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8-Nitro-cGMP Enhances SNARE Complex Formation through S-Guanylation of Cys90 in SNAP25

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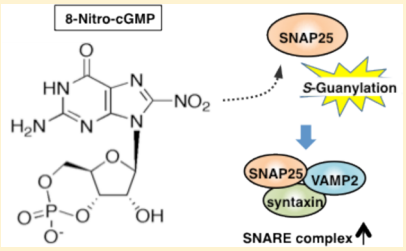
Supporting Information

ABSTRACT: Nitrated guanine nucleotide 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) generated by reactive oxygen/nitrogen species causes protein S-guanylation. However, the mechanism of 8-nitro-cGMP formation and its protein targets in the normal brain have not been identified. Here, we investigated 8-nitro-cGMP generation and protein S-guanylation in the rodent brain. Immunohistochemistry indicated that 8-nitro-cGMP was produced by neurons, such as pyramidal cells and interneurons. Using liquid chromatography-tandem mass spectrometry, we determined endogenous 8-nitro-cGMP levels in the brain as 2.92 ± 0.10 pmol/mg protein. Based on S-guanylation proteomics, we identified several S-guanylated neuronal proteins, including SNAP25 which is a core member of the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex. SNAP25 post-translational modification including palmitoylation, phosphorylation, and oxidation, are known to regulate neurotransmission. Our results demonstrate that S-guanylation of SNAP25 enhanced the stability of the SNARE complex, which was further promoted by Ca^{2+}-dependent activation of neuronal nitric oxide synthase. Using site-directed mutagenesis, we identified SNAP25 cysteine 90 as the main target of S-guanylation which enhanced the stability of the SNARE complex. The present study revealed a novel target of redox signaling via protein S-guanylation in the nervous system and provided the first substantial evidence of 8-nitro-cGMP function in the nervous system.



KEYWORDS: *8-Nitroguanosine 3′,5′-cyclic monophosphate, protein S-guanylation, redox signaling, SNAP25, SNARE complex, neuronal nitric oxide synthase*

Reactive oxygen/nitrogen species (ROS/RNS) are notorious toxic molecules that induce nonspecific damage of a wide array of biomolecules. Intracellular accumulation of ROS/RNS leads to oxidative stress, and, therefore, may be involved in the development of neurodegenerative diseases.[1,2] However, recent evidence suggests new physiological functions for ROS/RNS in the regulation of cell signal transduction. ROS/RNS-dependent signaling mechanisms and biological functions are tightly controlled by several endogenous moderate electrophiles generated by ROS/RNS during diverse physiological conditions or pathophysiological cellular responses.[3] In the brain, electrophile-induced redox modification of critical cysteine residues regulates the activity of proteins involved in neuronal function and is implicated in the development of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[1,2] Thus, recent studies have shown that S-glutathionylation of p53 augments oxidative stress and may be involved in Alzheimer's disease,[4] and that S-nitrosylation inhibits the activity of parkin, and contributes to the degeneration of dopamine neurons in Parkinson's disease.[5] S-Nitrosylation of *N*-ethylmaleimide-sensitive factor aids in the regulation of exocytosis,[6] while oxidation of synaptosomal-associated protein 25 (SNAP25) involved in synaptic transmission, inhibits neurotransmitter release[7] and synaptic plasticity.[8]

Nitric oxide (NO) is an endogenous signaling molecule and is involved in the regulation of diverse physiological processes. In neurons, NO produced by neuronal nitric oxide synthase (nNOS) acts as a modulator of neurotransmission[9] regulating neural development, neurotransmitter release, and synaptic plasticity in the brain.[8] Conversely, NO can promote neuronal damage and induce pathological effects promoting neuro-

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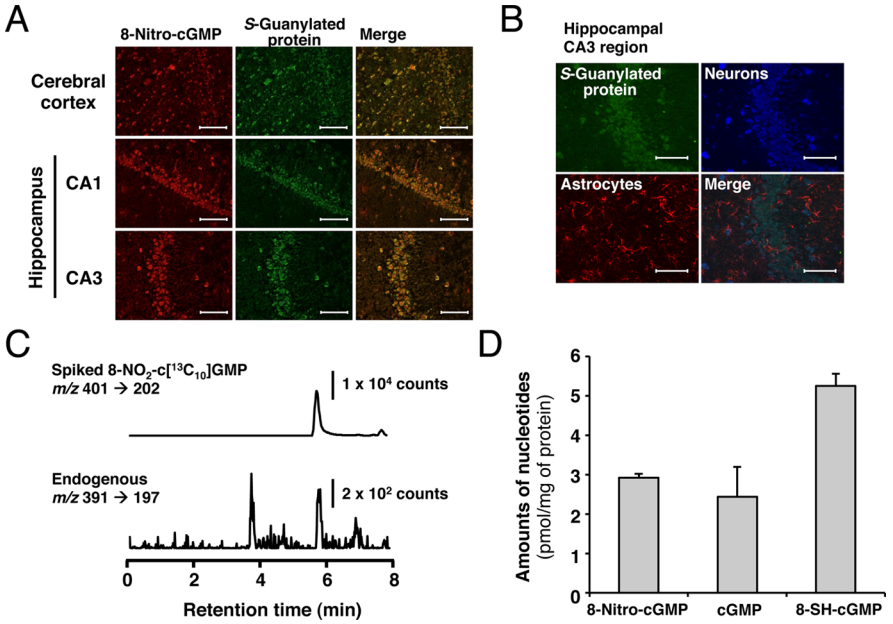

Figure 1. Detection of endogenous 8-nitro-cGMP generation in brain. (A) Brain tissues were stained with monoclonal anti-8-nitro-cGMP and polyclonal anti-S-guanylated protein (8-RS-cGMP) antibodies. Scale bars, 100 μm. (B) Brain tissues were immunostained with polyclonal anti-8-RS-cGMP and monoclonal anti-GFAP antibodies to detect astrocytes, and with Nissl stain to detect neurons. (C) Endogenous 8-nitro-cGMP in the mouse brain was analyzed by LC-MS/MS. Representative LC-MS/MS chromatograms of spiked ^{13}C-labeled authentic 8-NO_2-c[$^{13}C_{10}$]GMP and endogenous 8-nitro-cGMP are shown. (D) 8-Nitro-cGMP, cGMP, and 8-SH-cGMP from mouse brain tissues were quantified by methanol extraction and LC-MS/MS using a stable-isotope dilution method. Data represent the mean ± SE (n = 3).

degeneration.[2] There are two mechanisms underlying NO activity in the nervous system; cyclic guanosine monophosphate (cGMP)-dependent pathway[10,11] and chemical modification, including nitrosylation and nitration of proteins and lipids, which is induced by NO-derived RNS such as peroxynitrite ($ONOO^-$)[12] shown to participate in inflammatory response,[13] SNARE complex stability,[14] and neurotransmitter release.[15]

A novel nitrated cyclic nucleotide, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), was discovered as an endogenous second messenger of ROS/RNS redox signaling.[16] 8-Nitro-cGMP is generated via excessive endogenous production of NO coupled with oxidative stress[17−19] and is mainly synthesized by soluble guanylate cyclase (sGC).[20] 8-Nitro-cGMP has been characterized as a potent signaling molecule involved in diverse molecular mechanisms such as the canonical NO/cGMP pathway and noncanonical electrophilic signaling.[16]

Protein S-guanylation is a unique post-translational modification of protein thiol groups by electrophilic 8-nitro-cGMP detected in a variety of proteins such as kelch-like ECH-associated protein 1 (Keap1),[16,17] H-Ras,[21] and mitochondrial proteins.[22] Given that NO regulates multiple processes in neuronal tissues, it is likely that NO-generated 8-nitro-cGMP may play an important role in post-translational mechanisms occurring in the nervous system through post-translational protein S-guanylation.

Synaptic transmission via exocytotic neurotransmitter release is fundamental to signal transduction in the nervous systems. The process of neurotransmitter release depends on the exocytosis of presynaptic vehicles regulated by a highly conserved family of proteins, three SNARE proteins [SNAP25, syntaxin-1, and vesicle-associated membrane protein 2 (VAMP2)].[23] SNARE proteins have a conserved SNARE motif, elongated 60−70 amino acids, and form the SNARE complex consisting of four SNARE motif bundle.[24] Proteins assembled on the core complex, such as complexin and synaptotagmin, regulate synaptic vesicle fusion.[25]

SNAP25 is mainly localized in the axons and nerve terminals and is thought to be involved in neurotransmitter release, elongation of neurites,[26] and the regulation of ion channels.[27] SNAP25 functional activity is modulated by distinct post-translational modifications, including phosphorylation[27,28] and oxidation.[7] SNAP25 phosphorylation regulates the activity of Ca^{2+} channels,[27] and residue substitutions of phosphorylation sites resulted in the increase of anxiety-related behavior in mice.[29] Moreover, SNAP25 phosphorylation is upregulated in normal mice subjected to physical stress.[30] Previous findings suggest that changes in SNAP25 activity are involved in the modulation of synaptic plasticity[31] and memory,[32−34] and are implicated in various pathological conditions such as attention-deficit hyperactivity disorder[35] and Alzheimer's disease.[36] Notably, SNAP25 has high sensitivity to oxidative stress, as demonstrated by the existence of several modified forms generated through interactions with electrophiles such as ROS and RNS, affecting the tuning of neuronal functions.[7,15] Therefore, the elucidation of SNAP25-dependent regulatory pathways and their diverse roles in the nervous system would further understanding of the mechanisms underlying synaptic neurotransmission in health and disease.

