Identification of a Novel Fyn-associated Protein That is Concentrated in the Postsynaptic Density

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Contents

Abbreviations

BCIP : 5-bromo-4-chloro-indolyl phosphate

BPB : bromophenol blue

BSA : bovine serum albumin

CAM : cell adhesion molecule

CaMK-II : calmodulin kinase II

CBB : Coomassie brilliant blue

F1 : filial 1

GST : glutathione-S transferase

IPTG : isopropyl-β-D-thiogalactopyranoside

LTP : long-term potentiation

MAG : myelin associated glycoprotein

NBT : nitro-blue tetrazolium

NFS : N-terminal Fyn-specific region

NMDA : N-methyl-D-aspartate

pABSF : 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PSD : postsynaptic density

SDS : sodium dodecyl sulfate

SH2 : src homology 2

SH3 : src homology 3

TBS : Tris (hydroxymethyl) aminomethane buffered saline

Introduction

Recent advances in molecular and genetic studies of behaviors support a notion that most parts of animal behaviors, even in mammal, can be investigated at a molecular level. Genetical approaches have demonstrated that a single molecule can play a critical role in determining a certain type of behavior. Trials of mutagenesis of *Drosophila melanogaster* have produced many behavioral mutants, such as *period* which is defective in circadian rhythm (Zehring et al., 1984; Reddy et al., 1984; Kyriacou et al., 1990; Wheeler et al., 1991), *couch potato* (Bellen et al., 1992 a, b) which is defective in courtship and mating, and *dunce* and *rutabaga* (Siegel et al., 1984; Davis and Dauwalder, 1991) which are defective in olfactory learning. Genes responsible for these defects have been identified; they encode, respectively, a novel nuclear protein (*period*), a homologue of RNA-binding protein (*couch potato*), a cAMP-dependent phosphodiesterase (*dunce*) and a Ca^{2+}-dependent adenylyl cyclase (*rutabaga*). Of mouse, spontaneous mutants with abnormal behaviors have been isolated and some of the underlying chromosomal loci have been identified. Recent studies have revealed that *reeler* (*rl*) (D'Arcangelo et al., 1995) encodes a novel extracellular matrix protein, while *shaker-1* (*sh-1*) encodes myosin VII (Gibson et al., 1995). Another powerful approach using mouse genetics is the quantitative trait locus (QTL) analysis (Plomin and McClearn, 1993) (Crabbe et al., 1994), which takes advantage of recombinant inbred (RI) mouse strains; RI strains are the fully inbred descendants of an F2 intercross between two inbred strains, for example, BXD RI strains derived from C57BL/6 and DBA/2 strains. By examining the pattern of strain difference in the set of RI strains and referring the results to a database comprising the genetic map location of many marker loci, it is possible from the behavioral phenotype to infer the provisional chromosomal localization of the

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underlying genes without the need for new genotyping. The DBA/2 strain is well known for their behavioral abnormalities and has been extensively studied, especially in drug sensitivity, audiogenic seizures, and learning defect, particularly in comparison with behaviors of the C57BL/6 strain (Blum et al., 1983; Upchurch and Wehner, 1989; Neumann and Collins, 1991; Paylor et al., 1994). The QTL analysis of the BXD RI strains have revealed multiple genes underlying drug sensitivity and audiogenic seizures observed in the DBA/2 strain. The genetical approaches have identified and confirmed several tens of genes controlling behaviors up to now.

Recently the gene-targeting technique in mouse has allowed us to powerfully approach mammalian behaviors at a molecular level. It has been demonstrated that knockout of a single gene can cause behavioral defects. For example, defects in learning with impairment of synaptic formation and plasticity are produced by disruption of a gene encoding each of the following proteins; α-calmodulin kinase II (α-CaMK II), protein kinase Cγ (PKCγ), N-Methyl-D-aspartate receptor (NMDA-R) $\varepsilon 1$ and $\delta 2$ subunits, cyclic AMP responsible element binding protein (CREB), and Fyn (Silva et al., 1992; Chen et al., 1994; Abeliovich et al., 1993; Kashiwabuchi et al., 1992; Sakimura et al., 1995; Bourtchuladze et al., 1994; Grant et al., 1992; for review see Takahashi et al., 1994). The Fyn-deficient mouse strain shows several behavioral dysfunctions such as reduction in spatial learning in the Morris water maze (Grant et al., 1992), hyperresponsiveness to fear-inducing stimuli (Miyakawa et al., 1994), enhancement of audiogenic seizures (Miyakawa et al., 1995), and abnormality in suckling behavior (Yagi et al., 1993). Lack of Fyn also causes reduction in hippocampal long-term potentiation (LTP) (Grant et al., 1992) and malformation in hippocampal cell layers (Grant et al., 1992; Yagi et al., 1994). The behavioral defects of Fyn deficient mouse suggest that the Fyn play a critical role in determining behaviors.

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Fyn is a tyrosine kinase of non-receptor type of the Src family and is attached to the inner surface of the plasma membrane through a myristoyl anchor linked to the N-terminal glycine (Semba et al., 1986). It is assumed that Fyn transduces signals from outside to inside of the cell membrane and modulates functions of other proteins by tyrosine phosphorylation. Fyn has the N-terminal Fyn-specific region (NFS), which is not conserved among Src family kinases, and the Src homology (SH) regions, which consist of tyrosine kinase (SH1), SH2 and SH3 domains. The SH2 and SH3 domains are conserved in many signal-transducing and cytoskeletal proteins (Panchamoorthy et al., 1994). These domains are indispensable for specific protein-protein interactions and take part in cellular signal transductions and in protein localization (Pawson and Gish, 1992). Binding to the SH2 and SH3 domains of Fyn leads not only to phosphorylation of its substrates, such as focal adhesion kinase (FAK) and N-Methyl-D-aspartate (NMDA) receptors, but also to activation of enzymes, such as phospholipase Cγ, dynamin and 85 kDa-subunit of phosphatidylinositol 3-kinase (PI3-kinase) (Pleiman et al., 1993; Pleiman et al., 1994). The SH2 and SH3 domains, respectively, bind to phosphotyrosines and proline-rich sequences of the target molecules (Sadowski et al., 1986; Mayer et al., 1988; Cicchetti et al., 1992; Ren et al., 1993; Weng et al., 1994), whereas both domains are necessary for specific association with some target proteins, like large-myelin associated glycoprotein (L-MAG) and AFAP110. (Flynn et al., 1993; Umemori et al., 1994). The NFS region of Fyn has not been reported to bind any molecules, except the myristoyl anchor.

However, little is known what kind of molecules constitute the signaling pathway, which affect the determining behavior, upstream or downstream of Fyn. To address this question, I attempted to isolate Fyn-associated proteins expressed in mouse brain, which are assumed to participate in conveying signals. To isolate Fyn-associated proteins in the mouse brain, I used a recombinant Fyn protein as an

affinity ligand. The recombinant Fyn was designed to contain the NFS and the SH2 and SH3 domains. The kinase domain was excluded because this part is highly homologous among many tyrosine kinases. My initial plan was to isolate novel molecules that take part in the Fyn-signal transduction pathway and/or contribute to subcellular localization of Fyn. Here I have succeeded in identifying a novel Fyn-associated 130 kD-protein (named as p130), which was expressed only in the brain and highly concentrated in the postsynaptic density (PSD) fraction. Furthermore this p130 had a larger molecular weight in the behavioral-defect strain DBA/2 than that of C57BL/6.

Materials and Methods

Production and purification of GSTFynK⁻ fusion protein

Human Fyn cDNA, encoding amino acid residues 1-264 (Semba et al., 1986), was amplified by polymerase chain reaction (PCR) with *Pfu* thermostable DNA polymerase (Stratagene). The sense primer 5'-GGATCCATGGGCTGTGTGCAATGAA GGAT-3' and the antisense primer 5'-GATATCTCACGAGACATCTTTGGTTTTGAC-3' contained newly incorporated *Bam*HI and *Eco*RV sites, respectively (underlined). The *Bam*HI-*Eco*RV fragment of the amplified DNA was subcloned into pBluescript SK(+) vector (Stratagene) for sequencing. The *Bam*HI-*Eco*RV fragment of the verified phagemid was inserted between *Bam*HI and SmaI sites of the pGEX-2T vector (Pharmacia). The glutathione-S transferase-FynK⁻ fusion protein (GSTFynK⁻) was expressed in *E. coli* strain JM101. Cells were grown in 500 ml, and was incubated at OD_{600}=0.8 with 1 mM IPTG for 3 hr at 37 ˚C. Cells were pelleted and resuspended in 20 ml of *E. coli* lysis buffer (PBS containing 1 % TritonX-100, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, and 1 mM leupeptin). After sonicating 5 times for 30 s on ice, the lysate was cleared by centrifugation. The supernatant was incubated with 1 ml of 50 % glutathione-Sepharose 4B beads (Pharmacia) for 30 min at 4 ˚C. The beads were extensively washed 5 times with the lysis buffer, and the GSTFynK⁻ protein was eluted by glutathione elution buffer (5 mM glutathione (reduced form), 0.5 M NaCl, 10 mM Tris-HCl pH 8.0). The eluted sample was dialyzed against PBS and concentrated by ultrafiltration.

