**Altered gene expression in the subdivisions of the amygdala of Fyn-deficient mice
as revealed by laser capture microdissection and mKIAA cDNA array analysis**

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Size of the whole manuscript: 54 pages including 5 figures and 1 table.

Abstract

Fyn-tyrosine-kinase-deficient mice exhibit increased fearfulness and display enhanced excitability in the amygdala. To gain insight into the molecular changes associated with the increased excitability of the amygdala, we used a newly developed cDNA array system comprising mouse KIAA cDNA clones to identify novel genes differentially expressed in the amygdala of *fyn*(-/-) and *fyn*(+/-) mice following administration of N-methyl-D-aspartate (NMDA). Laser capture microdissection in combination with PCR-based cDNA amplification allowed us to analyze gene expression in each amygdalar subdivision. The statistical significance of the differential expressions was tested by one-way analysis of variance (ANOVA) by the false discovery rate controlling approach. Among the 805 mKIAA cDNA clones tested, only the expression level of mKIAA1577 (Zinc finger SWIM domain containing protein 6; gene name, *Zswim6*) showed statistically significant change in regard to the genotype and amygdalar subdivision. Namely, only the lowered expression of mKIAA1577 in the central nucleus of *fyn*(-/-) mice 1 hr after NMDA administration (2.1 fold lower relative to *fyn*(+/-) mice) was statistically significant. In situ hybridization analysis confirmed the

downregulation of the mRNA in the central nucleus of the *fyn*($-/-$) mice 1 hr after NMDA administration (3.2 fold lower relative to *fyn*($+/-$) mice). The NMDA-induced change in gene expression was partially blocked by the NMDA antagonist D-AP-5. These results suggest that Fyn deficiency was responsible for the NMDA-induced downregulation of a specific gene in the amygdalar central nucleus.

Classification terms

Theme D: NEUROTRANSMITTERS, MODULATORS, TRANSPORTERS, AND RECEPTORS

Topic: Gene expression

Key words: emotion, Fyn, amygdala, laser-capture microdissection, cDNA microarray

1. Introduction

The amygdala has been identified as a site for the convergence of input of emotional stimuli and for the output for behavioral responses [17], and transcriptional activation and protein synthesis in the amygdala has been reported to be essential for the consolidation of fear memory [4, 34, 37]. Since screening for genes induced in the amygdala by exposing animals to fear-inducing stimuli has recently led to the identification of various genes that exhibit altered expression in response to such stimuli [14, 38], altered gene expression in the amygdala may be involved in the mechanisms that control fear-related behavior by affecting neuronal activity in the amygdala.

Fyn is a non-receptor-type tyrosine kinase and is widely expressed in mouse brain, including in the amygdala [47]. Abnormal behavior related to expression of emotions, such as increased fearfulness, has been reported in *fyn*($-/-$) mice but not in *fyn*($+/-$) control mice [15, 25, 26]. Abnormal excitability of the amygdala in *fyn*($-/-$) mice has been suggested by the difference in numbers of c-Fos-immunoreactive neurons between *fyn*($-/-$) and *fyn*($+/-$) mice after N-methyl-D-aspartate (NMDA) administration [9] and after elicitation of a conditioned fear reaction [15]. More NMDA-induced

c-Fos-positive cells were present in the central and medial nuclei of the amygdala of the *fyn*($-/-$) mice than of the *fyn*($+/-$) mice, but the opposite was observed in the basolateral nucleus. Thus, the effect of Fyn deficiency on neuronal excitability is concluded to differ among the subdivisions of the amygdala.

The above findings suggest that changes in neuronal excitability of the amygdala of *fyn*($-/-$) mice increase fearfulness and cause abnormal behavior. At present, little is known about the cellular mechanism by which Fyn deficiency gives rise to changes in the neuronal excitability of the amygdala. Since each subdivision of the amygdala plays a different functional role in the processing of emotional information [24], a new approach that examines the gene expression profiles in each subdivision has been undertaken by means of laser-capture microdissection [49].

KIAA clones were originally obtained from a human cDNA sequencing project to accumulate protein-coding sequence information about unidentified human genes [29]. More than 2000 human long cDNA clones that have been newly identified and characterized by this project have been systematically designated “KIAA” plus a 4-digit number. KIAA clones are characterized by their long cDNA sequence (>4 kb) encoding

large proteins (>50 kDa) and isolation from the brain. A project to isolate and determine all the sequences of mouse cDNA clones corresponding to human KIAA is in progress. Use of mKIAA genes as research material that reflects a large population of proteins in the brain is advantageous for exploring the novel genes involved in specific neural functions. The structural features of mKIAA cDNA clones are being released through an Internet-based database (<http://www.kazusa.or.jp/rouge/>).

In the present study, we used a newly developed cDNA array system comprising mKIAA cDNA clones [12, 48], isolation of each amygdalar subdivision with laser-capture microdissection, and the preparation of hybridization probe from the dissected sections to identify novel genes differentially expressed in the subdivisions of the amygdala of *fyn*($-/-$) and *fyn*($+/-$) mice following NMDA administration. The gene expression data were tested by an analysis of variance (ANOVA) in conjunction with a false discovery rate (FDR) controlling procedure, and the differential expression of the candidate gene with statistically significant difference between the genotypes was confirmed by in situ hybridization analysis.