The expression of neuronal proteins with distinct roles such as structural axonal support or neurotransmission is tightly regulated by post-translational mechanisms. However, there have been few studies of protein S-guanylation occurring downstream of ROS/RNS signaling in the nervous system. Our group has recently developed a proteomic approach, S-guanylation proteomics, to identify S-guanylation targets using liquid chromatography-tandem mass spectrometry (LC-MS/MS).[22] In this study, we investigated the endogenous

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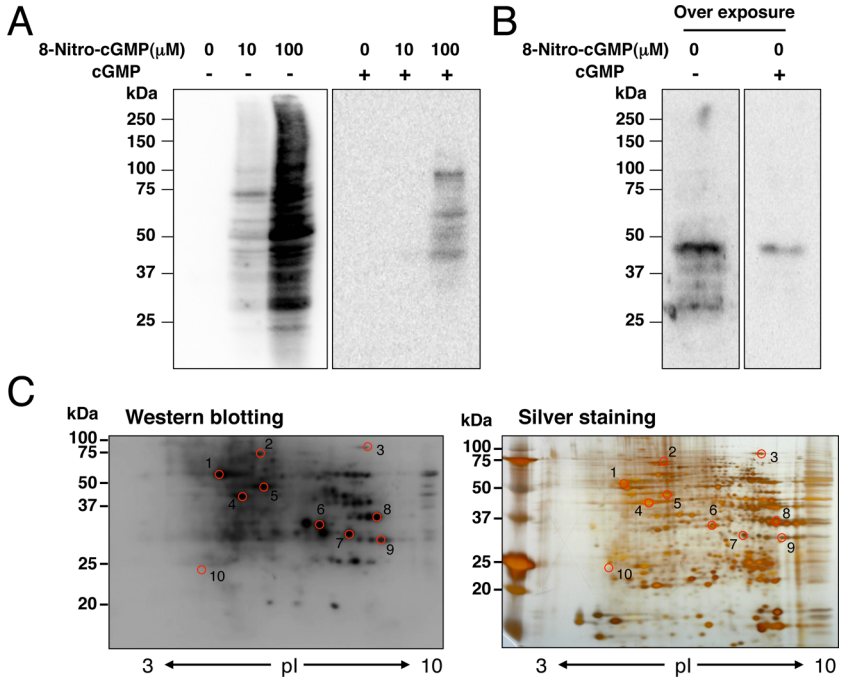

Figure 2. Identification of endogenously S-guanylated proteins in synaptosomes. (A) Rat synaptosomes were incubated with 8-nitro-cGMP (0, 10, 100 μM) for 1 h at room temperature. To assess S-guanylation, synaptosomal proteins were analyzed by Western blotting using anti-8-RS-cGMP antibody. To confirm antibody specificity, a competitive inhibitor (5 mM cGMP) of interaction between the antibody and S-guanylated protein was coincubated along with the antibody. (B) Nontreated synaptosomes were analyzed by Western blotting using anti-8-RS-cGMP antibody, in the presence and absence of a competitive inhibitor (5 mM cGMP). The membranes were overexposed to detect any weak signal, compared with the experiment (A). (C) Rat synaptosomes were treated with 8-nitro-cGMP (100 μM) for 1 h at room temperature. Synaptosomal proteins were analyzed by 2D-PAGE. S-Guanylated proteins were detected by Western blotting using anti-8-RS-cGMP antibody. Synaptosomal proteins were visualized by silver staining. Protein spots in the silver-stained gels were excised and subjected to in-gel digestion, followed by LC-MS/MS. Ten S-guanylated proteins were identified (red circle).

generation of 8-nitro-cGMP and S-guanylated proteins in the normal rodent brain and demonstrated that endogenous 8-nitro-cGMP causes S-guanylation of target proteins in the brain, including SNAP25, and enhances the stability of the SNARE complex.

RESULTS AND DISCUSSION

Although ROS/RNS are known for their involvement in pathological mechanisms, such as oxidative stress, they are now believed to play a role in regulating normal physiological processes. 8-Nitro-cGMP, recently discovered to be a second messenger of ROS/RNS signaling with a unique role in the formation of protein Cys-cGMP adducts, that is, S-guanylated proteins,[17,21,22] has been the focus of intense research. The formation of S-guanylated proteins depends on electrophilic 8-nitro-cGMP which thus mediates redox signal transduction. However, the information about 8-nitro-cGMP activity in the nervous system is limited. Therefore, we investigated the generation of endogenous 8-nitro-cGMP and S-guanylated proteins in normal rodent brain tissue.

Generation of Endogenous 8-Nitro-cGMP in the Brain. First, we examined the expression of S-guanylated proteins in the rat brain by immunohistochemistry using antibodies specific for 8-nitro-cGMP and S-guanylated protein (8-thioalkoxy-cGMP adducts; 8-RS-cGMP)[16] (Figure 1A). Strong immunostaining for 8-nitro-cGMP and 8-RS-cGMP was observed in the cerebral cortex, and the hippocampal CA1 and CA3 regions where both pyramidal cells and interneurons were strongly stained. In each region, the immunoreactivity for 8-nitro-cGMP and 8-RS-cGMP overlapped.

In order to determine which cell types generated 8-nitro-cGMP, we identified astrocytes by immunostaining with an antibody against astrocyte-specific glial fibrillary acidic protein (GFAP) and neurons by fluorescent Nissl staining (Figure 1B). 8-RS-cGMP immunostaining colocalized with neurons (Nissl staining) but not with astrocytes (GFAP staining) (Figure 1B), suggesting that, in the normal rat brain, 8-nitro-cGMP is generated mainly by neurons and may be involved in neuronal functions.

To further investigate 8-nitro-cGMP generation in the brain, we analyzed 8-nitro-cGMP content in the mouse brain tissue by LC-MS/MS using a stable-isotope dilution method.[17,18] Figure 1C shows a representative MS/MS chromatogram of 8-nitro-cGMP. Endogenous 8-nitro-cGMP was detected at the same retention time as isotope-labeled 8-NO_2-c[$^{13}C_{10}$]GMP; similar results were obtained from three mice (data not shown). The concentration of 8-nitro-cGMP was determined as 2.92 $\pm$ 0.10 pmol/mg protein which is similar to the levels of both cGMP and 8-SH-cGMP, an 8-nitro-cGMP thiol adduct (Figure 1D), and is consistent with our previous findings.[37] Overall, immunohistochemistry and LC-MS/MS analyses indicate that 8-nitro-cGMP was generated in the rodent brain under physiological conditions, supporting the notion that 8-nitro-cGMP is involved in neuronal activity.

Analysis of Protein S-Guanylation in Synaptosomes from the Rat Brain. Recently, we have developed a proteomic approach to investigate S-guanylation using a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and LC-MS/MS, which allowed the discovery of several 8-nitro-cGMP protein targets, including cytoskeletal

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proteins tubulin and actin, in the mitochondria of rat C6 glioma cells.[22] As 8-nitro-cGMP exerts its pathophysiological effects by targeting proteins for S-guanylation,[17,21] we analyzed S-guanylation levels in rat synaptosomes, the canonical nerve terminal model.

Rat synaptosomes were treated or not with 8-nitro-cGMP, and protein S-guanylation was examined by Western blotting using the 8-RS-cGMP-specific antibody (Figure 2A). Similar to our previous findings, a variety of S-guanylated proteins were identified, and the intensity of 8-RS-cGMP-specific bands depended on 8-nitro-cGMP concentration (Figure 2A). However, the 8-RS-cGMP signals were reduced in the presence of cGMP, a competitive inhibitor of antibody binding, indicating that the signals were specific for protein S-guanylated residues (Figure 2A). Furthermore, S-guanylation could be detected without 8-nitro-cGMP treatment (Figure 2B), which is consistent with immunochemistry results (Figure 1A) and suggests that several proteins were S-guanylated by endogenous 8-nitro-cGMP.

To identify the proteins targeted by 8-nitro-cGMP, we performed S-guanylation proteomic analysis of 8-nitro-cGMP-treated synaptosomes. As shown in Figure 2C, multiple spots were detected with the anti-8-RS-cGMP antibody. A total of 24 spots were analyzed and 10 proteins, including cytoskeletal proteins, glycolic enzymes, ion channels, and neuronal proteins, were identified (Table 1); two of them have been already

Table 1. S-Guanylated Proteins Identified in 8-Nitro-cGMP-Treated Rat Synaptosomes by 2D-PAGE and LC-MS/MS[a]

spot No.	protein name
1	*tubulin beta chains*
2	stress-70 protein, mitochondrial
3	aconitate hydratase
4	*actin, cytoplasmic 1*
5	creatine kinase B-type
6	malate dehydrogenase, cytoplasmic
7	voltage-dependent anion-selective channel protein 2
8	glyceraldehyde-3-phosphate dehydrogenase
9	voltage-dependent anion-selective channel protein 1
10	synaptosomal-associated protein 25

[a]Italicized proteins have also been identified as 8-nitro-cGMP targets in a recent study of mitochondria from rat C6 glioma cells.[22]

detected in our recent study[22] (Table 1, italicized), while the others were novel. Among the latter, a neuron-specific protein SNAP25 is known to regulate synaptic exocytosis of neurotransmitters via formation of the SNARE complex.[38,39]

S-Guanylation of SNAP25. SNAP25 is one of the three core SNARE proteins, including syntaxin-1 and VAMP2, which play an essential role in neuronal functions[39−41] also regulated via post-translational modifications such as phosphorylation[27,29] and oxidation.[7] SNAP25 is known to have high sensitivity to oxidative stress, and its modification by electrophiles affects its activity in neural tissues.[7,15]

To evaluate S-guanylation of SNAP25, we performed immunoprecipitation of synaptosome lysates using the anti-8-RS-cGMP antibody, and analyzed the results by Western blotting with antibodies against SNARE proteins syntaxin-1, SNAP25, and VAMP2. Immunoprecipitation showed that only SNAP25 was S-guanylated, whereas syntaxin-1 and VAMP2 were not (Figure 3A, upper panel). Conversely, S-guanylation of SNAP25 immunoprecipitated with the anti-SNAP25 antibody was detected in synaptosomes both with and without 8-nitro-cGMP treatment, indicating that some endogenous S-guanylation occurred (Figure 3A, lower panel).

