Functions of Fyn Tyrosine Kinase in Adult Brain

--epileptogenesis, akinesia and learning ability in fyn-transgenic mice

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1997

A thesis submitted to The University for Advanced Studies

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Summary

To find out functions of Fyn, a non-receptor tyrosine kinase, in the brains of adult animals, phenotypes of Fyn transgenic mice were sought.

The generation of fyn-transgenic mice which carry either native form of Fyn or constitutively active form of Fyn is described in the first part of this multi-part report.

The second part portrays biochemical substrates of Fyn tyrosine kinase. In the brains of adult fyn-transgenic mice, the tyrosine phosphorylation of at least five proteins was enhanced, one of which was identified to be the NMDA receptor modulatory subunit 2B (NR2B).

The third part describes characters of seizures that is a prominent phenotype of fyn-transgenic mice. Seizure characteristics were analyzed in two seizure-inducing experiments, the infusion of a convulsant drug pentylenetetrazol, and the electrical kindling stimulation. These experiments revealed that forebrain seizure was strengthened in both types of transgenic mice, meanwhile brainstem seizure was easily induced only in the active-Fyn transgenic mice. The acceleration of the forebrain seizure development was completely inhibited by the NMDA receptor antagonist MK-801. These results suggest that the tyrosine phosphorylation of the NMDA receptor enhances the development of the forebrain seizure.

The forth part of this study describes decreased spontaneous locomotor activity. The decreased locomotor activity was restored by the injection of MK-801, suggesting that the tyrosine phosphorylation of the NMDA receptor plays a role in the control of locomotor activity.

In the fifth part of this study, three kinds of learning and memory abilities are depicted. Performance of three kinds of tasks, each requires function mainly of the brain limbic region, was not different between wild-type control mice and fyn-transgenic mice.

In the general discussion section, I will describe the attempt to accomplish the purpose.

General Introduction

Fyn, a non-receptor tyrosine kinase, is expressed in the lymphocytes and neural system. In the brain, Fyn is considered to play a role in neural development; intrinsic Fyn expression begins well before the birth widely in neurons and glial cells (53), and Fyn is activated during the initial stages of myelination (49). Analysis by genetic interruption of fyn gene supports this idea; the lack of Fyn results in morphological defects, such as uncoordinated hippocampal cell arrangement (18, 53), impaired myelination (49) or decreased total brain mass (20).

Expression of Fyn reaches a peak during early embryogenesis and declines thereafter but remains after neural maturation (4). Knockout approach has shed light also on Fyn functions in the mature brain. In fyn-deficient mice, threshold for long term potentiation is raised and spatial learning ability was reported to be impaired (18). Increased fearfulness is also described (30). However, it is not clear whether the observed phenomena in adult fyn-knockout mice are direct results of the lack of Fyn in adult brains or by the indirect effects of lacking Fyn through the developmental period. To overcome this problem, Kojima has generated fyn-transgenic mice, in which transgene expression begins postnatally (21), and has showed that the introduction of fyn-transgene after the birth restored the impaired LTP but not the abnormal morphology in the hippocampas of fyn-deficient mice. These results suggest that the impaired LTP in fyn-deficient mice is not caused by the morphological abnormalities.

The functions of Fyn, other than in the synpatic plasticity, are largey unknown, however. To clarify Fyn's function in mature brain, I sought the behavioral changes caused by fyn-transgene expression . This paper consists

of five parts. The first part describes the generation of fyn-transgenic mice. The second part describes the biochemical effects of the transgene expression. The third part presents the characters of seizure, a clear phenotype of fyn-transgenic mice. The forth part depicts the decreased locomotor activity and its mechanism. The last part reports learning and memory abilities.

The last part, general discission part, is devoted for description of my effort for this thesis work. I wish the description of the flow of my idea, some cases resulted in success and some fell into failure, will help others in learning the experimental design.

Part 1. Generation of fyn-Transgenic Mice

Analysis of Fyn functions in adult mice using fyn-deficient mice suffers from the anatomical abnormality, which results from the absence in the neuronal developmental period. To overcome this problem, Kojima generated fyn-transgenic mice, in which transgenes expressed postnatally (21).

Methods

Construct

Construction of wild-type fyn-transgene was previously described (21). In brief, an *Eco*RI-*Dra*I fragment of brain type fyn cDNA (fynB cDNA; ref. (13)) was inserted into the vector, pNN265 (provided by N. Nakanishi, Harvard University), which was constructed by inserting a hybrid intron consisting of an adenovirus splice donor and an IgG splice acceptor (12) in 5' of the cloning site of the vector pcDNAI/Amp (Invitrogen) to give an efficient transgene expression. To add the CaMKIIα promoter to this construct, fynB cDNA fragment with 5' intron and simian virus 40 poly(A) signal sequences was excised using *Not*I and inserted into the pBluescript-based vector, pNN279 (provided by N. Nakanishi, Harvard University) into which the 8.5-kb fragment including the CaMKIIα promoter was inserted (26).

To construct the activated version of the fyn-transgene, the *Eco*RI-*Dra*I fragment of fynB cDNA was inserted into the vector, M13mp18. Single-stranded phage DNA was prepared, and mutation was introduced on codon 531 from TAT to TTT by the site-directed mutagenesis method (23) using the oligonucleotide 5'-CGGGCTGAAACTGGGGCTCTG-3'. The introduction of the point-mutation was confirmed by DNA sequencing. The mutated cDNA was inserted into the vector, pNN265 and then into pNN279.

The transgene was excised from the vector using *SalI*, and was then gel purified. The transgenic mice were generated by microinjecting the transgene into the pronuclei of fertilized eggs collected from C57BL6/CBA/F1 females; the injected eggs were then transferred into the oviduct of pseudopregnant females. Founder mice were screened by PCR of tail DNA using the transgene-specific primer-pair. Mouse lines were established by crossing founders with the C57BL/6Jx129Sv hybrid strain of hetero- or homozygous fyn-deficient mice (46). Offsprings were crossed with non-transgenic females, later with C57BL/6 females.

fyn-transgene expression

For ribonuclease protection assay, total RNA was prepared from forebrains using RNA isolation kit (Stratagene). A fragment of 5' sequence of the transgene, including transgene specific 5' noncoding sequence and a part of fyn cDNA fragment, was inserted into the vector, pGEM4Z (Promega). From the template DNA linearized at the *Eco*RI site, the antisense cRNA probe was synthesized using T7 RNA polymerase in the presence of [α-^{32}P]UTP. Ribonuclease protection assay was performed using a commercially available kit (RPAII kit, Ambion). Five micrograms of total RNA was hybridized with RNase A and T1. The protected bands were separated on a 3.5% denaturing acrylamide gel and detected by autoradiography.

For in situ hybridization experiment, brains were quickly removed and frozen in dry ice powder. Cryostat sections (12 μm-thick) were dried at 42°C and kept at -80°C until use. A fragment of transgene specific 3' noncoding sequence was inserted into the vector pGEM4Z (Promega). Antisense digoxigenin labeled RNA probe was transcribed using digoxigenin-11-UTP (Boehringer Mannheim) and T7 RNA polymerase (Promega). Procedures for pretreatment of sections, hybridization, and

detection were done according to the instruction manual of the supplier (Boehringer Mannheim).

Homogenization

The forebrains of adult mice were homogenized in 10 volume of RIPA buffer containing Tris-HCl, pH 7.5, 1% nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF), and were then centrifuged at 10,000 g for 10 min. Supernatants were rapidly frozen in liquid nitrogen and stored at -80°C until used. Protein concentration was determined using BCA protein assay kit (Pierce).

In vitro immune complex kinase assay

The forebrain extract (200 μg protein) was reacted with anti-Fyn antibody (Oncogene Science), then precipitated with ProteinG Sepharose (Pharmacia). The immunoprecipitate was incubated with 125 μM [γ-^{32}P]ATP (specific activity: 37 kBq/nmol) and 10 μg of the substrate peptide (KVEKIGEGTYGVVYK, UBI) at 30°C for 10 min. The reaction was stopped by addition of trichloroacetic acid solution. Phosphorylated peptide was spotted onto a P81 phosphocellulose paper. The filter was washed with 0.75% of phosphoric acid, and was then counted by liquid scintillation counter. The immunoprecipitate without anti-Fyn antibody was used as a background control.

Results

Four independent lines of transgenic mice carrying native-Fyn, f8, 39, 85 and 92, and six lines carrying active-Fyn, f(F_{531})3, 27, 47, 58, 78 and 84, were obtained.

Figure 2 shows temporal expression of transgenic and intrinsic fyn mRNA. Expression of transgenic fyn mRNA was weak on postnatal day 1,

increased on day 10, and further increased on day 21. The level of the intrinsic fyn mRNA expression was not altered by the expression of the transgene.

The transgene was expressed highly in the brain, and scarcely in non-neural tissues (data not shown). Figure 3 shows spatial expression of transgenic fyn mRNA in mature brains. As of CaMKIIα, the signal was detected preferentially in the neurons of the forebrains including the neocortex, hippocampus and amygdala (Figure 3B). In the neocortex, the transgene was detected strongly in layers II/III, moderately in layers IV through VI, but not detected in layer I. In the hippocampus, pyramidal cells in all subregions of Ammon's horn and dentate granule cells were uniformly stained. Neurons in the striatum and septum were moderately stained. By contrast, the signal was considerably weak in the thalamus, hypothalamus, cerebellum and brain stem, although some neurons, such as cerebellar Purkinje cells, were positive (Figure 3C). In spite of variable signal intensity, the spatial pattern of the transgene expression was essentially the same across lines.

Figure 4 shows kinase activities of Fyn proteins prepared from adult forebrains. The kinase activities varied among lines in native-form Fyn trasngenic group, f8 through f92. The kinase activities in three lines of active-Fyn transgenic group, f(F_{531})27, 58, 78, were lower than those of three lines of native-Fyn transgenic mice but higher than that of wild-type control mice.

Discussion

There are clear differences in the expression pattern between intrinsic Fyn and transgenic Fyn. Intrinsic Fyn is expressed widely in neurons and glial cells (53), while fyn-transgenes were detected mainly in neurons of the forebrain. Another difference is that the transgene expression is late-onset,

while expression of intrinsic Fyn peaks at embryonic day 7 (4). The effect of fyn-transgene expression on the neural development is considered to be small, supported by the following evidence: (1) the expression of fyn-transgene on postnatal day 1 was considerably weak (see the level of intrinsic fyn mRNA), (2) hippocampal malformation, which is characteristic to fyn-knockout mice, is not restored by introduction of fyn-transgene into fyn-knockout mice (21), and (3) no overt anatomical abnormalities were observed in fyn-transgenic mice. Therefore, the use of fyn-transgenic mice helps dissociate the roles of Fyn in adult animals from its roles in the developmental period.

Three lines of active-Fyn transgenic mice, f(F_{531})3,47,84, had high levels of transgene expression (data not shown) but died off as will be shown later. Fyn kinases extracted from three surviving lines of active-Fyn transgenic mice, f(F_{531})27,58,78 had lower kinase activities, when normalized by tissue weight, than those from native-Fyn transgenic mice. There is a possibility that inactive Fyn molecules in the brain became active in the process of purification, and the difference between active and inactive form was hindered. If so, the results show that native-Fyn transgenic mice have many Fyn molecules possibly in inactive form in the brain, while active-Fyn transgenic mice do not have many molecules, though most of them are in active form.

The domain structure of Fyn leads us to speculate that Fyn works, other than tyrosine kinase, as an adapter molecule. That is, since SH2 and SH3 domains have high affinity with phosphotyrosine and proline rich region of proteins respectively, Fyn might work as a 'bridge' between a phosphotyrosine protein and a protein possessing proline rich region. Comparison of phenotypes from two types of Fyn-transgenic mice may help dissociate Fyn's two roles, as an adapter molecule and as a tyrosine kinase.

Part 2. Substrates of Fyn Tyrosine Kinase

Fyn-transgenic mice offer good material in searching biological substrates of Fyn tyrosine kinase in the mammalian brain. First, an attempt to search phosphorylation substrates in non-mammalian cells may suffer from lacking phosphorylation pathway that is specific to the mammalian cells. Second, similar attempt using non-neural culture cell may suffer from lacking the neural specific biochemical signal pathway. Third, hyper-phosphorylated proteins in fyn-transgenic mice would be detected more easily than hypo-phosphorylated proteins in fyn-knockout mice. Therefore, to help identifying Fyn kinase substrates in mamalian brains, immunoblot detection, subcellular fractionation, and immuno-precipitation of phosphotyrosine proteins from fyn-transgenic mice were performed.

As a first step, immunoblot detection of phosphotyrosine-containing proteins was performed using anti-phosphotyrosine antibody.

A major 180kDa phosphotyrosine-containing protein in PSD fraction, Gp180, was identified as NR2B (31). The level of tyrosine phosphorylation of NR2B in fyn-transgenic mice's brains was examined.

Methods

Immuno-blotting and immuno-precipitation

The forebrain extract was obtained as described in the previous part. For immunoblotting of phosphotyrosine proteins, the extract (50 μg protein) was separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane (Schleicher & Schuell) and probed with anti-phosphotyrosine antibody (PT-66, Sigma). After incubation with HRP-conjugated anti-mouse IgG, signals were detected by ECL Western blotting detection reagents (Amersham).

