Neurochondrin Negatively Regulates CaMKII Phosphorylation, and Nervous System-specific Gene Disruption Results in Epileptic Seizure

Neurochondrin is a novel cytoplasmic protein and possibly involved in neurite outgrowth, chondrocyte differentiation, and bone metabolism. Our previous trial in disclosing its role by the loss of function in mice failed because of the lethality in utero. In this study, we eliminated the neurochondrin gene expression preferentially in the nervous system by the conditional knockout strategy. Our results showed that neurochondrin is a negative regulator of Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation and essential for the spatial learning process but not for the differentiation or neurite outgrowth of the neuron. In addition, the nervous system-specific homozygous gene disruption resulted in epileptic seizure.

EXPERIMENTAL PROCEDURES

Targeting Vector—The region covering the neurochondrin promoter and exon1, identical to the region encompassed by NaeI and AccI sites, was flanked by KpnI-loxP sequences (5'-AAAGTACCATGTCGT-AATATGATGCTACAGGTATTTATG-3') and neo-loxP cassette was placed downstream of the floxed promoter sequence, with 3.1- and 5.94-kbp homology arms placed next to it.

Generation of the Mice—The targeted embryonic stem (ES) cells were aggregated with eight cell-stage embryos of BSF1, a male sterile strain. A chimeric male mouse was obtained and intercrossed with nestin-cre transgenic female for germ line transmission.

PCR Analysis—An intact floxed allele was detected using primers A (5'TCTCTGCGGCGTGGATCAGC-3') and B (5'TCCGCCAAGCCCCG-GATCGC-3'). In the 20-μl reaction, containing 2.5 mM MgCl₂, 0.25 mM deoxyribonucleotide triphosphates, 1 unit of Taq polymerase (Takara), and 1 μM of the primers, PCR was carried out at 96 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 68 °C for 1 min, and a final extension at 72 °C for 10 min. The primer set amplifies 140- and 100-bp fragments for the targeted and wild-type alleles, respectively. A completely recombined allele was detected using primers A and C (5'ACAGACGGTCAAGTTCG-3'). This reaction and the thermal cycle are the same as those for the primers A and B. The set amplifies 200-bp and 2-kbp fragments for the recombined and intact floxed alleles, respectively.

Lysates and Western Blot Analysis—Whole brain and chondral tissues were homogenized with M2 lysis buffer (7) and rotated for 3 h at 4 °C, centrifuged, and then the supernatants were collected. Hippocampus tissue was homogenized with lysis buffer (20 mM HEPES, 5 mM MgCl₂, 5 mM EGTA, 100 mM NaCl, 10% glycerol, 1 mM dihydrotestol, 1 mM NaF, 1 mM Na₃VO₃, 0.1% Nonidet P-40, and protease inhibitors) by the procedure described above. The transfected cells were lysed by 30 μl of SDS sample buffer 27 h after the transfection. 10 μg of the cell lysates, 5 μg of whole brain lysates, and 20 μg of cartilage and hippocampus lysates were applied to the Western blot analysis.

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Minori Dateki‡, Takuro Horii§, Yoshitoshi Kasuya†, Reiko Mochizuki‡, Yasumitsu Nagao§, Junji Ishida‡, Fumihiro Sugiyama‡, Keiji Tanimoto‡, Ken-ichi Yagami, Hiroshi Imaï§, and Akiyoshi Fukamizu‡**
From the *Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan, §Laboratory of Reproductive Physiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, ¶Department of Biochemistry and Molecular Pharmacology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan, and ♥Laboratory Animal Source Center, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan

Neurochondrin (NC) is a cytoplasmic protein expressed in the neuronal, chondral, and bone tissues (1, 2). Expression of the gene in a cultured hippocampus slice is increased with tetraethylammonium-induced long-term potentiation (1). Forced neurochondrin expression in neural cell line Neuro2A leads to stimulated neurite outgrowth (1). Furthermore, neurochondrin protein localization in dendrite and somatic regions of neurons in the developing cerebellum is suggestive of the function associated with the dendrite outgrowth (3). In bone tissues, the neurochondrin gene is expressed in osteoblasts and osteocytes, and conditioned media of COS7 with forced neurochondrin expression induce bone marrow cells to absorb bone matrix (2). These facts demonstrate its possible involvement in bone metabolism. The finding that neurochondrin protein associates with semaphorin4C (a transmembrane-type semaphorin) and phosphatidic acid (a possible second messenger) indicates its role as a signal molecule (4, 5). Besides all these conceivable functions, our previous trial in disclosing its in vivo role by conventional loss of function strategy in mice failed because of its lethality in utero (6). In addition, hemizygous mutant mouse exhibited no obvious abnormality.