As illustrated in Figure 3B, SNAP25 has a cysteine-rich domain containing four cysteine residues in the linker region between coiled-coil forming SNARE motifs.[42] In order to identify S-guanylated residues, recombinant SNAP25 mutants containing cysteine-to-alanine mutations were generated by site-directed mutagenesis and analyzed for S-guanylation (Figure 3C). Compared to the wild-type SNAP25, the signal of the C90A SNAP25 mutant was significantly reduced (Figure 3D). In contrast, the reduction of other three mutants was smaller and significant difference was undefined (Figure 3D). This result indicates that cysteine 90 is the main target of 8-nitro-cGMP. To our knowledge, this is the first report of a functional S-guanylation in SNAP25.

nNOS-Dependent S-Guanylation of SNAP25 in Cultured Cells. 8-Nitro-cGMP is produced endogenously in various cells in an NO- and ROS-dependent manner, mainly by soluble guanylate cyclase.[20] In the nervous system, NO is synthesized by nNOS[43] which can also generate ROS when not coupled with a cofactor or substrate, that is, via uncoupling reactions.[16,17] Therefore, we examined whether SNAP25 was S-guanylated in a nNOS-dependent manner. In order to simplify the analysis of nNOS-dependent S-guanylation in SNAP25, we used nNOS-transfected HEK-293 cells (HEK-nNOS) as a model.[44] Soluble guanylate cyclase which is necessary to produce 8-nitro-cGMP was expressed both in HEK-293 and HEK-nNOS cells, whereas nNOS was expressed only in HEK-nNOS cells (Figure S1A). nNOS activity in HEK-nNOS cells was induced by a calcium ionophore A23187 (Figure S1B), indicating that it was calcium-dependent. An increase in 8-nitro-cGMP generation was detected by immunochemistry in A23187-treated HEK-nNOS cells (Figure 4A), which was further confirmed by Western blotting showing the upregulation of S-guanylated (8-RS-cGMP antibody-reactive) proteins (Figure 4B). To analyze the nNOS-dependent S-guanylation of SNAP25, FLAG-tagged SNAP25 was overexpressed in HEK-nNOS cells which were then treated with A23187 and examined for S-guanylation of immunoprecipitated SNAP25. As shown in Figure 4C, SNAP25 S-guanylation was increased in HEK-nNOS cells treated by calcium ionophore indicating that SNAP25 S-guanylation is regulated by nNOS activity.

Modulation of SNARE Complex Formation by 8-Nitro-cGMP. The core SNARE proteins produce a relatively stable complex which displays temperature-dependent resistance to sodium dodecyl sulfate (SDS) and is completely dissociated after boiling.[14,15] Therefore, we tested the ability of the three SNARE proteins to produce the complex in 8-nitro-cGMP-treated synaptosomes. SDS-resistant SNARE complexes were detected in unheated synaptosomes and completely dissociated after boiling (Figure 5A), while 8-nitro-cGMP improved the formation of the SNARE complex in a dose-dependent manner. Densitometry analysis revealed that 100 μM 8-nitro-cGMP significantly increased complex formation up to 123% compared with untreated synaptosomes (Figure 5B).

Next, we investigated the effect of SNAP25 S-guanylation on the assembly of the SNARE complex in pull-down assays using recombinant glutathione S-transferase (GST)-VAMP2, syntaxin-1, and S-guanylated SNAP25 proteins (Figure 5C) in order to selectively quantify the ternary SNARE complex. SNAP25 was pulled down with GST-VAMP2 (Figure 5C) and the amount of GST-VAMP2-bound SNAP25 was increased by 8-

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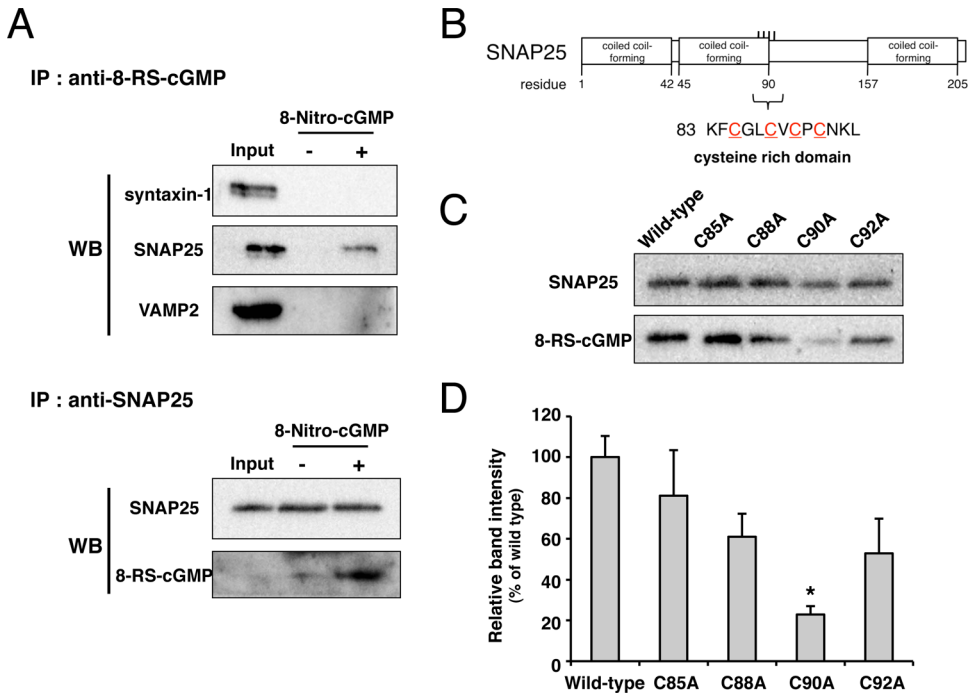

Figure 3. Identification of SNAP25 as a target for S-guanylation in synaptosomes. (A) Synaptosomes were treated with 100 μM 8-nitro-cGMP for 1 h at room temperature. The synaptosomal proteins were immunoprecipitated (IP) with anti-8-RS-cGMP or anti-SNAP25 antibodies. The precipitated proteins were identified by Western blotting using anti-syntaxin-1, anti-SNAP25, anti-VAMP2, or anti-8-RS-cGMP antibodies. (B) Schematic illustration of SNAP25 and amino acid sequence for the rat SNAP25B cysteine-rich domain. Residue numbers correspond to those in the amino acid sequence of rat SNAP25B. Cysteine residues are underlined. (C, D) In vitro S-guanylation of SNAP25 mutants. Recombinant SNAP25 wild-type and mutant proteins carrying C85A, C88A, C90A, and C92A substitutions were treated with 100 μM 8-nitro-cGMP for 3 h at room temperature, and analyzed for protein S-guanylation by Western blotting using anti-8-RS-cGMP or anti-SNAP25 antibodies (C). SNAP25 S-guanylation was quantified by densitometry as the ratio of 8-RS-cGMP to total SNAP25, shown as its percentage relative to the wild-type SNAP25. Data are presented as the mean ± SE (n = 3). One-way ANOVA with Dunnett's multiple comparison posthoc test was used for statistical analysis. *p < 0.01 was considered significant, where the values were compared with the wild-type values (D).

nitro-cGMP pretreatment to 120% of that in untreated SNAP25 as revealed by densitometry analysis (Figure 5D). These results suggest that S-guanylation of SNAP25 improved the stability of the ternary complex with VAMP2 and syntaxin-1.