2. Materials and Methods

2.1. Animals

Fyn-tyrosine-kinase-deficient mice were generated by inserting the beta-galactosidase gene (*lacZ*) into the reading frame of the *fyn* gene [46] of C57BL/6 mice. Adult homozygote Fyn-deficient mice, *fyn*(-/-), heterozygote mice, *fyn*(+/-), and wild type mice, *fyn*(+/+), were obtained by crossing heterozygotes, and the *lacZ* introduced was found to be expressed in the *fyn* (-/-) and *fyn* (+/-) mice [46, 47]. *Fyn* (+/-) mice were used as the control instead of *fyn* (+/+) mice in order to compensate for the possible effect of expression of *lacZ* as a foreign gene, and thus, the results obtained in the *fyn*(+/-) and *fyn*(-/-) mice were compared. In order to check the gene-dosage effect, the results obtained from *fyn*(+/+) mice were also compared with other genotypes in the quantitative in situ hybridization experiments. Genotypes were analyzed by the polymerase chain reaction. Adult male mice were used for all experiments at 8 weeks of age.

All animals were maintained at the National Institute of Neuroscience (Kodaira, Tokyo, Japan) under standard laboratory conditions (23±1°C, a 12:12-h light/dark

regime). All procedures in the animal experiments in this study were approved by the Animal Care Committee of the National Institute of Neuroscience.

2.2. Drug treatment

Mice were intraperitoneally injected with NMDA (70 mg/kg body weight) in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl (PBS). The NMDA dose was limited to the subthreshold dose for seizure induction [9], and the effect of pretreatment with a competitive NMDA antagonist, D-(–)-2-amino-5-phosphonovaleric acid (D-AP-5, Sigma, St. Louis, MO), was investigated to confirm that the NMDA-induced differential expression of the gene was mediated by activation of the NMDA receptor. D-AP-5 was administered intraperitoneally (7.5 mg/kg body weight) 20 min prior to the NMDA injection. After a single injection of NMDA, each mouse was decapitated and the brain was quickly removed at the specified time. Each brain was rapidly frozen in crushed dry ice within 1 min after decapitation, and stored at –85 °C until used.

2. 3. Laser microdissection and mRNA purification

Coronal sections (8 µm thick) of mouse forebrain (bregma -1.70 mm) were prepared on a cryostat (Leica Microsystems, Shinagawa, Tokyo, Japan) and collected onto

membrane-coated glass slides (Leica, 90FOIL L-SL25). The sections were immediately fixed in 100% ethanol for 1 min and then air-dried for 30 seconds. After staining the sections for 30 seconds with 0.3% toluidine blue in diethyl pyrocarbonate (DEPC)-treated water, they were washed in DEPC-treated water for 1 minute, immersed in 70% ethanol for 20 seconds, and air-dried. The AS Laser Microdissection (LMD) System (Leica) was used to dissect tissues of the central-, lateral/basolateral-, and medial nucleus of the amygdaloid complex from the cryostat sections, and they were stored at -85°C in 50 μl of the lysis solution in a RNA extraction kit (RNAqueous-4PCR, Ambion, Austin, TX). Single section per animal and area has been taken for the laser capture, and the hybridization probe for cDNA microarray analysis was successfully prepared from the single section in this study. The lysis solution containing dissected tissue was thawed at 37°C for 30 seconds and mixed with 50 μl of the lysis solution supplemented with 20 ng of poly-cytidylic acid (Amersham Biosciences, Piscataway, NJ). RNA isolation, including DNase treatment, was performed according to the manufacturer's protocol (RNAqueous-4PCR, Ambion, Austin, TX).

2. 4. cDNA array analysis

2. 4. 1. cDNA amplification

A cDNA amplification procedure was performed as described previously [30], with some modifications. Briefly, the first-strand cDNA was synthesized with Sensiscript reverse transcriptase (RT) (QIAGEN, Valencia, CA), and after second-strand synthesis and end-filling, bead-fixed cDNA was ligated with an adaptor/linker (1 µg) containing a T7 RNA polymerase promoter (upper sequence:

5'-CCGGAATCGATTAATACGACTCACTATAGGGAGATGTCTAGATC-3', and

lower sequence:

5'-pGATCTAGACATCTCCCTATAGTGAGTCGTATTAATCGATTCC-3'). After

ligation, the sense-strand cDNA was isolated and reconverted to the double-stranded

form by using an oligo (dT)₂₄ primer containing the SP6 promoter sequence and

Sensiscript RT. A 2 µl aliquot of the cDNA mixture was used for amplification by

two-step PCR (15 + 13 cycles). Amplicons were purified on a PCR purification column

(QIAGEN) according to the manufacturer's directions.

