Neuronal nitric-oxide synthase (nNOS) has a PSD-95/Dlg/ZO-1 (PDZ) domain that can interact with multiple proteins. nNOS has been known to interact with PSD-95 and a related protein, PSD-93, in brain and with α1-syntrophin in skeletal muscle in mammals. In this study, we have purified an nNOS-interacting protein from bovine brain by affinity-purification on a CH-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). GST fusion proteins were expressed in Escherichia coli and affinity-purified on glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

**EXPERIMENTAL PROCEDURES**

**GST Fusion Proteins**—GST-α1-syntrophin-(31–90), GST-α1-syntrophin-(69–201), and GST-nNOS-(1–230) constructs were generated by cloning corresponding sequences, which were amplified by reverse transcription-polymerase chain reaction from rat brain cDNA, and introduced into the pGEX vector (Amersham Pharmacia Biotech, Uppsala, Sweden). GST fusion proteins were expressed in Escherichia coli and affinity-purified on glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

**Antibodies**—Anti-α1-syntrophin polyclonal antibody was raised in a rabbit against GST-α1-syntrophin-(31–90) fusion protein and was affinity-purified on a CH-Sepharose column (Amersham Pharmacia Biotech) coupled with GST-α1-syntrophin. For Western blotting and immunohistochemistry, this antibody was diluted in TWEEN/BSA (0.1% v/v) and 10% fetal bovine serum.

**RESULTS**

Interaction of Neuronal Nitric-oxide Synthase with α1-Syntrophin in Rat Brain*

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Akiko Hashida-Omura†‡§, Nobuaki Okumura†, Akihiro Iwamatsu, Ruud M. Buijs**,
Hermes J. Romijn†, and Katsuya Nagai‡

From the †Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamada-Oka, Suita, Osaka 565-0871, Japan; ¯Central Laboratories for Key Technology, Kirin Brewery Company, Limited, 1-13-5 Puhara, Konanawaku-Yokohama, Kanagawa 236-0004, Japan, and the **Netherlands Institute for Research, Meibergdreef 33, 1105 AZ, Amsterdam Zuidoost, Netherlands

Nitric oxide is a major endogenous mediator involved in many physiological and pathological functions such as vasodilation, neurotransmission, and cytotoxicity (1–3). In brain, NO is synthesized mainly by nNOS (4), which is expressed in various brain regions including the cerebellum, olfactory bulb, and several hypothalamic nuclei (5). One of the nNOS-positive nuclei in the hypothalamus is the suprachiasmatic nucleus (SCN) (6), which has a circadian oscillator to create circadian rhythms in hormonal secretions, enzyme activities, and behaviors. The SCN also controls energy metabolism through the regulation of the autonomic nervous system (7). We have previously shown that Nω-methylarginine, an inhibitor of NOS, disturbs the circadian rhythm of drinking behavior in rats, suggesting that NO is involved in the generation and/or synchronization of the circadian rhythm (8).

nNOS is one of three known isoforms of nitric-oxide synthase. Although nNOS does not have a transmembrane domain, subcellular fractionation experiments showed that ~60% of the total NOS activity in brain was found in the particulate fraction, suggesting that nNOS is associated with membranes by interacting with some other membrane proteins (9). The N-terminal domain of nNOS is unique to this isoform, having a PDZ motif, which is found in various structural proteins (10). This domain of nNOS is reported to interact with PDZ motifs in PSD (postsynaptic density)-95 and PSD-93 (11) to form macromolecular signaling complexes at postsynaptic sites and possibly to modulate synaptic transmission.

nNOS is expressed not only in neuronal cells, but also in several other tissues such as the fast-twitch fibers of skeletal muscle (12). In skeletal muscle, nNOS is targeted to sarcolemmal membranes by association with another PDZ-containing protein, α1-syntrophin, through PDZ-PDZ interactions (11). The syntrophins are a multigene family of proteins including α1, β1, and β2 isoforms, each of which has one PDZ domain and three pleckstrin domains (13). In mammalian skeletal muscle, syntrophins are components of the dystrophin complex at sarcolemmal membranes, and are thought to function as adaptors that recruit signaling proteins to the membranes (14). α1-Syntrophin is also expressed in brain (15). However, the interaction of nNOS with α1-syntrophin in brain has not been precisely investigated yet.