Modulation of SNARE Complex Formation by SNAP25 S-Guanylation. SNAP25 cysteine residues are known to be modified with palmitate, forming palmitate-adducts which provide anchorage to the plasma membrane important for the function of the SNARE complex.[42]

Here, we investigated whether SNAP25 cysteine residues are targeted by 8-nitro-cGMP and whether SNAP25 S-guanylation increased the stability of the SNARE complex. For this, neuroblastoma SH-SY5Y cells were transfected with the wild-type and C90A mutant SNAP25 and analyzed for SNARE complex assembly. In cells overexpressing the wild-type SNAP25, the SNARE complex was detected in unheated cell lysates (Figure 6A), consistent with the data obtained in rat synaptosomes (Figure 5). Densitometry analysis revealed a 180% increase in complex assembly in cells treated with 10 μM 8-nitro-cGMP compared to the untreated control (Figure 6B); however, in cells overexpressing the C90A SNAP25 mutant, substituted for main target cysteine of protein S-guanylation, the effect of 8-nitro-cGMP was not observed (Figure 6C, D). Under the experimental conditions employed in this study, the bands belonging to monomeric SNAP25 were not saturated (data not shown). These results suggest that the improved stability of the SNARE complex was a result of SNAP25 S-guanylation at cysteine 90. Thus, SNAP25 function may be regulated by the modification of thiol groups in the cysteine-rich domain by S-guanylation as well as palmitoylation. A recent study has reported that cysteines 85 and 88 are S-palmitoylation sites essential for the anchorage of SNAP25 to the plasma membrane.[42] These and our results suggest that SNAP25 may be simultaneously modified at different cysteine residues by S-palmitoylation and S-guanylation which occur independently. SNARE complexes are existed in multiple conformations before the triggering of exocytosis,[23−25] which is consistent with the multiple bands of SNARE complexes in Figures 5A and 6A. In this study, we could not determine whether SNAP25 S-guanylation affects only the fully assembled SNARE complex formation. Further work is in progress to clarify the function of S-guanylated SNAP25.

In conclusion, we detected 8-nitro-cGMP generation in the rodent brain and identified several synaptosomal proteins, including SNAP25, as targets of S-guanylation. Moreover, we determined that 8-nitro-cGMP affected SNARE complex formation by S-guanylation of SNAP25. To date, there are limited data on ROS/RNS signaling mediated via 8-nitro-cGMP in the nervous system. This is the first study to demonstrate that ROS/RNS signaling in the brain at individual-level is coupled to 8-nitro-cGMP generation and S-guanylation of SNAP25 (Figure 7). S-Guanylation proteomics enables identification of proteins potentially targeted by this post-translational modification. Further research is needed to

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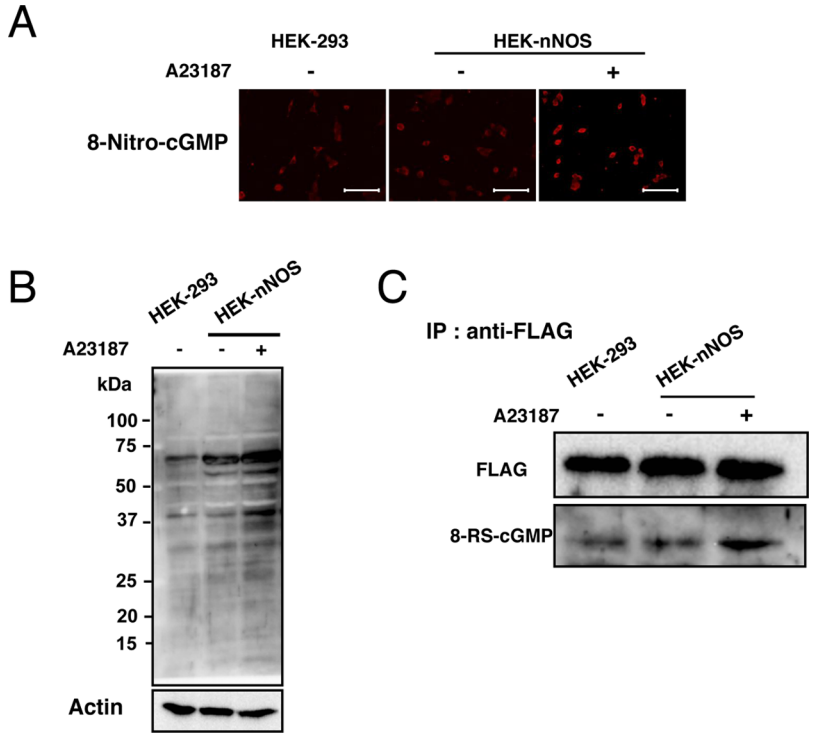

Figure 4. Induction of SNAP25 S-guanylation in HEK-nNOS cells by Ca^{2+}-dependent nNOS activation. HEK-293 and HEK-nNOS cells were stimulated with 5 μM A23187 for 12 h. (A) Cells were immunostained with an anti-8-nitro-cGMP antibody. Scale bars, 100 μm. (B) Cell lysates (50 μg protein) were analyzed by Western blotting using the anti-8-RS-cGMP antibody. β-actin was used as a loading control. (C) Cell lysates were immunoprecipitated with an anti-FLAG antibody and analyzed by Western blotting using anti-FLAG and anti-8-RS-cGMP antibodies.

identify other S-guanylated proteins and to reveal the mechanisms regulated by 8-nitro-cGMP in the nervous system.

METHODS

Materials. The mouse monoclonal anti-8-nitro-cGMP (clone 1G6) and rabbit polyclonal anti-8-thioalkoxy-cGMP (8-RS-cGMP) antibodies were obtained as described previously.[16] Anti-syntaxin-1A, anti-SNAP25, and anti-VAMP2 antibodies were generated as described previously.[45] HiLyte Fluor 555-conjugated goat antimouse IgG and HiLyte Fluor 647-conjugated goat anti-rabbit IgG antibodies were purchased from Ana Spec Inc. (Fremont, CA). NeuroTrace 435/455 Blue Fluorescent Nissl Stain was obtained from Thermo Fisher Scientific (Waltham, MA), BlockAce was from Snow Brand Milk Products Co., Ltd. (Tokyo, Japan), and Can Get Signal Immunoreaction Enhancer Solution was from TOYOBO (Osaka, Japan). Blocking One, protease inhibitor cocktail, and *N*-ethylmaleimide (NEM) were purchased from Nacalai Tesque (Kyoto, Japan), peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from GE Healthcare UK Ltd. (Buckinghamshire, U.K.), and A23187 was from Cayman Chemical (Ann Arbor, MI). Anti-GFAP and anti-FLAG antibodies, and penicillin-streptomycin were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT), and Dulbecco's modified Eagle's medium (DMEM) and anti-DYKDDDDK-tag antibody beads were purchased from Wako Pure Chemical Industries (Osaka, Japan). cGMP was purchased from ICN Biomedicals (Irvine, CA), and 8-nitro-cGMP and 8-mercapto-cGMP (8-SH-cGMP) was synthesized as previously described.[16,20] All other reagents used in this study were of the same purity as or higher purity than the guaranteed reagent.

Animals. This study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University, Japan. Adult Wistar rats and C57BL/6 mice (Kiwa Laboratory Animals Co., Ltd.; Wakayama, Japan) were reared at 24 ± 1 °C and with a 12 h light/12 h dark cycle. The animals were fed commercial pellets CE-2, Clea Japan Inc.; Tokyo, Japan) and provided water ad libitum.

Fluorescence Immunostaining. Immunostaining analysis was performed as previously described.[17,18,46] Briefly, for brain, the rat brains were removed, embedded in OCT compound (Sakura Finetek USA Inc.; Torrance, CA), and immediately stored at −80 °C. Brain coronal sections (10 μm thick) were cut using a cryostat (Leica; Wetzlar, Germany) and fixed in 4% paraformaldehyde at 4 °C for 15 min. For cells, cells were fixed in Zamboni solution (4% paraformaldehyde and 10 mM picric acid in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 16 h. After washing with phosphate-buffered saline (PBS), the preparations were permeabilized with 0.3% TritonX-100 for 5 min, washed, and incubated in BlockAce overnight at 4 °C to block nonspecific antigenic sites. After washing with PBS, the sections were incubated overnight at 4 °C with the primary anti-8-nitro-cGMP monoclonal antibody (1 μg/mL), anti-8-RS-cGMP polyclonal antibody (1 μg/mL), or anti-GFAP antibody (1 μg/mL) diluted in Can Get Signal Solution I. The preparations were then washed three times with PBS, and incubated with HiLyte Fluor 555-conjugated antimouse IgG antibody (1 μg/mL) or HiLyte Fluor 647-conjugated antirabbit IgG (1 μg/mL) in Can Get Signal Solution II for 1 h at room temperature; nuclei were stained with Hoechst 33258 (Dojindo Laboratories; Kumamoto, Japan). The preparations were washed three times with PBS and mounted on glass slides using fluorescent mounting medium (KPL Inc.; Gaithersburg, MD). Images were acquired using an ECLIPSE T*i*-S inverted microscope (Nikon Instruments Inc.; Tokyo, Japan) equipped with an ORCA-R2 camera (Hamamatsu Photonics; Hamamatsu, Japan).