Preparation of Fyn-associated proteins

Brains of newborn (P0) ICR strain mice were homogenized with Downce homogenizer in 8 vol of BS buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 1 mM pABSF, 1

mM aprotinin, 1 mM pepstatin, and 1 mM leupeptin). The homogenate was centrifuged at 1,000g for 10 min at 4 °C. The supernatant was collected and centrifuged at 13,800g for 20 min at 4 °C. The pellet was rehomogenized and centrifuged at 13,800g for 20 min at 4 °C. The pellet (P2 fraction) was homogenized with Downce homogenizer in 5 vol of CHAPS lysis buffer (2 % CHAPS, 0.14 M NaCl, 20 mM KCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, and 1 mM leupeptin). The lysate was left on ice for 15 min and centrifuged at 28,000g for 20 min. To remove non-specific binding, the obtained supernatant was precleared with 100 μl of 50 % suspension of glutathione-Sepharose 4B beads for 1 h at 4 °C by gentle rotating. The precleared supernatant was incubated with glutathione-Sepharose 4B beads prebound to GSTFynK⁻ fusion protein for 2 h at 4 °C using a rotating shaker. The beads were centrifuged at 1,000g at 4 °C for 2 min and washed 5 times with 10 vol of the CHAPS lysis buffer. The associated proteins were eluted with 2xSDS sample buffer for SDS-PAGE analysis.

Production of monoclonal antibodies

Anti-Fyn monoclonal antibody was raised against the GSTFynK⁻ fusion protein. 8-week-old male Fischer rats were immunized with 100 μg of the GSTFynK⁻ fusion protein in footpads every two days 3 times. Next day of the last immunization, lymphocytes were collected from inguinal lymphnodes and fused to P3UI myeloma cells by polyethylene glycol method (Köhler et al., 1980). Fused cells were selected in HAT medium (RPMI-1640 containing 10 % FCS, 100 μM hypoxianthine, 100 μM aminopterine, 10 μM thymidine, and recombinant mouse interleukin-6) in 96-well plates for two weeks, and colonies of survived cells were placed into 24-well plates. Specificity of the antibodies was examined by comparing staining patterns of Western blotting of brain extracts of Fyn-deficient and wild-type mice. Thus monoclonal

antibody γC3 was obtained.

The affinity-purified GSTFynK⁻-binding proteins were separated by SDS-PAGE, and protein bands were visualized by CBB staining. The bands larger than GSTFynK⁻ were excised by razor blade, and proteins were electrophoretically eluted from the gel. This solution was used as the antigen for each injection. Monoclonal antibodies against Fyn-associated proteins were produced by immunization of 8-weeks-old female BALB/C mice intraperitoneally every 2 weeks for 3 times, and the fourth immunization was done by direct injection of antigen into spleen. Lymphocytes were collected from spleen on the next day of the final immunization. The myeloma cell line used and the procedures of cell fusion and selection were the same as described above. Screening of the antibodies was done by Western blotting of mouse brain extracts. Anti-Fyn polyclonal antibody was described previously (Yagi et al., 1994). Anti-NMDA receptor ε1 and ε2 antibodies were kindly provided by Dr. K. Sakimura, Niigata University.

Immunoprecipitation of Fyn and Fyn-associated proteins

Mouse brains were homogenized in 10 vol of RIPA buffer (0.15 M NaCl, 1 % TritonX-100, 0.2 % deoxycholic acid, 20 mM Tris-HCl pH 7.5, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin, and 1 mM Na_3VO_4) in Downce homogenizer and left on ice for 15 min, followed by centrifugation at 15,000g for 30 min at 4 °C. The supernatant was collected and incubated with 100 μl of 50 % suspension of protein G-Sepharose 4B beads for 2 h at 4 °C by gentle rotating to remove proteins bound to the beads nonspecifically. The supernatant was collected and incubated with anti-Fyn antibody bound to protein G-Sepharose 4B beads for overnight. The beads were centrifuged at 1,000g at 4 °C for 2 min and washed five times with 20 vol of RIPA buffer. The bound proteins were eluted by 50 μl of 2x

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SDS sample buffer for SDS-PAGE.

SDS-PAGE

Mouse tissues were homogenized in 5 vol of RIPA buffer (0.15 M NaCl, 20 mM Tris-HCl pH 7.5, and 1% Triton X100) or SDS sample buffer (3 % SDS, 20 mM Tris-HCl pH 6.8, and 10 % sucrose), and centrifuged at 15,000g for 30 min at 4 °C. The protein concentration of the supernatant was quantified by micro BCA assay (Pierce) using BSA as standard. 1/5 vol of 5x SDS sample buffer (50 % sucrose, 15 % SDS, 15 % 2-mercaptoethanol, 0.1 M Tris-HCl pH 6.8, and 1.5 % BPB) was added and 100 µg of each sample was boiled for 3 min, and applied to a SDS-PAGE. For studying subcellular distribution, 10 µg of protein was applied to each lane. SDS-polyacrylamide gel electrophoresis was performed using an 8 % gel for p130 and a 10 % gel for Fyn, in a discontinuous Tris-glycine buffer system (Laemmli, 1970). The blots were developed in AP buffer (10 mM ethanolamine pH 9.5, and 5 mM MgCl$_2$) containing NBT and BCIP. The size markers were obtained from Pharmacia and Bio Rad. Gels were stained with Coomassie brilliant blue (CBB) or silver by silver staining kit (Daiichi pure chemicals Co. LTD.)

Western blotting

Proteins were electrophoretically transferred from an SDS-polyacrylamide gel to a nitrocellulose filter by the method of Towbin et al. (Towbin et al., 1979). The nitrocellulose filter was treated with 10 % skim milk in TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.5) and incubated with antibodies for 1 hr at room temperature, followed by treatment with biotinylated sheep anti-mouse IgG or biotinylated sheep anti-rat IgG (Amersham) for 1 hr at room temperature. After the immunoreaction, the filter was washed with TBS for 10 min 3 times and incubated for 30 min with streptoavidine-

conjugated alkaline phosphatase (Amersham) in 10 % skim milk-TBS. The filter was washed in TBS for 15 min 3 times.

Subcellular fractionation of brain

P2 fractions (see above) of C57BL/6, DBA/2 and Fyn-deficient mice were rehomogenized with Downce homogenizer in 3 vol of solution B (0.32 M sucrose, 1 mM $NaHCO_3$ pH 8.3, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin). The rehomogenized P2 fraction was further purified with discontinuous sucrose density gradient. 8 ml of the rehomogenized P2 fraction was layered on the top of 10 ml each of 0.85, 1.0 and 1.2 M sucrose solutions (all containing 1 mM $NaHCO_3$ pH 8.3, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin), and centrifuged at 82,500g for 2 hr at 4 °C (Beckman SW 27 swing rotor). The band between 1.0 and 1.2 M sucrose was collected as synaptosome fraction (si) and diluted with 5 vol of ice-cold 80 mM Tris-HCl pH 8.0. An equal vol of ice-cold 1 % TritonX-100 was added, the diluted material was rotated for 15 min at 4 °C, and centrifuged at 201,800g for 1 hr. The pellet was resuspended in 0.5 % TritonX-100 buffer (0.5 % TritonX-100, 40 mM Tris-HCl pH 8.0) and centrifuged at 201,800g for 1 hr. The pellet was used as postsynaptic density (PSD) fraction.

Chromosome mapping

Brains of the BXD recombinant inbred strain mice were homogenized in RIPA buffer, and Western blotting was performed with 1B6 monoclonal antibody which recognizes p130. The results were referred to strain distribution patterns of loci (Lyon and Searle, 1989), and the locus of p130 was determined.

Mouse strains

ICR, C57BL/6, and DBA/2 mice strains were obtained from Charles Liver. BXD strains and *shaker-1* mutant mice were obtained from Jackson Laboratory. Wild mice and other laboratory mice were kindly provided by Dr. T. Shiroishi, National Institute of Genetics (Mishima, Japan). Fyn-deficient mice were produced by Dr. T. Yagi (Yagi et al., 1993), and founders were backcrossed onto a C57BL/6 background.

Results

Isolation of a Fyn-associated protein p130

To understand the function of Fyn in brain, I planned to identify proteins which are associated with Fyn in mouse brain using an affinity column method. An affinity column was prepared with a recombinant glutathione S-transferase (GST)-Fyn fusion protein (GSTFynK⁻), which contains SH3 and SH2 motifs, and the amino terminal region unique to Fyn, but lacks its kinase domain (Figure 1 A). Proteins extracted from neonatal mouse brains were incubated with affinity beads coupled with the GSTFynK⁻ fusion protein, and binding proteins were analyzed by SDS-PAGE. Many proteins specifically bound to the GSTFynK⁻ fusion protein, but not to GST itself (Figure 1B). Associated proteins of 150 kDa, 130 kDa, and 65 kDa were detected as major bands (Figure 1B).

To characterize Fyn-associated proteins purified with the GSTFynK⁻ affinity column, I prepared monoclonal antibodies by immunizing two mice with proteins that bound to the GSTFynK⁻ affinity column and were larger in molecular weight than GSTFynK⁻. I selected antibodies that could react to proteins in neonatal mouse brain judged by Western blotting. 1B6, one of the monoclonal antibodies, recognized three proteins in brain extract of C57BL/6 mice, whose sizes were approximately 130 kDa, 80 kDa and 75 kDa by Western blotting (Figure 1C).

To ensure association of Fyn and the proteins recognized by 1B6, firstly I examined whether these proteins were present in the GSTFynK⁻ affinity column fraction. Western blotting of this fraction with monoclonal antibody 1B6 showed that only the protein of 130 kDa (p130) was detected. The 80 and 75 kDa proteins present in whole brain extract were not detected in the GSTFynK⁻ affinity column fraction (Figure 2B lane GSTFynK⁻).