Immunoprecipitation of NMDA receptor subunit 2B (NR2B) was carried out essentially according to Rosenblum et al. (42). The LP1 fraction (500 μg protein) was resuspended in 10 mM Tris-Cl, pH 8.0, containing 1 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF, mixed 1:1 with 2% SDS, and was then boiled for 5 min. The sample was diluted 1:10 in 10 mM Tris-Cl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF, incubated with 1 μg of antibody (PY20 or anti-NR2B, Transduction Laboratories) at 4°C for overnight, then with 5 μg of anti-mouse IgG for an additional 60 min. The immunoreactive was precipitated with 50 μl of Protein G-Sepharose (50% of slurry, Pharmacia). The sample was washed with 500 μl of RIPA buffer three times, separated on 7.5% of SDS-PAGE gel and was then blotted with either anti-NR2B or anti-phosphotyrosine antibody, PT-66.

Subcellular fractionation

Subcellular fractionation was performed essentially according to Hirano et al. (19). Forebrains were homogenized in 9 volume of ice-cold 0.32 M sucrose in phosphate-buffered saline containing 1 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF in a glass-Teflon homogenizer. All centrifugation was done at 4°C. The homogenate was centrifuged at 1,000 g for 10 min and the supernatant was centrifuged at 10,000 g for 20 min yielding P2 fraction. S2 fraction was obtained by centrifuging the supernatant at 100,000 g for 60 min. The P2 pellet was resuspended in 10 mM Tris-Cl, pH 8.0, containing 1 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF, then centrifuged at 100,000 g for 20 min. The lysate pellet (LP1) was resuspended, then centrifuged at 100,000 g for 60 min to yield LS2 and LP2 fractions. Each fraction (50 μg proteins) was separated on 7.5% of SDS-PAGE gel and blotted with anti-phosphotyrosine antibody.

Results

Figure 5A shows immunoblot detection of phosphotyrosine proteins in the forebrains. Five proteins (designated as PY190, 180, 120, 110 and 100 by their relative electro-mobility) were hypo-phosphorylated in fyn-knockout mice, and were hyper-phosphorylated in both types of fyn-transgenic mice. The tyrosine phosphorylation of those proteins was at approximately the same level among four lines of transgenic mice. The tyrosine phosphorylation of a 60-kDa protein, which was identified to be Fyn in another experiment (not shown), was stronger in native-Fyn transgenic mice than in active-Fyn transgenic mice.

Figure 5B shows subcellular distribution of Fyn and five phosphotyrosine proteins, PY190, PY180, PY120, PY110 and PY100. PY110 was enriched in the crude plasma membrane fraction (LP1). PY120 was highly enriched in the light membrane and crude synaptic vesicle fraction (LP2). PY180, PY100 and Fyn were enriched in LP1 and LP2 fractions. In particular, PY180 and 110 were enriched in the postsynaptic density fraction (data not shown). PY190 was abundant in the soluble fractions (S2 and LS2).

Upper left panel in Figure 6 shows that phosphotyrosine proteins (PY180, 120, 110 and 100) are immunoprecipitated by PY20. NR2B was also immunoprecipitated by PY20 and the intensity of the band was weak in fyn-knockout mice and strong in fyn-transgenic mice. Lower left panel shows that the level of NR2B protein was the same across groups. Lower right panel shows that the level of tyrosine-phosphorylated NR2B was low in fyn-knockout mice and high in both types of fyn-transgenic mice. Therefore, a protein composing PY180, which are major phosphotyrosine proteins in the plasma membrane, is NR2B and hyper-phosphorylated in both types of fyn-transgenic mice.

Discussion

Five proteins, PY190, 180, 120, 110, 100, were hyper-phosphorylated in fyn-transgenic mice. There remains the possibility that these five proteins are phosphorylated secondarily by other tyrosine kinases. However, the evidence that these same five proteins were conversely hypo-phosphorylated in fyn-knockout mice suggests that PY190, 180, 120, 110 and 100 are indeed the substrates of the Fyn tyrosine kinase.

Fyn is not exclusively but heavily expressed in post-synaptic density (53), and is considered to be anchored to the plasma membrane (5). Fyn is, therefore, considered to mediate the synaptic signal from plasma membrane to inside the cell. In line with this expectation, four proteins, PY180, 120, 110 and 100, existed primarily in the membrane fraction.

Native NMDA receptor is thought to be composed of heteromers of NR1 (ζ1) and one or more NR2A-D (ε1-4) subunits (35). Heterologous co-expression studies reveal that NR2 subunits do not comprise an essential part for channel activity, but have a modulatory function. In the cultured spinal cord neurons, tyrosine kinase inhibitor, genistein, reduces NMDA current, and intracellular application of c-Src increases NMDA current (51). Salter and his colleagues recently reported that the activation of endogenous Src specifically increased the activity of NMDA receptor (54). Since NR2A and 2B but not NR1 subunits are targets for tyrosine phosphorylation (25, 31, 47), it is speculated that the modulatory effect of tyrosine kinases on the channel activity is mediated through tyrosine phosphorylation of NR2A or 2B or both. Köhr and Seeburg have demonstrated, in co-expression experiments of cultured cells, that tyrosine phosphorylation of NR2A rather than NR2B contributes to the modification of NMDA current (24). In addition, the finding that NMDA current is normal in fyn-knockout hippocampus (18) suggests that hypo-phosphorylation of NR2B does not

affect NMDA channel activity. Therefore, tyrosine phosphorylation of NR2B may not contribute directly to the alteration of NMDA channel activity. Instead, since NR2B has direct interaction with PSD-95 (also referred as SAP90) or SAP102, which are thought to regulate clustering of NMDA receptors (22, 34), the tyrosine phosphorylation of NR2B might work in clustering and localizing the NMDA receptors to active synapses.

In conclusion, the tyrosine phosphorylation of NR2B was enhanced in the brain of fyn-transgenic mice, and is likely to be a substrate of Fyn kinase. Four other un-identified proteins, PY190, 120, 110, 100, which are candidates as the substrates of the Fyn tyrosine kinase, have not been identified. The information offered in this report, however, will help in identification in the future.

Part 3. Epileptogenesis

Epileptogenesis, the clearest phenotype of fyn-transgenic mice, is analyzed in this part of paper.

I occasionally observed spontaneous seizures and resulting death in Fyn transgenic mice. To reveal the seizure characteristics in Fyn transgenic mice, death rate was observed. Furthermore, seizure inducing experiment was done to characterize the seizure susceptibility. The seizure characteristics at lighter stages were analyzed by two types of seizure inducing experiments. An intravenous infusion of pentylenetetrazol (PTZ) with a constant flow rate elicits a series of stereotyped seizure responses, beginning with facial twitching, go on to clonic phase of seizure, followed by tonic extension and death (48). Another type of seizure inducing experiment, kindling experiment, is widely used to analyze the characteristics of clonic seizures. In these seizure-inducing experiments, the seizure progressions of wild-type mice and fyn-transgenic mice were observed and compared.

Methods

Observation of spontaneous seizures

Juvenile mice were separated from their parents at 3 weeks of age. Tail was cut at this time for genotyping and mice were marked on their fingers for individual identification. The viability of 3 weeks old transgenic offspring was surveyed up to 20 weeks.

Pentylenetetrazol infusion

Eleven wild-type control mice, 21 native-Fyn transgenic mice and 12 active-Fyn transgenic mice were used. All mice were 2-6 months of age and weighed 23-40 g. Animals were loosely held in a plastic bag and subjected a constantaneous infusion (0.1 ml/min) of 1.0% pentylenetetrazol in saline into the tail vein. Progression of seizure was monitored and the time

latencies exhibited facial twitch and tonic extension were recorded. Doses required for the twitch and extension were calculated by the recorded time latencies and corrected for body weight. Data were analyzed using one-way ANOVA each for the dose inducing twitch and the dose eliciting extension after inducing the first twitch.

Kindling protocol

Operation

Male mice, 2.5-4 months of age and weighed 23-36 g, were anesthetized with 15 μl/g B.W. of 2.5% Avertin solution: 0.25% (w/v) tribromomethyl alcohol, 0.25% (v/v) tertiary amyl alcohol. A bipolar electrode was stereotaxically implanted into the left basolateral amygdala (2.4 mm anterior to interaural zero, 2.8 mm lateral to the midline, 4.8 mm ventral to the skull surface) according to Cain and Dexergommeaux (8). The electrode was made of twisted Teflon-insulated stainless steel wires (each wire 75 μm in diameter, Plastic One), and served for both stimulation and recording.

Stimulation and evaluation

After 10 days of recovery period, trains of sine-wave pulses (60 Hz for 1.0 sec) were applied through the electrode. The stimulus intensity was measured by monitoring the voltage drop across a 3 kΩ resistor in series with the electrode. Electrical neural activities were picked up from the left amygdala through the same electrode, digitized at 1 kHz through A-D converter (MacLab, ADInstruments) and stored on the personal computer for later analysis. Stimulation was given repeatedly with increasing intensity until afterdischarge (AD), defined as high amplitude spike or polyspike epileptiform activity, was observed for 3 seconds or longer. Inter-train interval was at least five minutes to avoid poststimulus hyper-inhibition. Behavioral seizures were classified according to a modified version of Racine's criteria (38, 39) as follows: 1, chewing; 2, head nodding; 3, unilateral

forelimb clonus; 4, bilateral forelimb clonus or rearing; 5, hindlimb clonus or falling; 6, running or bouncing seizure; 7, tonic seizure; 8, tonic extension culminating in death.

Following the determination of AD threshold and evaluation of initial response was the once-daily stimulation to analyze development of seizure responses. For this phase of the experiment, mice showed strong seizure response, AD of 20 sec or longer or convulsion stage of 2 or more, were excluded. Mice were defined as fully kindled when greater than stage 4 convulsions were elicited for four days consecutively.

Examination of electrode position

After the completion of the experiment, electrical current of square shape pulse was applied through the electrode at 1 mA for 1 second. After the sacrifice, frozen section was prepared and stained for Nissl substance or cresyl violet to examine the position of the electrode tip. Data from mice in which the electrode tip missed the basolateral amygdala were omitted from analysis.

Injection of MK801 in kindling experiment

To assess the involvement of NMDA receptor activity in accerelated kindling development in fyn-transgenic mice, NMDA receptor antagonist MK-801 (Sigma) dissolved in saline was injected intraperitoneally 60 to 120 minutes before the kindling stimulation in days 2 through 11. The rates of seizure response development during day 1 through 10 were statistically analyzed at first among all four groups using two-way ANOVA with repeated measures (days), and were then compared between each pair using the same procedure.

Results

Spontaneous seizures

Figure 7 shows the survival rate. All mice in three lines of active-Fyn transgenic mice, f(F_{531})3, 47 and 84, died off in 15 weeks. Relatively high transgene expression was detected in lines f(F_{531})3 and 84 by in situ hybridization and Northern blotting (data not shown). In the remaining three active-Fyn transgenic lines, f(F_{531})27, 58 and 78, the death rate was considerably high. Sudden death in these lines was generally observed after four weeks of age. In contrast, the lethality of native-Fyn transgenic mice at 20 weeks was less than 10%. In concert with the death rate, the spontaneous seizures were observed more frequently in active-Fyn transgenic mice than in native-Fyn transgenic mice.

Pentylenetetrazol induced seizures

Figure 8 presents the drug dose required for the first twitch and tonic extension for wild-type control mice, native-Fyn transgenic mice and active-Fyn transgenic mice. No significant variation was observed among groups in the dose for the first twitch ($F_{2,41}$=2.33, p>0.1). The significant variation was observed in the drug dose required for tonic extension after eliciting the first twitch ($F_{2,41}$=3.44, p=0.042). Significant difference was observed between wild-type control mice and active-Fyn transgenic mice (Post hoc Fisher's PLSD test; p=0.012).

Kindling induced seizures

The threshold for afterdischarge (AD) generation did not differ among groups (in μA): , 42.2 ± 4.1 for wild-type mice, 39.1 ± 11.7 for f8, 39.5 ± 9.4 for f(F_{531})27, and 35.8 ± 5.3 for f(F_{531})78.

Figure 9 shows the first response of each animal to kindling stimulation. Figure 9A shows examples of ADs from one wild-type control mouse and one active-Fyn transgenic mouse. The mouse of the upper trace showed no observable behavioral seizure response, while the mouse of lower expample showed bilateral forelimb clonus ans rated as stage 3 accordingly. Figure 9B shows distribution of the AD duration and convulsion stage. The

first ADs were no longer than twenty seconds in all of wild-type mice, while AD of longer than 40 seconds was observed in at least one animal in each transgenic group. No mice in wild-type and f39, in which transgene expression was weak, showed obvious convulsive responses, while apparent behavioral responses were observed in considerable number of mice in f8 and two active-Fyn transgenic groups. Brainstem seizure, scored as stage 6 or higher, was observed only in active-Fyn mutant.

Figure 10 shows rate of kindling development. Both AD duration and convulsion stage progressed more rapidly in line f8 and mice expressing active-Fyn (data from f(F_{531}) 27 and 78 were combined) than those in wild-type control.