In this study, we eliminated the neurochondrin gene expression preferentially in the nervous system by conditional knockout strategy. Our results showed that neurochondrin is a negative regulator of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Thr-286) phosphorylation and essential for the spatial learning process but not for the differentiation or neurite outgrowth of the neuron. In addition, nervous system-specific homozygous gene disruption resulted in epileptic seizure.

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phospho-CaMKII (Thr-305) (Chemicon), CaMKII (Stressgen), and microtubule-associated protein 2 (MAP2) (Neomarkers) were used in the dilution of 1:1000 with 1% skim milk/TBS (1% Tween-20).

Histological and Immunohistochemical Analysis—4% paraformaldehyde-fixed, paraffin-embedded brains from 2-month-old NClox/loxCre and wild-type littermates were cut into 7-μm serial sections and applied to Bodian’s staining (8). 4% paraformaldehyde-fixed whole brain of 10-month-old epileptic NClox/loxCre and age matching NClox/wtCre mice were sliced into 30-μm serial sections, and then applied to the immunostaining with primary antibody, monoclonal anti-GFAP (glial fibril-
lary acidic protein) antibody (Sigma), monoclonal anti-S100β antibody (Sigma), or polyclonal anti-doublecortin antibody (Santa Cruz Biotechnology) by the procedures described previously (9, 10). The sections were stained with hematoxylin or 4’6-diamidino-2-phenylindole (blue fluorescence). For Timm’s staining, brains were soaked in 0.4% Na₂S·9H₂O in phosphate buffer (pH 7.4) at 4 °C for 2 h and then fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight. After overnight soaking in 30% sucrose, the frozen brains were cut into 30-μm serial sections and applied to the staining by the procedure described previously (11).

**Motor Function Analysis**—A wire-hanging test was performed as described previously (12). The rod working test was performed using an iron rod 70 cm long and 90 mm in diameter wrapped with a masking tape. The experiment was performed as described elsewhere (13).

**Morris Water Maze**—The pool was circular with a diameter of 90 cm and a height of 35 cm. The platform was 18 cm high and 10 cm in diameter. The pool was filled with 25 °C water to a depth of 19.5 cm. The center of the columnar platform was set at a distance of 15 cm from the edge of the pool. The training was repeated for seven days, three sessions/day. The experimental procedure is described elsewhere (14). In the probe test, the swimming paths were recorded for 1 min.

**Calcineurin Assay**—Calcineurin assay kit (Calbiochem) was used for determination of phosphatase activity following the manufacturer’s instructions. The protein serine/threonine phosphatase assay kit (Calbiochem) was used for determination of phosphatase activity following the manufacturer’s instructions. The protein serine/threonine phosphatase assay kit (New England Biolabs) was used for determination of phosphatase activity following the manufacturer’s instructions.

**RESULTS AND DISCUSSION**

**Generation and Basic Characterization of the Mutant Mice**—To avoid early embryonic death, we generated the neural tissue-specific neurochondrin-deficient mice by the Cre-loxP system. We have previously identified the promoter and the cell type-specific regulatory regions of the mouse neurochondrin gene (15). Because functional importance of the semaphorin4C or phosphatidic acid binding domains in the neurochondrin protein has not yet been fully investigated (4, 5), we decided to target the promoter as well as the regulatory region for the Cre-loxP-mediated deletion. In the targeting vector, we floxed the 1.4-kbp promoter and regulatory region in the first exon (Fig. 1A), the precise location of which is described in the “Experimental Procedures.” Following linearization, the targeting vector was electroporated into TT2 ES cells, and the homologous recombination event as well as the intactness of the inserted loxP sites were verified by Southern blot analysis of EcoRI- and KpnI-digested tail genomic DNA, respectively. Diagnostic 7.9-kbp EcoRI and 1.4-kbp KpnI fragments were detected by probes A and B, respectively, in correctly targeted ES cells (Fig. 1B). Chimeric male mice, generated by aggregating the targeted ES cells and eight cell-stage embryos of BSF1 (F1 from intrastrain cross-breeding between C57BL6J and SPRET/Ei), were mated with Nestin-cre transgenic females (16) and found no remarkable deficiency, either in the dendrite formation of the cerebellar Purkinje cell or the axon formation of the cerebrum pyramidal cell (Fig. 1C). Using the primers A and B, a 200-ng restriction fragment was amplified from the genomic DNAs of the brains of NClox/wtCre mice was reduced compared with that of the tail (Fig. 1C). Using the primers A and C, a 200-ng DNA product was amplified from the fully recombined allele. The 200-ng DNA product from the genomic DNAs of the brains was increased as compared with that of tail (Fig. 1C).