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As a further confirmation, I performed immunoprecipitation using anti-Fyn polyclonal antibody and anti-Fyn monoclonal antibody γC3: this monoclonal antibody was newly generated against GSTFynK⁻ expressed in *E.coli*. γC3 strongly reacted with proteins in IPTG-induced *E. coli* extracts (Figure 2A lane 2, 4), whereas only weakly reacted with proteins in preinduced extracts (Figure 2A lane 1, 3). γC3 detected a protein of ~60 kDa in adult Fyn +/+ mouse brain extract (figure 2A lane 7) but not in adult Fyn -/- mouse brain extract (Figure 2A lane 8). The immune complexes of neonatal mouse brain extracts with γC3 or with anti-Fyn polyclonal antibody were resolved by SDS-PAGE and transferred to a nitrocellulose filter. The Western blotting was probed with monoclonal antibody 1B6. As shown in Figure 2B, both immune complexes prepared with monoclonal antibody γC3 and with anti-Fyn polyclonal antibody contained a 130 kDa protein that was recognized by monoclonal antibody 1B6. Its size was identical to that of p130 in the GSTFynK⁻ affinity column fraction.

p130 protein is detected only in brain

To understand the function of the p130 protein, I examined the expression of p130 in various tissues of adult mice by Western blotting using 1B6 monoclonal antibody. As shown in Figure 3A, p130 was abundantly expressed in the brain. In other tissues, 1B6 recognized proteins of different sizes, 65 kDa in the lung, 47 kDa in the kidney, and 60 and 58 kDa in the testis. Because p130 was a brain-specific protein, I next examined the regional expression of p130 in the brain of C57BL/6 and Fyn -/- strains. As shown in Figure 3B, p130 was detected in all brain regions I examined. The regional expression pattern of p130 was similar to that of Fyn. The p130 was also detected in all the regions of Fyn -/- mouse brain.

p130 is highly concentrated in the postsynaptic density

In mouse brain, Fyn is relatively enriched in the postsynaptic density (PSD) (Grant et al., 1992). Since p130 was identified as a Fyn-associated protein, I examined expression of p130 in PSD. Figure 4 shows that Western blotting of synaptosome fraction and PSD fraction of C57BL/6 and the Fyn-/-. To estimate the purity of the prepared PSD fraction, NMDA receptor $\varepsilon 1$ and $\varepsilon 2$ subunit proteins were used as markers because they have been shown to be highly concentrated in the PSD fraction (Moon et al., 1994). In both C57BL/6 (B6 in Figure 4) and Fyn -/- strains, p130 was highly concentrated in the PSD fraction as compared with in synaptosome fraction (approximately 10 fold). Although p130 was a Fyn-associated protein, absence of Fyn did not influence the concentration of p130 in the PSD fraction.

Developmental change of p130 expression

I prepared Western blots with equivalent amounts of brain homogenates of mice of embryonic days 11.5, 15, 18, and postnatal days 0, 5, 10, 15, 20, 30, and 60 (Figure 5). The expression of p130 was unambiguously detected from P0 to P60. Relative amount of p130 protein increased between P0 and P20. This period coincides with the time when synaptogenesis rapidly occurs in the developing brain. The amount of p130 was maintained through to P60 (Figure 5). This time course paralleled that of the PSD-95 and calmodulin kinase II in PSD. These data of the abundant expression in PSD and the developmental time course supported the hypothesis that p130 was involved in synaptogenesis and synaptic functions.

p130 is different between C57BL/6 and DBA/2 strains

A mouse strain, DBA/2, has well-characterized behavioral phenotypes, for example, audiogenic seizure-prone, defect of spatial learning and emotional abnormalities.

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These phenotypes are similar to the behavioral abnormalities observed in Fyn-deficient mice. Since it was possible that DBA/2 strain had abnormality in proteins related to signal transduction pathway of Fyn, I examined the properties of p130 of DBA/2 strain. Brain extracts of C57BL/6, DBA/2 and filial 1 (F1) of C57BL/6 and DBA/2 were separated by SDS-PAGE, transferred to a nitrocellulose filter, and blotted with monoclonal antibody 1B6. Surprisingly 1B6 monoclonal antibody recognized a 140 kDa protein in DBA/2, which was different in size from p130 of C57BL/6 (Figure 6A). The 80 and 75 kDa bands of DBA/2 were identical to those of C57BL/6. In F1 mice of C57BL/6 and DBA/2 strains, two types of p130 were detected (Figure 6A lane F1 BXD).

Next, I examined regional expression in the brain of the p130 and Fyn in DBA/2 strain by Western blotting. p130 and Fyn were detected in all brain regions I examined. Although the size was different from that of C57BL/6, no quantitative or regional differences of the p130 were observed between C57BL/6 and DBA/2 strain (Figure 6B upper), as was the case for the distribution of Fyn (Figure 6B lower).

Western blotting of PSD proteins showed that p130 of DBA/2 strain was as much concentrated in the PSD as that of C57BL/6 (Figure 6C). Fyn was also concentrated in the PSD of DBA/2 mice and no significant quantitative difference was observed between DBA/2 and C57BL/6.

Chromosome mapping of p130

Over 30 recombinant inbred strains between C57BL/6 and DBA/2 have been maintained as BXD strains. I used these strains for mapping the chromosomal locus of the p130 protein by Western blotting. If the p130 gene was derived from the chromosome of DBA/2 mouse, the p130 protein should be detected as the larger band. If derived from C57BL/6, p130 should be smaller. Brain extracts from 18

BXD strains were resolved by SDS-PAGE and transferred to nitrocellulose filters, and Western blottings were probed with 1B6 monoclonal antibody to detect the p130 protein. Each BXD strain expressed either type of p130, C57BL/6 or DBA/2 type (Figure 7A). As referred to strain distribution pattern of loci typed in BXD recombinant inbred strains (Lyon and Searle, 1989), only the *hbb* locus in chromosome 7 showed the same pattern of p130 (Figure 7A and 7B BXD 1, 5, 28 and 29). These results indicated that p130 locus was closely linked to the *Hbb* locus, which located between *Mod-2* and *Odc-7* on mouse chromosome 7 (Figure 7B).

p130 is different between shaker-1 mutant mouse and hetero mouse

As the result of chromosome mapping, the p130 locus was localized close to the *hbb* locus. Near this locus, the gene locus of the neurological mutant mouse *shaker-1* (*sh-1*) was mapped (Gibson et al., 1995). I examined the p130 protein in *sh-1* mutant mice purchased from Jackson laboratory. Brain extracts of the heterozygote and homozygote mice of *sh-1* were Western blotted with 1B6 monoclonal antibody. As shown in Figure 7C, two bands, 130 and 140 kDa, were detected in *sh-1* /+ whereas only the smaller band was detected in *sh-1* /*sh-1*. These results suggested that the p130 locus was very close or identical to the *sh-1* locus. Recently it was reported that *sh-1* gene encodes myosin VII (Gibson et al., 1995). Expression of *sh-1* was reported to be detected in lung, kidney, and testis, but not in brain, judged by Northern blotting. Because this pattern of expression did not correspond to that of p130, the *sh-1* and p130 loci were not identical although very close to each other.

Distribution of two types of p130 in various mouse strains

I examined the size of p130 in many strains of laboratory and wild mice. Brain extracts of various strains were separated by SDS-PAGE, and transferred to

nitrocellulose filters. The type of p130 was determined by Western blotting with 1B6 monoclonal antibody. The results were summarized in Figure 7D. In laboratory mice, p130 of DBA/2 type was detected only in six strains, DBA/1, DBA/2, CBA, P/J, AU/SsJ, and WB/ReJ (Lyon and Searle, 1989). All of these six strains derived from an identical origin of laboratory mice. On the other hand, in wild mice, p130 of DBA/2 type was detected in as many strains as p130 of C57BL/6 type (Figure 7D). The distribution of DBA/2-type wild mice were restricted to Asia. The ancestor of DBA/2 strain might be derived from such an Asian wild mouse.

Discussion

In this work, I have identified and characterized a novel 130 kDa-protein, p130, in the mouse brain. The main results obtained for p130 are as follows: (1) p130 is associated with Fyn in the mouse brain. (2) p130 is expressed exclusively in the brain and its expression is rapidly increased between postnatal days 15 and 20. (3) p130 is concentrated in the PSD fraction. (4) An aberrant form of p130 is expressed in DBA/2 mice and some other strains. (5) The chromosomal locus of p130 is located on the 7th chromosome, very close to the *sh-1* locus.

p130 is identified as a 130 kDa-protein recognized by monoclonal antibody 1B6 that was raised against proteins prepared with the GSTFynK⁻ affinity column. 1B6 also recognizes 80 kDa- and 75 kDa-proteins besides p130. In the fraction purified with the GSTFynK⁻ column, p130 is present but the 80 kDa- and 75 kDa-proteins were not detected. Furthermore, only p130 is present in immunoprecipitates formed with anti-Fyn monoclonal and polyclonal antibodies. These results indicate that p130 recognized by 1B6 is associated with Fyn in the mouse brain.

In mouse tissues outside the brain, 1B6 recognizes proteins of different sizes; 65 kDa in the lung, 47 kDa in the kidney, and 50 kDa and 58 kDa in the testis. Variety in size of proteins recognized by 1B6 suggests that these proteins are unlikely to be isoforms which share functional properties. Since 1B6 is a monoclonal antibody which should recognize a single epitope, however, these proteins may have an identical amino acid sequence recognized by 1B6.