The number of mice experienced brainstem seizure before reaching the fully kindled state was the following; two out of 17 in wild-type control, one out of eight in f8, zero out of three in f39, four out of eight in f(F_{531})27, and four out of eight in f(F_{531})78. Fisher's exact test revealed marginally high frequencies of brainstem seizure in two active-Fyn transgenic lines compare to wild-type control group ($p=0.059$ for both between wild-type control and f(F_{531})27, and between wild-type control and f(F_{531})78). Two active-Fyn transgenic mice suffered from spontaneous tonic extension and culminated in death (stage 8) after the completion of the experiment.

Once mice reached a fully generalized kindled state, the seizure susceptibility persisted, that is, the electrographic and behavioral responses did not become weak when re-stimulated after 2 months of resting period (data not shown).

Figure 11 shows the development of seizure responses in the presence of MK-801 during day 2 through 11. Pre-administration of MK-801 in wild-type mice effectively suppressed the development of AD duration ($F_{9,216}=3.37$, $p=0.0007$) and convulsive response ($F_{9,216}=8.78$, $p<0.0001$). This effect was also observed in line f8 both in AD duration ($F_{6,54}=2.88$,

p=0.017) and in convulsive response ($F_{9,81}$=4.43, p<0.0001). As a consequence, the rate of the development became the same between wild-type and line f8 by MK-801 injection (compare filled circles and filled squares) both in AD duration ($F_{9,126}$=1.41, p=0.19) and in convulsive response ($F_{9,126}$=0.13, P=1.00). AD duration and convulsion stage on day 10 were not significantly different: 17.2 ± 6.0 sec and 2.0 ± 0.9 in f8 (n=7), 19.0 ± 2.9 sec and 1.8 ± 0.3 in control mice (n=9), respectively. The inhibitory effect of MK-801 on seizure development was reversible, since both AD duration and behavioral response developed quickly by the subsequent daily stimulation without MK-801 (day 12 and later).

Discussion

Overall appearance of fyn-transgenic mice was indistinguishable from that of non-transgenic litter mates. No abnormality was observed in reproduction and child-rearing. Histochemical examination of the brain, Nissl staining, Zimm staining, and in situ hybridization for glutamate decarboxylase as a marker for GABAergic neurons, did not show any overt abnormalities at the light microscopic level (data not shown). Obvious lethality was not observed at the embryonic or preweaning period. These are in line with the evidence that the transgene expression was not detected in the hypothalamus, brainstem, and spinal cord. Nevertheless, mature mice expressing mutant Fyn were prone to death from unknown reason. Deaths were not preceded by any noticeable health problems, such as weight loss or dehydration, and autopsies showed no evidence of hemorrhage, infarction or ischemia that might be associated with cardiovascular failure or stroke. Hindleg extension was observed, however, in many dead bodies. Furthermore, spontaneous seizures and resulting deaths were sometimes observed. This observation suggests that the deaths were caused by spontaneous seizures. Indeed, There seemed to be a good correlation between the death rate and incidence of observed spontaneous seizures, i.e.

the death rate increased through juvenile period when the fyn transgene expression increased.

Pentylenetetrazol (PTZ), which is believed to act at the picrotoxin site on the GABA-benzodiazepine receptor complex (45), has been widely used as a convulsant drug. Facial twitch is a type of clonic seizure responsible for the motor cortex. Tonic extension is assumed to be caused by the epileptic neural activity in the brainstem. The dose to develop the seizure from forebrain origin to brainstem origin was greatly reduced in active-Fyn transgenic mice and in a lesser extent in native-Fyn transgenic mice. This finding suggests that the spread of epileptic EEG is enhanced in some way by Fyn and that the number of active Fyn kinase molecules rather than the total number of Fyn molecules has influence on spread of epileptic EEG. Since I could not observe the seizure responses that might have appeared between the first facial twitch and tonic extension, it was not clear whether the difference lied in the development of the forebrain seizure after the first twitch, or in the brainstem seizure before the tonic extension. Development of forebrain seizure, however, can be precisely observed in another seizure inducing protocol called kindling experiment. The next section describes the details of seizure characteristics revealed by the kindling experiment.

Pharmacological studies provide evidence that NMDA receptor activity is involved in molecular mechanism for kindling development (15, 17, 28, 43). There is a good correlation between the development of amygdaloid kindling and tyrosine phosphorylation of NR2B. Development of amygdaloid kindling was retarded in fyn-knockout mice (9) and accelerated in fyn-transgenic mice (in this paper). Tyrosine phosphorylation of NR2B was enhanced in fyn-transgenic mice and weakened in fyn-knockout mice (part 2 in this paper). Furthermore, the tyrosine phosphorylation of the NMDA receptor increases the NMDA current (51). The finding that pre-administration of MK-801 canceled acceralted kindling development of fyn-

transgenic mice suggests that acceleration of kindling development is mediated by NMDA receptor activation.

In fyn-knockout mice the kindling development was retarded, despite having the normal parameters in the basal synaptic transmission (9). The development of forebrain kindling seizure was, in contrast, faster in both types of fyn-transgenic mice than in wild-type mice.

Though highly speculative, Fyn's involvement in the development of forebrain seizure may be mediated thorough Fyn's role as an adapter molecule rather than as a tyrosine kinase. Fyn's role as a tyrosine kinase may be reflected in the spread of epileptic from forebrain to brainstem.

As a mechanism of seizure development, axonal sprouting of the granule cell in the dentate gyrus was proposed: (1) blocking the nerve growth factor (NGF) activity by anti-NGF antiserum retards amygdaloid kindling associated with inhibition of mossy fiber sprouting (50), (2) intraventricular administration of NGF accelerates kindling development and enhances mossy fiber sprouting (1), and (3) over-expression of GAP-43 enhanced both mossy fiber sprouting and seizure activity (2). Axonal sprouting is not seemed to underlie the epileptogenesis of fyn-transgenic mice, since there is no evidence showing enhancement of Zimm staining in either native-Fyn or active-Fyn transgenic mice (data not shown).

Genetic manipulation, either disruption or overexpression, often leads to epileptogenesis (reviewed in (36)). Those genes are, potassium channel (37), glutamate receptor (Brusa et al., 1995), synapsin I/ II (41), CaMKIIα (7) or serotonin receptor 5-HT_{2C} (48). However, pharmacological manipulation on those receptors not always results in epileptogenesis. This consideration gives us an insight that sustained, but not transient, imbalance in the system is critical for inducing epileptogenesis. Overexpression of Fyn, among manipulation of those genes, offers good material to investigate the mechanisms of epileptogenesis, since (1) epileptogenesity and expression

level are correlated, (2) the accelerated development of the forebrain seizure in fyn-transgenic mice was completely suppressed by NMDA receptor antagonist.

The findings by Lu and his colleagues that the effect of bicuculline on EPSP amplitude is reduced in hippocampal slices expressing active-Fyn, but not in slices expressing wild-type Fyn, suggest that GABAergic system is weakened by constitutive activation of Fyn (personal communication). Therefore, the phenotypes in active-Fyn transgenic mice may be mediated, in part, by reduced tone of GABAergic inhibition.

Part 4. Spontaneous Locomotor Activity

Phenotypes of fyn-transgenic mice at whole body level, other than the epileptic phenotype, were sought. Overall behavior observed in an open field was not different from that of wild-type control mice. Nevertheless, locomotor activity appeared to be low in fyn-transgenic mice. Distance moved in 120 seconds in open-field was slightly shorter (736 ± 73 cm) than wild-type (818 ± 53 cm), though statistically not significant. In a novelty preference test, transgenic mice crossed the border between familiar box and novel box less often (12.3 ± 4.1 times) than wild-type did (16.8 ± 6.9 times). The artificial and new environment in the experiments above, however, leaves the possibility of having different sensitivity to unfamiliar condition. To exclude this possibility and to examine the locomotor activity in more direct way, the locomotor activity in the home cage was measured.

It is generally accepted that glutamatergic neuronal activity at the basal ganglia suppresses locomotor activity (reviewed by (11)). As can be seen in Part 2 in this paper, it is likely that NMDA receptors are constitutively active in fyn-transgenic mice's brains. To find out whether the tyrosine phosphorylation of NMDA receptors affects locomotor activity, effect of NMDA receptor antagonist on locomotor activity was also tested.

Methods

Fifty-five male mice, aged 3 to 5 months at the beginning of the experiment, were used. The room illumination was lit on at circadian time 06:00 and off at 18:00. The room temperature was set to 24 ± 1°C. The room used in this experiment was dedicated for this experiment and disturbance during experiment was kept minimal. Subjects were housed individually and an infrared beam sensor was attached to the lid of each cage. Beginning at 3 days after attacing the sensor, the locomotor activity was measured for

48 hours through the sensor and records were stored on a personal computer to be analyzed later (ABsystem, Neuroscience).

To investigate the effect of the NMDA receptor activity on the locomotor activity, saline or each dose of MK-801 (RBI) dissolved in saline was injected intraperitoneally between circadian time 11:30 and 11:35. The locomotor activity was measured for 10 minutes beginning at 30 minutes after the injection. Each animal was used only once.

Results

Left panel of Figure 12 presents the mean count for 24 hours in each group. A non-paired two-tailed *t* test on these data revealed that the locomotor activity of Fyn-transgenic mice was significantly lower than that of wild-type control mice (t_{30}=2.89, p=0.0071). Right panel of Figure 12 shows the count in each time zone. The activity pattern observed in wild-type, highest at the beginning and end of the dark period, was observed in fyn-transgenic mice as well.

Figure 13 shows the effect of MK-801 on locomotor activity in wild-type control mice and active-Fyn transgenic mice. Saline injection intraperitoneally did not affect the locomotor activity. Injection of NMDA receptor antagonist MK-801 increased locomotor activity in a dose dependent manner. Two-way ANOVA revealed no significant effect of transgene expression ($F_{1,49}$=3.45, p=0.069), a significant increase in counts across drug doses ($F_{2,49}$=13.48, p<0.0001), and significant interaction between transgene expression and drug doses ($F_{2,49}$=6.26, p=0.0038). This indicates that the effect of the drug is stronger on transgenic mice than on wild-type.

Discussion

Two evidence were obtained. First, the spontaneous locomotor activity of fyn-transgenic mice was lower than that of wild-type mice. Since the overall behavior was normal as far as observed, the Fyn activity is possibly related to the gain control of the motor system rather than to the initiation or tuning of the specific behavior.

Second, the effect of MK-801 on locomotor activity was stronger in transgenic mice than in wild-type mice. Two models have been presented for the mechanism how the glutamatergic activity modulates the spontaneous locomotor activity. One supposes the modulation of GABAergic activity, and the other presumes modulation of dopaminergic activity, both in the striatum. Huntington's disease, characterized by chorea, decreased tone, and dementia, is closely related to reduced glutamic acid decarboxylase and GABA activity, and treated with dopamine antagonists. Parkinson's disease, characterized by akinesia or involuntary movement, is closely related to degeneration of dopaminergic neurons in the substantia nigra (16). Furthermore, glutamatergic activity has been shown to modulate dopamine release (52). I do not suppose the reduced locomotor activity in fyn-transgenic mice is mediated through suppressed GABAergic activity, since the mRNA level of glutamic acid decarboxylase, examined by in situ hybridization, of fyn-transgenic mice was not different from that of wild-type control mice (data not shown). Therefore, it may be that the active NMDA receptors in fyn-transgenic mice suppress the locomotor activity by decreasing dopaminergic neural activity. Injection of MK-801 not only restored the suppressed locomotor activity in fyn-transgenic mice to the wild-type level, but also reversed. I hypothesize that the reduced locomotor activity in fyn-transgenic mice resulted from sustained suppression of dopaminergic activity by the constitutively activated NMDA receptors, and that the blocking of the NMDA receptor resulted in rebound-like hyperactivity of the dopaminergic pathway. It is tempting to test this

possibility by measuring the dopaminergic activity in the striatum in the basal state and after MK-801 injection.

Part 5. Learning and Memory Ability

Two phenotypes, epileptogenesis and akinesia, were both mediated, at least in part, by the activated NMDA receptors. This implicates close relation of Fyn tyrosine kinase and NMDA receptor. Hence, I speculated that fyn-transgenic mice possessed other phenotypes, which were related to the NMDA receptor functions. Plenty of evidence suggest that long term potentiation (LTP) of synaptic transmission is a mechanism of behavioral learning, and that NMDA receptor activation plays a crucial role in some type of LTP and behavioral learning. Taken together, the NMDA receptor activity possibly modulates the learning process through the LTP of synaptic transmission. Three brain regions, the hippocampus, cerebral cortex, and amygdala, express fyn-transgene and have NMDA receptor dependent neural plasticity. Lu and his colleagues have observed that the threshold for LTP in hippocampal CA1 region was lower in active-Fyn transgenic mice than that in control mice (personal communication). Speculating that the tyrosine phosphorylation of the NMDA receptor modifies the learning and memory process, I assigned three behavioral tasks to evaluate the ability of learning and memory, which were related to the functions of the limbic system.

Methods

Fear conditioning

The apparatus consists of a box and a shock generator. The box consists of a smaller, clear Plexiglas chamber (start box) and a larger, darkened, black Plexiglas chamber (shock box). The larger compartment is accessible to the subject through a circular opening in a black Plexiglas wall separating the two chambers. The floor of the box consists of a grid of metal

rods, 2 mm in diameter, placed about 6 mm apart (center-to-center). The grids in the larger chamber are wired to the shock generator.

