prone humans or laboratory animals [33], it is now possible to ask if a known gene is involved with epilepsy using gene-targeting. Gene-targeting involves engineering a defined mutation into the mouse germ line and thereby creating mutant mice that can be neurologically evaluated [16]. An important advantage of gene-targeting is that the interpretation of the seizure phenotype is simplified by the prior knowledge of the neural expression pattern and biochemical function of the targeted gene. Moreover, this approach makes it possible to identify genes that are required for normal epileptogenesis, which will be revealed in mutant mice that show a resistance rather than a predisposition to epileptogenesis.

Electrical kindling of the amygdala provides a robust animal model for temporal lobe epilepsy [3,29], and is readily studied in mice [5,21]. A brief low-intensity electrical stimulus applied once daily evokes progressively more severe electrographic and

* Corresponding author.

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behavioral responses, culminating in a generalized seizure. The kindled state is highly stable and may persist for months to years [10] indicating that long-term modification of neural function is induced by the kindling stimuli.

Kindling shares a number of physiological and pharmacological properties with a form of synaptic plasticity known as long-term potentiation [1] (LTP), which suggests that kindling and LTP may share common cellular and molecular mechanisms. The induction of LTP requires a cascade of signaling steps in the pre- and post-synaptic cells [1]. The activation of post-synaptic glutamate receptors results in calcium influx and signalling requiring the activity of protein tyrosine [15,22] and serine–theonine kinases [23]. A specific tyrosine kinase that is required for the induction of LTP was identified by examining mice carrying mutations in non-receptor tyrosine kinases Fyn, Src, Yes or Abl [14]. Only *fyn* mutants showed a deficit in LTP, in the form of an increase in the threshold for the induction of LTP by tetanic stimulation. In addition to the deficit in LTP, the *fyn* mutants also revealed spatial learning deficits and structural changes in the hippocampus (see below). Thus, mice carrying a mutation of the *fyn* gene show impaired synaptic plasticity in the hippocampus and impairments in hippocampal dependent forms of learning [14].

We asked whether Fyn tyrosine kinase is also involved with epileptogenesis. To address this issue we examined whether *fyn* mutants show impaired induction of kindling and whether the persistence of the kindled state is affected in *fyn* mutants.

2. Methods

The *fyn* mutation was engineered by homologous recombination into mouse embryonic stem cells and resulted in no detectable expression of Fyn in the brain [14,31]. Since kindling is influenced by genetic background [4,21] we examined both wild-type (control) and *fyn* mutant mice in the inbred C129Sv strain. Control mice were age and sex matched from the Jackson Labs. The *fyn* mutant group consisted of three males and three females; non-littermate controls consisted of six males and five females. Mice in both groups ranged in age from 3.5 to 6 months at surgery. All *fyn* mutant mice were confirmed to be homozygous by genomic southern blotting.

Administration of 10 mg xylazine and 60–80 mg/kg pentobarbital was followed by implantation of indwelling bipolar wire electrodes bilaterally into the basolateral amygdala at the following coordinates, using standard stereotaxic techniques [6] determined using pilot animals: Anterior–posterior +2.5 mm relative to interaural zero; medial–lateral 3.3 mm; 5.3 mm ventral to skull surface. The electrodes were constructed of twisted teflon-insulated Nichrome wire 127 μm in diameter soldered to gold-plated connector pins. A miniature connector and leads attached the pins to brain stimulation and recording equipment. Histological examination confirmed the accuracy of the electrode placements.

After 10 days recovery, trains of biphasic square wave pulses (1.0 ms each, 60 pulse-pairs per second, for 1.0 s) were applied through the electrode at increasing intensity to determine AD threshold. Once-daily stimulation at 110% AD threshold was applied beginning 24 h later until three generalized convulsions occurred. Stimulation was applied between 10:00 am and 2:00 pm at the same time for both groups. Electrographic records were obtained from the electrodes before and after each stimulation using a Grass polygraph. AD (Fig. 2) occurred in response to each suprathreshold stimulation. All of the kindling sessions of the first three mice studied in each group were videotaped, and the tapes were replayed for scoring of the convulsions using Racine's categories [25], with stage 1 indicating brief behavioral immobility with ear flattening or twitching of the facial musculature and stage 5 indicating generalized convulsions. This procedure aided in the identification of the early and late landmarks of kindling. For subsequent mice, sessions through the development of the first stage 1 convulsion, and sessions involving stage 4 and 5 convulsions were videotaped and replayed for scoring.