In this study, I have concluded that p130, recognized by monoclonal antibody 1B6, is a novel Fyn-associated protein for the following reasons: (1) As Fyn-associated proteins of ~130 kDa in size, only three proteins have been identified; focal adhesion kinase (p125FAK), AFAP 120 and p130^{Cas}. Western blot analysis with an anti-FAK

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antibody has shown that FAK is smaller in size than p130 (data not shown). AFAP120, obtained from the chick brain, is known to be smaller than FAK (AFAP120 is composed of 721 amino acid residues, while FAK is composed of 1053 amino acid residues). $p130^{Cas}$ is expressed not only in the brain but also in other tissues, indicating that $p130^{Cas}$ is a molecule different from p130. (2) p130 is concentrated in the PSD fraction. It is known that the PSD fraction contains a number of proteins, but only few of them have been studied. To our knowledge, no proteins of ~130 kDa in size have been isolated or characterized. (3) Analysis of the chromosomal locus suggested that the chromosomal locus of p130 may be identical to *sh-1*. But the observation that *sh-1* encodes myosin VII indicates that *sh-1* and the p130 locus cannot be identical, because myosin VII is expressed abundantly in the lung, kidney and testis, and scarcely in the brain, whereas p130 is exclusively and abundantly expressed in the brain.

Tissue distribution

I demonstrated that p130 is a brain-specific protein. Fyn, which is associated with p130, is abundantly expressed in the brain, thymus, and testis. Thymic abnormality of Fyn-deficient mice is known to be caused by disruption of signal of T-cell receptor which leads to an increased intracellular calcium concentration in T lymphocytes (Appleby et al., 1992; Stein et al., 1992). Since it has been shown that Fyn is linked specifically to the ζ-chain of T-cell receptor complex (Samelson et al., 1990; Timson Gauen et al., 1992), disruption of this link is supposed to be the molecular basis of the thymic abnormality of Fyn-deficient mice. In Fyn-deficient mice, abnormalities are observed not only in the immune system but also in the brain. Brain defects of Fyn-deficient mice are grouped into three categories: (1) Impairment in architecture of hippocampus and olfactory glomerulus. (2) Impairment of myelination, ascribed

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to reduced production of myelin basic protein (MBP). (3) Impairment in induction of long-term potentiation (LTP). Defects of myelination is explained by the observation that Fyn is associated with large MAG, a myelin-specific protein of immunoglobulin superfamily exclusively expressed in oligodendrocytes, and that Fyn participates in the initial events of myelination as a signaling molecule downstream of large MAG (Umemori et al., 1994). In neurons, FAK, N-CAM, AFAP120, p130^{Cas}, and Efs are known or supposed to be Fyn-associated proteins (Beggs et al., 1994; Cobb et al., 1994; Schaller et al., 1994; Sakai et al., 1994; Flynn et al., 1995; Ishino et al., 1995). These proteins, except FAK and N-CAM, have not been well characterized for their function and distribution in brain. The relation of these three proteins to impairments found in Fyn-deficient mice is not clear. In neural cells, FAK is distributed in the cell body, dendrite, and axon, as is the case for Fyn. FAK activity is regulated by its phosphorylation specifically effected by Fyn (Grant et al., 1995). Therefore it is possible that deficiency in phosphorylation of FAK leads to abnormality in formation and function of the brain. But because FAK is not concentrated in the PSD fraction where Fyn and p130 are concentrated, FAK may not be a critical component of a postsynaptic mechanism involved in LTP. In culture system, it has been demonstrated that N-CAM-dependent neurite outgrowth is regulated by Fyn-signaling pathway (Beggs et al., 1994). As described above, there are several lines of evidence at a molecular level to support involvement of Fyn-associated signaling pathway in T-cell activation, myelin formation and brain development, but there is no report on a molecule that is critically involved in synaptic plasticity, exemplified by LTP. I am ,therefore, interested in p130 as a candidate for the molecule involved in synaptic plasticity, because p130 is exclusively expressed in the brain and is concentrated in the PSD fraction as are the NMDA receptor subunit proteins.

Presence of p130 in the PSD fraction

I demonstrated that p130 is concentrated in the PSD fraction. It is known that the PSD is submembranous cytoskeletal elements of the postsynaptic structure of central synapses, which are observed under electron microscopy. The PSD can be purified by treating the synaptosomal fraction with detergents (Carlin et al., 1980; Cho et al., 1992). This PSD fraction contains many proteins important for synaptic functions, which include NMDA receptor ε-1 and ε-2 subunits, αCaM kinase II, PKCγ, and Fyn and others (Kennedy et al., 1983; Grant et al., 1992; Suzuki et al., 1993; Moon et al., 1994; for see review Kennedy 1993). Furthermore, analysis of gene-targeted mice have shown that disruption of genes of those proteins can lead to reduction of LTP and abnormalities in learning and behavior, indicating that those proteins present in the PSD fraction are critical in synaptic plasticity. Since I demonstrated that p130 is concentrated in the PSD fraction as is Fyn, I believe that p130 plays an important role in synaptic plasticity. It is also an intriguing question whether p130 is associated with proteins, other than Fyn, located in the PSD.

Developmental increase of p130 during brain formation

I observed that p130 level increases during the period of rapid brain growth after birth. The increase in p130 level is especially remarkable between postnatal days 15 and 20. Also the time course of increase in p130 level correlates with that of maturation of synaptic structure observed under electron microscopy and with those of level of synapsin I, calmodulin kinase II and PSD-95 (Kennedy et al., 1983) (Cho et al., 1992), which are also present in synapse.

Myelin formation, which is defective in Fyn-deficient mice, also becomes very active after birth. Because it is reported that Fyn becomes activated and functional at the early phase of myelin formation (postnatal days 4 to 8) (Umemori et al., 1994),

and because subcellular fractionation analysis showed that p130 is more concentrated in the PSD fraction than the synaptosomal fraction, developmental increase of p130 is more likely caused by synaptogenesis than myelin formation. The observation that p130 expression remains high in the adult brain suggests a possibility that p130 is also involved in maintenance of the synaptic structure and in synaptic transmission.

Different form of p130 in some mouse strains

Western blot analysis with monoclonal antibody 1B6 showed that not the 130 kDa- but the 140 kDa-band is detected in brain extracts of DBA/2 mice. Two proteins were observed in the F1 mice between C57BL/6 and DBA/2 mice. This result indicates that the additional molecular size of p130 in DBA/2 mice is not due to post-translational modification, such as phosphorylation and glycosylation, but is caused by alternation of the gene itself. Furthermore, the result showing that each of 18 recombinant inbred mouse strains, BXD, which are generated by crossing C57BL/6 and DBA/2, has either type of p130 indicates that the 130 kDa- and 140 kDa- proteins are product of an identical gene. It is interesting to note that DBA/2 mice, whose p130 has a different size, show abnormal behaviors, which include enhancement of audiogenic seizure, reduction of spatial learning in the Moris water maze, emotional behavior, and acceleration of ethanol sensitivity; all these phenotypes are similar to those of Fyn-deficient mouse. The phenotypical similarity between DBA/2 and Fyn-deficient mice suggest a possibility that p130 takes part in the Fyn-mediated signal pathway and that alternation of p130 causes alteration of the Fyn-signaling pathway. In such a case, I assume that the behavioral abnormalities of DBA/2 mice are caused by defective Fyn-signaling pathway, and therefore are similar to those of Fyn-deficient mice. I could not demonstrate further evidence, however, to support the hypothesis that the different form of p130 causes behavioral abnormalities by itself, because its

distribution and amount of expression in brain and the concentration ratio in the PSD fraction of DBA/2 mice are not different from those observed for C57BL/6 mice. I think it unlikely that all the phenotypical abnormalities of DBA/2 mice can be ascribed to the alternation of p130, because it is known that behavioral abnormalities of DBA/2 mice are not determined by a single locus, but because each abnormal behavior is determined by a set of chromosomal loci. It has been reported that one of the chromosomal loci of ethanol sensitivity is located on the 7th chromosome.

Analysis of the p130 form of other strains of mouse revealed that only six laboratory mouse strains out of 18 strains studied have the p130 of DBA/2 type. All of these six strains are substrains derived from a common origin, DBA. Interestingly, DBA/1 mice, like DBA/2 mice, are known to show enhanced audiogenic seizures, emotional behaviors, and acceleration of ethanol sensitivity. Unfortunately, no data are available for behavior abnormalities in other five mouse strains. I also studied p130 of wild mice, since laboratory mice were established by crossing wild mice of European and Asian origins. In wild mice, the p130 of DBA/2 type is observed as frequently as the p130 of C57BL/6 type. The p130 of DBA/2 type is almost confined to wild mice obtained in Asia. This result suggests that generation of the aberrant p130 may accompanied differentiation of wild mice into subspecies. It has been observed that behavioral alternations of sexual behaviors of *Drosophila* accompany subspecies differentiation.

The facts that wild mice have different types of p130 in the PSD fraction and that each subspecies of the wild mice has either type of p130 raise the possibility that mutation of p130 caused changes in behavior of wild mice, leading to subspecies differentiation. In this sense, p130 is an interesting molecule in studying a molecular basis of mammalian evolution.

Chromosomal locus of p130 and its relation to *sh-1*

There are a considerable number of mutant strains of mouse, and the chromosomal loci have been determined for many of those mutant mice. Determination of the chromosomal loci is important because it may reveal relationship to mutations already analyzed. I took advantage of using recombinant inbred strains, BXD and determined the chromosomal locus of p130 by Western blotting. The results indicated that the locus which determines the size of p130 is located near the *Hbb* locus, on the 7th chromosome. *sh-1*, a locus responsible for a neurological disorder, has been mapped near the Hbb locus (Lyon and Searle, 1989). The *sh-1* mutant mice have been maintained by crossing heterozygous siblings. The homozygous *sh-1* mice have a single band of p130, while heterozygous mice has two bands of p130 when analyzed by Western blotting. Although this result strongly suggested that the p130 locus is identical to *sh-1* it was reported last year that the *sh-1* locus encodes myosin VII, whose expression pattern is completely different from that of p130 (Gibson et al., 1995). From those observations, I concluded that the p130 locus is located very close to *sh-1*, but is not *sh-1* itself.