Quantification of cGMP Derivatives by LC-MS/MS. Endogenous levels of 8-nitro-cGMP, 8-SH-cGMP, and cGMP were measured by LC-MS/MS using a stable-isotope dilution method.[17,18,37] The mouse brains were removed, washed twice with ice-cold PBS, and homogenized in a 10 volumes of methanol containing 5 mM NEM and 1 μmol ^{13}C-labeled authentic 8-NO_2-c[$^{13}C_{10}$]GMP[16,17] for 8-nitro-cGMP quantification, or methanol containing 2% acetic acid, authentic 8-SH-c[$^{13}C_{10}$]GMP and c[$^{15}N_5$]GMP for 8-SH-cGMP and

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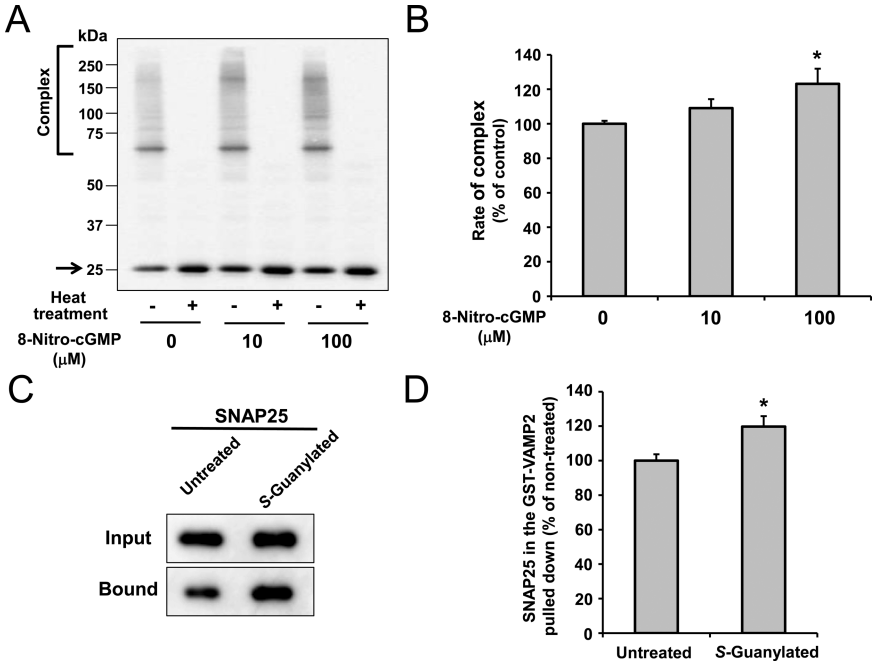

Figure 5. Modulation of SNARE complex formation by 8-nitro-cGMP. (A, B) Synaptosomes were treated with 8-nitro-cGMP (0, 10, 100 μM) for 1 h at room temperature and analyzed by Western blotting using anti-SNAP25 antibody. Samples were boiled for 3 min (heat treatment, +) or incubated at room temperature (heat treatment, −). The arrow indicates monomeric SNAP25, which was the only SNAP25 form present in the boiled sample (total SNAP25) (A). The change in the SDS-resistant SNARE complex was assessed based on the ratio of SNAP25 in the SNARE complex to the total SNAP25, quantified by densitometry and shown as the percentage of 8-nitro-cGMP-untreated synaptosomes (control). Data are presented as the mean ± SE (n = 7) One-way ANOVA with the Tukey's multiple comparison posthoc test was used for statistical analysis (B). (C, D) Binding of SNAP25 to other SNAREs was evaluated in an in vitro pull-down assay. Recombinant rat SNAP25 was S-guanylated with 8-nitro-cGMP (500 μM) for 3 h at room temperature, and preincubated with syntaxin-1A for 3 h at room temperature to form a dimeric complex. GST-VAMP2 bound to Glutathione Sepharose was then added to the mixture to form a ternary complex. The coprecipitated SNAP25 was detected by Western blotting using anti-SNAP25 antibody (C). The graph shows the band intensity of GST-VAMP2-precipitated SNAP25, calculated as a percentage of the nontreated SNAP25. Data are presented as the mean ± SE (n = 5). Student's t test was used for statistical analysis. *p < 0.05 was considered statistically significant when compared with values for the control (D).

cGMP quantification, respectively. The homogenate was centrifuged at 5,000g at 4 °C, and the resultant supernatant was dried in vacuo, and redissolved in distilled water for 8-nitro-cGMP analyze. On the other hand, for 8-SH-cGMP and cGMP analyze, the supernatant was collected and subjected to anion-exchange purification with Oasis WAX cartridge (Waters; Milford, MA). After the cartridge was washed with methanol, cGMP derivatives were collected into the elution with 1 mL of methanol containing 15% aqueous ammonia. Eluted sample was dried in vacuo and then redissolved in distilled water. LC-MS/MS was performed using a TSQ Vantage electrospray ionization triple-quadrupole (Q) mass spectrometer (Thermo Fisher Scientific) after reverse-phase high-performance liquid chromatography (HPLC). The observed ion masses (parent → daughter ions) were m/z 391 → 197 and m/z 401 → 202 for endogenous 8-nitro-cGMP and spiked 8-nitro-c[$^{13}C_{10}$]GMP, m/z 378 → 184 and m/z 388 → 189 for endogenous 8-SH-cGMP and spiked 8-SH-c[$^{13}C_{10}$]GMP, and m/z 346 → 152 and m/z 351 → 157 for endogenous cGMP and spiked c[$^{15}N_5$]GMP, respectively; collision energies of 17, 19, and 23 V were applied for 8-nitro-cGMP, 8-SH-cGMP, and cGMP, respectively. The signals of endogenous cGMP derivatives were identified simultaneously with respective ^{13}C or ^{15}N-derivative.

Isolation of Synaptosomes and 8-Nitro-cGMP Treatment. Synaptosomes were isolated from adult Wistar rats as described previously.[47] Rats were decapitated under anesthesia; the brain was removed and transferred immediately into ice-cold buffer containing 0.32 M sucrose, 5 mM HEPES, and 1% Protease Inhibitor Cocktail (Nacalai Tesque), pH 7.4. The tissue was homogenized by a rotor-stator homogenizer (Tokyo Rikakikai Co., Ltd.; Tokyo, Japan) and centrifuged at 800g for 10 min. The supernatant was recovered obtained and centrifuged again at 10 000g for 30 min, and then the pellet was resuspended in 0.32 M sucrose buffer and fractioned by discontinuous sucrose density gradient centrifugation. The band between 0.8 and 1.2 M sucrose assumed to be the synaptosome fraction was collected. Synaptosomes were diluted in three volumes of 5 mM HEPES (pH 7.4) containing 150 mM NaCl and centrifuged at 10 000g for 30 min. The resulting pellet was resuspended in low-K^+ solution (2.3 mM $CaCl_2$, 154 mM NaCl, 5.6 mM KCl, 5 mM HEPES, 5.6 mM D-glucose, and 5 mM $NaHCO_3$, pH 7.4), and was referred to as the synaptosome fraction.

The synaptosome fraction was treated with 8-nitro-cGMP (10 or 100 μM) for 1 h and centrifuged at 10 000g for 30 min. The pellet was solubilized in PBS containing 1% TritonX-100 and protease inhibitors, and centrifuged at 10 000g for 10 min. The supernatant was referred to as the solubilized synaptosomes.

Western Blotting. For Western blotting analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare UK Ltd.). The membrane were washed with TTBS (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6), blocked with Blocking One at room temperature for 30 min, rinsed, and incubated with primary antibodies diluted in TTBS containing 20% Blocking One at room temperature for 1 h. After washing three times in TTBS, the membranes were incubated with a peroxidase-conjugated secondary antibody at room temperature for 1 h, rinsed three times in TTBS, and immunoreactive bands were detected by enhanced chemiluminescence (Millipore; Bedford, MA, and Thermo Fisher Scientific) using a luminescent image analyzer (LAS-1000; Fuji Film; Tokyo, Japan).

Two-Dimensional PAGE. Two-dimensional PAGE (2D-PAGE) was performed as described previously.[22] Solubilized synaptosomal proteins (100 μg) were treated using the 2-D Clean-Up Kit (GE Healthcare UK Ltd.) according to the manufacturer's instructions and

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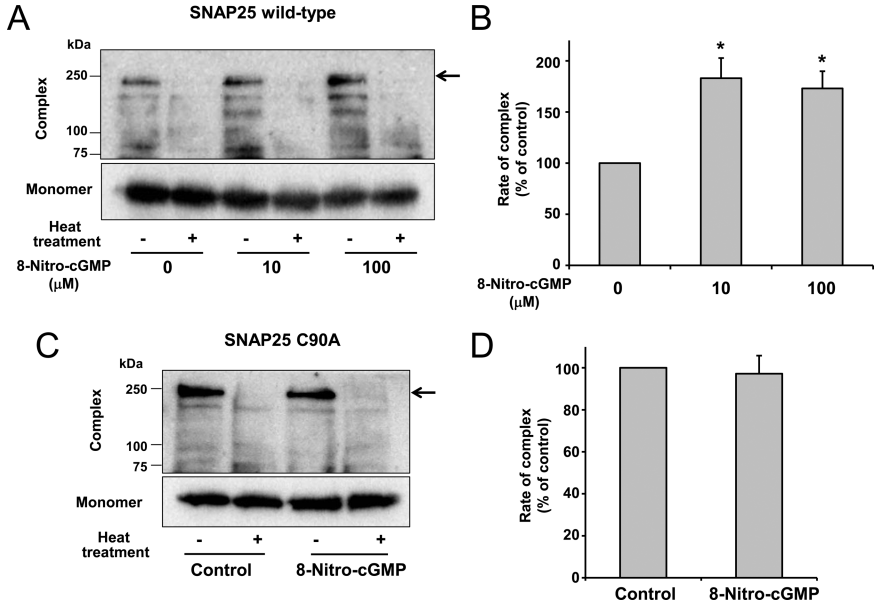

Figure 6. Modulation of SNARE complex formation by SNAP25 S-guanylation. Intracellular modulation of SNARE complex formation by 8-nitro-cGMP was evaluated by using SH-SY5Y neuroblastoma-transfected with FLAG-tagged SNAP25 [wild-type (A, B) and C90A (C, D)]. SH-SY5Y cells were treated without or with 10 and 100 μM (wild-type) or 100 μM (C90A) 8-nitro-cGMP at 37 °C for 3 h. Cell lysates were boiled for 3 min (heat treatment, +) or incubated at room temperature (heat treatment, −) and analyzed by Western blotting using the anti-FLAG antibody. The arrows indicate complexes with other SNARE proteins (A, C). Graphs show the rate of SNARE complex formation, quantified by densitometry and presented as a percentage of the control (mean ± SE). Data represent means ± SE (n = 4). Kruskal−Wallis test with the Dunn's Multiple Comparison test was used for statistical analysis. *p < 0.01 was considered statistically significant when compared with values for the control (B). Data represent means ± SE (n = 6). Student's t test was used for statistical analysis (D).