Spontaneous behavioral activity was measured using an automated behavior monitoring device (Digiscan, Omnitech) that consisted of six monitors (40 $\times$ 40 $\times$ 30 cm), each of which contained two tiers of infrared sensors to measure horizontal (locomotion) and vertical (rearing) movements. The pattern of beam interruptions was recorded and analyzed by an Omnitech Analysis Unit, and stored on disk by a

microcomputer. A total of 12 behavioral activity variables was then obtained from the pattern of beam interruptions (for details of apparatus and technique see [18]). Mice were observed during a 60-min observation period.

After the completion of behavioral testing all mice were prefused through the heart with a formalin–saline solution, and the brains were removed, sectioned on a freezing microtome, mounted on glass slides, stained for Nissl substance, and examined microscopically.

Statistical analysis was accomplished using analysis of variance.

3. Results

The *fyn* mutants were severely retarded in their development of kindled convulsions (Fig. 1). Control mice required a mean of 9.0 daily ADs (± 0.4 S.E.M.; $n = 11$) to reach the generalized convulsions of stage 5 (Fig. 1A,B). By contrast, *fyn* mutant mice required a mean of 23.5 daily ADs (± 4.0; $n = 6$). Thus, the *fyn* mutants required 2.6 times as many ADs to kindle to stage 5 as the control mice. The range of kindling rates to stage 5 in the *fyn* mutant group does not overlap with that of the controls, and repeated measures analysis of variance of seizure stage progression revealed a highly significant group difference ($F_{1,15} = 27.8$, $P < 0.0001$). The behavioral convulsions of both control and mutant mice were alike and similar to those described previously for mice [5] and rats [25] kindled in the amygdala. An added feature of the convulsion, which was present in all animals, was the occurrence of brief vocalization during the stage 5 convulsions.

The increase in the number of ADs required to reach stage 5 in the *fyn* mutants appeared to be a specific physiological change in that *fyn* mutants and control mice were otherwise similar in several other physiological parameters. These included: (i) a