2. 4. 2. Target preparation and hybridization

An Ampliscribe transcription kit (Epicentre, Madison, WI) was used according to the manufacturer's directions to produce sense-strand cRNA from the amplified cDNA (100 ng) by in vitro transcription with T7 RNA polymerase. A 5 µg volume of amplified mRNA was mixed with 1.8 µg of random nonamer (TAKARA BIO, Ohtsu, Shiga, Japan), incubated at 70°C for 10 min, and then chilled on ice. The RT reaction was performed by adding 5 µl of 5× reaction buffer (Invitrogen), 2.5 µl of 0.1 M DTT, and 2.5 µl of dNTP mixture (5 mM of each dATP, dGTP, dTTP and 1.5 mM dCTP) to the RNA/primer mixture. Next, 5 µl of 92.5 TBq/mmol [α -³³P] dCTP (Amersham), 22.5 U of RNaseOUT (Invitrogen), and 150 U of the Superscript II RT (Invitrogen) was added, and the samples were incubated for 90 min at 43°C. The reaction was stopped by adding 5 µl of 0.5 M EDTA, and then the solution was incubated at 65°C for 10 min. RNA was removed by adding 60 U of RNase H (TAKARA) and 70 U of RNase A (Sigma) and incubating for 10 min at 37°C. Radiolabeled cDNA targets were purified with a Minielute PCR purification kit (QIAGEN) and heat-denatured (100°C, 5 min) before adding to the hybridization solution.

The cDNA array analysis was performed with an mKIAA cDNA array [12, 48], which contains a total of 810 mouse brain full-length cDNAs spotted on a nylon membrane. The array consists of 64 known cDNAs and 746 cDNA homologues of human genes cloned by the Kazusa DNA Research Institute, and they are described as genes whose biological function is unknown (<http://www.kazusa.or.jp/rouge/index.html>). Eighteen microarray hybridizations (2 genotypes×3 animals×3 amygdalar subdivisions) were performed.

The cDNA arrays were incubated for 1 h at 68°C in 800 µl of PerfectHyb hybridization solution (TOYOBO, Osaka, Japan), and then hybridized with radiolabeled cDNA targets ($\sim 1 \times 10^7$ cpm) overnight at 68°C in hybridization solution containing 50 µg of mouse Cot-1 DNA (Amersham) and 2 µg of polyadenylic acid (Amersham). Membranes were washed twice in 2×SSC containing 1% SDS at 68°C for 15 min, and then at 68°C for 30 min with 0.2×SSC containing 1% SDS. The washed membranes were vacuum-dried for 60 min at 80°C with a gel drier (Bio-Rad, Hercules, CA), and a BAS-SR imaging plate (Fuji film, Tokyo, Japan) was exposed to them for 70 hr. Finally, the screens were scanned with a BAS-5000 image analyzer (Fuji film) at 25-µm

resolution.

2. 4. 3. Microarray data analysis

Hybridization signals were converted into raw intensity values by using Array Gauge software (Fuji film, Ver.2.0), and the data obtained were transferred to MS Excel spreadsheets. The data filtering and statistical analysis were performed within Excel, and the intensity value of the background (non-spotted area) was subtracted from intensity of the individual cDNA spots for each membrane. The data for five cDNAs were excluded from further analysis, because their intensity was below the background level on at least one membrane.

Z-score transformation methods have been used in microarray data analysis [3, 45]. The Z-score transformation corrects data internally within a single hybridization, and intensity values for individual genes are expressed as units of SD (standard deviation) from the normalized mean of zero. Comparison across microarray results from multiple experiments is then performed on equivalently transformed data. The raw data for the 805 retained genes were log₁₀-transformed and normalized by Z-score transformation [5] for each membrane, and the normalized data (n=3) for each amygdalar subdivision

were tested by one-way analysis of variance (ANOVA) to compare *fyn*(-/-) and *fyn*(+/-) mice. The FDRalgo program available at <http://www.math.tau.ac.il/~ybenja> was used to adjust *p*-values by the false discovery rate (FDR) controlling procedure [32].

2. 5. In-situ hybridization histochemistry

2. 5. 1. Preparation of the cRNA probe

PCR primers (forward primer:

5'-CATACGATTTAGGTGACACTATAGAATACTGATGTCCAACAGCACCATAG-3',

reverse primer:

5'-CATACGTAATACGACTCACTATAGGGCGAGCTCACTGCAAAGGCGTAA-3')

were constructed to amplify mKIAA1577 cDNA [31] comprising a 1451 bp fragment that contains the SP6 RNA polymerase promoter at the 5'-end and the T7 RNA polymerase promoter at the 3'-end. Full-length mKIAA1577 cDNA [31] was used as a template for the PCR with rTaq (TAKARA) according to the manufacturer's instructions. The amplified cDNA was purified with a Minielute Gel Extraction kit (QIAGEN) from agarose gel after electrophoresis. The in vitro transcriptions from the cDNA fragment were performed with the DIG RNA labeling kit (Roche, Basel, Switzerland). SP6 and

T7 RNA polymerase generated the sense- and antisense-cRNA probes, respectively.

2. 5. 2. Staining procedures

Coronal sections (8 μ m thick) of mouse forebrain through the amygdala (bregma -1.70 mm) were cut on a cryostat and thaw-mounted on silane-coated slides. Three cryostat sections were prepared per animal at 50 μ m intervals through the rostro-caudal levels for each amygdalar subdivision. The sections were air-dried, surrounded with a liquid-repellent slide marker pen (PAP pen, Daido Sangyo, Tokyo, Japan), and stored at -80°C until processing for in situ hybridization histochemistry. Hybridization and detection procedures were performed as described elsewhere [35] with slight modification. Sections were used for the hybridization without a prehybridization procedure. Hybridization with hybridization buffer containing the probe (300 ng/ml) was carried out for 16 h at 65°C in a chamber humidified with 5×SSC/50% formamide without cover-slipping the slides. No labeling was detectable in the control sections, which were hybridized with the sense cRNA probe (data not shown).