To examine whether the PDZ domain of nNOS interacts with proteins other than PSD-95 in brain, we purified nNOS-interacting proteins from bovine brain lysate. In this report, we show that one of the nNOS-interacting proteins in brain is α1-syntrophin. We also investigated the localization of nNOS and α1-syntrophin in primary cultured neurons from rat brain and in neurons from the hypothalamus to gain insight into the physiological functions of nNOS and nNOS-associated proteins in the central regulation of metabolism.

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† To whom correspondence should be addressed. Tel.: 81-6-6879-8632; Fax: 81-6-6879-8633; E-mail: hashida@protein.osaka-u.ac.jp.

‡ The abbreviations used are: nNOS, neuronal nitric-oxide synthase; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; GST, glutathione S-transferase; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PDZ, PSD-95/Dlg/ZO-1.
bound proteins were eluted by addition of 10 mM glutathione. Eluted proteins were fixed with 4% paraformaldehyde 10 days after plating, cryoprotected in 30% sucrose at 4 °C for 5 days, and cut into 20-µm sections with a microslicer. The sections were treated for 3 h in phosphate-buffered saline containing 3% bovine serum albumin and 0.3% Triton X-100; incubated overnight with primary antibodies to α-syntrophin or nNOS, which were diluted into TWEEN/TBS; and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG. Signals were visualized with 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan). For confocal microscopy, signals were visualized with fluorescein isothiocyanate-labeled anti-mouse IgG and rhodamine-labeled anti-rabbit IgG and were then observed on a Bio-Rad Micro Radiance confocal scanning system.

**RESULTS**

**Purification and Identification of an nNOS-interacting Protein**—To examine whether the PDZ domain of nNOS in brain interacts with proteins other than PSD-95 and PSD-93, we purified nNOS-interacting proteins by affinity chromatography using glutathione-Sepharose beads coupled with GST-nNOS-(1–230) fusion protein. We also used glutathione-Sepharose coupled with GST as a control. Crude extracts from bovine brain were loaded on each column, and proteins were eluted with glutathione. We found a protein of ~60 kDa that was eluted from the GST-nNOS affinity column, but not from the GST column, indicating that this protein was selectively bound to the N-terminal region of nNOS (Fig. 1A). Because the eluate contained a large amount of GST-nNOS fusion protein (55 kDa) and its proteolytic products, proteins with molecular masses <55 kDa were not analyzed.

To identify the protein, it was subjected to amino acid sequencing (Fig. 1B). The protein was digested with *Achromobacter* protease I on a polyvinylidene difluoride membrane, digested with *Achromobacter* protease I, and the fragments were separated by reversed-phase HPLC. Sequences of three of the fragments (AP-1, AP-2, and AP-3) were determined by peptide microsequencing and aligned with those of α-syntrophin deduced from its cDNA sequence.
Fig. 2. Interaction of nNOS with α1-syntrophin. A, characterization of anti-α1-syntrophin antibody. An antibody was raised against GST-α1-syntrophin-(31–90) in a rabbit and applied to Western blotting of a crude brain extract (lane 1). As a control, the extract was treated in the same manner without the primary antibody (lane 2). The asterisk denotes α1-syntrophin. B, analysis by pull-down assay. Crude brain extract was incubated with glutathione-Sepharose coupled with GST-nNOS-(1–230) fusion protein. After washing the beads, bound proteins were subjected to SDS-PAGE and analyzed by Western blotting with anti-α1-syntrophin antibody. To confirm that the immunoprecipitation (IP) was successful, the immunoprecipitates were analyzed by Western blotting with anti-α1-syntrophin antibody (lane 3). In the same gel, purified GST (lane 2) and GST-α1-syntrophin-(31–90) fusion protein (lane 1) were run to confirm the specificity of the antibody. The same series of samples was also subjected to Western blotting with anti-α1-syntrophin antibody preabsorbed with its antigen (lanes 4–6).