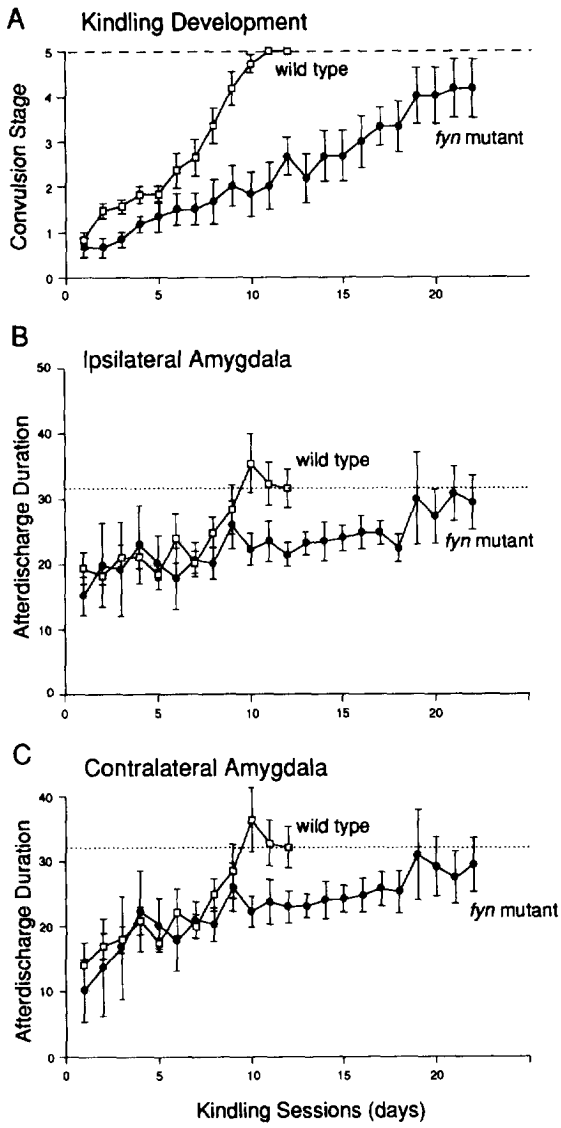

Fig. 1. (A) Amygdala kindling is severely retarded in *fyn* mutant mice. Convulsion stages are from Racine [24]; stage 1 indicates ear flattening or twitching of the facial musculature and stage 5 indicates a generalized behavioral convulsion. The first 22 sessions are shown for the *fyn* mutants, which required 23.5 ± 4.0 (mean $\pm$ S.E.M., $n = 6$) after-discharges to kindle to stage 5. The control mice required 9.0 ± 0.4 (mean $\pm$ S.E.M., $n = 11$) after-discharges to kindle to stage 5; the range of the control data does not overlap with that of the mutants. (B) After-discharge duration in the ipsilateral amygdala does not differ between *fyn* mutant and control mice during the first 9 days. By Day 10 most (9/11) control mice had reached stage 5, which is associated with a marked increment in after-discharge duration [2]. Here and in Fig. 1C) the dotted line indicates the stage 5 after-discharge duration of control mice. Each point represents the mean $\pm$ S.E.M. (C) After-discharge duration in the contralateral amygdala does not differ between *fyn* mutant and control mice during the first 9 days.