2. 5. 3. Quantitative measurement of mKIAA1577 mRNA and data analysis

Micrographs of sections subjected to in situ hybridization were captured and

digitized with a CCD camera mounted on a light microscope. The digitized images were processed with US National Institutes of Health Image software (version 1.63). The hybridization signals were quantitatively analyzed according to the previously reported protocol [1, 6], with slight modifications. The means of the hybridization signals (optical densities) of three sections at 50 μ m intervals through the rostro-caudal levels for each amygdalar subdivision were used for quantitative measurement for each animal (n=3). Pixels in each subdivision were counted when the density values were more than 2.0 S.D greater than the background values of the section. Data were reported as relative integrated density in arbitrary units divided by total area, and they reflect both the signal intensity and the numbers of pixels above background. Normalized data were analyzed by a two-factor ANOVA.

3. Results

In the present study we investigated gene expression profiles by means of cDNA microarrays in the three amygdalar subdivisions of *fyn*(-/-) and *fyn*(+/-) mice 1 hr after NMDA administration, and the profiles in each subdivision were compared between the

two genotypes. Three mice of each genotype were used to eliminate variance among subjects and to obtain more reliable profiles within the genotype-matched group. The hybridization signals of each gene were obtained by calculating the average of the values obtained from three mice in each experimental group.

First, we focused on the changes in expression profiles between the genotypes in the three subdivisions. The scattergrams of the expression levels (Fig. 1) showed more than a 2-fold difference in expression of about 5% of the genes between the *fyn*(-/-) and *fyn*(+/-) mice in all subdivisions. The list of gene expression data showing more than 2-fold differences is shown in Table 1. Expression of one of these genes, the cAMP responsive element modulator (CREM) gene in the lateral/basolateral nuclei, showed a 2.2-fold difference between the genotypes. CREM gene expression has been reported to show a significant change in the central nucleus of the amygdala of the rat following LiCl administration, an unconditioned stimulus in the conditioned taste aversion training [16]. This change in CREM gene expression following the manipulation of neural activity in the amygdala confirms the reliability of the array analysis of amygdalar gene expression in the present study.

Next, we analyzed differences between the expression level of each gene in the *fyn*(-/-) and *fyn*(+/-) mice 1 hr after NMDA administration. An ANOVA between the genotypes of the data for the 805 cDNAs was performed by the FDR controlling procedure to avoid false identification (type-one error) [32]. We adjusted the *p*-value so that FDR was ≤ 0.05 , and based on this criterion found a highly statistically significant difference between the *fyn*(-/-) and *fyn*(+/-) mice in the expression level of one cDNA, mKIAA1577, in the amygdalar central nucleus alone [$F(1,4)=523.81$, $p=2.16 \times 10^{-5}$]. The average Z-score ($\pm$ S.E.M. $n=3$) of mKIAA1577 hybridization signals in the amygdalar central nucleus was -1.04 ± 0.14 in the *fyn*(-/-) mice and 0.55 ± 0.14 in the *fyn*(+/-) mice.

The functional significance of this clone is not known at present. The structural information predicts the possible significance of this molecule. mKIAA 1577 corresponds to Zinc finger SWIM domain containing protein 6; clone name mbh02526, EMBL accession number, AK122528; gene name, Zswim6. The predicted amino acid sequence of mKIAA1577 consisted of 827 amino acid residues having a molecular mass of 93 KDa, and it was highly conserved between mice and humans, with 94%

identity. Though the predicted amino acid sequence showed no homology with any proteins whose function was known, zinc finger SWIM domain containing protein 4 (mFLJ00044, 733 amino acid, 53% homology) and zinc finger SWIM domain containing protein 5 (mKIAA 1511, 1207 amino acid, 71% homology) have been found as the homologue of mKIAA 1577. A motif search revealed that the cDNA had a predicted zinc-finger motif, SWIM [22], in its 5'-terminal portion. No motifs predictive of membrane spanning were found in the cDNA sequence.

As above mentioned, the strategy to survey novel genes in the downstream of Fyn-mediated signal transduction was based on the strict criteria of statistical significance to select the differentially expressed genes between the *fyn*(-/+) and *fyn*(-/-) mice. Among the apparent difference in the expression between the genotypes, only mKIAA1577 met the standard of selection in the present study.

Based on the results of the cDNA microarray analysis, we performed an in situ hybridization analysis of mKIAA1577 mRNA in the amygdala of the *fyn*(-/-) and *fyn*(+/-) mice 1 hr after NMDA administration. The mRNA levels 1 hr after PBS administration were compared between the *fyn*(-/-) and *fyn*(+/-) mice at the same time to

determine whether the difference in mRNA levels depended on both the genotypes and NMDA administration. The hybridization signal was stronger in the central nucleus than in other subdivisions in the PBS group of both genotypes (Fig. 2a, c), and no significant difference was observed between the *fyn*(-/-) and *fyn*(+/-) mice in the central nucleus of the PBS groups (Fig. 2a-d). mKIAA1577 mRNA expression in the central nucleus of the NMDA group had decreased much more in the *fyn*(-/-) mice (Fig. 2g, h) than in the other groups (Fig. 2a-f).