Association of Fyn and p130

In this work, I demonstrated that p130 is associated with Fyn in the mouse brain. This association is likely effected by binding of p130 to the SH2 and SH3 domains of Fyn, because p130 is present in the GSTFynK⁻ affinity column fraction. It is known that the SH2 domain binds phosphorylated tyrosines, while the SH3 domain binds amino acid sequences rich in proline content. Many of the Fyn-associated proteins so far identified are phosphorylated at their tyrosines, suggesting they are substrates for Fyn. The observation that antibody pY20, which recognizes phosphorylated tyrosines, failed to recognize p130, however, suggests that p130 is

unlikely to be a substrate for Fyn (data not shown), although I cannot totally exclude this possibility because not all phosphorylated tyrosines are detected by pY20 (Umemori et al., 1994).

Besides functioning as a substrate for Fyn, p130 may function as; (1) a protein located upstream of Fyn, conveying extracellular and intracellular signals to Fyn, (2) a protein involved in subcellular localization of Fyn, i.e. transport to the PSD, (3) a protein which modulates phosphorylation activity by binding substrate proteins of Fyn. Although the function of p130 is not known yet, I think p130 is a very interesting molecule, because p130 is identified as a Fyn-associated protein present in the PSD, where Fyn is assumed to be actively functional and because Fyn has been demonstrated to play a critical role in brain formation and in function from analyses of Fyn-deficient mice. I believe that further characterization of p130, including analyses of its relationship to abnormal behaviors, can lead to new insights into synapse formation related to behavior controls and molecular mechanisms of synaptic plasticity .

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Figure 1.

A

Fyn

| Unique | SH3 | | SH2 | | Kinase |

GSTFynK⁻

| GST | Unique | SH3 | | SH2 | |

B

GST   GSTFynK⁻

- 200k ➤
- 97k ➤
- 69k ➤
- GSTFynK⁻ ➤
- 46k ➤
- GST ➤

C

CBB   1B6

- 200k ➤
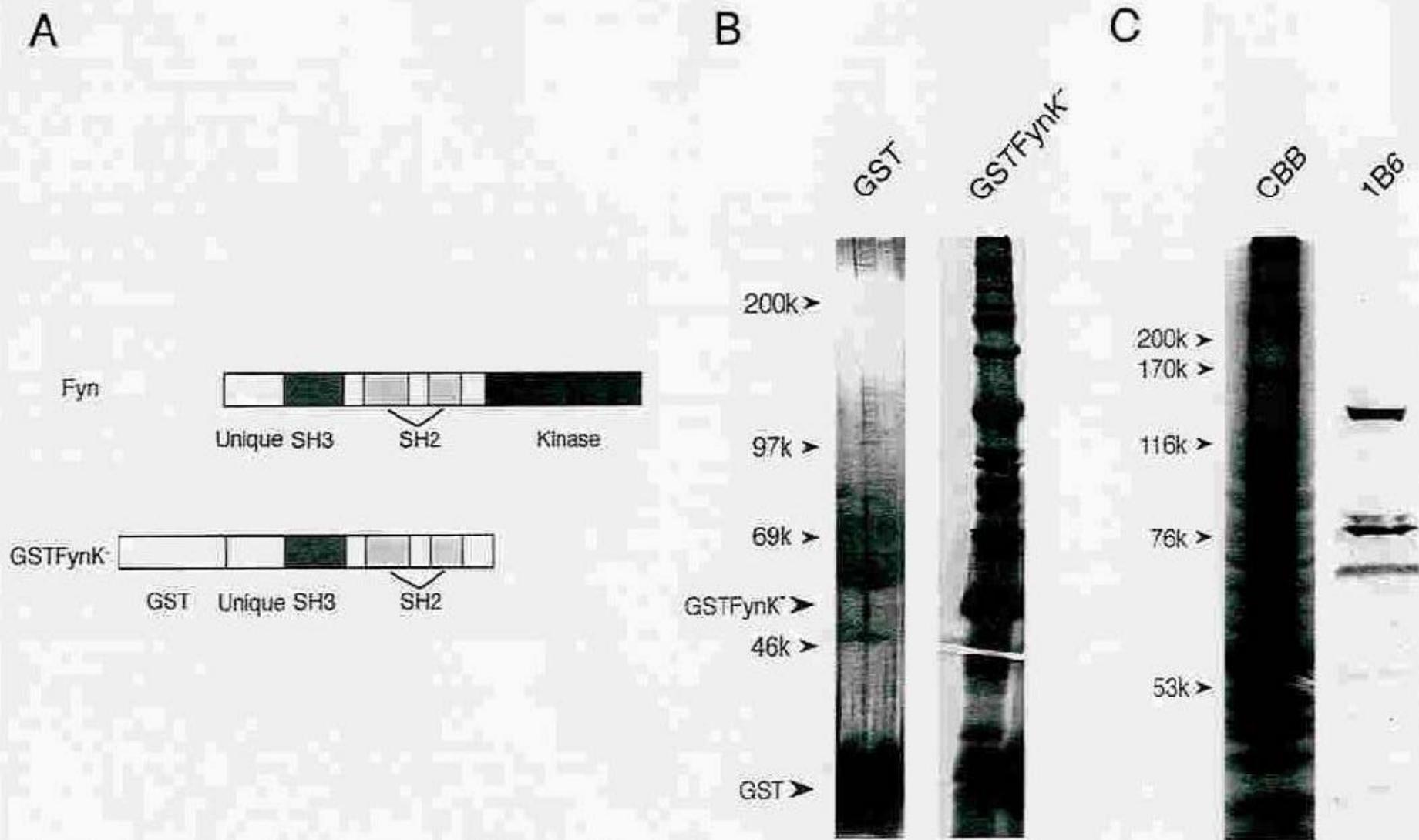- 170k ➤
- 116k ➤
- 76k ➤
- 53k ➤

Figure 1.

(A) Schematic domain structures of Fyn and the GSTFyn fusion protein (GSTFynK⁻). GSTFynK⁻ contains the N-terminal unique region, the SH3 motif, and the two domains of SH2 motif of Fyn.

(B) Purification of Fyn-associated proteins by affinity chromatography. Mouse brain P2 fractions, solbilized in CHAPS lysis buffer, were incubated with affinity beads coupled with GST (left lane) or coupled with GSTFynK⁻ (right lane). The eluted proteins were analyzed by SDS-PAGE and stained with coomassie brilliant blue (CBB).

(C) Characterization of monoclonal antibody 1B6. Brain extracts of C57BL/6 mice was analyzed by SDS-PAGE and stained with CBB (left lane). Proteins were transferred to a nitrocellulose filter and probed with monoclonal antibody 1B6 (right lane) 1B6 reacts with ~130 kDa-, 80 kDa-, and 75 kDa-proteins. The band of 65 kDa results from the secondary antibody used in this experiment (mouse internal immunoglobulin).

Figure 2.