The neurochondrin gene was considered to participate in the neurite outgrowth or neural cell differentiation (1, 6). We therefore examined the neurite formation in the NClox/loxCre mice and found no remarkable deficiency, either in the dendrite formation of the cerebellar Purkinje cell or the axon formation of the cerebrum pyramidal cell (Fig. 1E, arrowheads). Expression of the differentiation marker of neuron, MAP2, and neuron-specific class III β-tubulin in the brains of NClox/loxCre mice did not differ from that of NClox/loxCre and wild-type littermates (Fig. 1F). We also evaluated motory ability, which is reflective of developmental abnormality in the nervous system, in NClox/lox Cre mice by rod-walking and wire-hanging tests and found no significant differences between 2–3-month-old NClox/loxCre, NClox/wtCre, and wild-type mice (Fig. 1G). These findings dem-
onstrated that the neurochondrin gene played no essential role in the neurite outgrowth and neural cell differentiation in the developmental process.

**Learning Defect of the NClox/loxCre Mice**—It is suggested that the rat neurochondrin gene was involved in a hippocampal synaptic plasticity (1). Hence, we investigated the spatial learning ability by Morris water maze test. Because we found no obvious phenotype in conventional hemizygotes (6), NClox/wtCre mice were used as a control in this study. In the training session, NClox/loxCre mice spent more time in searching the submerged platform than NClox/wtCre control mice (Fig. 2A, p < 0.05 on the second day’s session). In the probe test, NClox/wtCre mice spent significantly longer time (45% of the total trail time, p < 0.01) in the A quadrant than in the other quadrants, as expected, because it formerly contained the platform (Fig. 2B). In contrast, time spent by NClox/loxCre mice in the A quadrant was not significantly different from that in the other two quadrants (B, C) and was only significantly different from that in the D quadrant (Fig. 2B). Thus, the neural tissue-specific neurochondrin deficiency in mice resulted in a serious spatial learning defect, indicating its essential role in the process of spatial learning.

**Epileptic Seizure of the NClox/loxCre Mice**—We noticed that some mice displayed epileptic seizure (~24% of NClox/loxCre) when they aged over 6 months (Fig. 3A, supplemental movie). The epileptic seizures were often observed when we placed the mice in the new cages, and in some cases, the fit resulted in death. Seizures are sometimes observed in Alzheimer disease (17). Because motory ability is remarkably impaired in neurodegenerative disease, we therefore analyzed this in the 6-month-old NClox/loxCre mice (17). The scores of NClox/loxCre mice in both wire-hanging and rod-walking tests were not significantly different from those of the control NClox/wtCre mice (Fig. 3B). We found no apoptotic cell death in the 6-month-old NClox/loxCre mouse brain (data not shown), suggesting that the epileptic seizure observed in NClox/loxCre mice was not attributable to the neurodegeneration.
Morphological alteration of the hippocampus is a prominent example of epileptic seizure, because it is observed in animal models of epilepsy and also in human epileptic patients (18, 19, 20). Immunostaining with anti-doublecortin polyclonal antibody revealed that newborn neuroblasts generated from the subgranular zone of dentate gyrus were rather increased in the epileptic NClox/loxCre mouse (Fig. 3C). Likewise, ectopic innervation of the mossy fibers into the stratum oriens in the CA3 region was observed (Fig. 3D, arrows). These findings showed good agreement with the previous report of pilocarpine-induced status epilepticus (11). Furthermore, in the hilus of the dentate gyrus of epileptic NClox/loxCre mice (Fig. 3E), a number of reactive astrocyte-like cells were observed, which was highly immunoreactive to the antibody against GFAP and S100β, an acidic calcium binding protein. Under a high power field microscope, both GFAP- and S100β-expressing cells clearly showed hypertrophic profiles with the thick processes from the large cell body when compared with NClox/wtCre mice. Astrocytes play a major support role for a wide range of neural processes, including neural growth and sprouting, and are observed to be activated in the process of epileptogenesis (18, 19, 20). Taken together, these age-dependent phenotypes are related at least to the progressive neurochondrin deficiency by neural cell turnover, although some other pathophysiological factor possibly affecting the rate of incidence is still unclear.