In order to make clearer the difference in the mKIAA1577 expression among each amygdalar subdivision, we have performed the quantitative in situ hybridization analysis of the mKIAA1577 expression 1 hr after NMDA administration (Fig. 3), showing that the intense expression and also NMDA-induced difference in the expression between genotypes are restricted to the central nucleus among the amygdalar subdivisions. Statistical analysis of the hybridization signal revealed that the mKIAA1577 mRNA level was significantly lower in the central nucleus of the *fyn*(-/-) mice than those of the *fyn*(+/-) and *fyn*(+/+) mice (Fig. 3). This finding was highly

consistent with the data obtained in the cDNA array analysis. The difference between the effect of NMDA administration on the *fyn*(-/-) and other genotypes was obvious.

To confirm that the reduction of the mKIAA1577 mRNA level is mediated by the NMDA receptor activation, a competitive NMDA receptor antagonist, D-AP-5 was injected prior to the NMDA administration. The expression level was compared among the groups of NMDA-, PBS (vehicle)-, and D-AP-5 plus NMDA-administration in the *fyn*(-/-) and *fyn*(+/-) genotypes (Fig. 4). NMDA administration significantly reduced the mRNA level in the *fyn*(-/-) mice compared to PBS administration. Pretreatment with D-AP-5 markedly reversed the NMDA-induced reduction of the mRNA level in the *fyn*(-/-) mice. NMDA administration apparently reduced the expression level of the mRNA in the *fyn*(+/-) mice, but the reduction was not statistically significant.

In order to examine the mode of mKIAA1577 expression in relation to the emotional neural circuit, in which c-Fos expression following fear response is enhanced in Fyn-deficient mice [15], quantitative in situ hybridization analysis was performed in the non-amygdalar brain regions involved in the emotional system such as the hypothalamus (paraventricular nucleus; PVN and lateral hypothalamic area; LH) and

midbrain periaqueductal gray; PAG as the targets for the amygdalar projections as well as the thalamus (ventral posterolateral nucleus; VPL and medial geniculate body; MGB) as the input system of the emotion-related sensory information and the hippocampus (CA1-3 and dentate gyrus; DG) as the contextual memory system. Consequently, the % expression level of mKIAA1577 (integrated density/area sampled) in these areas of the wild type mice was as follows in comparison to the amygdalar central nucleus as 100%; PVN, $22.7 \pm 2.8\%$; LH, $21.6 \pm 1.1\%$; PAG, $18.3 \pm 1.2\%$; VPL, $25.2 \pm 3.3\%$; MGB, $28.6 \pm 7.1\%$; CA1-3, $17.9 \pm 1.2\%$; DG, $5.1 \pm 0.4\%$. Thus, the mKIAA1577 expression in the non-amygdalar regions in the emotional circuit was much lower than that in the amygdalar central nucleus. Furthermore, each non-amygdalar region exhibited no statistically significant changes in the expression level of mKIAA1577 1 hr after the NMDA administration in comparison to the basal level of each genotype (data not shown). Little difference in the mode of expression was also noticed in the above each non-amygdalar area between the genotypes (not illustrated), suggesting that mKIAA1577 plays a role mainly in the central amygdalar nucleus.

The time course of the expression of mKIAA1577 in the amygdalar central nucleus

after NMDA administration was examined in the *fyn*(+/+), *fyn*(+/-) and *fyn*(-/-) mice. As represented in Fig. 5, the expression level reached the peak 2hr after NMDA administration and the pattern was very similar between the three genotypes. However, there was a temporal shift in mKIAA1577 induction, with low levels in *fyn* (-/-) mice at 1hr and 2hr, but slightly increased at 3hr after injection.

4. Discussion

The cDNA array approach in the present study identified a gene, mKIAA1577, that was downregulated in the amygdalar central nucleus of *fyn*(-/-) mice 1 hr after NMDA administration. The data obtained from direct comparison of hybridization signals from the microarray as raw data are represented in the Table 1. Thus, many genes appear to be differentially expressed between the genotypes, and these genes are the candidates to be further examined in detail. Therefore, the statistical significance of the differential expression was tested by one-way analysis of variance (ANOVA) (n=3) by the false discovery rate controlling approach by setting the strict statistical criteria. Consequently, only mKIAA1577 clone met the criteria in regard to the genotype difference, amygdalar

subdivision and NMDA administration. The function of the most clones in the mKIAA cDNA array has not been elucidated including mKIAA1577, and the structural characteristics of mKIAA1577 suggested the significance in the control of neuronal activity through intracellular signal transduction. Thus, we have performed further analysis to confirm and extend the result of cDNA array analysis with quantitative in situ hybridization study in regard to the NMDA-induced change in the expression of this clone.