A

B
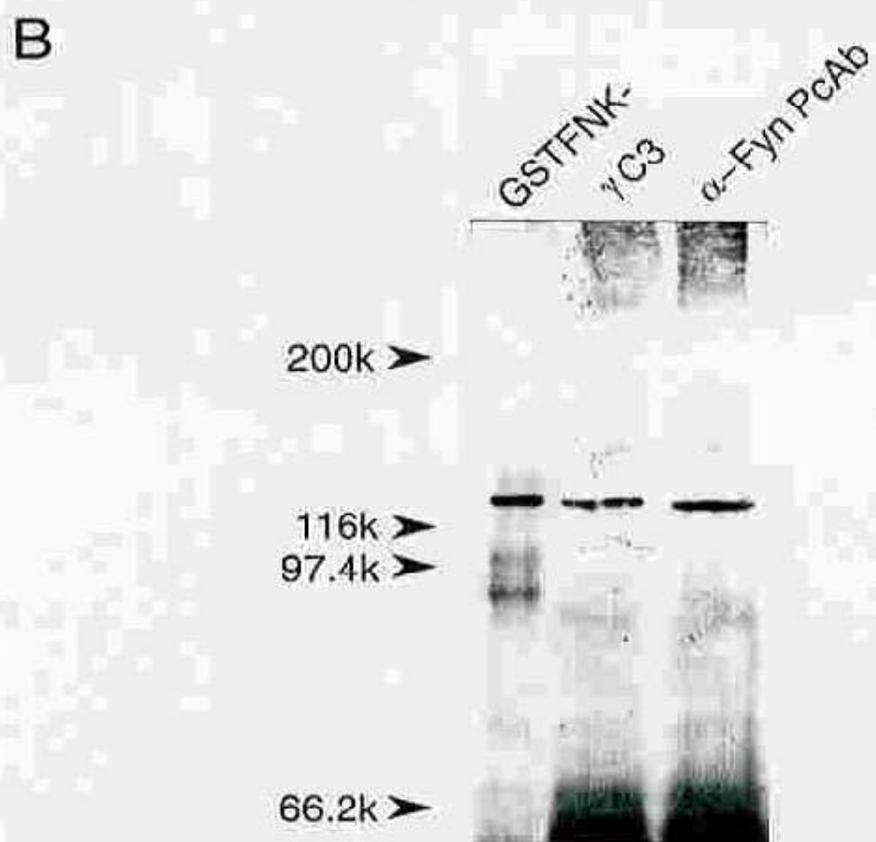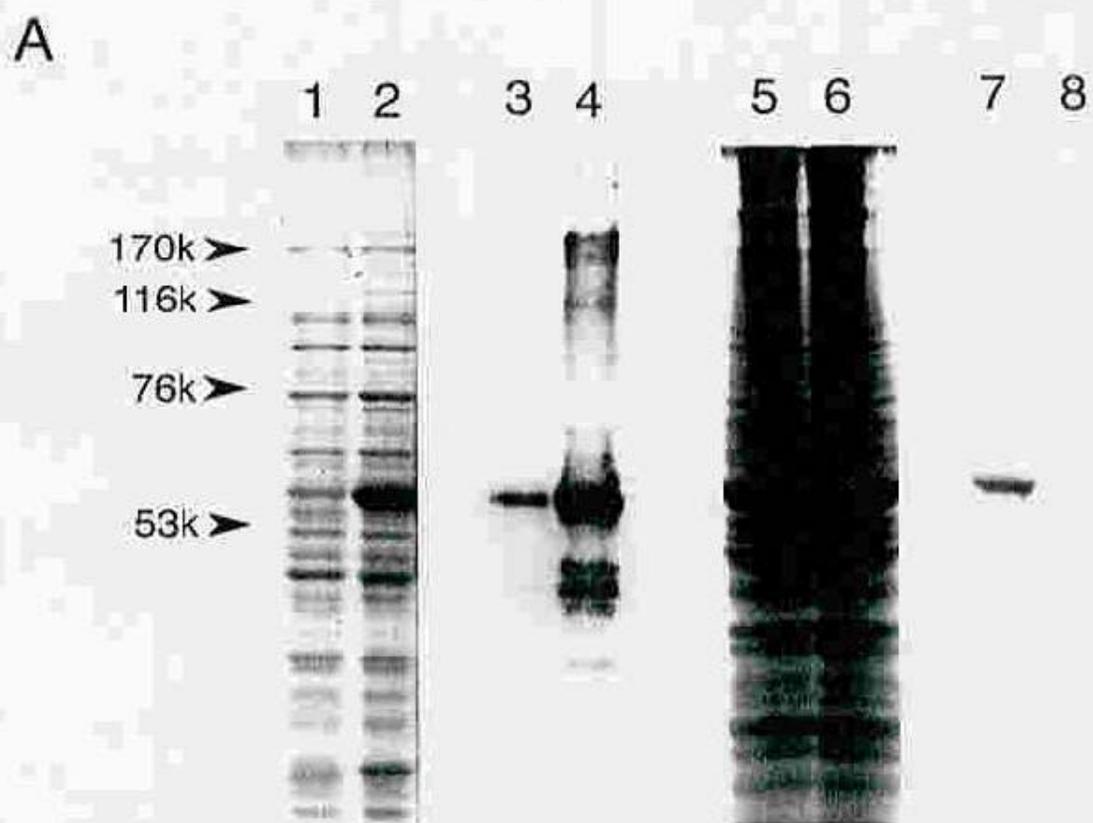
Figure 2.

(A) Specificity of anti-Fyn monoclonal antibody γC3. SDS-PAGE analysis of extracts of GSTFynK⁻-producing *E. coli* before induction (lane 1) and three hours after induction (lane 2), stained with CBB. Western blot analysis probed with γC3 of *E. coli* extracts before (lane 3) and after induction (lane 4). SDS-PAGE analysis of brain extracts of Fyn+/+ (lane 5) and Fyn-/- mice (lane 6). Western blot analysis of brain extracts of Fyn+/+ (lane 7) and Fyn-/- mice (lane 8) probed with γC3. In lane 7 and lane 8, γC3 also detected a band of ~120 kDa protein weakly. A band like background of membrane was observed at 76 kDa in lane 8.

(B) Coimmunoprecipitation of p130 with Fyn. Mouse brain homogenates in RIPA buffer were incubated with protein G beads coated with GSTFynK (left lane), anti-Fyn monoclonal antibody γC3 (middle lane), and anti-Fyn polyclonal antibody (right lane). Immunoprecipitates were analyzed by Western blotting using monoclonal antibody 1B6. Both monoclonal and polyclonal ant-Fyn antibodies coimmunoprecipitated p130.
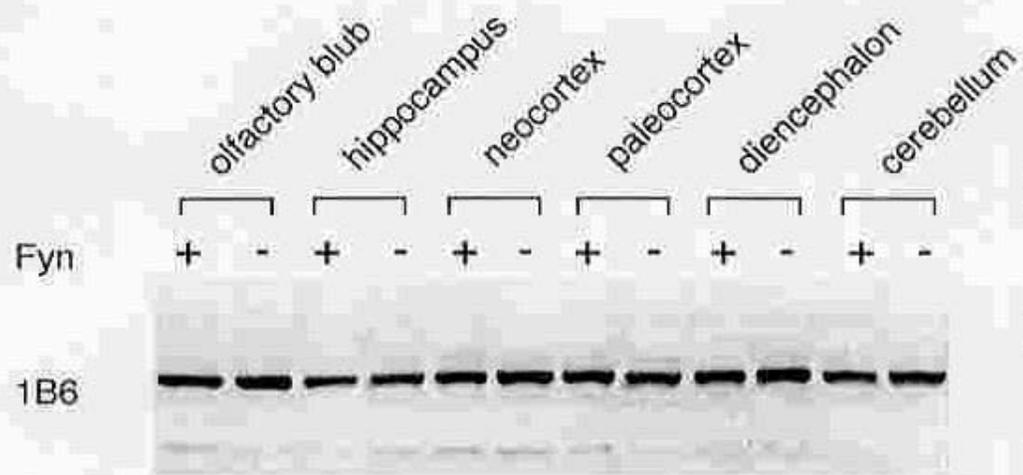
Figure 3.
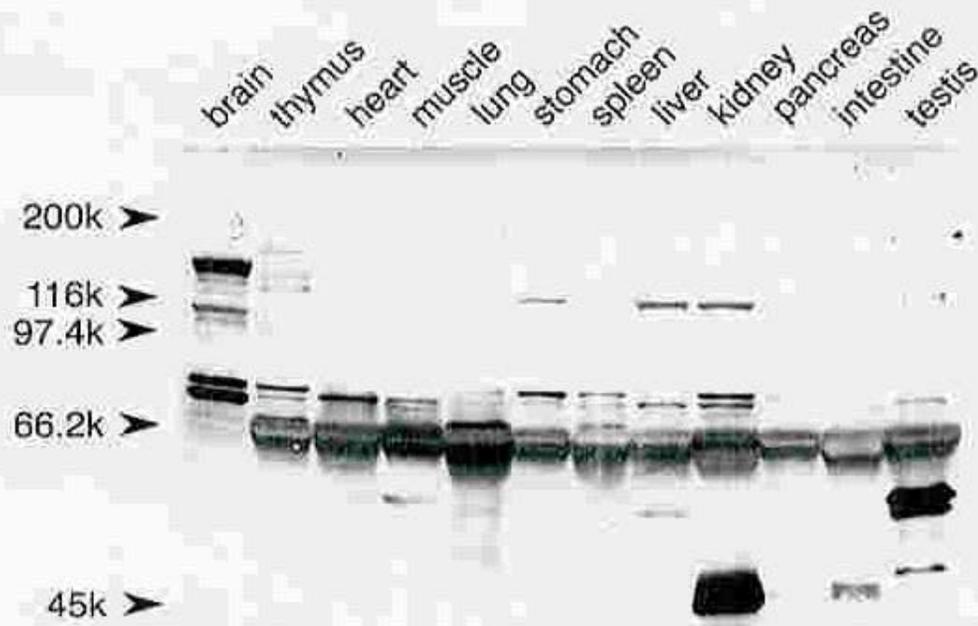
Figure3. Expression pattern of p130. 100 μg of proteins were loaded to each lane, and p130 was detected by Western blotting with monoclonal antibody 1B6.

(A) Tissue distribution of p130 was examined by Western blotting. Tissues from 4-week-old mice were homogenized in RIPA buffer. p130 was detected only in brain. The 80 kDa-protein present in the brain (Figure 2B) was also detected in the thymus, stomach, and kidney, and in smaller amounts, in the lung, spleen, pancreas, and intestine. The 75 kDa-protein was detected strongly in brain. A 66 kDa-protein in the lung, a 45 kDa-protein in the kidney, 60 and 58 kDa-proteins in testis were abundantly expressed, although they were not detected in the brain .