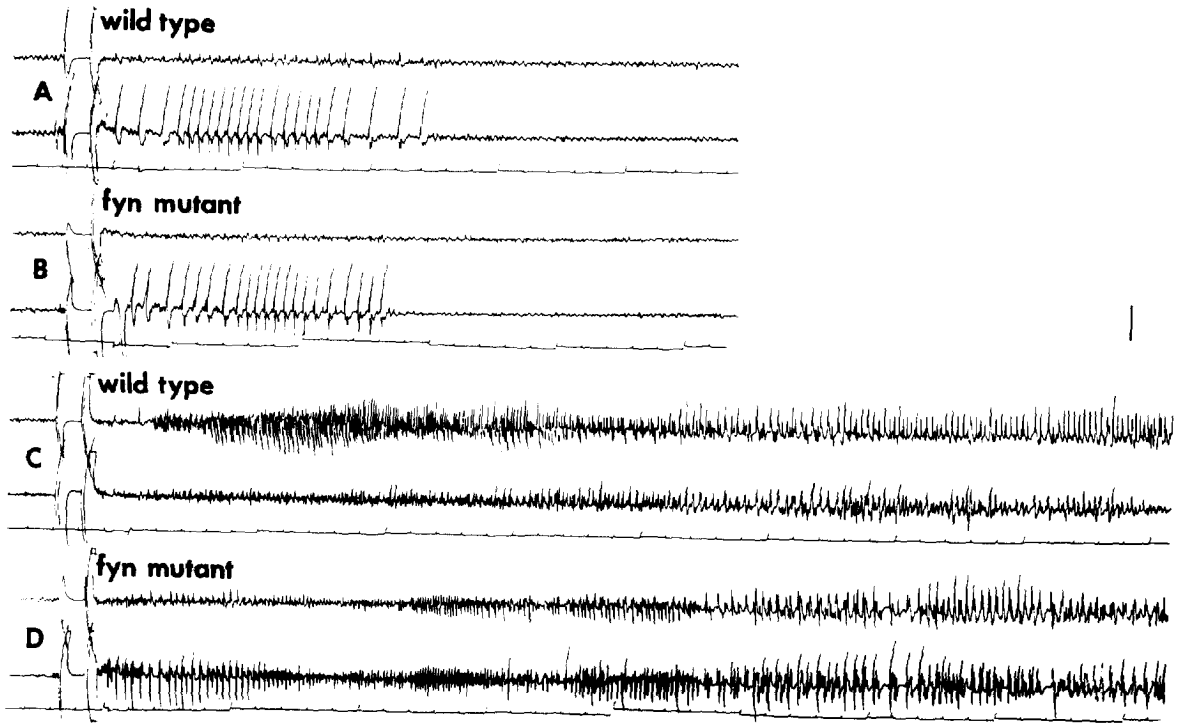

Fig. 2. Normal electrographic response in *fyn* mutant mice. Record of the first after-discharge (A) and the first stage 5 seizure (C) in a control mouse. Record of the first after-discharge (B) and the first stage 5 seizure (D) in a *fyn* mutant mouse. The responses are comparable between groups. The waxing and waning of spike amplitude in (C) and (D) is similar to the pattern that is typical for amygdala kindling in rats [24]. Stimulation was delivered to the right amygdala (lower trace of each pair) during the flattening of the trace near the left side of the records. The response of the left amygdala is shown in the top trace of each pair. Time marker: 1 s; calibration: 0.5 mV.

similar threshold current required to elicit AD (*fyn*: 40.8 μA; control 40.5 μA), (ii) similar electrophysiological patterns of seizure (Fig. 1B and Fig. 2); (iii) similar convulsive behavior (both overall pattern and vigor); (iv) similar propagation of the AD to the contralateral amygdala (Fig. 1C); (v) the expected sudden increase in duration of the AD when stage 5 was attained [2] (session prior to first stage 5, *fyn*: 25.5 ± 0.6 s, control: 24.7 ± 3.2 s; first stage 5, *fyn*: 39.5 ± 5.3 s, control: 37.8 ± 4.2 s); and (vi) the expected postictal behavioral depression after the stage 5 convulsions[11] (*fyn*: 177.5 ± 25.9 s; control: 187.8 ± 17.6 s). Analysis of variance carried out on the data in i, v, and vi failed to reveal a significant group difference on any measure ($P > 0.05$). Pearson product moment correlations calculated between age and number of ADs required to reach the first stage 5 convulsion for the combined control and *fyn* mutant groups or for the control

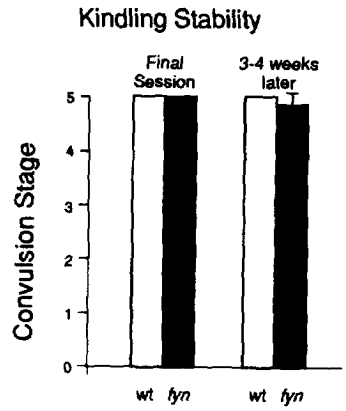

Fig. 3. Normal retention of the kindled state in *fyn* mutant mice. Convulsion stage on the last session before a 3–4-week rest (Final Session), and on the first session after a 3–4-week rest (3–4 weeks later). The absence of error bars is indicative of zero variability.

group alone were small ($R < -0.15$) and not significant ($P > 0.05$).

To examine the persistence of kindling, the kindled mice were rested for 3–4 weeks after the third generalized convulsion and then restimulated at the same current intensity. We found no significant reduction in the convulsive (Fig. 3) or electrographic response (AD duration on last session before rest period, *fyn*: 34.6 ± 3.5 s, control: 37 ± 4.7 s; duration of AD on the first stimulation after the rest period, *fyn*: 50.0 ± 5.8 s, control: 38.2 ± 2.1 s; $P > 0.05$).