Fig. 3. Analysis of the interaction between nNOS and α1-syntrophin by overlay assay. Rat brain extracts were immunoprecipitated with protein G-Sepharose coupled with or without anti-nNOS antibody. To confirm that the immunoprecipitation (IP) was successful, the immunoprecipitates were analyzed by Western blotting with anti-nNOS antibody (lanes 1 and 2). The same samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and overlaid with an antibody against α1-syntrophin (lanes 3 and 4) or GST (lanes 5 and 6). The membranes were then incubated with anti-GST antibody followed by horseradish peroxidase-labeled anti-rabbit IgG and developed with chemiluminescence reagent.

and three peptide fragments (AP-1, AP-2, and AP-3) were analyzed on a peptide sequencer. A homology search analysis showed that all these sequences were identical to the fragments of Mus musculus α1-syntrophin except for the fifth amino acid in the peptide AP-3 (Fig. 1B). The molecular mass of α1-syntrophin calculated from its cDNA sequence was 58 kDa, which was close to that of the nNOS-interacting protein estimated by SDS-PAGE. We therefore concluded that the nNOS-interacting protein was bovine α1-syntrophin.

Interaction of nNOS with α1-Syntrophin in Brain—To confirm whether nNOS can interact with α1-syntrophin, we raised an antibody against α1-syntrophin-(31–90). We chose this region as an antigen because it has >50% sequence homology to two other isoforms, β1 and β2. The antibody was affinity-purified with CH-Sepharose coupled with GST-α1-syntrophin-(31–90). Immunoblotting of a crude rat brain extract with the antibody showed that it specifically reacted with a 60-kDa protein (Fig. 2A, lane 1). Some other bands under 45 kDa were also detected, but they seemed to be nonspecific signals of the secondary antibody used because they were detected even when the primary antibody was omitted (Fig. 2A, lane 2).

Fig. 4. Analysis of nNOS-interacting proteins using various kinds of buffers. A, purification of nNOS-interacting proteins by GST-nNOS affinity column chromatography using various kinds of buffers. Adult rat brains were extracted with the following: buffer a, 1% Nonidet P-40, 500 mM NaCl, 50 mM Tris, pH 7.4, and 1 mM EDTA (lanes 1 and 2); buffer b, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 500 mM NaCl, 50 mM Tris, pH 7.4, and 1 mM EDTA (lanes 3 and 4); buffer c, 1% Triton, 500 mM NaCl, 50 mM Tris, pH 7.4, and 1 mM EDTA (lanes 5 and 6); and buffer d, 0.5% deoxycholate, 100 mM NaCl, 50 mM Tris, pH 7.4, and 1 mM EDTA (lanes 7 and 8). Each extract was loaded onto a glutathione-Sepharose column previously coupled with GST (lanes 1, 3, 5, and 7) or GST-nNOS-(1–230) (lanes 2, 4, 6, and 8) and analyzed as described in the legend to Fig. 1A. The arrow denotes α1-syntrophin. B, affinities of nNOS for α1-syntrophin and for PSD-95. Rat brain lysates with various kinds of buffers as described above were precipitated with GST or GST-nNOS and then analyzed by Western blotting with PSD-95 (upper panel) or α1-syntrophin (lower panel). Arrows denote PSD-95 (upper panel) and α1-syntrophin (lower panel).
nNOS Interacts with α1-Sytrophin

The specificity of the antibody was further confirmed in Fig. 2B. The antibody reacted with GST-α1-syntrophin (Fig. 2B, lane 1), but not with GST (lane 2). In addition, binding of the antibody to GST-α1-syntrophin was completely abolished when it was preincubated with the antigen (Fig. 2B, compare lanes 1 and 4). These results suggest that the antibody specifically reacts with α1-syntrophin.