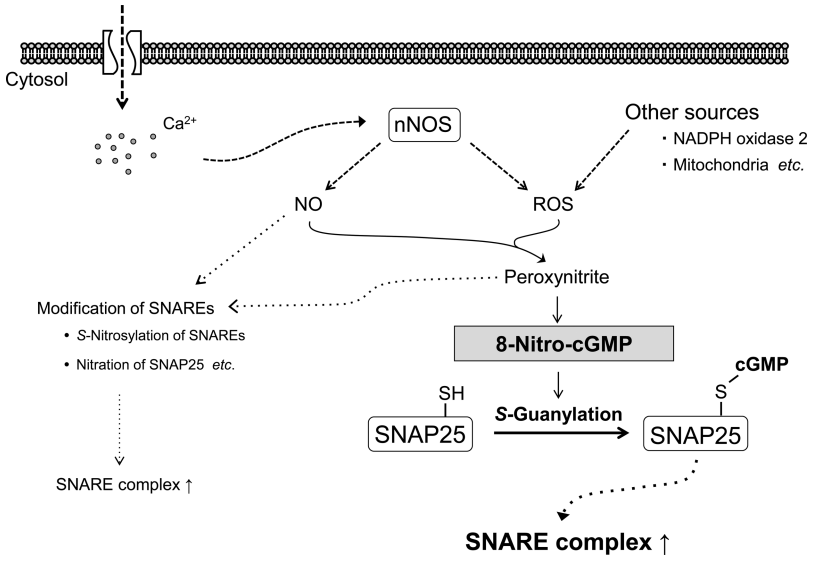

Figure 7. Schematic model for the regulation of redox signaling by SNAP25 S-guanylation in neurons. Elevation of intracellular Ca^{2+} levels activates nNOS. NO generated by nNOS and ROS derived from nNOS uncoupling reactions and other sources, rapidly react to form peroxynitrite, which is essential the production of 8-nitro-cGMP.[16,17,19] 8-Nitro-cGMP reacts with SNAP25, producing S-guanylated SNAP25, which stabilizes the SNARE complex.

dissolved in rehydration buffer containing 2 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, and 0.5% immobilized pH gradient (IPG) buffer, pH 3−10 NL (GE Healthcare UK Ltd.). IPG strips (7 cm, pH 3−10 NL; GE Healthcare UK Ltd.) were loaded with protein samples for 10 h at 10 °C using the Ettan IPGphor 3 apparatus (GE Healthcare UK Ltd.), and isoelectric focusing was performed at 300 V for 4 h; 1000 V (gradient) for 30 min; 5000 V (gradient) for 1.5 h; and 5000 V for another 3 h. The IPG strips were then incubated in equilibration buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 1% SDS, and 0.25% dithiothreitol for 15 min, and then in the same buffer containing 4.5% iodoacetamide for 15 min. The strips were then placed on a 12% polyacrylamide, and proteins were separated by SDS-PAGE and visualized by silver staining (Silver Stain MS Kit; Wako Pure Chemical Industries) or analyzed for S-guanylation by Western blotting. To identify spots containing S-guanylated proteins, the images of silver-stained gels were overlain with those of S-guanylated proteins detected by Western blotting[22] using Microsoft Power Point (Microsoft Corporation; Redmond, WA). The identified spots were subjected

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to in-gel digestion as previously described and analyzed by LC-MS/MS.[22]

Identification of S-Guanylated Proteins. S-Guanylated proteins were identified as previously described.[22] Briefly, peptide samples obtained by 2D-PAGE were analyzed using an Agilent 6510 electrospray ionization quadrupole time-of-flight tandem mass spectrometer (Agilent Technologies, Inc.; Santa Clara, CA). A microfluidic reverse-phase HPLC chip (5 μm particle size, 75 mm i.d., 43 mm length; Zorbax 300SB-C18, Agilent Technologies) was used for peptide separation. Protonated molecular ions were fragmented in the auto-MS/MS mode starting with the initial collision energy voltage of 3 V which was then increased to 3.7 V per 100 Da. The m/z range was 300−2400 Da in the MS mode and 59−3000 Da in the MS/MS mode. Mass lists in the form of Mascot files were used to perform Mascot MS/MS ion searches of the National Center for Biotechnology Information nonredundant (NCBI nr) database.

Plasmids. Plasmids containing rat *SNAP25B*, *syntaxin-1A*, and *VAMP2* cDNA were constructed as described previously.[48] The *SNAP25B* and *syntaxin-1A* plasmids were used as a template for PCR amplification of the respective genes. The following primers were used. *SNAP25B*: forward, 5′-CACCATGGCCGAGGACGCAGAC-3′ and reverse, 5′-TTAACCACTTCCCAGCATCT-3′. *syntaxin-1A*: forward, 5′-CACCATGAAGGACCGAACCCAG-3′ and reverse, 5′-TTACTTCTTCCTGCGTGCCTT-3′. The *VAMP2* gene was amplified from rat cerebellar granule cell cDNA using primers: forward, 5′-CACCATGTCGGCTACCGCTGC-3′ and reverse, 5′-TTACTTGAGGTTTTTCCACCAGT3′. The amplified *SNAP25B* gene was cloned into the pENTR/D-TOPO vector using the directional TOPO cloning system (Gateway Cloning Technology, Thermo Fisher Scientific), yielding the wild-type *SNAP25B* entry clone and was used to generate mutant *SNAP25B* clones in which cysteine residues were substituted for alanine.[49] For mutagenesis, the following forward and reverse primers, respectively, were used. C85A: 5′-GCCGGGCTTTGTGTGTGTCC-3′ and 5′- GAATTTTCCTAGGTCCGTCA-3′; C88A: 5′-GGCTTGCTGTGTGTCCCTGT-3′ and 5′-CGCAGAATTTTCCTAGGTC-3′; C90A: 5′-AAGCTTAAATCCAGTGATGC-3′ and 5′-GTTACAGGGAGCCACACAAAGCC-3′; C92A: 5′- CGCTAACAACCTTAAATCCAGTGATGC-3′ and 5′- GGACACACACAAAGCCCG-3′; mutated sites are underlined. All resulting clones were confirmed by sequencing. The rat *SNAP25B* wild-type and cysteine mutants, and *syntaxin-1A* into the pET30a-DEST (for His-tagged protein) expression plasmid by the LR reaction (Thermo Fisher Scientific). *VAMP2* entry clones were subcloned into the pDEST15 (for GST-tagged protein) expression plasmid; *SNAP25B* clones were also subcloned into pcDNA3.2/nFLAG-DEST.

Protein Expression and Purification. The expression plasmids were transferred into *Escherichia coli* BL21 Codon Plus (Agilent Technologies) grown in Luria−Bertani medium, and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside at OD_{600} = 0.8. Bacteria were pelleted, resuspended in PBS containing 1% TritonX-100 and protease inhibitor cocktail (Nacalai tesque), sonicated and centrifuged at 10,000g for 10 min. The cleared lysate was loaded onto a Ni-Sepharose 6 Fast Flow column (GE Healthcare UK Ltd.) for SNAP25B and syntaxin-1A purification, or a Glutathione Sepharose 4B column (GE Healthcare UK Ltd.) for VAMP2 purification. Ni^{2+} Sepharose columns were washed with wash buffer (20 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) and eluted with elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4). Glutathion Sepharose columns were washed with 50 mM Tris-HCl, pH 8.0 and eluted with 50 mM Tris-HCl, 10 mM reduced GSH, pH 8.0. The purity of eluted proteins was confirmed by SDS-PAGE.