(B) Regional expression of p130 in the brain. Brain extracts from Fyn +/+ (marked with '+') and Fyn -/- mice (marked with '-') were analyzed by Western blot probed with 1B6. p130 was uniformly expressed in all brain regions; olfactory bulb (lanes 1 and 2), hippocampus (lanes 3 and 4), neocortex (lanes 5 and 6), paleocortex (lanes 7 and 8), diencepharon (lanes 9 and 10), and cerebellum (lanes 11 and 12).
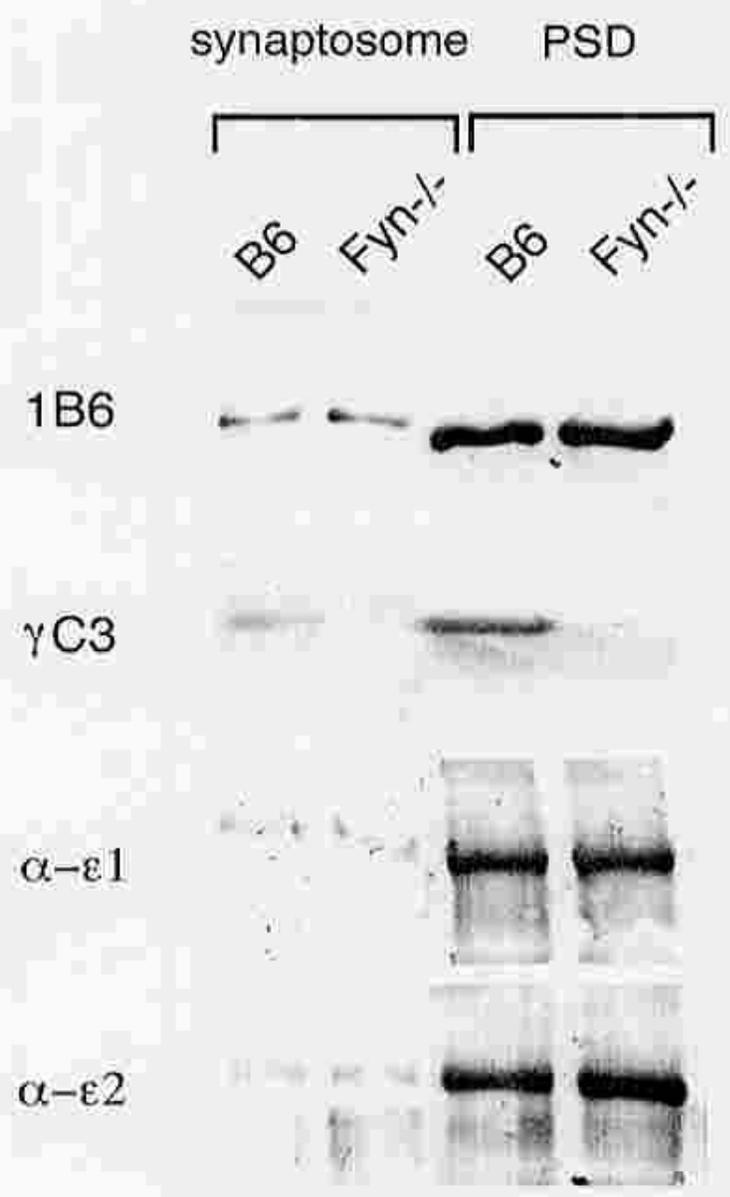
Figure 4.

synaptosome   PSD

B6   Fyn-/-   B6   Fyn-/-

1B6

γC3

$\alpha-\varepsilon 1$

$\alpha-\varepsilon 2$

Figure 4. Subcellular localization of p130. Synaptosomal fractions of C57BL/6 (labeled as B6), and Fyn -/- mice (labeled as Fyn-/-) were prepared by discontinuous sucrose density ultracentrifugation. The PSD fractions were prepared from the synaptosomal fractions as described in Materials and Methods. 10 µg of samples were loaded. Identical blots were probed with monoclonal antibody 1B6 for p130 (top panel), with monoclonal antibody γC3 for Fyn (second panel), with a polyclonal antibody against the NMDA-R ε1 subunit (third panel), and with a polyclonal antibody against the NMDA-R ε2 subunit (bottom panel).

Figure 5.

1B6

E11.5 E15 E18 P0 P5 P10 P15 P20 P30 P60
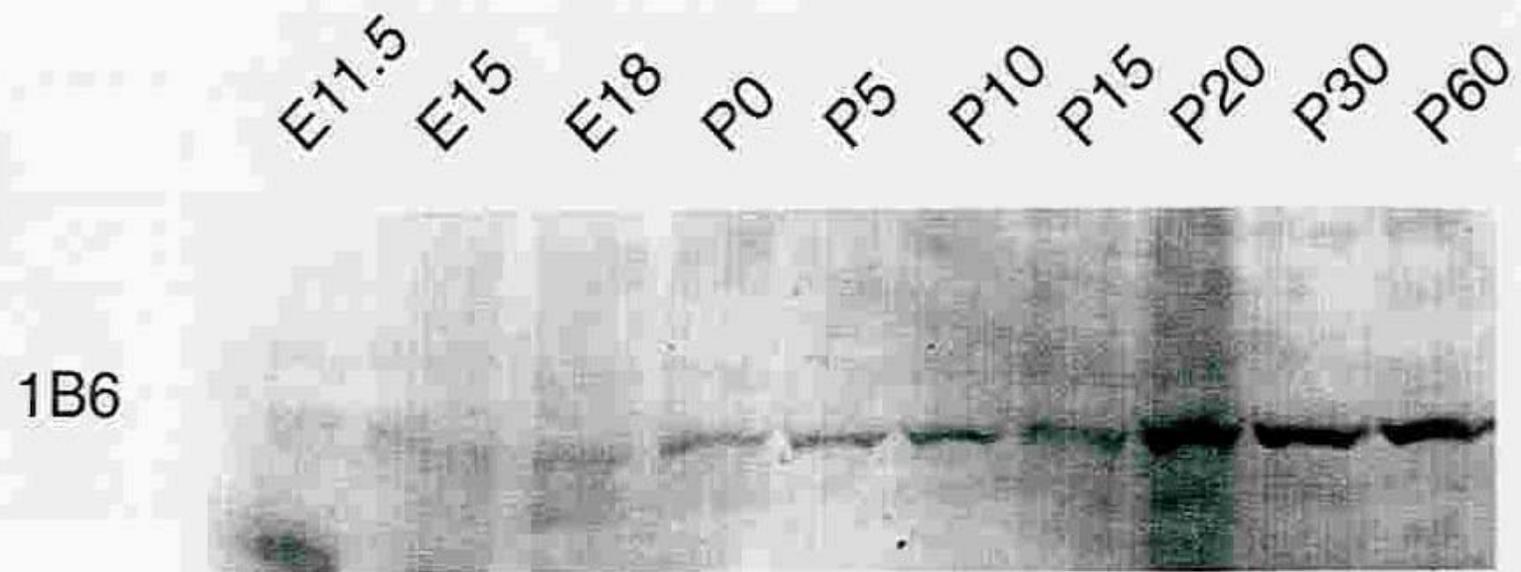
Figure 5. Developmental profile of p130 expression. 100 μg of whole brain extracts at embryonic days 11.5, 15, 18, and postnatal days 0, 5, 10, 15, 20, 30, and 60 were loaded, and the p130 was detected by Western blotting with 1B6. The expression is first detected clearly at P0, and markedly increases between P0 and P20. The expression may slightly decrease after P20.
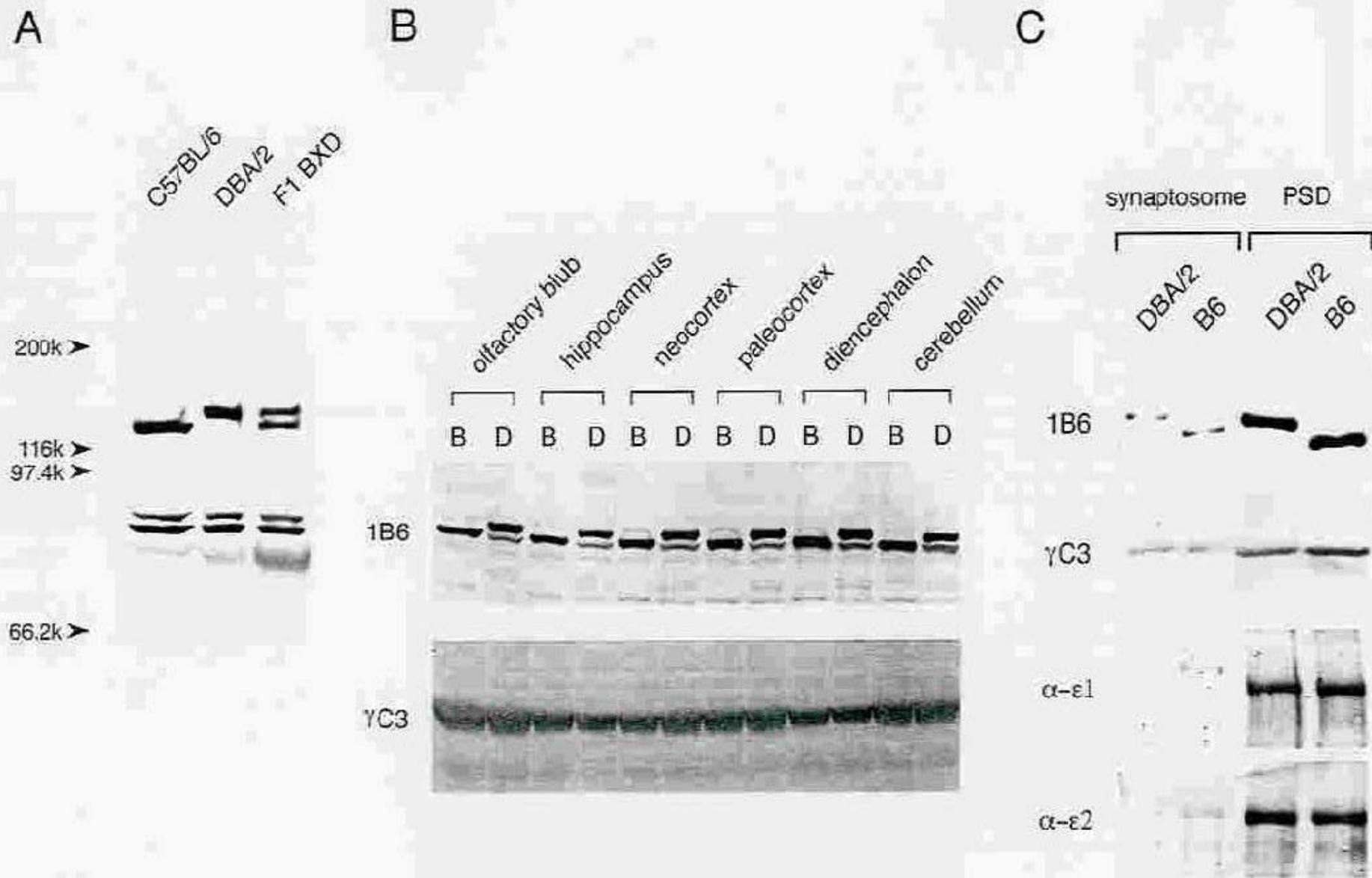
Figure 6.