Next, we analyzed the interaction of nNOS with α1-syntrophin in brain by pull-down assay. Glutathione-Sepharose beads coupled with GST-nNOS-(1–230) were incubated with a rat brain lysate and then precipitated. Western blotting with anti-α1-syntrophin antibody showed that α1-syntrophin was retained by GST-nNOS-conjugated Sepharose (Fig. 2B, lane 3), but not by GST-Sepharose (data not shown). The 60-kDa band was not detected when the antibody was preabsorbed with antigen (Fig. 2B, lane 6). These results suggest that nNOS interacts with α1-syntrophin in rat brain.

Analysis of the Interaction between nNOS and α1-Sytrophin by Overlay Assay—To confirm the interaction of nNOS with α1-syntrophin and to examine whether binding of nNOS to α1-syntrophin is direct or indirect, we performed protein overlay assays using GST-α1-syntrophin-(69–201), which contains the PDZ domain, as a probe. Rat brain extracts were immunoprecipitated with or without anti-nNOS antibody (Fig. 3). Western blotting with anti-nNOS antibody confirmed that nNOS was precipitated with the antibody (Fig. 3, lane 1). The same samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and overlaid with GST-α1-syntrophin (Fig. 3, lanes 3 and 4) or GST (lanes 5 and 6). The probes were detected with anti-GST antibody and visualized by enhanced chemiluminescence. The band corresponding to nNOS was detected when the immunoprecipitated materials were overlaid with GST-α1-syntrophin-(69–201) (Fig. 3, lane 3), but not with GST (lane 5). These results indicate that nNOS can directly interact with α1-syntrophin in rat brain.

Assessment of the Affinity of nNOS for α1-Sytrophin—We further examined the affinity of nNOS for α1-syntrophin in the presence of different kinds of detergents (Fig. 4). Nonidet P-40 (1%) was used as the detergent in other analyses, but 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS (buffer b) or 1% Triton X-100 (buffer c) also gave similar results as detected by silver staining (Fig. 4A) and Western blotting (Fig. 4B). Deoxycholate (1%) (buffer d) was the strongest condition in which interaction of nNOS with α1-syntrophin was partly perturbed, whereas the interaction was not perturbed when deoxycholate was used in combination with 1% Nonidet P-40 and 0.1% SDS (buffer b).

Because nNOS has been known to interact with PSD-95, we compared the affinity of nNOS for α1-syntrophin with that for PSD-95 (Fig. 4B). When detergent lysates from rat brain were precipitated with GST-nNOS-conjugated Sepharose, α1-syntrophin was highly concentrated in the precipitated fractions. PSD-95 was also solubilized with all the buffers tested and

**Fig. 5.** Immunofluorescent staining for α1-syntrophin, nNOS, and synaptotagmin in primary cultures of fetal rat brain neuronal cells. Primary cultures of neuronal cells were prepared from embryonic day 18 rat brain and cultured for 10 days. Cells were then fixed with paraformaldehyde and subjected to immunofluorescent staining as follows. Double-labeling experiments with anti-α1-syntrophin polyclonal antibody (A) and anti-nNOS monoclonal antibody (B), those with anti-α1-syntrophin polyclonal antibody (C) and anti-synaptotagmin monoclonal antibody (D), and those with anti-nNOS polyclonal antibody (E) and anti-synaptotagmin monoclonal antibody (F) were carried out.

**Fig. 6.** Distribution of α1-syntrophin and nNOS in rat brain. Rat brains were cut into 0.5-mm slices, and tissues were excised from various regions in the slices as indicated. Extracts from the tissues (10 μg/lane) were analyzed by Western blotting with anti-nNOS (upper panel) or anti-α1-syntrophin (lower panel) antibody. The data are representative of five independent experiments.