Each animal was at first allowed to explore the box for 100 sec. The door on the circular opening was open and shock generator was disconnected during this habituation trial. Twenty-four hours later, the shock trial was given. The animal was placed at the far end of the smaller chamber, with its head pointing away from the circular opening. After all four paws entered the larger chamber, the door was closed and the shock generator was made active to deliver the shock of 25 volts for 2 seconds.

The retention test was given 24 hours later. This procedure was undertaken in a similar manner to the shock trial but no shock was given. The latency to enter the larger chamber was measured.

Spatial learning

The apparatus consists of a circular pool, a platform, and several visual landmarks. The pool is 120 cm diameter by 50 cm high, with the water level set at a height of 30 cm above the base. The water temperature was set to 24 ± 1°C. Nontoxic white paint was added to make the water opaque and to make the platform invisible. The platform is 10 cm diameter and 1 cm below the water surface. The location of the platform was fixed throughout the training for a given animal, but randomly assigned in a given group to have a counterbalance across subjects. The visual landmarks, posters and objects, were posted on the wall or hanged from the ceiling to serve as the spatial cues.

A training trial began with the experimenter lowering the animal into the pool, the animal facing and close to the side wall at three quadrants excepting that the platform lied in. Maximal time for each trial was 60 sec: if a mouse had not found and climbed onto the platform by this time, it was placed on the platform by hand. The animal remained on the platform for 30

sec before being picked up and placed back into the home cage where the subject was left for 5 minutes. Then second trial began, this time at a different starting location. The sequence of starting locations was such that a mouse started twice each from three locations over the six training trials each day.

The spatial bias was assessed in single transfer trial. The platform was removed from the pool and the animal placed in again for the 60-sec trial. The swimming of the mouse was videotaped from above and the amount of time spent in each quadrant of the pool was measured later by monitoring the swimming path from videotape record.

Odor discrimination learning

All training and testing were performed in the home cage. Mice were first 'shaped' to obtain bits of buried sweet cereal by digging through a 50:50 clean sand and ground chow mixture contained in a plastic cup of 3.8 cm diameter by 2.6 cm high.

In subsequent training, the subject was presented with two scented cups. The odors used were coffee, turmelic, nutmeg, cinnamon, basil, and laurel. Preliminary experiment showed that none of these odours was particularly favored or deterred by mice. The reward cereal was always buried in a cup of one odor but not in the other. The odor pairing assignments were counterbalanced across subjects, and the left-right positions of the choice cups followed a pseudorandom trial sequence. Each day, maximum of 50 trials was conducted until subjects reached a criterion of 18 correct in 20 consecutive trials. Once in every 10 trial, neither choice cup was baited; instead the reward was dropped on the surface of the sand mixture following the choice response, to confirm that subjects were not solving the task by directly detecting buried rewards. After reaching the

criterion, second set of training was started. Single test trial was presented to the subject on 28 days after reaching the criterion.

Results

Figure 14 shows time latencies to enter the darker chamber in fear conditioning experiment in the habituation phase and in the test phase. The latencies in the habituation period was not different between two groups, suggesting that preference of the dark place was not altered by expression of active-Fyn transgene. After the conditioning, the latencies increased about 6-hold in both groups. Two-way ANOVA with repeated measure as conditioning phase revealed that there was no effect of subject groups ($F_{1,22}<0.01$, $p=0.98$), significant effect of conditioning phase ($F_{1,22}=22.8$, $p<0.0001$), and no signigicant effect of interaction between subject groups and conditioning phase ($F_{1,22}=0.017$, $p=0.090$), indicating that both groups of mice were conditioned to avoid the darker chamber successfully and that the extent of the conditioning was the same.

Upper panel of Figure 15 shows the learning curves of wild-type control mice and active-Fyn transgenic mice in the Morris watermaze. Escape latency was gradually decreased in both groups, and learning curves in two groups were not different. Lower panel of the Figure 15 shows the time spent in each quadrant of the pool in the transfer test. Mice's swims were spatially biased to the target quadrant in both groups. These results suggest that fyn-transgenic mice have the same level of the spatial learning ability as in wild-type control mice.

Left panel of Figure 16 shows the number of trials required for task acquisition. Numbers of trials required for the task acquisition were not different between wild-type control mice and active-Fyn transgenic mice. Right panel shows the correct rate in percentage at the test trial. Correct rate in the retention test was not different in two groups.

Discussion

The NMDA receptor activity and LTP in the amygdala are likely to underly fear conditioning; the lateral and basolateral amygdaloid nuclei contain high densities of NMDA receptors (14), infusion of NMDA receptor antagonist into the amygdala blocks acquisition in fear conditioning (29), and fear conditioning induces LTP in the amygdala (27, 40). Therefore, I expected that some kind of alteration existed in the ability to learn fearful condition in fyn-transgenic mice, and examined accordingly.

Passive avoidance procedures are widely used to measure cognitive alterations follwing drug administration, lesions and behavioral manipulations. Several authors have discussed passive avoidance: the logic is that animals will 'remember' that a certain response terminated in an unpleasnat event, and will therefore hesitate to repeat it in the future. The consequent increase in response latency is thought to reflect the strength of the memory trace for the aversive event. Despite its popularity, the passive avoidance procedure should be used carefully if the subjects were collected from different groups such as two genetically distinct pool as in this experiment. Since animals are collected from different groups, preference to the dark place or the sensitivity to the electronic shock might be different. In this research, two groups of mice seemed to have the same level of light-dark preference, since the time latencies of the first entry into the darker box in the habituation phase were not different. It is not clear whether two groups had the same level of the sensitivity to the shock. The number of mice vocalized after the shock seemed similar. This objective impression is not suffice to rule out entirely the possibility that fyn-transgenic mice have different sensitivity to the shock. Therefore, it worths examining the learning and memory ability in less stresfull task.

Since then the watermaze was developed by Morris, the procedure has been used widely to evaluate the spatial learning ability in rats and mice.

The ablation of the hippocampus results in the deterioration in the learning (33) but does not influence on the visible version of the watermaze that does not require the spatial learning ability but require the normal level of the motivation and sensorimotor ability. The administration of the NMDA receptor blocker abrogated the learning ability (32). These results seemed to suggest that the NMDA receptor activity in the hippocampus was indispensable in the learning process. One should be noted that in experiments that involved a detailed behavioral analysis of watermaze learning in naive rats given NMDA receptor antagonists, performance of the required behaviors was impaired by the sensorimotor disturbances that result from NMDA antagonism, such as thigmotaxic and slowed swimming, or swimming over or deflecting off the platform (10). Furthermore, two reports showed that rats, if pretrained, could perform the task even in the presence of NMDA receptor blockade, and not having some type of LTP (3, 44). These results may suggest that NMDA receptor activity has a fundamental role in learning the strategy of the task, but not required in the spatial learning. Alternatively, some kinds of LTP-like potentiation of the synaptic transmission indeed underlie the spatial learning but not have been observed yet.

There was no detectable difference both in learning curve and in the performance in the transfer test. Considering that fyn-transgenic mice are sensitive to new environment and that watermaze task inflicts much stress to the subjects, the result presented in this paper may not reflect the actual spatial learning ability. Therefore, it might worth examining the ability possessed by the hippocampus by another task, less stressful than watermaze.

A behavioral task relating olfaction, a primary sense of rodents, enables us to examine the learning and memory abilities in the least stressful condition. it is a natural habit for them to associate a specific odor

with reward. The experimental procedure adopted by Bunsey and Eichenbaum (6) is one such a task.

Contrary to the prediction, three types of learning and memory tests revealed no differences in the performance between wild-type control mice and fyn-transgenic mice. This can be explained in, at least, four ways. One is that the behavioral tasks, which are believed to be NMDA receptor dependent, are not really dependent on NMDA receptor functions. The second interpretation is that the tyrosine phosphorylation of the NMDA receptors is not the only way of modulating the NMDA receptor functions. In other words, the tyrosine phosphorylation results in the modification of the receptor in some occation but not in other occasion. The third possibility is that the tyrosine phosphorylation of the NMDA receptors is effective in modulating the NMDA receptors, but not influential in a short period, for example a few days, as used in this paper. This means that the the sustained phosphorylation of the NMDA receptor induced epileptogenesis in a long time span, while the same tyrosine phosphorylation was not influential on the learning and memory processes since the time window required for consolidating learning traces was too short. This possibility can be tested by using other learning and memory tasks which require long period, for example 4 weeks, for training. The last, but not least, possibility is that the tyrosine phosphorylation of the NMDA receptors is indeed involved in the learning and memory processes, however, hyper-phosphorylation of the receptor does not bring beneficial effects more than necessary. In any case, further research focusing on the relation between the NMDA receptor functions and the phenotypes of fyn-transgenic mice will clarify not only the functions of Fyn, but also the mechanism how the tyrosine phosphorylation relates to the functions of the NMDA receptor.

General discussion

I have described the generation, biochemical substrates of Fyn tyrosine kinase, epileptogenesis, akinesia and learning and memory ability in fyn-transgenic mice. I had three main purpose; searching biochemical substrates of Fyn tyrosine kinase, behavioral effect of Fyn expression, and the relation of these two.

The first purpose, searching the substrates, was partially succesful. The NR2B subunit was shown to be tyrosine phosphorylated in vitro (47). In their experiment, purified Fyn proteins were added to postsynaptic density fraction and the resulting phosphotyrosine proteins were detected. One of the proteins enhanced in tyrosine phosphorylation was NR2B, indicating that the NR2B could be tyrosine phosphorylated by Fyn. It is not clear, however, whether the NR2B subunit is the real substrate in the brain. I have shown in this report that the tyrosine phosphorylation of NR2B is less in fyn-deficient mice and more in fyn-transgenic mice than in wild-type control mice. Although there remains the possibility that the NR2B is tyrosine phosphorylated by tyrosine kinases other than Fyn, it is reasonable to speculate that the weak and strong tyrosin phosphorylation of NR2B are caused by lackness of Fyn protein in fyn-deficient mice and overexpression of Fyn in fyn-transgenic mice, respectively. By combining the results from and from this paper, I consider the NR2B is the substrate of Fyn in the mice's forebrains.

The second purpose was to search the effect of Fyn transgene expression on the behavior. The start point for this purpose was clearly visible since spontaneous seizures were frequently observed. The frequency of the spontaneous seizures was not high enough to make me to monitor all mice continuously to measure the occurence of spontaneous seizures, but high enough to lead me explore further about epileptogenesis of fyn-transgenic mice. Considering the frequency of the spontaneous seizures, it is

the only to way to induce seizure experimentally in analyzing the seizure characteristics.

After the detailed analysis, I worked for the third purpose, investigation of the relationship of tyrosine phosphorylation of the substrate, NR2B, and the behavioral change, epileptogenesis. Among the seizure characterisitcs revealed by seizure inducing experiments, the development of the clonic seizure was selected fot this purpose by the following reason; (1) generation of the spontaneous seizures should be inhibited by continuous injection of antagonist, which is inpractical, (2) the first response is compared only once and cannot be compared within each animal. The strategy adopted here enables to compare the effect of the drug in animals having the same response intensity eithout the drug.

Further, other behavioral phenotypes were sought. Open-filed observation revealed that fyn-transgenic mice have tendencies of increased fearfulness and decreased locomotor activity. Since staying in the open-filed is a stresfull situation for the subjects, it was necessary to measure the locomotor activity in less stresfull environment than in the open-field. Thus, the spontaneous locomotor activity was measured in each subject's home cage. While the count of the spontaneous locomotion of fyn-transgenic mice was significantly lower than that of wild-type control mice, circadian rhythm of the locomotor activity was not different from that of control mice. Furthermore, general behavior was not different between two groups. As I did in the analysis of epileptic characteristics, I injected MK-801 and analyzed the relation between NMDA receptor activity and the akinesia. The administration of NMDA receptor antagonist MK-801 dose-dependently recovered the hypokinesia in fyn transgenic mice, showing that the locomotor-enhancing effect of the drug was stronger in fyn-transgenic mice than in wild-type control mice. These results suggest the involvement

of the tyrosine phosphorylation of NMDA receptors in the gain control of total locomotor activity, but not in the initiation of specific behavior.

Since the activated NMDA receptors, at least in part, mediated both epileptogenesis and hypokinesia in fyn-transgenic mice, I inferred close relation between Fyn tyrosine kinase and NMDA receptors, and thus speculated fyn-transgenic mice possessed other phenotypes, which were mediated through the NMDA receptor functions. I, therefore, examined learning and memory abilities thought to depend on the NMDA receptor activity. I aimed at examining the learning and memory ability in the least stresful condition. The procedure adopted by Bunsey and Eichenbaum offers a good exapmle (6). They showed that the hippocampus was the place for inference ability in rats. While paired-association learning was not impaired by hippocampal lesion, an ability of inference from already-learned material was impaired by the hippocampal lesion. I applied their experimental procedure for fyn-transgenic mice and wild-type control mice. I did not succeed in paired association learning. At first, I assigned paired association in random order. The subjects did not learn. Later, I assigned one pair consecutively, and after the learing has reached, the other pair was presented. When the pair flipped, the subject selected the previously rewarded choice. Eventualy, they learn correct choice. I felt that mice paid attention to the choice odor but not to the 'key' odor. Since I did not examine consecutive trials, I can not entirely conclude in this way, however.