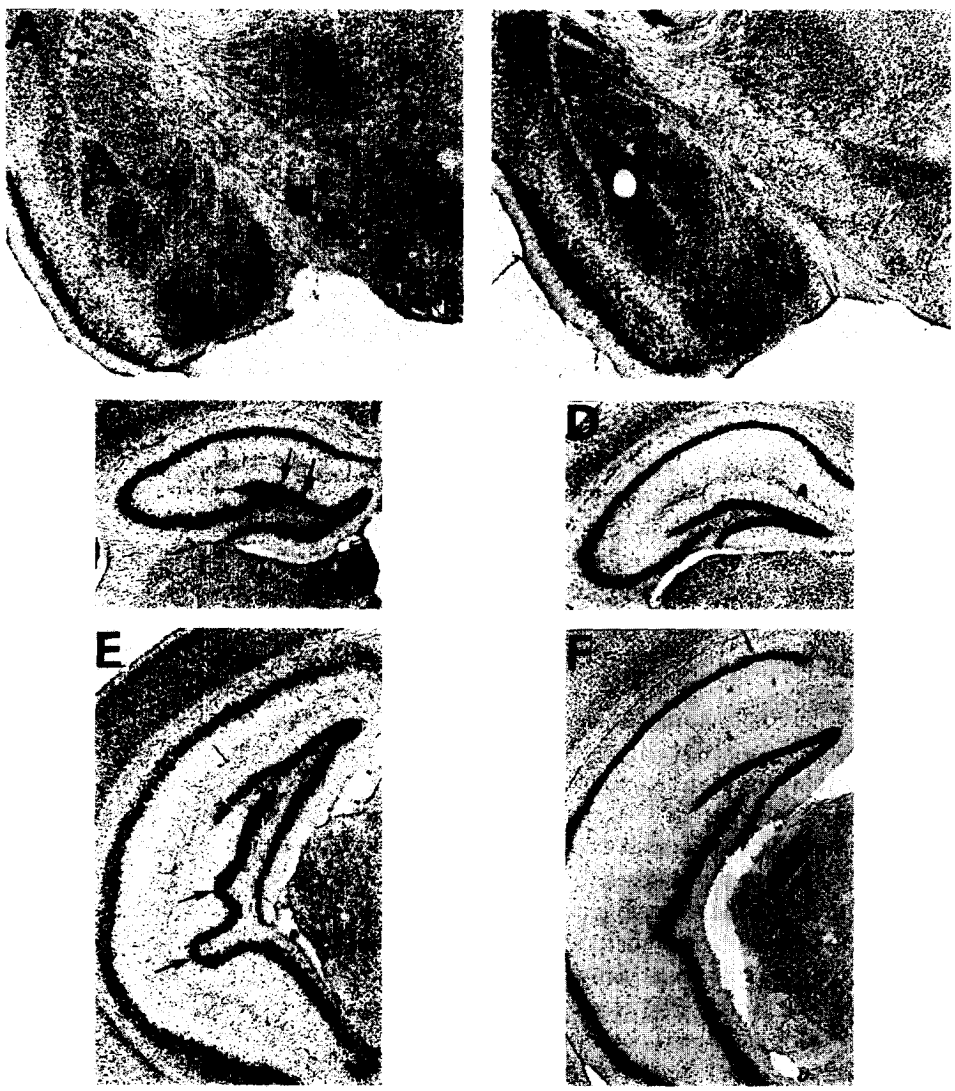

Fig. 4. Coronal section showing architecture of the amygdala in fyn mutant (A) and control (B) mice at the level of the basolateral nucleus. The gross structure of the amygdala and surrounding areas does not differ between the groups. A portion of the electrode track shows near the border between La and BL in (B). Dorsal hippocampus from the same coronal section as (A) showing undulations in dentate granule cell layer of fyn mutant (C). Dorsal hippocampus from the same coronal section as (B) showing dentate granule cell layer of control mouse (D). Posterior hippocampus showing undulations in CA3 region of fyn mutant (E). Posterior hippocampus showing CA3 region of control mouse (F). Abbreviations: BL, basolateral amygdaloid nucleus; BM, basomedial amygdaloid nucleus; Ce, central amygdaloid nucleus; Co, cortical amygdaloid nucleus; La, lateral amygdaloid nucleus; Me, medial amygdaloid nucleus.

There were no group differences among 11 of the 12 behavioral activity variables [18] ($P > 0.05$). The only variable that differed between groups was mean speed of movement ($F_{1,24} = 6.0$, $P < 0.05$), which was 22% less in the *fyn* mutants than in the controls.

Examination of Nissl-stained sections from *fyn* mutant and control mice revealed no systematic group differences in the overall size or position of the nuclei of the amygdala or in surrounding structures (Fig. 4A,B). Examination of anterior and posterior hippocampus revealed the expected [14] undulations in the granule cell layer of the anterior dentate gyrus and the pyramidal cell layer of the posterior hippocampal CA3 region (Fig. 4C–F).