**Fig. 7.** Photomicrographs of brain sections stained with anti-α1-syntrophin and anti-nNOS antibodies. Rat brain sections including the hypothalamus were immunostained with antibodies against α1-syntrophin (A and B) and nNOS (D and E). Control experiments using the antibodies preincubated with GST-α1-syntrophin (C) or GST-nNOS (F), respectively, were done. OC, optic chiasm; 3V, third ventricle. Scale bars indicate 200 μm.
detected in the lysates by Western blotting. But, in contrast to α1-syntrophin, only a small amount of PSD-95 was precipitated with GST-nNOS. These results suggest that the affinity of nNOS for α1-syntrophin is higher than that for PSD-95.

**Colocalization of nNOS with α1-Syntrophin in Primary Cultures of Neuronal Cells**—We next examined whether nNOS and α1-syntrophin colocalized in neuronal cells by immunocytochemistry. Primary cultures of neuronal cells were prepared from fetal rat brain and maintained for 7–10 days in serum-free medium. Most neurons were double-labeled with anti-nNOS and anti-α1-syntrophin antibodies with similar subcellular distribution. α1-Syntrophin-like immunoreactive substances were present in both neuronal cell bodies and neurites (Fig. 5, A and C). nNOS-like immunoreactivity was also detected in both neuronal cell bodies and neurites (Fig. 5, B and E). All these immunoreactivities became very weak when the primary antibodies were preincubated with the respective antigens (data not shown).

We further examined the distribution of synaptotagmin, an essential component of the synaptic membranes, to determine the location of presynaptic structures in these cells. Synaptotagmin-like immunoreactivity was found as punctate signals along the neurites (Fig. 5, D and F) and was not detected in their cell bodies in most neurons. Double staining with anti-synaptotagmin and anti-α1-syntrophin antibodies confirmed that syntrophin was not restricted to synapses (Fig. 5, C and D). Double staining with anti-synaptotagmin and anti-nNOS antibodies showed that a fraction of nNOS was colocalized in synapses, but the majority of nNOS seemed to be present outside of synapses (Fig. 5, E and F).

**Distribution of nNOS and α1-Syntrophin in the Hypothalamus**—Relative amounts of nNOS and α1-syntrophin localized in various brain regions were examined by Western blotting with the respective antibodies. nNOS was highly expressed in the cerebellum and olfactory bulb as reported previously (5), but low level expression was detected in all other regions tested, including the striatum, cerebral cortex, SCN, and hypothalamic paraventricular nucleus (PVN) (Fig. 6). α1-Syntrophin was also present in all regions tested, with the highest expression observed in the PVN (Fig. 6).

Immunohistochemical staining of rat hypothalamic sections using anti-nNOS and anti-α1-syntrophin antibodies was done. In the hypothalamus, α1-syntrophin-like immunoreactive substance was observed in the SCN, PVN, and anterior hypothalamic area (Fig. 7, A and B). nNOS-positive neurons were also detected in the SCN and PVN (Fig. 7, D and E), consistent with previous studies (5, 6). In the SCN, nNOS- and α1-syntrophin-like immunoreactive substances were most concentrated in the dorsomedial region, whereas weak signals were also detected in the ventrolateral region (Fig. 7, A, B, D, and E). In the PVN, α1-syntrophin- and nNOS-like immunoreactive substances were detected mainly in the magnocellular part. No immunostaining was detected when primary antibodies were preincubated with their respective antigens, confirming the specificity of immunolabeling (Fig. 7, C and F).

Fine distributions of nNOS and α1-syntrophin in the dorsomedial region of the SCN were observed using a confocal microscope. In the SCN, nNOS-like immunoreactive substance was detected in the cell matrix of neuronal cell bodies, but not in the nuclei (Fig. 8A). α1-Syntrophin also showed a subcellular distribution similar to nNOS (Fig. 8B). Superimposing fluorescence images for nNOS and α1-syntrophin gave a yellow color, suggesting that nNOS and α1-syntrophin are colocalized in the same regions of the same SCN neurons (Fig. 8C). In the PVN, the colocalization of nNOS with α1-syntrophin was also observed (data not shown).