Conclusion

In this paper, I have described the effects of fyn-transgene expression. Biochemically, tyrosine phospshorylation of at least five proteins were enhanced. One of which was NMDA receptor modulatory subunit 2B. Behaviorally, two differences were found. Both epileptogenesis and akinesia were, at least partly, mediated through NMDA receptor activation. This implicates strong relation of Fyn tyrosine kinase and NMDA receptor activity. In contrast, the learning and memory ability was not different, suggsting that the tyrosine phosphorylation does not always enhance the functions of NMDA receptor. I have obtained two conclusion. First, the functions of Fyn is partly mediated through the tyrosine phosphorylation of NMDA receptors. Second, the tyrosine phosphorylation of the NMDA receptors does not always result in the enhancement of the receptor activity.

Acknowledgements

The author thanks Drs. Kunihiko Obata, Kei Maruyama, Nobuhiko Kojima and Yoko Yamagata for invaluable advice for planning of experiments and analyzing experimental results, Drs. Tadashi Isa, Kazuhiro Ikenaka, Shunichi Yamagishi and Yoshio Sakurai for critical review of the maniscript. The author also thanks Tuyoshi Ozaki for technical advice for EEG recording and Hiroko Kuzume for doing in situ hybridization experiment.

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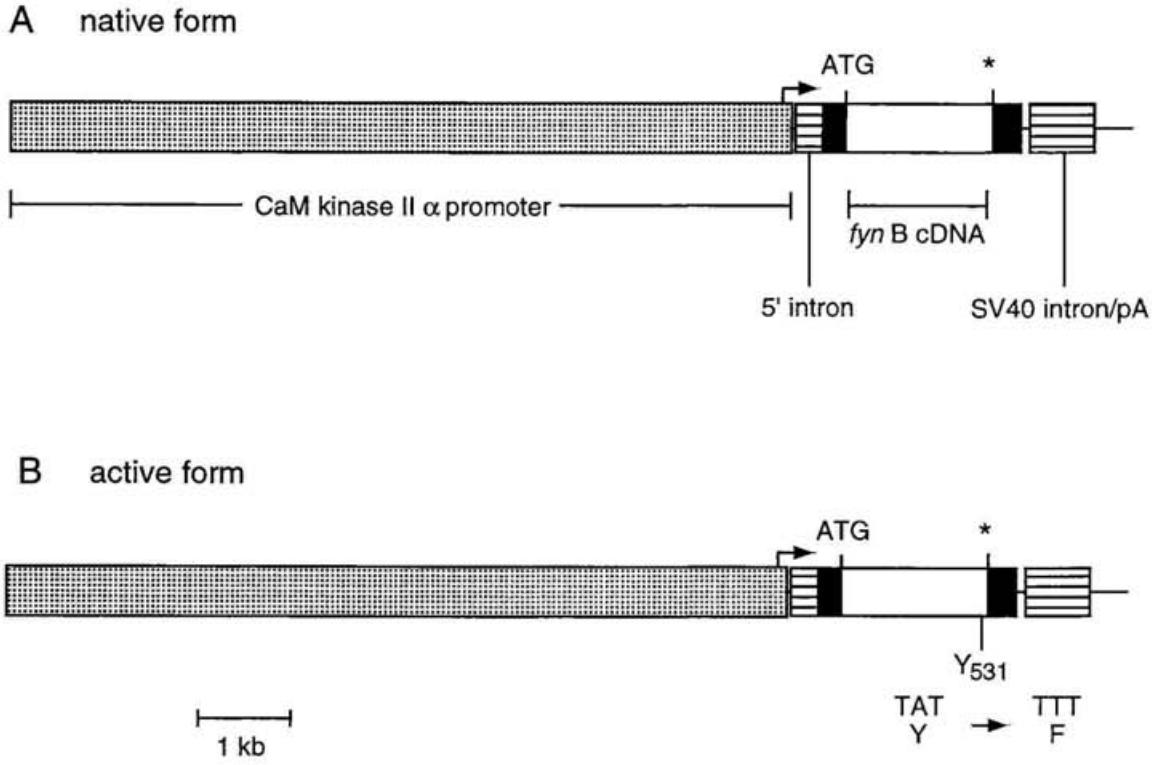

Figure 1. Constructs for fyn-transgenes. Coding region (white region) of brain-type fyn cDNA (fynB cDNA), either as a native form (A) or a mutant form (B), was connected to the promoter for calcium-calmodulin kinase II α. In the active form of the construct, tyrosine residue of codon 531 was replaced by phenylalanine.

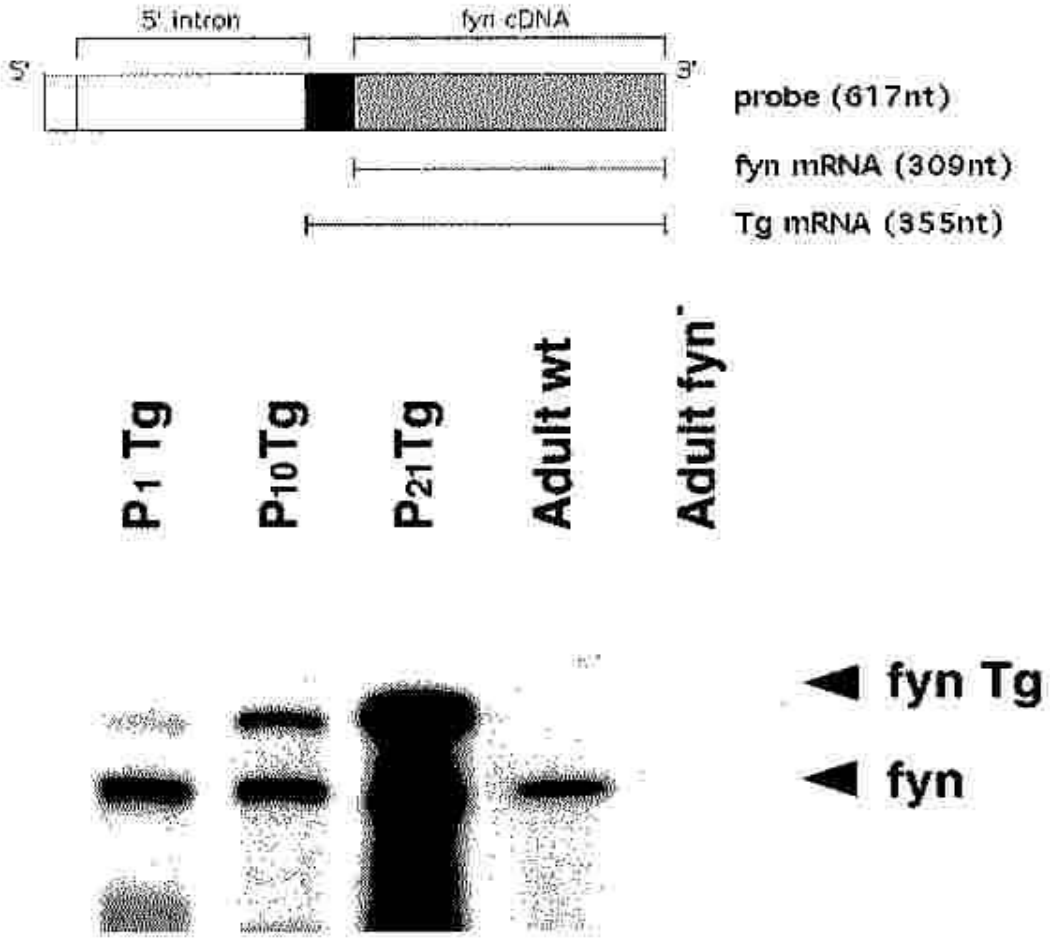

Figure 2. Temporal expression pattern of fyn-transgenes in the forebrains. Trasngenic fyn mRNA (upper band) and intrinsic fyn mRNA (lower band) were detected with ribonuclease protection assay. Samples of total RNA were prepared from transgenic mice of postnatal day 1, 10 and 21, adult wild-type mice, and adult fyn-knockout mice.

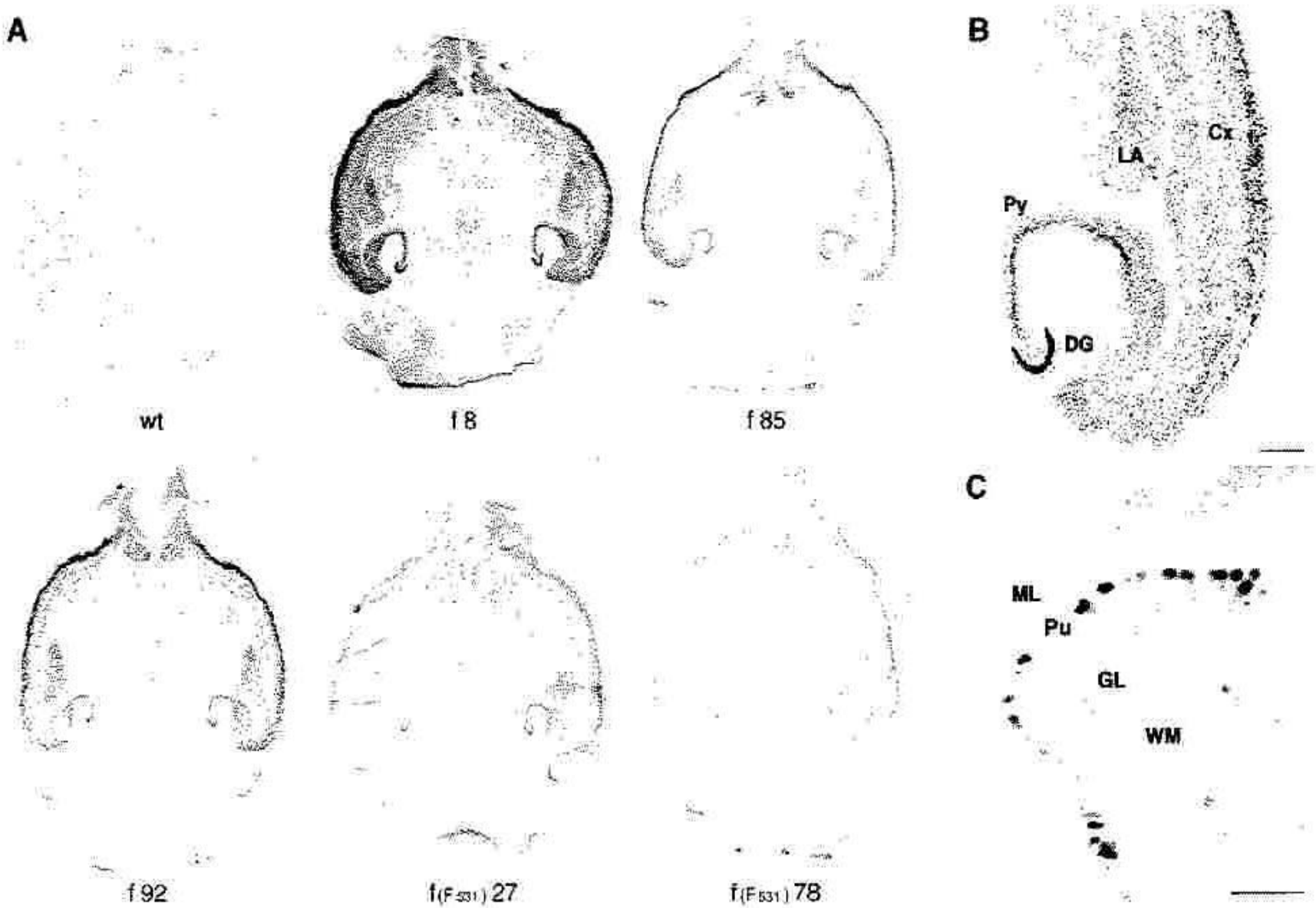
A
wt
f 8
f 85
f 92
f(F531) 27
f(F531) 78
B
Cx
LA
Py
DG
C
ML
Pu
GL
WM

Figure 3 (previous page). Spatial expression pattern of fyn-transgenes in the brain. Fyn-transgene was detected by in situ hybridization of horizontal brain sections with a digoxigenin-labeled transgene-specific cRNA probe. Abbreviation: Cx, neocortex; DG, dentate granular layer; Py, hippocampal pyramidal layer; LA, lateral amygdala nucleus. Lines f8, f85 and f92 are transgenic mice having native form of Fyn, and lines f(F_{531})27 and f(F_{531})78 are transgenic mice carrying active-form of Fyn.

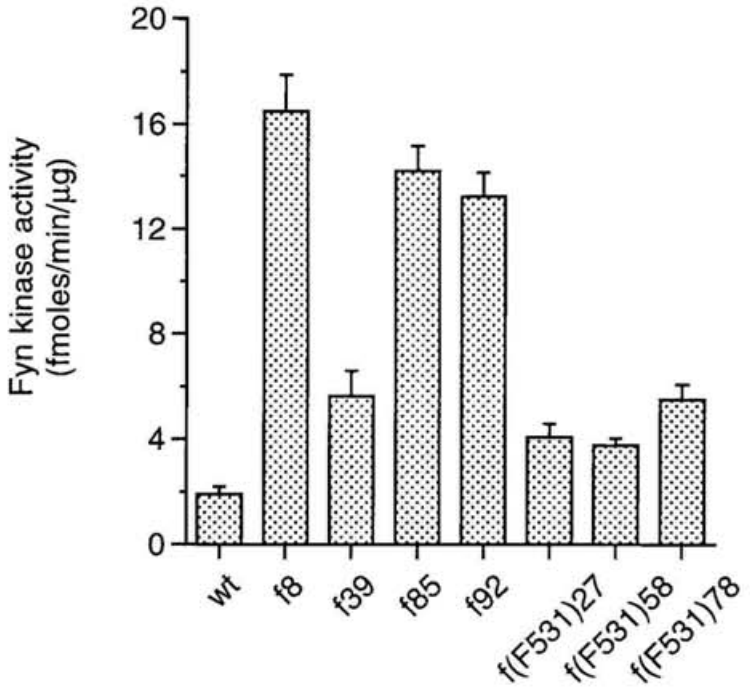

Figure 4. In vitro Fyn kinase activity. Fyn kinase activity was measured from the brain extracts by in vitro immune complex kinase assay using anti-Fyn antibody. Lines f8 through f92 are transgenic mice having native form of Fyn, and lines f(F_{531})27 through f(F_{531})78 are transgenic mice containing active-form of Fyn. Each bar represents the mean ± SEM.