A

C57BL/6   DBA/2   F1 BXD

200k ➤

116k ➤
97.4k ➤

66.2k ➤

B

synaptosome   PSD

olfactory blub   hippocampus   neocortex   paleocortex   diencephalon   cerebellum

B D   B D   B D   B D   B D   B D

1B6

γC3

C

DBA/2   B6   DBA/2   B6

1B6

γC3
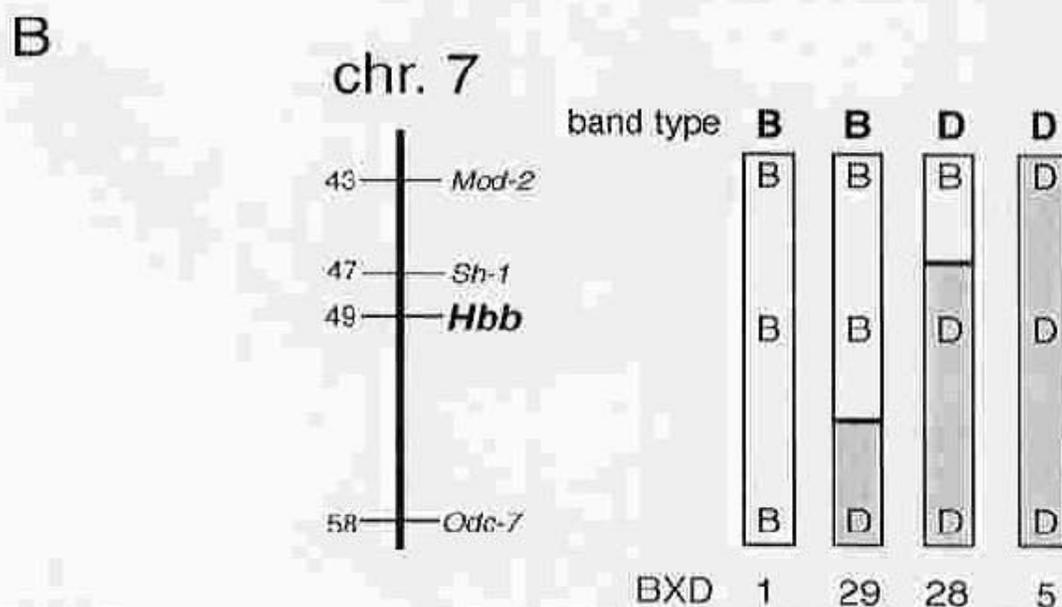
α-ε1

α-ε2

Figure. 6 Characterizing p130 of DBA/2 type

(A) Monoclonal antibody 1B6 reacts with 130 kDa-, 80 kDa-, and 75 kDa-proteins in brain extracts from adult C57BL/6 mice (left lane). The 130 kDa-band shifts upward in brain extracts from DBA/2 mice, while the 80 kDa- and 75 kDa-proteins are of the same sizes as those of C57BL/6 (middle lane), The F1 mice between C57BL/6 and DBA/2 strains have two types of bands, each related to C57BL/6 and DBA/2 (right lane).

(B) Synaptosomal fractions of C57BL/6 (labeled as B6) and DBA/2 (labeled as DBA/2) strains were prepared by discontinuous sucrose density ultracentrifugation. The PSD fractions were prepared from the synaptosomal fractions as described in Materials and Methods. 10 μg samples were loaded to each lane. Identical blots were probed with monoclonal antibody 1B6 for p130 (top panel), with monoclonal antibody gC3 for Fyn (second panel), with a polyclonal antibody against the NMDA-R e1 subunit (third panel), and with a polyclonal antibody against the NMDA-R e2 subunit (bottom panel).

(C) Regional expression of p130 in the C57BL/6 (marked with 'B') and DBA/2 (marked with 'D') brains. Although p130 of DBA/2 has an aberrant size, p130 is detected in both strains by Western blotting, uniformly in all brain regions (upper panel); olfactory bulb (lanes 1 and 2), hippocampus (lanes 3 and 4), neocortex (lanes 5 and 6), paleocortex (lanes 7 and 8), diencepharon (lanes 9 and 10), and cerebellum (lanes 11 and 12). A faint band below p130 in DBA/2 strains is background caused by a lot of secondary a........y.

Expression of Fyn is not different between C57BL/6 strain and DBA/2 (lower panel).

Figure. 7

A

BXD No.

1 4 5 8 9 11 13 14 15 18 19 21 22 25 28 29 30 32

1B6

B

chr. 7

43 — Mod-2

47 — Sh-1

49 — **Hbb**

58 — Odc-7

band type	**B**	**B**	**D**	**D**
	B	B	B	D
	B	B	D	D
	B	D	D	D
BXD	1	29	28	5

C

Sh-1/+   Sh-1/Sh-1

200k ➤

116k ➤
97.4k ➤

66.2k ➤
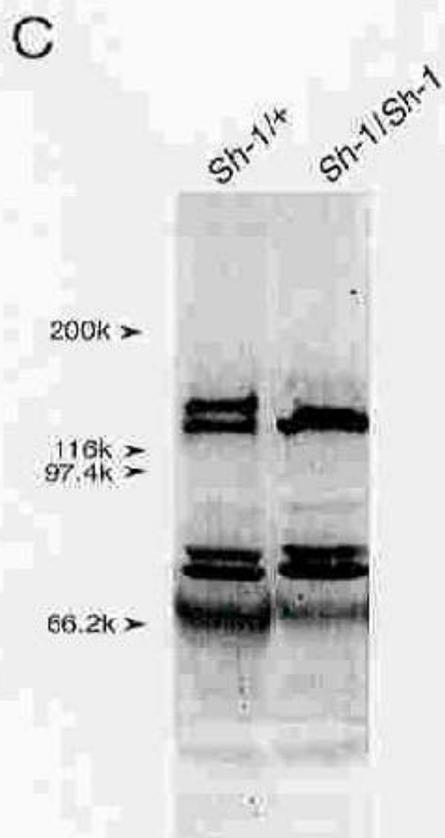
D

band type	B	D
laboratory mouse	129/SVJ6s	DBA/1
	BALB/AaKn	DBA/2
	C58/J	CBA
	N2B/BlN	P/J
	C57L/J	AU/SsJ
	CE/J129	WB/ReJ
	A/WySnJ	
	C57BR/cdJ	
	AKR/J	
	PT/J	
	C57BL/10SnJ	
	C57BL6J	
	SM/J	
	SJL/J	
	A2G/OLA/HSD	
	PL/J	
	RIIIS/J	
	SWR/J	
	BALB/c	
wild mouse	M.DOM-PGN2 (Canada)	M.Bac-Iran (Iran)
	M.Bac-Avz3 (Iran)	Japanese Mouse (Japan)
	M.sub-CHD (China)	M.SUB-SHI-1 (China)
	M.Mus-NJL (Denmark)	M.SUB-Bjn4 (China)
	CAST-Ei (Jax)	KJR/Msf (Korea)
	HM1Msf (Taiwan)	SWN/Msf (Korea)
	BFM/2Msf (France)	MSM/Msf (Japan)
	MAL/Msf (Malaysia)	MOM (Japan)
	BLG2/Msf (Bulgaria)	

Figure.7 Chromosomal mapping of p130 and typing of p130 in various mouse strains.

(A) Western blotting of brain extracts from eighteen BXD RI strains was probed with monoclonal antibody 1B6. The numbers above the panel indicate strains of BXD. BXD strains 1, 4, 8, 11, 19, 21, 22, 29, and 30 have p130 of C57BL/6 type, whereas BXD strains 5, 9, 13, 14, 15, 18, 25, 28, and 32 have p130 of DBA/2 type.

(B) A schematic representation of chromosomal loci on the chromosome 7 (left). Scales of loci are given in centimorgans (cM) from the centromere.
The result of Western blotting experiment of Figure. 7A was referred to a database comprising the genetic map location of many marker loci typed in BXD, inferring the chromosomal localization of p130 close to *Hbb* on the chromosome 7. In BXD1, *Mod-2*, *Hbb*, and *Odc-7* are derived from C57BL/6. In BXD 29, *Mod-2*, and *Hbb* are derived from C57BL/6, *Odc-7* is derived from DBA/2. In BXD 28, *Mod-2* is derived from C57BL/6, *Hbb*, and *Odc-7* are derived from DBA/2. In BXD 5, *Mod-2*, *Hbb*, and *Odc-7* are derived form DBA/2. Only *Hbb* locus showed the same pattern of Western blotting (left).

(C) 1B6 reacts with 130 kDa-, 80 kDa-, and 75 kDa-proteins in brain extracts from adult homozygous *sh-1* mice (right lane) But two types of p130 were detected in heterozygous *sh-1* mice (left lane) .

(D) Typing p130 in various mouse strains. The results of Western blotting of p130 were summarized. 'B' indicates the C57BL/6 type and 'D' indicates the DBA/2 type, respectively

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Acknowledgments