**DISCUSSION**

In this study, we have purified an nNOS-binding protein from bovine brain and identified it as α1-syntrophin. We further demonstrated that nNOS- and α1-syntrophin-like immunoreactive substances showed similar subcellular distribution in primary cultures from fetal rat brain. Finally, the two proteins were expressed at relatively high levels and colocalized in the PVN and SCN in hypothalamic sections of adult rat brains. These results suggest that nNOS interacts with α1-syntrophin in specific neurons in brain.

nNOS was originally found in mammalian brain, but was
later shown to be present also in skeletal muscle, lung epithelial cells, and certain endocrine glands (12, 19). In skeletal muscle, nNOS is localized at the sarcolemmal membranes by association with syntrophins, a component of the dystrophin complex (11, 20). The dystrophin complex is a membrane cytoskeletal structure that links the sarcolemmal membranes to extracellular matrix proteins and intracellular actin fibers. In brain, on the other hand, nNOS has been shown to be associated with PSD-95 and PSD-93 (11), which are localized at the synapses as a component of the postsynaptic density structures (10). But the distribution of nNOS is found not only in the synapses, but also in the entire surfaces of cell bodies and neurites in several types of neurons such as those in the PVN of the hypothalamus (5). These findings indicate that there are different mechanisms that define the intracellular localization of nNOS depending on cell types.

Our present data showed that α1-syntrophin was associated with nNOS in vitro even in the presence of strong detergents such as Nonidet P-40, deoxycholate, and SDS. The binding affinity of nNOS for α1-syntrophin seemed to be much higher than that for PSD-95 as estimated by pull-down assay. In addition, nNOS and α1-syntrophin were colocalized in cell bodies, neuronal processes, and synapses in cultured neurons from fetal rat brains. These two proteins were also colocalized in neuronal cells in the SCN and PVN. From these results, we propose that α1-syntrophin also contributes to determining the subcellular localization of nNOS in certain regions of the brain.

α1-Syntrophin was expressed in most regions in adult rat brain judging from Western blotting with anti-α1-syntrophin antibody. The present immunohistochemical data showed that the level of α1-syntrophin was relatively high in several neurons, including the PVN and SCN in the hypothalamus (Fig. 7). These two nuclei have been known to contain nNOS. These results support the possibility that nNOS and α1-syntrophin interact with each other in certain brain regions and imply that they have functional relationships. The immunohistochemical distribution of α1-syntrophin is not completely consistent with its mRNA distribution previously shown by in situ hybridization (21), but the discrepancy might be elicited by the stability of mRNA and protein.

In skeletal muscle, syntrophins have been shown to be a component of the dystrophin complex and to function as molecular adaptors that recruit signaling proteins to the membrane (14). Dystrophin is also present in brain and has been reported to localize at postsynaptic densities (22). However, nNOS is unlikely to interact with dystrophin in brain because nNOS is membrane-associated even in the brains of mdx mice that lack dystrophin (20). Rather, nNOS might be associated with membrane proteins such as other dystrophin family proteins via α1-syntrophin.

The SCN and PVN have key roles in the regulation of metabolism and behavior through controlling the autonomic nervous system and endocrine functions in mammals. The PVN regulates various neuroendocrine hormones through the hypothalamic-hypophysial system, and NO has been suggested to be implicated in its function (23). The SCN is the nucleus containing the circadian oscillator responsible for circadian rhythms (24) and a mechanism controlling the autonomic nervous system (7). We have previously shown that infusion of N\textsuperscript{G}-methyl-arginine into the third ventricle in rats disrupts the circadian rhythm of drinking behavior (8), suggesting that NO might be involved in the generation and/or synchronization of the circadian oscillator. nNOS- and α1-syntrophin-like immunoreactive substances were detected at relatively high levels in the dorsomedial region of the SCN, where vasopressin-containing neurons exist, and it was shown that these vasopressin neurons are involved in the regulation of secretion of adrenal glucocorticoid (25). Therefore, it will be interesting to examine the coexistence of nNOS and α1-syntrophin in the SCN to obtain further information on the physiological functions of nNOS and α1-syntrophin in the SCN.

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