**Effect of neurochondrin on the CaMKII phosphorylation.**

A, phosphorylation of CaMKII in the NClox/loxCre (non-epileptic) and NClox/wtCre hippocampus lysates. Aliquots (20 μg) from NClox/loxCre and NClox/wtCre hippocampus lysates were pooled (n = 9 for both), and then 20 μg of lysates from the pools were applied to Western blot analysis (left). Relative ratio of CaMKII (Thr-286) phosphorylation (normalized with the total CaMKII protein) is showing stimulated CaMKII phosphorylation (n = 9) (right). B, relative ratio of CaMKII activities in the NClox/loxCre and NClox/wtCre hippocampus. C, decreased CaMKII (Thr-286) phosphorylation in the NIH3T3 cells transfected with NC-pcDNA3. Western blot analysis (left) and relative ratio of phospho-CaMKII (Thr-286)/CaMKII from six experiments (right). D, relative ratio of PP1 (NClox/loxCre, n = 5; NClox/wtCre, n = 3) and PP2A (n = 4) activity in hippocampus. Error bars in the figures indicate S.E. n.s., not significant.

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**Effect of neurochondrin on CaMKII Phosphorylation**—Defects of several components of the signal cascade related to the synaptic plasticity could cause learning defects or epileptic seizure.
(21, 22, 23, 24). We examined the protein level and the phosphorylation status of CaMKII, a critical mediator of synaptic plasticity, in the hippocampus of 8–9-month-old N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre and N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre mice. We used non-epileptic N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre mice for the following assays to exclude the possibility of epileptic seizure-augmented CaMKII activity. Phosphorylation of threonine 286 of CaMKII was remarkably increased in the hippocampus of N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre in comparison to the control mice but not of threonine 305, an inhibitory phosphorylation site (25) (Fig. 4A). In accordance with these results, the activity of CaMKII also increased in the hippocampus of N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre mice (Fig. 4B). Thus, the neurochondrin gene might act as a negative regulator of CaMKII (Thr-286) phosphorylation. To address this notion, we transiently overexpressed the neurochondrin gene in NIH3T3 cells, in which its endogenous gene expression is negligible (15). A significant reduction in the phosphorylation of endogenous CaMKII was clearly shown (Fig. 4C). These results suggest that a decrease in neurochondrin simply leads CaMKII activation in N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre mice. Then, we further examined whether neurochondrin negatively regulates CaMKII phosphorylation through PP1 and PP2A, because it is well established that these phosphatases are the major regulators for CaMKII phosphorylation (26) and involved in synaptic plasticity (27). The activity of PP1 and PP2A in the hippocampus lysates of 8–9-month-old N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre and N\textsuperscript{lox/lox}\textasciitilde\textasciitilde\textasciitilde\textasciitilde Cre mice were compared (Fig. 4D) and showed no significant difference, suggesting that neurochondrin negatively regulates CaMKII, not in the two-phosphatase-dependent manner, but by other direct or indirect pathways existing in the downstream of the neurochondrin. The site-specific manner of neurochondrin for CaMKII (Thr-286) is of much interest but requires further precise investigation.

Transgenic mice carrying the constitutive active form of CaMKII \(\alpha\) subunit show learning defects and long term potentiation abnormality (28, 29). The CaMKII \(\alpha\) subunit-deficient mice display the impairment of long term potentiation and the spatial learning defect (30, 21), and the mice are epileptic (22). Appropriate regulation of CaMKII phosphorylation must be required for normal synaptic plasticity and neural transmission. The phenotypes observed in the tissue-specific neurochondrin-deficient mice, such as learning defects and epileptic seizures, could therefore be attributed to the increase of CaMKII (Thr-286) phosphorylation. The level of neurochondrin gene expression is correlated with the long term potentiation-like synaptic potentiation induced by tetraethylammonium (1). Thus, it is highly plausible that the neurochondrin gene is a negative regulator for CaMKII activity.

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