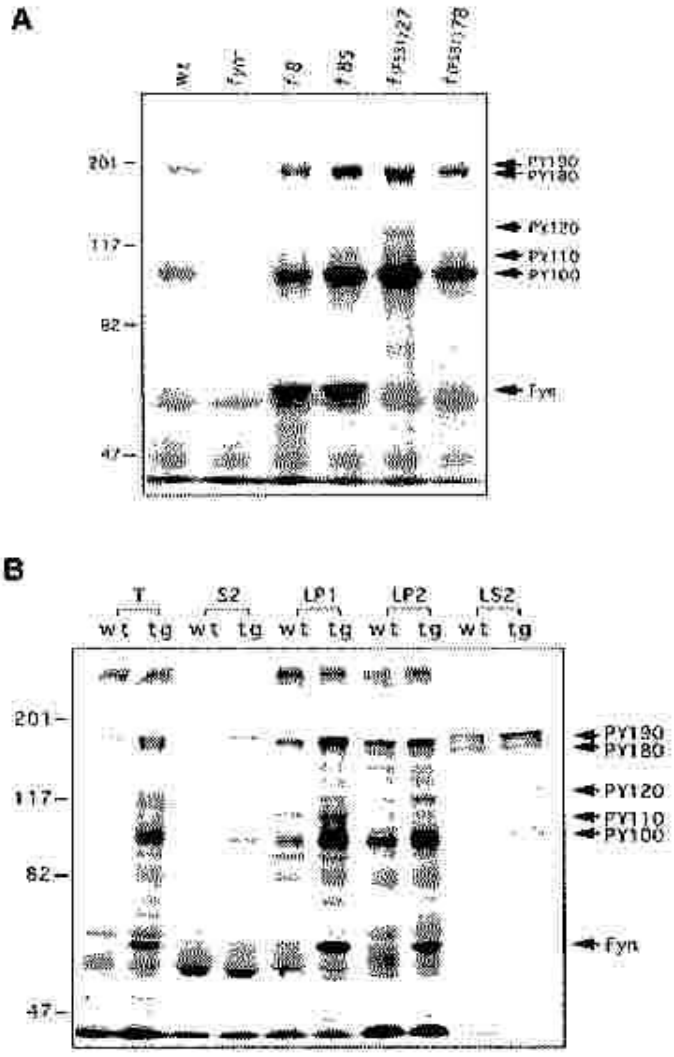

Figure 5. Immunoblot detection of phoshphotyrosine proteins. A: The forebrain extracts were separated on 7.5% SDS-PAGE and blotted with anti-phosphotyrosine antibody. Arrows indicate proteins that are hyper-phosphorylated in fyn-transgenic mice. B; Subcellular distribution of phoshphotyrosine proteins. Samples were prepared from wild-type (wt) and line f8 (tg). T, total homogenate; S2, cytosolic fraction; LP1, crude plasma membrane fraction; LP2, crude synaptic vesicle fraction; LS2, synaptosomal cytosolic fraction. Molecular weight markers (in kilodaltons) are indicated on the left.

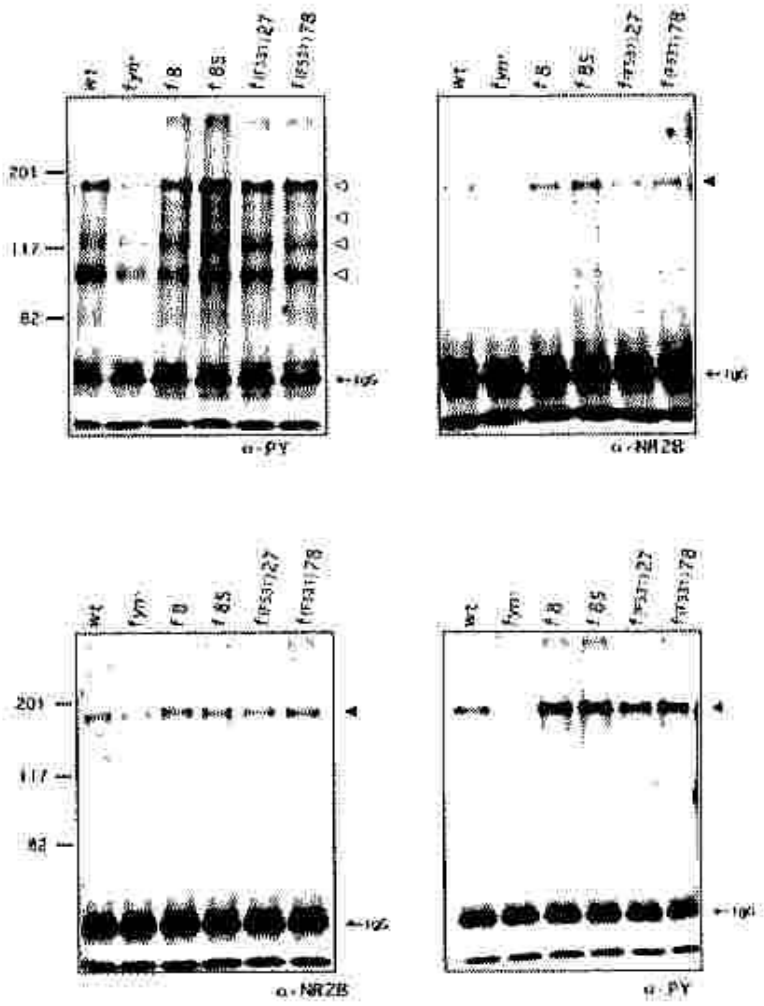

Figure 6. Tyrosine phosphorylation of NMDA receptor subunit 2B (NR2B). Upper panels: Immunoprecipitated phosphotyrosine (PY) proteins were blotted with either anti-PY antibody (PT-66, left panel) or anti-NR2B antibody (right panel). Open triangles indicate PY-containing proteins that are hyper-phosphorylated in fyn-transgenic mice. Closed triangle indicate NR2B. Lower panels: Immunoprecipitated NR2B was blotted with either anti-NR2B antibody (left panel) or anti-PY antibody (right panel). NR2B is indicated by closed triangles. Molecular weight markers (in kilodaltons) are indicated on the left.

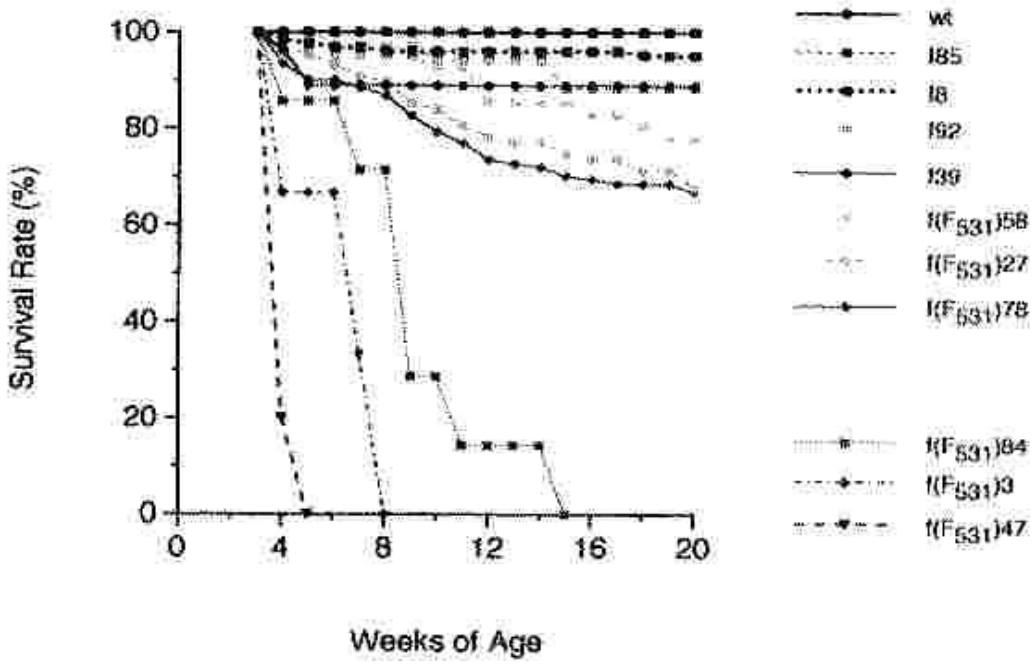

Figure 7. Survival rate of fyn-transgenic mice. The survival rate of three-week old transgenic offspring was surveyed up to twenty weeks and represented as a percentage.

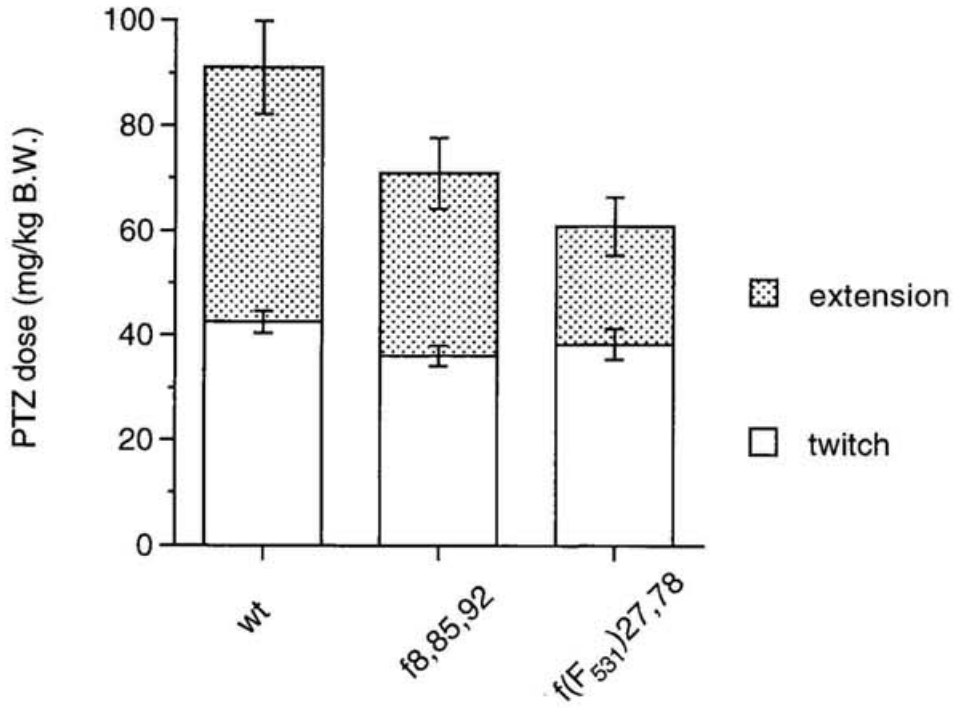

Figure 8. Pentylenetetrazol-induced seizure. Seizure progression induced by intravenous infusion of pentylenetetrazol (PTZ) was monitored. The time exhibiting first twitch and tonic extension were corrected for body weight and converted to the corresponding PTZ dose. Each bar represents mean ± SEM from wild-type control (n=11), native-Fyn transgenic mice, which are the total of f8 (n=10), f85 (n=6) and f92 (n=5), and active mutant mice, which are the total of f(F_{531})27 (n=8) and f(F_{531})78 (n=4).