Cell Culture, Transfection, and Stimulation. HEK-nNOS cells were obtained as described previously.[44] SH-SY5Y, HEK-293, and HEK-nNOS cells were cultured in DMEM with 10% FBS in a 5% CO_2 atmosphere at 37 °C. For immunoprecipitation, HEK-293 and HEK-nNOS cells were seeded at the density of 3.0×10^6 cells/dish in 100 mm culture dishes. For immunocytochemistry, cells were seeded on circular coverslips at the density of 1.0×10^5 cells/well in 24-well plates (AGC Techno Glass Co.; Shizuoka, Japan). Cells were transfected with *SNAP25B*/pcDNA3.2/nFLAG using polyethylenimine (PEI) Max (Polysciences, Inc.; Warrington, PA) and examined 48 h after transfection. To determine protein S-guanylation under the conditions of Ca^{2+}-dependent NO generation, cells were stimulated with 5 μM of a calcium ionophore A23187 for 12 h at 37 °C, washed three times with PBS, and solubilized in PBS containing 1% TritonX-100 and protease inhibitors. The lysate was cleared by centrifugation at 15 000g for 10 min, and the resulting supernatants were designated as HEK-293 and HEK-nNOS solubilized cell extracts. SH-SY5Y cells were seeded at the density of 6.0×10^5 cells/dish on 35 mm culture dishes, transfected with *SNAP25B*/pcDNA3.2/nFLAG using PEI Max, and examined 24 h after transfection. Cells were treated with 8-nitro-cGMP (10 and 100 μM) for 3 h, washed three times with PBS, and solubilized with PBS containing 1% TritonX-100 and protease inhibitors. The lysate was cleared by centrifugation at 15 000g for 10 min, and was referred to as SH-SY5Y solubilized cell extract.

Immunoprecipitation. Solubilized synaptosomes (300 μg) were incubated with the anti-8-RS-cGMP antibody bound to Protein A beads (Bio-Rad; Hercules, CA) or the anti-SNAP25 antibody bound to Protein G beads (Bio-Rad) at room temperature for 1 h. Immunocomplexes were collected by centrifugation and washed three times with 1% TritonX-100 in PBS. The bound proteins were eluted with SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8) separated in a 12% SDS-PAGE gel, and analyzed by Western blotting with the respective antibodies. HEK-293 and HEK-nNOS cell lysates were incubated with anti-DYKDDDDK (FLAG) antibody bound to magnetic beads (Wako Pure Chemical Industries) at 4 °C overnight. Immunocomplexes were collected using a magnetic stand (Thermo Fisher Scientific), washed three times with 1% TritonX-100 in PBS, and bound proteins were eluted with 0.1 M glycine-HCl buffer, pH 3.0. The eluted samples were immediately neutralized with 1.5 M Tris-HCl buffer, pH 8.8, separated in a 12% SDS-PAGE gel, and analyzed by Western blotting with the anti-FLAG and anti-8-RS-cGMP antibodies.

In Vitro S-Guanylation and Pull-Down Assays. The recombinant wild-type and mutant SNAP25B proteins were S-guanylated with 100 μM 8-nitro-cGMP for 3h at room temperature; S-guanylation was confirmed by Western blotting with the anti-8-RS-cGMP antibody. Pull-down assays were performed as described previously.[50] Recombinant rat SNAP25B was S-guanylated with 500 μM 8-nitro-cGMP for 3 h at room temperature, and preincubated with syntaxin-1A in binding buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet-P40, 0.5 mg/mL bovine serum albumin, and 0.01% sodium azide) for 3 h at room temperature to form dimeric complex. Purified GST-VAMP2 was bound to Glutathion Sepharose in binding buffer for 3 h at room temperature, washed three times with binding buffer. GST-VAMP2 bound to glutathione sepharose was then added to the mixture to form a ternary complex, and incubated for 3 h at room temperature. The beads were washed three times, resuspended in SDS-PAGE sample buffer, and eluted proteins were analyzed by Western blotting with anti-SNAP25 antibody.

SDS-Resistant Complexes. The detection of SDS-resistant complexes was carried out as described previously.[14,15] In brief, solubilized synaptosomes and cell extracts were mixed with SDS-PAGE sample buffer and aliquots were diluted in the same buffer. Paired samples were either boiled to dissociate the SNARE complex or incubated at room temperature for 3 min, and 3 μg of synaptosomal proteins or 50 μg of total cell proteins per gel lane were resolved by SDS-PAGE and analyzed by Western blotting with anti-SNAP25 or anti-FLAG antibodies.

Statistical Analysis. The data presented as the mean ± SE of individual experiments performed at least three times. Statistical significance was determined by one-way analysis of variance (ANOVA), Kruskal−Wallis test or the Student's t test using the GraphPad Prism Software (GraphPad Software, Inc.; La Jolla, CA).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00196.

Methods for cell culture and stimulation and figure showing Ca^{2+}-depenent nNOS activation in HEK-nNOS cells (PDF)

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Author Contributions

K.K., T.S., N.G., M.T., T.A., and H.I. designed research. K.K., H.T., T.I., Y.K., S.K., and M.I. performed experiments, analyzed, and interpreted the results. H.I. conducted the experiments. K.K., T.S., T.A., and H.I. contributed to data interpretation and manuscript writing.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Hsieh, H. L., and Yang, C. M. (2013) Role of redox signaling in neuroinflammation and neurodegenerative diseases. *BioMed Res. Int. 2013*, 484613.

(2) Okamoto, S., Nakamura, T., Cieplak, P., Chan, S. F., Kalashnikova, E., Liao, L., Saleem, S., Han, X., Clemente, A., Nutter, A., Sances, S., Brechtel, C., Haus, D., Haun, F., Sanz-Blasco, S., Huang, X., Li, H., Zaremba, J. D., Cui, J., Gu, Z., Nikzad, R., Harrop, A., McKercher, S. R., Godzik, A., Yates, J. R., 3rd, and Lipton, S. A. (2014) S-nitrosylation-mediated redox transcriptional switch modulates neurogenesis and neuronal cell death. *Cell Rep. 8*, 217−228.

(3) Akaike, T., Nishida, M., and Fujii, S. (2013) Regulation of redox signalling by an electrophilic cyclic nucleotide. *J. Biochem. 153*, 131−138.

(4) Di Domenico, F., Cenini, G., Sultana, R., Perluigi, M., Uberti, D., Memo, M., and Butterfield, D. A. (2009) Glutathionylation of the pro-apoptotic protein p53 in Alzheimer's disease brain: implications for AD pathogenesis. *Neurochem. Res. 34*, 727−733.

(5) Chung, K. K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J. C., Marsh, L., Dawson, V. L., and Dawson, T. M. (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science 304*, 1328−1331.

(6) Matsushita, K., Morrell, C. N., Cambien, B., Yang, S. X., Yamakuchi, M., Bao, C., Hara, M. R., Quick, R. A., Cao, W., O'Rourke, B., Lowenstein, J. M., Pevsner, J., Wagner, D. D., and Lowenstein, C. J. (2003) Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell 115*, 139−150.

(7) Giniatullin, A. R., Darios, F., Shakirzyanova, A., Davletov, B., and Giniatullin, R. (2006) SNAP25 is a pre-synaptic target for the depressant action of reactive oxygen species on transmitter release. *J. Neurochem. 98*, 1789−1797.

(8) Kakizawa, S., Shibazaki, M., and Mori, N. (2012) Protein oxidation inhibits NO-mediated signaling pathway for synaptic plasticity. *Neurobiol. Aging 33*, 535−545.

(9) Bredt, D. S., Hwang, P. M., and Snyder, S. H. (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature 347*, 768−770.

(10) Madhusoodanan, K. S., and Murad, F. (2007) NO-cGMP signaling and regenerative medicine involving stem cells. *Neurochem. Res. 32*, 681−694.

(11) Taqatqeh, F., Mergia, E., Neitz, A., Eysel, U. T., Koesling, D., and Mittmann, T. (2009) More than a retrograde messenger: nitric oxide needs two cGMP pathways to induce hippocampal long-term potentiation. *J. Neurosci. 29*, 9344−9350.

(12) Radi, R. (2004) Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U. S. A. 101*, 4003−4008.

(13) Schopfer, F. J., Baker, P. R., and Freeman, B. A. (2003) NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? *Trends Biochem. Sci. 28*, 646−654.

(14) Meffert, M. K., Calakos, N. C., Scheller, R. H., and Schulman, H. (1996) Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron 16*, 1229−1236.

(15) Di Stasi, A. M., Mallozzi, C., Macchia, G., Maura, G., Petrucci, T. C., and Minetti, M. (2002) Peroxynitrite affects exocytosis and SNARE complex formation and induces tyrosine nitration of synaptic proteins. *J. Neurochem. 82*, 420−429.

(16) Sawa, T., Zaki, M. H., Okamoto, T., Akuta, T., Tokutomi, Y., Kim-Mitsuyama, S., Ihara, H., Kobayashi, A., Yamamoto, M., Fujii, S., Arimoto, H., and Akaike, T. (2007) Protein S-guanylation by the biological signal 8-nitroguanosine 3′,5′-cyclic monophosphate. *Nat. Chem. Biol. 3*, 727−735.

(17) Fujii, S., Sawa, T., Ihara, H., Tong, K. I., Ida, T., Okamoto, T., Ahtesham, A. K., Ishima, Y., Motohashi, H., Yamamoto, M., and Akaike, T. (2010) The critical role of nitric oxide signaling, via protein S-guanylation and nitrated cyclic GMP, in the antioxidant adaptive response. *J. Biol. Chem. 285*, 23970−23984.

(18) Ihara, H., Ahtesham, A. K., Ida, T., Kasamatsu, S., Kunieda, K., Okamoto, T., Sawa, T., and Akaike, T. (2011) Methodological proof of immunochemistry for specific identification of 8-nitroguanosine 3′,5′-cyclic monophosphate formed in glia cells. *Nitric Oxide 25*, 169−175.

