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Neuregulin 1 as an endogenous regulator of nicotinic acetylcholine receptors in adult major pelvic ganglion neurons

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ABSTRACT

We investigated whether endogenous neuregulin 1 (NRG1) is released in a soluble form (called sNRG1) and upregulates expression of nicotinic acetylcholine receptor (nAChR) in autonomic major pelvic ganglion (MPG) neurons of adult rats. To elicit the release of sNRG1, either the hypogastric nerve or the pelvic nerve was electrically stimulated. Then, the MPG-conditioned medium (CM) was subjected to western blotting using an antibody directed against the N-terminal ectodomain of NRG1. Both sympathetic and parasympathetic nerve activation elicited the release of sNRG1 from MPG neurons in a frequency-dependent manner. The sNRG1 release was also induced by treatment of MPG neurons with either high KCl or neurotrophic factors. The biological activity of the released sNRG1 was detected by tyrosine phosphorylation (p185) of the ErbB2 receptors in MPG neurons. When MPG neurons were incubated for 6 h in the CM, the protein level of the nAChR α3 subunit and ACh-induced current (I_{ACh}) density were significantly increased. The CM-induced changes in I_{ACh} was abolished by a selective ErbB2 tyrosine kinase inhibitor. Taken together, these data suggest that NRG1 functions as an endogenous regulator of nAChR expression in adult MPG neurons.

1. Introduction

Peripheral autonomic ganglia serve not only as a relay of the central command signals but also as an integration center where multiple extrinsic and intrinsic neuronal inputs are processed before being transmitted to various target organs including the heart and the bladder [1,2]. Synaptic transmission in autonomic ganglia is mediated primarily by acetylcholine (ACh) which activates nicotinic ACh receptor (nAChR) in postganglionic neurons. Previous studies have shown that activity of the ganglionic nAChR can be acutely modulated in a non-genomic way by substances derived from various sources within the ganglia [3,4]. To date, however, it remains unclear how nAChR is regulated over the long-term in adult autonomic ganglia.

Neuregulin 1 (NRG1) belongs to a family of growth and differentiation factors that are crucial for development and plasticity of the nervous system [5]. Multiple NRG1 isoforms are generated by alternative splicing and specific promoter sites and are classified into three major groups (type I, II, and III) based on the N-terminal sequence of their extracellular domain. Most NRG1 isoforms are initially synthesized as large membrane-spanning precursors. By proteolytic cleavage, the extracellular domains of types I and II NRG are free of the plasma membrane and secreted as soluble mature forms. In contrast, the cleaved type III NRG is tethered to the membrane by the hydrophobic cysteine-xrich domain for juxtacrine signaling. NRG1 isoforms activate the ErbB family of receptor tyrosine kinases (ErbB2, ErbB3 and ErbB4). Soluble or membrane-bound forms of NRG1 share an EGF-like domain for binding either ErbB3 or ErbB4, which stimulates receptor dimerization to form heterodimeric ErbB2/ErbB3 or homodimeric ErbB4/ErbB4, respectively [6]. Receptor dimerization leads to phosphorylation of specific tyrosine residues within the cytoplasmic tail of receptors by activating receptor tyrosine kinase intrinsic to ErbB2 and ErbB4 and then activates multiple downstream signaling for producing various biological activities.

One of the biological activities exerted by NRG1 is regulating expression of nAChR in skeletal muscles during development [7,8].

Abbreviations: ACh, acetylcholine; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CM, conditioned medium; HGN, hypogastric nerves; nAChR, nicotinic acetylcholine receptor; MPG, major pelvic ganglia; NGF, nerve growth factor; PVN, pelvic nerves; NRG1, neuregulin 1; sNRG1, soluble NRG1.

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Likewise, NRG1 is also known to play critical roles in the formation of nicotinic synapses by increasing the expression of nAChR subunits in embryonic sympathetic neurons [9]. We recently demonstrated that expression of NRG1 and its receptors (ErbB2/ErbB3) is retained in mature autonomic neurons and that the recombinant NRG1β increases nAChR currents (I_{ACh}) regardless of cell phenotype (i.e., sympathetic and parasympathetic) [10]. These findings have suggested that NRG1 is an endogenous regulator of nAChR expression in adult autonomic synapses. Accordingly, in the present study, we tested whether different stimulants release the soluble form of NRG1 (sNRG1) from the major pelvic ganglia (MPG) of adult rats, and whether the released sNRG1 is capable of upregulating protein levels of nAChR subunits as well as I_{ACh} density through tyrosine phosphorylation (p185) of ErbB receptors.