In situ hybridization analysis 1 hr after NMDA administration confirmed a much lower level of mKIAA1577 mRNA in the central nucleus of the *fyn*(-/-) mice than of the *fyn*(+/-) mice. This finding suggested that NMDA receptor activation induced a different response in the *fyn*(-/-) and *fyn*(+/-) mice in regard to the regulated expression of mKIAA1577 in the central nucleus. During neural transmission, Fyn is co-localized with NMDA receptors at the postsynaptic density [41], and it is involved in the regulation of receptor channel activity [13] through tyrosine phosphorylation of the subunits [28, 39]. Thus, it is likely that Fyn regulates mKIAA1577 gene expression through control of NMDA receptor function and that loss of the kinase reduces the

mKIAA1577 mRNA level following NMDA receptor activation at the specific time.

The results confirmed little difference due to the gene dosage between *fyn*(+/-) and *fyn*(+/+) in regard to the mode of mKIAA1577 expression in response to NMDA administration.

The in situ hybridization data also indicated no statistically significant difference between the PBS-administered *fyn*(-/-) mice and *fyn*(+/-) mice in mKIAA1577 mRNA level. This result showed that Fyn deficiency has hardly any effect on maintenance of the mKIAA1577 mRNA level, and that NMDA receptor activation is essential to downregulation of the mKIAA1577 mRNA level in the *fyn*(-/-) mice, because prior administration of the NMDA receptor antagonist D-AP-5 strongly inhibited the NMDA-induced downregulation of mKIAA1577 mRNA level in the *fyn*(-/-) mice.

It is widely accepted that the amygdala is involved in the processing of emotion-related information and that the central nucleus is the output system to effector systems involved in the expression of emotional behavior, such as the fear response and autonomic responses [17]. Our previous study showed enhanced NMDA-induced c-Fos expression in the central nucleus of Fyn-deficient mice [9]. The results of c-Fos study

strongly suggest that the response of the central nucleus to excitatory stimuli is much more enhanced in the mutant. Since the excitatory stimulus decreased the mKIAA1577 mRNA level in the central nucleus of the mutant mice at the specific time, 1 hr after NMDA administration in the present study, the enhanced downregulation of mKIAA1577 mRNA may also be involved in the neural basis for the defective emotional response of Fyn-deficient mice. Expression of some genes is known to decrease after glutamate receptor activation. The mRNA level of the glutamate transporter GLAST, which removes glutamate from the synaptic cleft, has been reported to be downregulated by activation of AMPA receptors in glial cells [20], and expression of ligatin mRNA, a marker of membrane-vesicle transport systems, is also downregulated by NMDA receptor activation [10]. These features of the mRNA expression profile may provide clues for investigation of mKIAA1577 function.

Glutamate receptor activation has been reported to induce expression of a series of immediate early genes (IEGs) such as c-Fos [18, 43]. The entire time course of mKIAA1577 expression in the central nucleus after NMDA administration exhibited very similar pattern between *fyn* (+/+), *fyn* (+/-) and *fyn* (-/-), reaching the peak at 2hr and

without difference in the height of the peak. Taking into consideration that c-Fos expression in the central nucleus of *fyn*(-/-) mice is significantly upregulated relative to *fyn*(+/-) mice at the peak [9], the expression of mKIAA1577 and c-Fos may be regulated differentially.

The biological function of mKIAA 1577 is unknown, because the predicted amino acid sequence of the cDNA shows no homology with any other proteins. A database scan of the deduced amino acid sequence has shown that the amino-terminal end contains a SWIM zinc-finger motif [22] consisting of a CxCx(n)CxH pattern of predicted zinc-chelating residues. The motif has been identified in a variety of prokaryotic and eukaryotic proteins, and is predicted to possess DNA-binding capacity and various functions in protein-protein interactions. Especially since the vertebrate mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase 1 (MEKK-1), which is part of the MAPK cascade, contains a SWIM domain at the N-terminal region and interacts with other MAPK cascade components, such as 14-3-3 proteins and c-Jun N-terminal kinase, through the N-terminal SWIM domain [8, 22], mKIAA1577 protein may be involved in the control of the interaction between

MEKK-1 and other members of the MAPK signaling cascade. Moreover, since the MAPK signal transduction system is thought to play an important role as the molecular mechanism involved in fear memory consolidation in the amygdala [36], Fyn-deficiency- and NMDA-administration-dependent downregulation of mKIAA1577 gene expression may be related to the altered signal transduction in the amygdala of Fyn-deficient mice.

The expression of myelin-associated genes was found to be downregulated in the adult hippocampus of the *fyn*(-/-) mice by means of cDNA microarray analysis [7]. The downregulation of specific genes in the hippocampus and amygdala of the Fyn-deficient mice suggests that these genes are the transcriptional targets of Fyn-mediated signal transduction system, and some common mechanisms might operate to control the expression of these genes in the downstream of Fyn both in the hippocampus and amygdalar central nucleus. Some delay in the time course of NMDA-induced mKIAA1577 gene expression as noticed in the *fyn* (-/-) mice (Fig. 5) suggests the delay in the transcriptional initiation of mKIAA1577 gene in response to the NMDA administration. Taking into account that Fyn is involved in the control of brain-specific

gene expression such as myelin basic protein at the transcriptional [42] and posttranscriptional [21] levels, it is considered that Fyn is involved in the transcriptional control of mKIAA1577 gene and the time course of the expression might be delayed due to the defective transcriptional control due to Fyn deficiency. Reportedly, Fyn is also involved in the control of receptor-mediated activation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) system that leads to transcriptional activation of specific genes in the vascular smooth muscle cells [44]. Accordingly, Fyn might control the expression of mKIAA1577 gene in the brain similarly through such signal transduction pathways as modify the transcriptional machinery, since JAK/STAT system in the neuron has been also reported to be controlled by tyrosine-phosphorylation cascade [11].