(19) Ahmed, K. A., Sawa, T., Ihara, H., Kasamatsu, S., Yoshitake, J., Rahaman, M. M., Okamoto, T., Fujii, S., and Akaike, T. (2012) Regulation by mitochondrial superoxide and NADPH oxidase of cellular formation of nitrated cyclic GMP: potential implications for ROS signalling. *Biochem. J. 441*, 719−730.

(20) Sawa, T., Ihara, H., Ida, T., Fujii, S., Nishida, M., and Akaike, T. (2013) Formation, signaling functions, and metabolisms of nitrated cyclic nucleotide. *Nitric Oxide 34*, 10−18.

(21) Nishida, M., Sawa, T., Kitajima, N., Ono, K., Inoue, H., Ihara, H., Motohashi, H., Yamamoto, M., Suematsu, M., Kurose, H., van der Vliet, A., Freeman, B. A., Shibata, T., Uchida, K., Kumagai, Y., and Akaike, T. (2012) Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydration. *Nat. Chem. Biol. 8*, 714−724.

(22) Rahaman, M. M., Sawa, T., Ahtesham, A. K., Khan, S., Inoue, H., Irie, A., Fujii, S., and Akaike, T. (2014) S-guanylation proteomics for

DOI: 10.1021/acschemneuro.5b00196
ACS Chem. Neurosci. 2015, 6, 1715−1725

redox-based mitochondrial signaling. *Antioxid. Redox Signaling 20*, 295−307.
(23) Brunger, A. T., Weninger, K., Bowen, M., and Chu, S. (2009) Single-molecule studies of the neuronal SNARE fusion machinery. *Annu. Rev. Biochem. 78*, 903−928.
(24) Halemani, N. D., Bethani, I., Rizzoli, S. O., and Lang, T. (2010) Structure and dynamics of a two-helix SNARE complex in live cells. *Traffic 11*, 394−404.
(25) Sudhof, T. C. (2013) Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron 80*, 675−690.
(26) Wang, Y., Dong, Y., Song, H., Liu, Y., Liu, M., Yuan, Y., Ding, F., Gu, X., and Wang, Y. (2012) Involvement of gecko SNAP25b in spinal cord regeneration by promoting outgrowth and elongation of neurites. *Int. J. Biochem. Cell Biol. 44*, 2288−2298.
(27) Pozzi, D., Condliffe, S., Bozzi, Y., Chikhladze, M., Grumelli, C., Proux-Gillardeaux, V., Takahashi, M., Franceschetti, S., Verderio, C., and Matteoli, M. (2008) Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. *Proc. Natl. Acad. Sci. U. S. A. 105*, 323−328.
(28) Kataoka, M., Kuwahara, R., Matsuo, R., Sekiguchi, M., Inokuchi, K., and Takahashi, M. (2006) Development- and activity-dependent regulation of SNAP-25 phosphorylation in rat brain. *Neurosci. Lett. 407*, 258−262.
(29) Kataoka, M., Yamamori, S., Suzuki, E., Watanabe, S., Sato, T., Miyaoka, H., Azuma, S., Ikegami, S., Kuwahara, R., Suzuki-Migishima, R., Nakahara, Y., Nihonmatsu, I., Inokuchi, K., Katoh-Fukui, Y., Yokoyama, M., and Takahashi, M. (2011) A single amino acid mutation in SNAP-25 induces anxiety-related behavior in mouse. *PLoS One 6*, e25158.
(30) Yamamori, S., Sugaya, D., Iida, Y., Kokubo, H., Itakura, M., Suzuki, E., Kataoka, M., Miyaoka, H., and Takahashi, M. (2014) Stress-induced phosphorylation of SNAP-25. *Neurosci. Lett. 561*, 182−187.
(31) Antonucci, F., Corradini, I., Morini, R., Fossati, G., Menna, E., Pozzi, D., Pacioni, S., Verderio, C., Bacci, A., and Matteoli, M. (2013) Reduced SNAP-25 alters short-term plasticity at developing glutamatergic synapses. *EMBO Rep. 14*, 645−651.
(32) Hou, Q., Gao, X., Zhang, X., Kong, L., Wang, X., Bian, W., Tu, Y., Jin, M., Zhao, G., Li, B., Jing, N., and Yu, L. (2004) SNAP-25 in hippocampal CA1 region is involved in memory consolidation. *Eur. J. Neurosci. 20*, 1593−1603.
(33) Hou, Q. L., Gao, X., Lu, Q., Zhang, X. H., Tu, Y. Y., Jin, M. L., Zhao, G. P., Yu, L., Jing, N. H., and Li, B. M. (2006) SNAP-25 in hippocampal CA3 region is required for long-term memory formation. *Biochem. Biophys. Res. Commun. 347*, 955−962.
(34) McKee, A. G., Loscher, J. S., O'Sullivan, N. C., Chadderton, N., Palfi, A., Batti, L., Sheridan, G. K., O'Shea, S., Moran, M., McCabe, O., Fernandez, A. B., Pangalos, M. N., O'Connor, J. J., Regan, C. M., O'Connor, W. T., Humphries, P., Farrar, G. J., and Murphy, K. J. (2010) AAV-mediated chronic over-expression of SNAP-25 in adult rat dorsal hippocampus impairs memory-associated synaptic plasticity. *J. Neurochem. 112*, 991−1004.
(35) Baca, M., Allan, A. M., Partridge, L. D., and Wilson, M. C. (2013) Gene-environment interactions affect long-term depression (LTD) through changes in dopamine receptor affinity in Snap25 deficient mice. *Brain Res. 1532*, 85−98.
(36) Greber, S., Lubec, G., Cairns, N., and Fountoulakis, M. (1999) Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease. *Electrophoresis 20*, 928−934.
(37) Ida, T., Sawa, T., Ihara, H., Tsuchiya, Y., Watanabe, Y., Kumagai, Y., Suematsu, M., Motohashi, H., Fujii, S., Matsunaga, T., Yamamoto, M., Ono, K., Devarie-Baez, N. O., Xian, M., Fukuto, J. M., and Akaike, T. (2014) Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proc. Natl. Acad. Sci. U. S. A. 111*, 7606−7611.
(38) Bark, I. C., Hahn, K. M., Ryabinin, A. E., and Wilson, M. C. (1995) Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. *Proc. Natl. Acad. Sci. U. S. A. 92*, 1510−1514.
(39) Graham, M. E., Washbourne, P., Wilson, M. C., and Burgoyne, R. D. (2002) Molecular analysis of SNAP-25 function in exocytosis. *Ann. N. Y. Acad. Sci. 971*, 210−221.
(40) Mohrmann, R., de Wit, H., Verhage, M., Neher, E., and Sorensen, J. B. (2010) Fast vesicle fusion in living cells requires at least three SNARE complexes. *Science 330*, 502−505.
(41) Sorensen, J. B., Matti, U., Wei, S. H., Nehring, R. B., Voets, T., Ashery, U., Binz, T., Neher, E., and Rettig, J. (2002) The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. *Proc. Natl. Acad. Sci. U. S. A. 99*, 1627−1632.
(42) Greaves, J., Prescott, G. R., Fukata, Y., Fukata, M., Salaun, C., and Chamberlain, L. H. (2009) The hydrophobic cysteine-rich domain of SNAP25 couples with downstream residues to mediate membrane interactions and recognition by DHHC palmitoyl transferases. *Mol. Biol. Cell 20*, 1845−1854.
(43) Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D. A., and Giuffrida Stella, A. M. (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat. Rev. Neurosci. 8*, 766−775.
(44) Song, T., Sugimoto, K., Ihara, H., Mizutani, A., Hatano, N., Kume, K., Kambe, T., Yamaguchi, F., Tokuda, M., and Watanabe, Y. (2007) p90 RSK-1 associates with and inhibits neuronal nitric oxide synthase. *Biochem. J. 401*, 391−398.
(45) Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito, T., and Takahashi, M. (1992) HPC-1 is associated with synaptotagmin and omega-conotoxin receptor. *J. Biol. Chem. 267*, 24925−24928.
(46) Izawa, T., Takenaka, S., Ihara, H., Kotani, T., Yamate, J., Franklin, R. J., and Kuwamura, M. (2008) Cellular responses in the spinal cord during development of hypomyelination in the mv rat. *Brain Res. 1195*, 120−129.
(47) Iida, Y., Yamamori, S., Itakura, M., Miyaoka, H., and Takahashi, M. (2013) Protein phosphatase 2A dephosphorylates SNAP-25 through two distinct mechanisms in mouse brain synaptosomes. *Neurosci. Res. 75*, 184−189.
(48) Kataoka, M., Kuwahara, R., Iwasaki, S., Shoji-Kasai, Y., and Takahashi, M. (2000) Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells: a possible involvement in the regulation of SNAP-25 localization. *J. Neurochem. 74*, 2058−2066.
(49) Kasamatsu, S., Watanabe, Y., Sawa, T., Akaike, T., and Ihara, H. (2014) Redox signal regulation via nNOS phosphorylation at Ser847 in PC12 cells and rat cerebellar granule neurons. *Biochem. J. 459*, 251−263.
(50) Taniguchi, M., Taoka, M., Itakura, M., Asada, A., Saito, T., Kinoshita, M., Takahashi, M., Isobe, T., and Hisanaga, S. (2007) Phosphorylation of adult type Sept5 (CDCrel-1) by cyclin-dependent kinase 5 inhibits interaction with syntaxin-1. *J. Biol. Chem. 282*, 7869−7876.

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