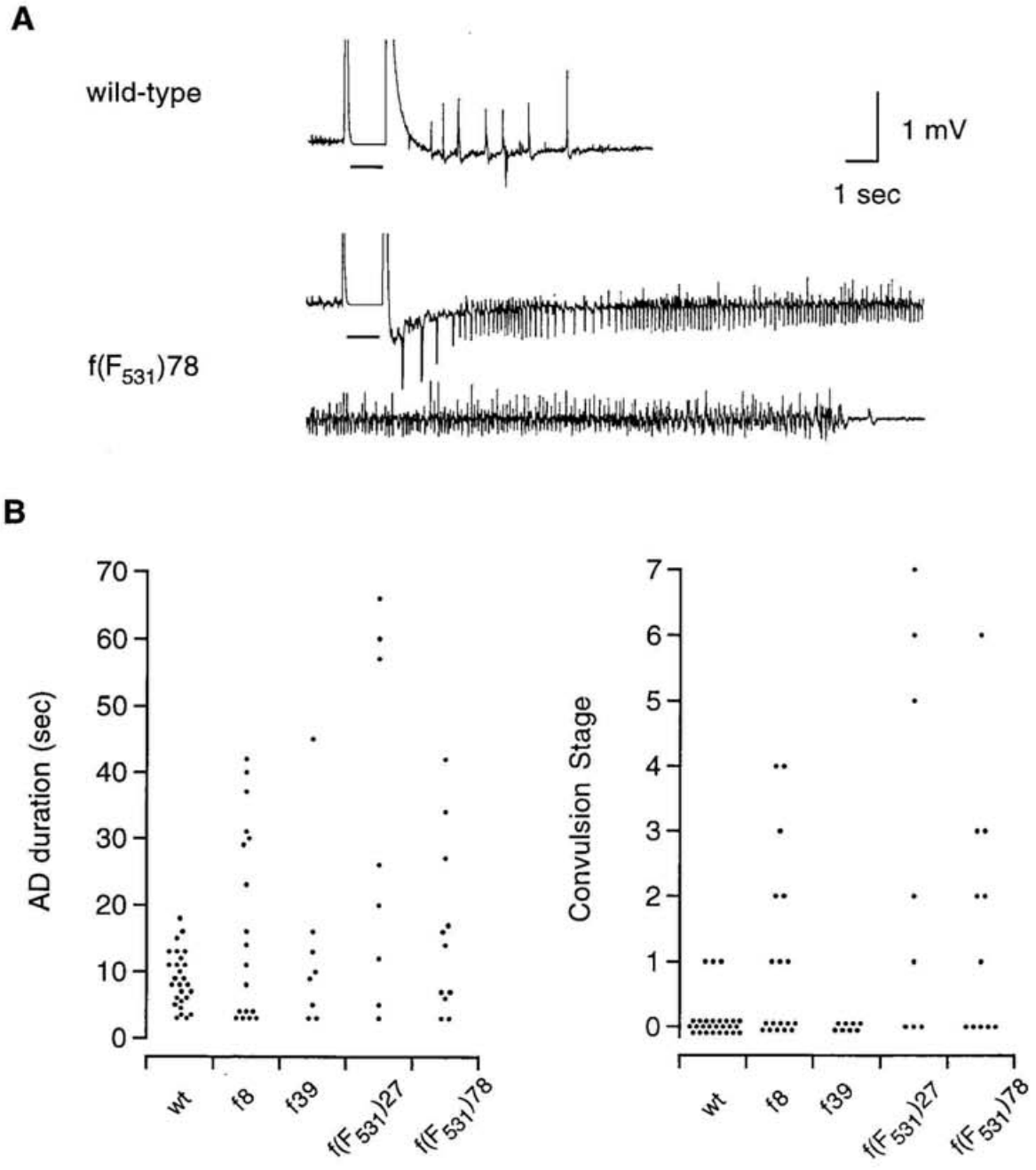

Figure 9. Records of the first kindling responses. A: Electrographic responses from a wild-type control mouse (top) and a mouse expressing active-Fyn transgene (bottom). Bars indicate periods of the electrical stimulation. The mouse in the top example did not show any observable behavioral seizure, while the one in the bottom example exhibited bilateral forelimb clonus. B: Scatter plotts of afterdischarge (AD) durations (left) and convulsion stages (right). Convulsion responses were rated according to the modified Racine's classification (see Methods).

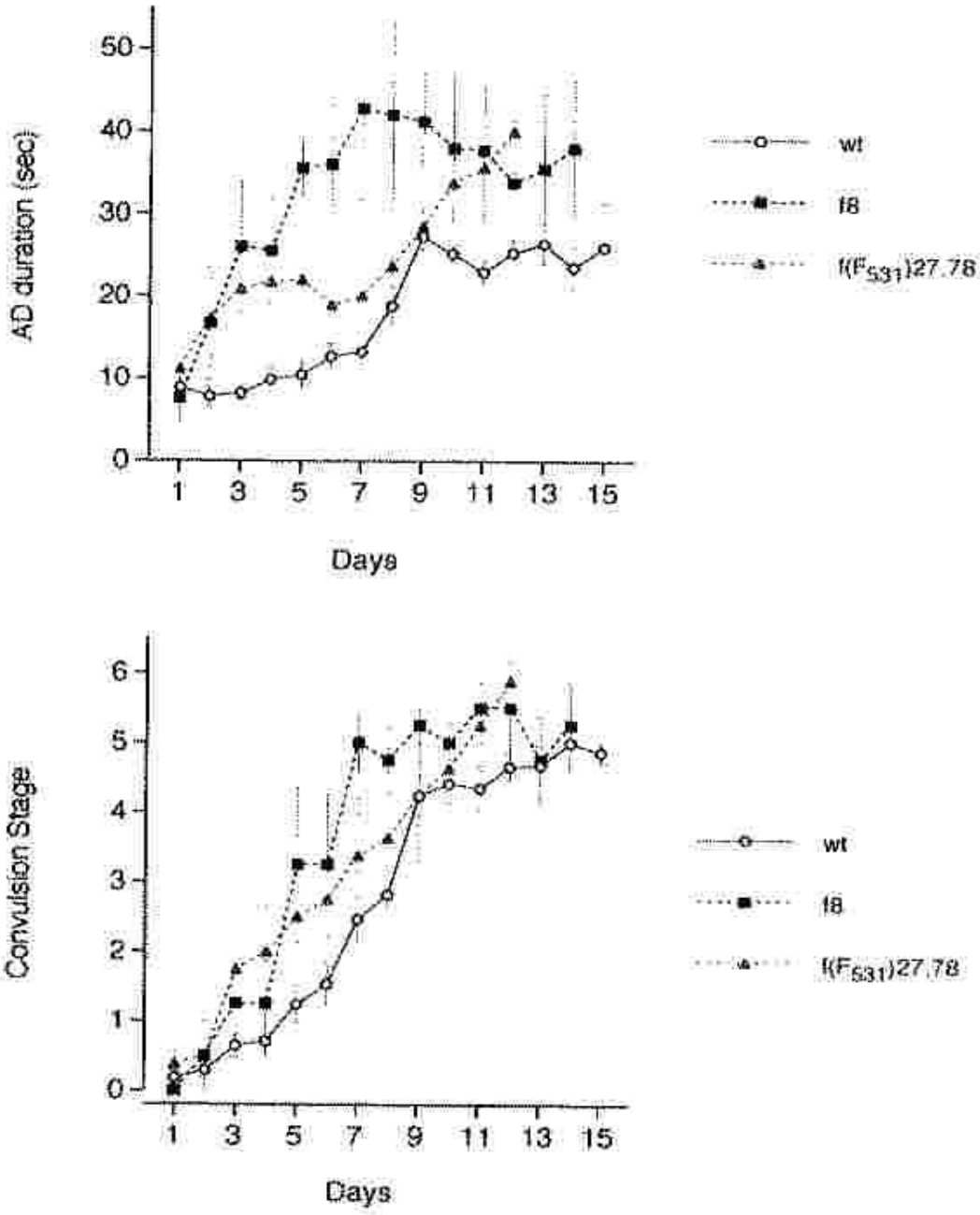

Figure 10. Rate of kindling development. The development of AD duration (top) and convulsion stage (bottom) by once-daily stimulation. Mice showed AD of longer than 20 seconds or convulsion of stage 2 or more at the first stimulation were excluded. Data were from wild-type mice (wt, n=17), native-Fyn transgenic mice (f8, n=4) and active-Fyn transgenic mice (f(F_{531})27,78, n=8). Each point represents mean ± SEM.

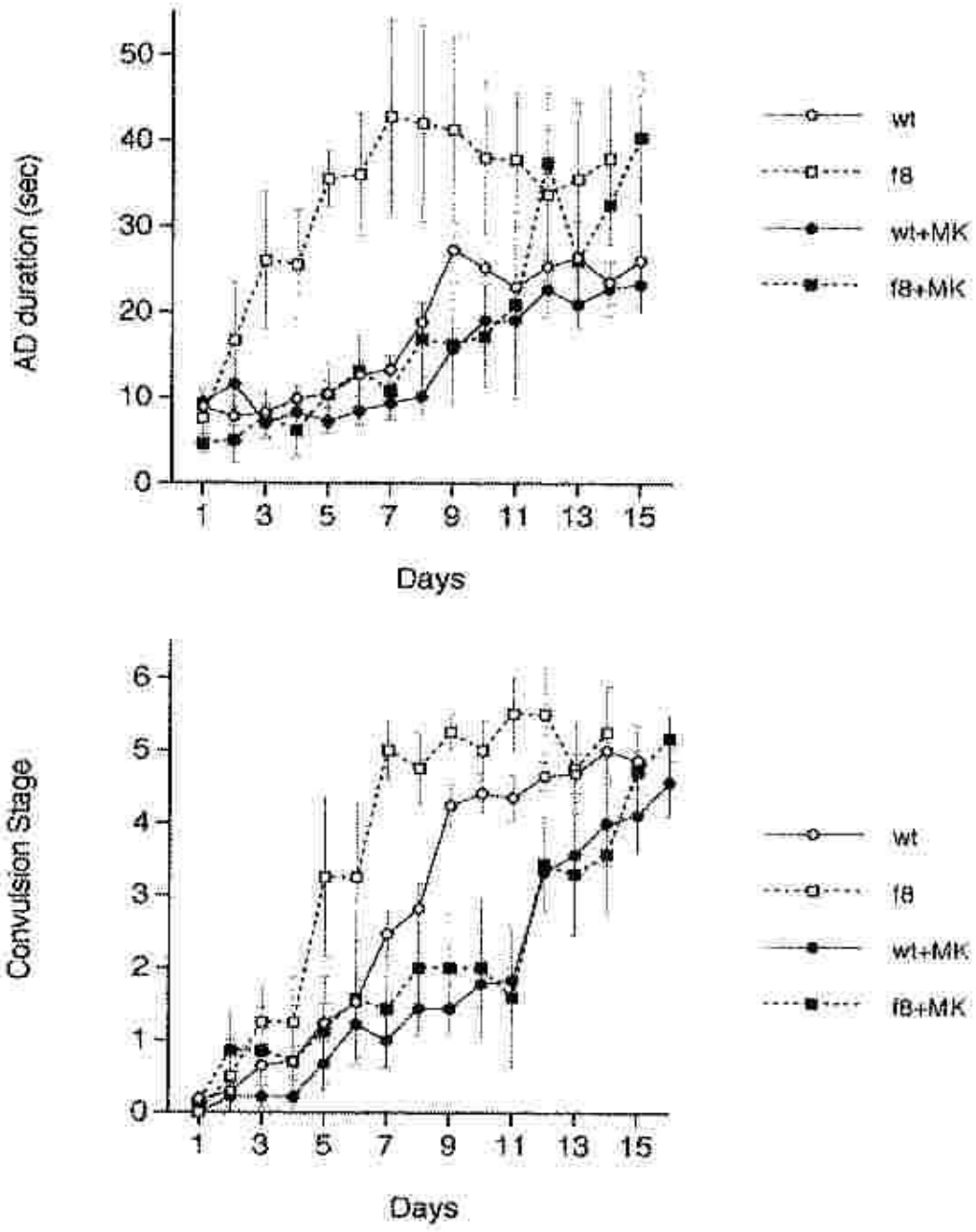

Figure 11. Effect of MK-801 on the rate of kindling development. Open circle (wild-type) and open square (f8) are the same results as in Figure. 10. Represented as filled circle (wild-type) and filled square (f8) are from two other groups of mice being administered MK-801 prior to the stimulation during the period indicated by bar. Each value represents mean ± SEM. Top: AD duration. Bottom: convulsion stage.

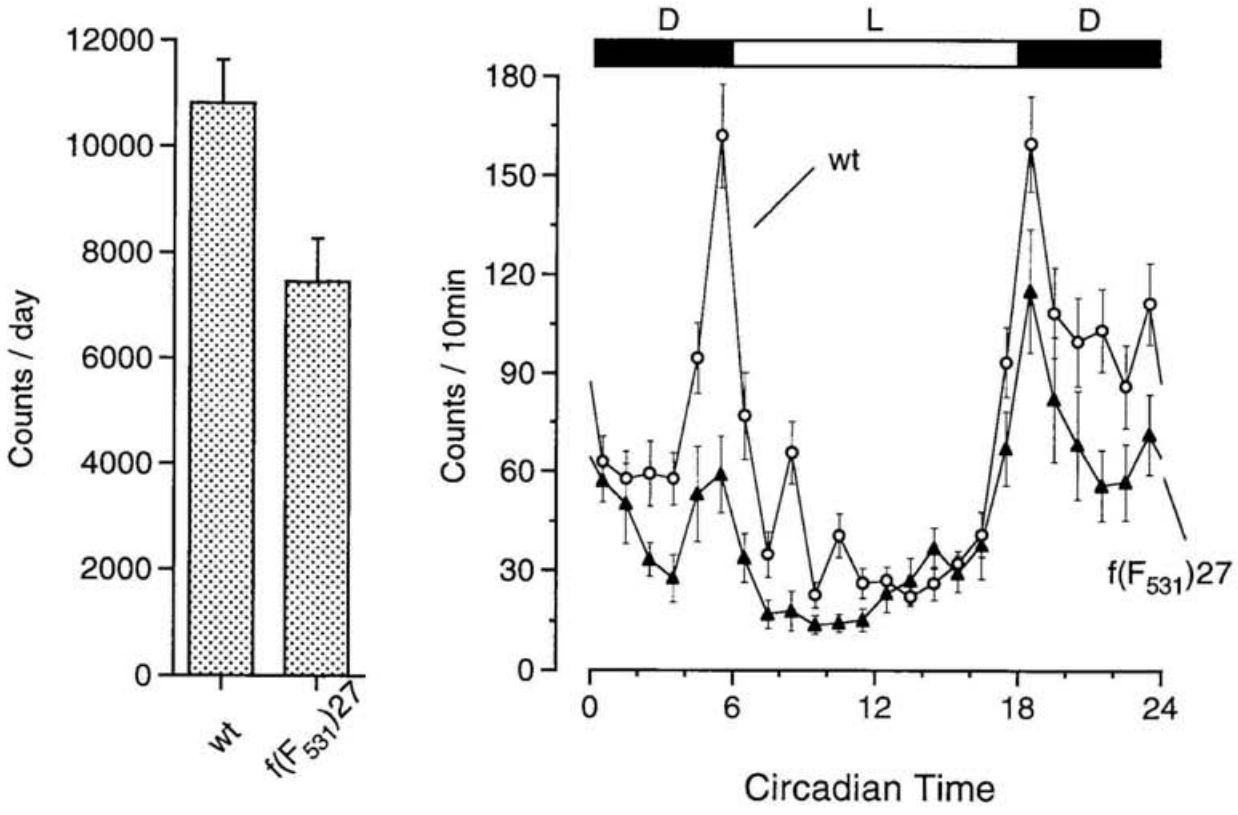

Figure 12. Spontaneous locomotor activity. Left: Counts of locomotor activity in each time zone. Room ilumination was lit on during circadian time 06:00 and 18:00 and represented as 'L' in the bar above the data. Right: Total counts of locomotor activity in 24 hours.