4. Discussion

Among the kindling measures obtained in this study, the only one that distinguished mutant from control mice was the rate of development of generalized convulsions. Mean AD threshold, AD characteristics, and behavioral pattern and vigor of seizure expression were similar in mutant and control mice. Thus the difference in the rate of kindling does not appear to be due to a difference in initial seizure susceptibility or epileptic responsiveness to the stimulation. There was no reduction in the convulsive or electrographic response after a 3–4-wk rest. Therefore it appears that Fyn is necessary for the normal induction of kindling but not in maintaining the kindled state.

The mechanism by which Fyn participates in kindling is unknown. The absence of Fyn may lead to a change in kindling rate that is secondary to a developmental defect, although the available electrophysiological, behavioral, and neuroanatomical evidence seems less consistent with this interpretation. Objective measurement of spontaneous activity, an index of amygdala damage in rats [30], revealed no group differences among 11 of 12 behavioral activity variables. The reduction in mean speed of movement that was found for the *fyn* mutants could reflect a developmental defect, but this finding is contrary to the expected hyperactivity that results from amygdala damage [30]. Histological examination of the amygdala and surrounding regions implicated in amygdala kindling such as the pyriform cortex [26] revealed no obvious defects in *fyn* mutants at the light microscopic level. Although the presence of fine structural defects in these and other brain regions of *fyn* mutants cannot be excluded, gross developmental defects in this structure were not seen in our histological material.

fyn mutants show ectopic pyramidal neurons and a change in architecture of the dentate gyrus and CA3 and CA1 regions of the hippocampus, although no gross abnormalities were detected in other regions of the brain [14]. These changes in neuronal architecture were observed also in our *fyn* mutants, raising the question whether hippocampal function in the mutants was normal, and whether any abnormality could have contributed to the retardation of amygdala kindling in the mutants. Evidence from kindling studies with rats indicates that the hippocampal complex is one of the most slowly kindling structures in the brain [8,12,27], and that damage to the dentate gyrus or hippocampus proper either does not affect or accelerates kindling of the amygdala and surrounding structures [17,20,26,32]. The undulations observed in the dentate gyrus granule cell layer and the pyramidal cell layer in CA3 reflect a 25% increase in the number of these cells in *fyn* mutants, and the pathway connecting the granule cells with area CA3 appears normal in its overall projections [14]. Synaptic transmission and two short term forms of synaptic plasticity in the pathway connecting CA3 pyramidal cells with area CA1 were normal in *fyn* mutants. Taken together, presently available data do not seem consistent with the idea that a developmental defect of the hippocampus readily accounts for the retardation of amygdala kindling in *fyn* mutants. However, we cannot exclude this possibility.

Alternatively, Fyn may be involved directly in a biochemical process that is required for normal amygdala kindling. Fyn is a nonreceptor tyrosine kinase associated with the cytoplasmic side of the plasma membrane. Nonreceptor tyrosine kinases are often activated by transmembrane signalling molecules [31] and may be involved in a signalling cascade that mediates the postsynaptic response of the cell. An active signalling role for Fyn in LTP is supported by the finding that tyrosine kinase inhibitors on normal hippocampus slices phenocopy the *fyn* mutant defect in the induction of LTP [24]. Recent studies indicate that Fyn is necessary to

phosphorylate a number of brain proteins including another non-receptor tyrosine kinase, the Focal Adhesion Kinase (FAK) [13]. Both Fyn and FAK may phosphorylate a number of relevant substrates that have been implicated in kindling [7,9], including neurotransmitter receptors [23] and ion-channels [19,28,34].

There are striking similarities between the described deficits in amygdala kindling and hippocampal LTP [14] in *fyn* mutant mice. First, the induction but not the stability of both forms of plasticity is affected. Second, both in LTP and kindling basic electrophysiological parameters are unaffected. These similarities suggest that *fyn* may be part of a basic biochemical mechanism that sets the threshold or determines the rate of development of both forms of long-term plasticity.

In addition to implicating a role for *fyn* tyrosine kinase signal transduction pathways in kindling, this study suggests that gene-targeting may be a useful approach for the identification of genes involved with epilepsy. The availability of other mutants with similar phenotypes may help in identification of the multiple components of the signal transduction pathways that regulate neuronal excitability and epileptogenesis.

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