Some experimental approaches to elucidate the functional significance of mKIAA1577 in regard to the amygdalar functions could be proposed taking into account the restricted expression of this clone in the central nucleus among the amygdalar subdivisions. Some insight into the function of mKIAA1577 could be obtained from the influence of fear conditioning and extinction [19] on the mKIAA1577 expression in the

amygdala. Diazepam, an anxiolytic agent has been known to specifically induce c-Fos in the central nucleus, although the mechanism of this paradoxical action is not known at present [2, 23]. Accordingly, some clue might be provided by the expression pattern of mKIAA1577 in comparison with c-Fos expression after diazepam administration. Central nucleus is also involved in the ethanol addiction [33], and ethanol administration activates Fyn and enhances the tyrosine phosphorylation of NMDA receptor NR2B subunit [27]. Thus, the expression of mKIAA 1577 following the development of ethanol dependence might also suggest the functional implication. Furthermore, zinc is released in the amygdala concomitant with glutamate and control the emotional response [40]. The zinc-binding domain of this clone might interact with the increased zinc in the central nucleus and be involved in the control of transcriptional activity. Zinc chelating agent, diethyldithiocarbamate [40] is expected to influence the mKIAA1577 expression in the amygdala. Another approach to stereotactically transfected the RNAi of mKIAA1577 into the central nucleus and examine the effect of the knock down of mKIAA1577 expression on the emotion-related behavior such as fear conditioning, diazepam action and ethanol tolerance would be useful for the elucidation

of the function of this clone.

No genes in the medial nucleus or lateral/basolateral nuclei were expressed with statistically significant difference between *fyn*(-/-) and *fyn*(+/-) mice. There are at least two possible explanations for this finding. First, none of the other genes tested in this study may be affected by Fyn deficiency in these subdivisions, and, second, the change in gene expression may occur within a restricted subpopulations of cells in these subdivisions of the *fyn*(-/-) mice. The difference may be not enough to overcome the variance in expression level among subjects to produce a statistically significant difference. To investigate the above possibility, it will be necessary to perform a cDNA array analysis of restricted neuronal subsets, such as c-Fos-positive cells, in these subdivisions. Our cDNA array analysis in conjunction with laser microdissection and PCR amplification techniques may be helpful in investigating differences in gene expressions between such neuronal subsets.

Many genes appear to be differentially expressed between the genotypes in the amygdalar subdivisions as shown in Table 1, and these genes are the candidates to be further examined in detail, although the changes in the expression levels showed no

statistical significance except for mKIAA1577. The classification of the genes into several groups suggests their functional implication in the NMDA-induced changes in gene expression and neuronal activity. Some clones which are presumed to be involved in such neuronal control were classified into three categories, i.e. 1) clones involved in the control of gene expression at the transcriptional or translational level, 2) those involved in the intracellular signal transduction, and 3) those involved in the neural development and plasticity.

As for the category 1, mKIAA 0127 and 0978 are putative transcription factors and mKIAA0835 and 1802 harbor zinc finger motif. The changes in the expression of these clones might be under the control of Fyn signal following NMDA stimulation and implicated in the transcriptional control of a variety of genes related to emotional neural activity. In the category 2, the clones, mKIAA 0694, 1322 and 1478 encode GTPase activating protein, and mKIAA 1331 is involved in the Wnt signaling pathway. The changes in the expression of these clones under the control of Fyn signal might exert a wide influence on G-protein-coupled signal transduction pathways following NMDA stimulation. In the category 3, mKIAA 0858 and 1400 encode cadherin-related

molecules and mKIAA 0938 is implicated in axonal growth. These genes might be involved in the fear-induced plasticity of synapse and circuit under the control of Fyn signal. Further investigation of the expression of these novel clones in the restricted subsets of amygdalar neurons and related neural circuit following NMDA stimulation would reveal new aspects of molecular mechanisms of emotional control.

Acknowledgments

This study was supported in part by Grants to S. Y. from the Ministry of Health, Labour and Welfare of Japan (research grants 9B-4 and 15B-3 for Nervous and Mental Disorders), the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) Program from Japan Science and Technology Corporation (JST), the Futaba Electronics Memorial Foundation, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (grant 05-32), and the Long-range Research Initiative (LRI) by Japan Chemical Industry Association (JCIA).

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Figure legends

Fig. 1. Scattergram analysis of microarray data. The average value of gene expression for each of the 805 cDNAs was compared between Fyn-deficient and control mice in the central nucleus (a), medial nucleus (b), and the lateral/basolateral nuclei (c). The two parallel lines indicate a 2-fold difference in transcript levels between the two genotypes. The value for mKIAA1577 in (a) is circled.

Fig. 2. Representative mKIAA1577 mRNA expression in the amygdalar subdivisions 1h after PBS (a,c) and NMDA (e,g) administration to *fyn*(+/-) (a,e) and *fyn*(-/-) (c,g) mice. Higher magnification of the central nucleus after PBS (b,d) and NMDA (f,h) administration to the *fyn*(+/-) (b,f) and *fyn*(-/-) (d,h) mice. BLA/LA, basolateral and lateral nuclei; BM, basomedial nucleus; CeA, central nucleus; Co, cortical nucleus; MeA, medial nucleus. Scale bars: a, c, e, g=1 mm; b, d, f, h= 0.2 mm.