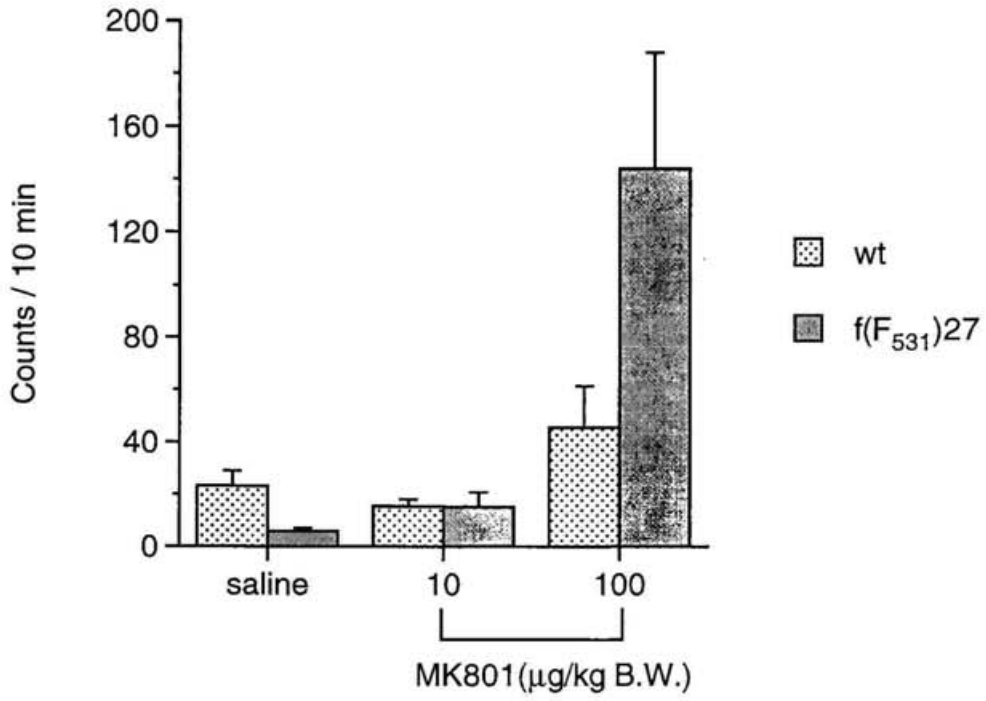

Figure 13. Effect of NMDA receptor antagonist MK801 on locomotor activity. Locomotor activity was measured for 10 minutes beginning at 30 minutes after intraperitoneal injection of saline, 10 or 100 μg/kg B.W. of MK801.

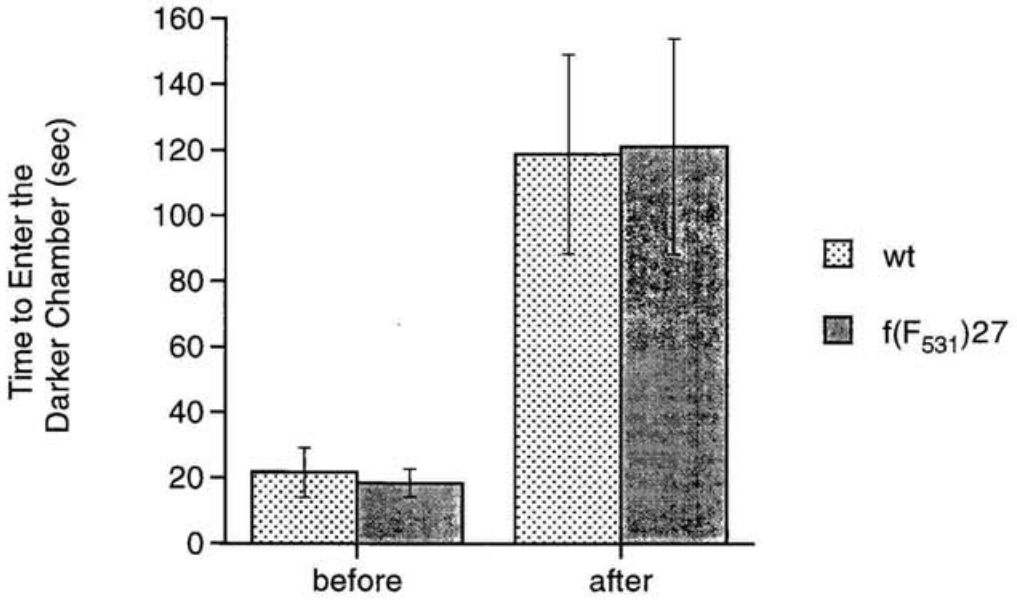

Figure 14. Fear conditioning. Each bar represents mean time ± SEM to enter the darker chamber in the habituation phase (before) and test phase (after).

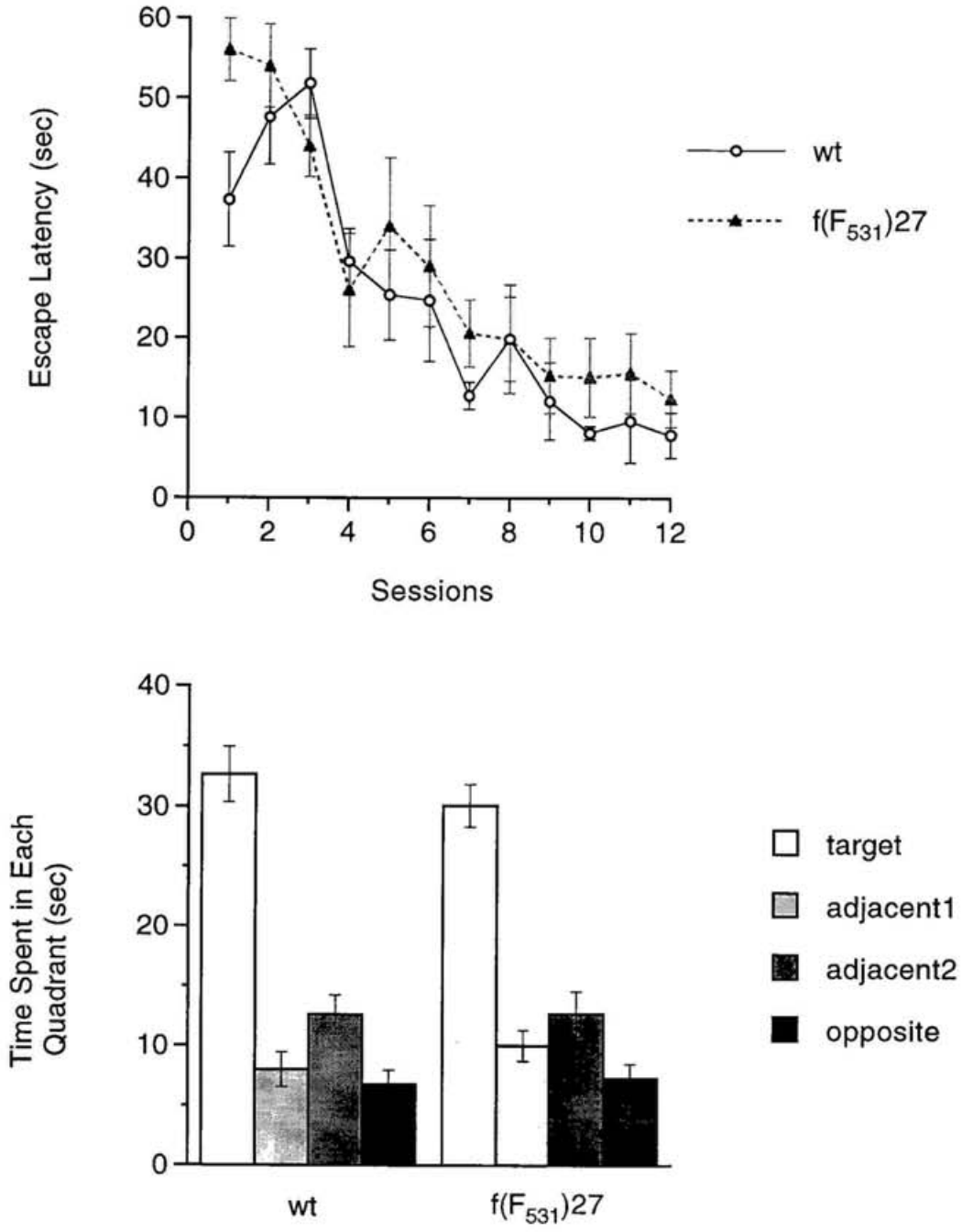

Figure 15. Spatial learning ability. Top: Mean escape latency from the water in the Morris watermaze. Each session consists of two trials. Bottom: Mean time spent in each quadrant in the transfer test. Target quadrant designates the space where the platform lied in the training phase.

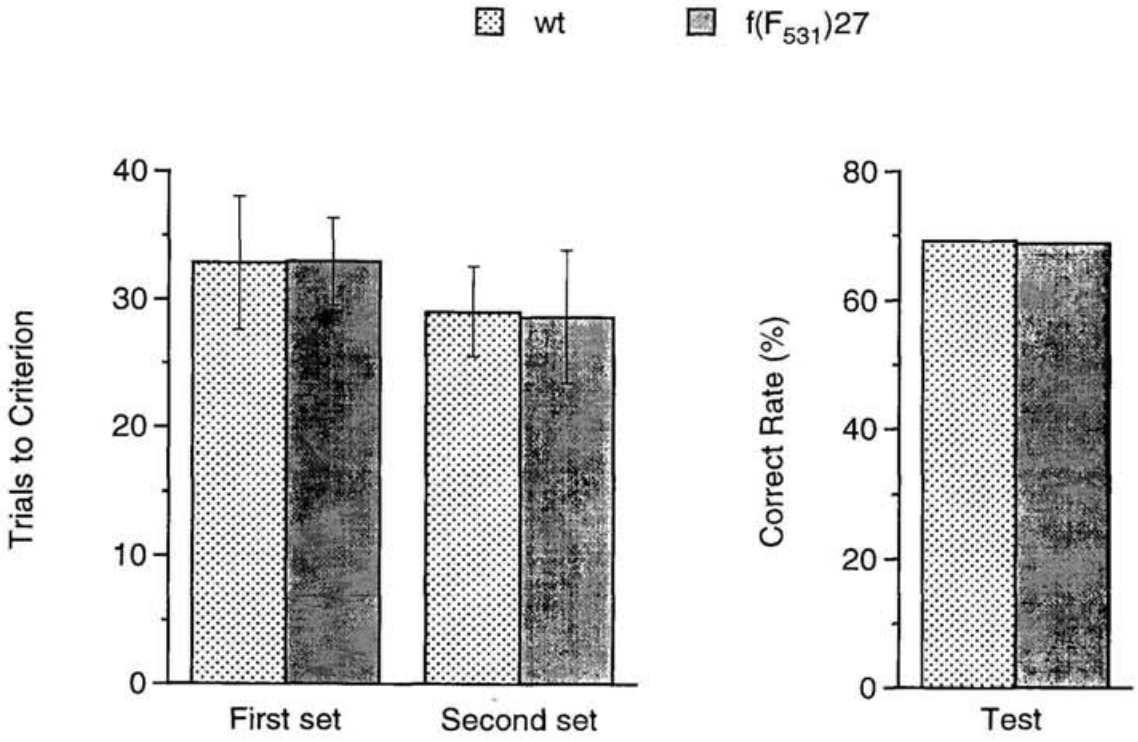

Figure 16. Odor discrimination learning. Left: Number of trials to reach the criterion. Second set of training was started after the completion of the first set. Each value represents mean ± SEM. Right: Correct rate in the retention test, which was given on 28 days after the completion of the training.