Fig. 3. Quantitative analysis of mKIAA1577 mRNA level 1h after NMDA administration in the amygdalar subdivisions of *fyn*(+/+), *fyn*(+/-) and *fyn*(-/-) mice.

Statistically significant downregulation in the mKIAA1577 expression is evident in the central nucleus of the *fyn*(-/-) mice in contrast to the *fyn*(+/+) and *fyn*(+/-) mice. Each value represents the mean $\pm$ S.E.M. (n=3). **P<0.01.

Fig. 4. Quantitative analysis of mKIAA1577 mRNA level in the central nucleus of the *fyn*(-/-) and *fyn*(+/-) mice 1h after PBS, NMDA, and D-AP-5 plus NMDA administration.

A two-way ANOVA indicated a significant main effect of treatment [$F(2,12)=12.53$, $p<0.002$] and a significant interaction between treatment and genotype [$F(2,12)=4.325$, $p<0.05$]. The simple effect of genotype was significant for the NMDA-administered animals [$F(1,4)=7.75$, $p<0.05$], but was not for either the PBS-administered [$F(1,4)=1.66$, $p>0.2$] or D-AP-5 + NMDA-administered [$F(1,4)=0.87$, $p>0.4$] animals.

The analysis of the simple effect of treatment on each genotype revealed that the mRNA level of the NMDA-administered *fyn*(-/-) mice was significantly lower than that of the PBS- administered mice [$F(1,4)=24.9$, $p<0.01$] and the D-AP-5 + NMDA-administered [$F(1,4)=31.1$, $p<0.01$] mice. Each value represents the mean $\pm$ S.E.M. (n=3). *P<0.05;

**P<0.01.

Fig. 5. Time course of the expression of mKIAA1577 after NMDA administration in the amygdalar central nucleus of *fyn*(+/+), *fyn*(+/-) and *fyn*(-/-) mice. The expression of mKIAA1577 reached the peak 2h after NMDA administration similarly in the three genotypes. However, there was a temporal shift in mKIAA1577 induction, with low levels in *fyn* (-/-) mice at 1h and 2h, but slightly increased at 3h after injection. Each value represents the mean±S.E.M. (n=3).

Figure

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Table 1

Genes whose hybridization signals showed more than 2-fold change between *fyn*(+/-) and *fyn*(-/-) mice in the cDNA microarray analysis

Subdivisions	mKIAA# or gene name	Descriptions	Fold change: <i>fyn</i> (-/-)/ <i>fyn</i> (+/-)
central nucleus	0127	Sertad2 protein	↓ 3.70
	1577	Zinc finger SWIM domain containing protein	↓ 2.13
	0207	Grb10 protein	↑ 2.02
	1331	Sentrin-specific protease 2	↑ 2.00
lateral/basolateral nuclei	1031	ubiquitous tetratricopeptide containing protein	↓ 2.86
	0694	Dedicator of cytokinesis protein	↓ 2.44
	0548	Attractin precursor	↓ 2.38
	0316	unnamed protein product	↓ 2.27
	1698	unnamed protein product	↓ 2.22
	0442	Auts2 protein	↓ 2.17
	Crem	CAMP responsive element modulator	↓ 2.17
	1013	GRP1-binding protein	↓ 2.17
	1802	unnamed protein product	↓ 2.13
	1322	TBC1 domain family	↓ 2.13
	0172	Ankyrin repeat domain protein	↓ 2.13
	1891	Ubiquitin carboxyl-terminal hydrolase 38	↑ 2.09
medial nucleus	1500	Fras1 protein	↓ 2.13
	1017	Hermansky-Pudlak syndrome 5 protein homolog	↑ 3.20
	0938	neuron navigator 3	↑ 2.98
	1455	odz, odd Oz/ten-m homolog 3	↑ 2.82
	3010	unnamed protein product	↑ 2.50
	1400	OL-protocadherin isoform	↑ 2.48
	0945	Bahd1 protein	↑ 2.42
	0858	unnamed protein product	↑ 2.40
	0532	similar to Cohen syndrome 1 protein isoform 1	↑ 2.40
	1316	similar to CG2747-PA	↑ 2.36
	1122	Probable ATP-dependent DNA helicase	↑ 2.24
	1577	Zinc finger SWIM domain containing protein	↑ 2.20
	1567	Eukaryotic translation initiation factor 2C 4	↑ 2.17
	1478	Rac GTPase-activating protein 1	↑ 2.14
	1294	FERM domain containing 4A	↑ 2.13
	0774	unnamed protein product	↑ 2.12
	0835	neural zinc finger protein NZF-2b	↑ 2.12
	1626	2810441C07Rik protein	↑ 2.09
	0156	tumor-rejection antigen SART3	↑ 2.05
	0978	Putative Polycomb group (PcG) protein	↑ 2.04
	1511	Zinc finger SWIM domain containing protein	↑ 2.04
	1329	VPS10 domain-containing receptor SorCS2 precursor	↑ 2.02