2. Materials and methods

2.1. Chemicals and antibodies

Rabbit polyclonal anti-neuregulin 1α/β1/2 (C-20) and anti-neuregulin 1 (H-210) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-nAChR-α3 and anti-nAChR-β4 antibodies were from Santa Cruz Biotechnology and Research Diagnostics (Flanders, NJ, USA), respectively. Rabbit polyclonal anti-ErbB2 (Neu, C-18) and anti-pErbB2 (Tyr1248) antibodies were from Santa Cruz Biotechnology and Cell Signaling Technology (Beverly, MA, USA), respectively. Rabbit monoclonal anti-β-actin antibody was from Santa Cruz Biotechnology. Nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and brain-derived neurotrophic factor (BDNF) were from Sigma Chemical Co (St Louis, MO, USA). Recombinant NRG1-1β (amino acid residues 176–246) was from R&D System (Minneapolis, MN, USA). AG-825 and genistein were from Calbiochem (Beeston, UK).

2.2. Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju College of Medicine (approval No. YWC-110330-1). Adult male Sprague–Dawley rats (200–300 g) were obtained from OrientBio Co. (Seongnam-Si, Republic of Korea) and were housed in separate cages under a 12:12 h light/dark cycle with controlled temperature (21–22 °C) and *ad libitum* access to food and water.

2.3. Electrical stimulation for sNRG1 release from MPG

The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p) and placed on a heating pad to maintain body temperature. The pelvic nerve (PVN) and hypogastric nerve (HGN) were identified at the lateral surface of the prostate after a midline abdomen incision. Either of the nerves was introduced into a suction electrode filled with 0.9% NaCl and connected to a stimulator (Harvard Apparatus, England) via an isolation unit. The nerve was electrically stimulated at different frequencies ranging from 0 to 30 Hz with a square pulse (1 ms, 1 V) for 10 s. To ensure selective stimulation of the PVN or the HGN, we also monitored intracavernous pressure [11]. Immediately after stimulation, MPG were isolated and transferred to minimum essential medium (MEM) containing 1% L-glutamine and 1% penicillin-streptomycin for 2 h at 37 °C. The conditioned medium (CM) was harvested by centrifugation at 13,200 rpm for 5 min at 4 °C.

2.4. Western blotting analysis

Proteins from whole ganglia were prepared as previously described [10]. After boiling in sodium dodecyl sulfate (SDS) buffer for 5 min, MPG lysates (20 μg) and CM samples (20 μl) were electrophoresed on 1.0% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were blocked with 0.1% Tris-buffered saline/Tween-20 (TBST) containing 5% bovine serum albumin (BSA, Sigma) for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies against nAChR-α3 (H-100) (1:200), nAChR-β4 (1:200), neuregulin 1α/β1/2 (C-20) (1:400), neuregulin 1 (H-210) (1:400), ErbB2 (1:200), pErbB2 (1:200), and β-actin (1:2000). Membranes were washed several times with cold 0.1% TBST and incubated with horseradish-peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. After intensive washing, immunoreactive bands were visualized using Luminata™ forte Western HRP substrate (Millipore) on FluorChem FC-2 (Alpha Innotech CO, San Leandro, CA, USA). As confirmed in previous studies [12], immunoblotting of MPG lysates with the N-terminal antibody revealed pro-NRG1 (135 kDa) and N-terminal fragments of NRG1 at 48–55 kDa and 68~72 kDa which are cleaved from type I and type III NRG1, respectively (data not shown).

2.5. Electrophysiological recordings

MPG neurons were enzymatically dissociated as previously described [10,13]. For current recording, neurons were plated in culture dishes (35-mm) coated with poly-L-lysine (Sigma) and maintained in a humidified 95% air/5% CO_2 incubator at 37 °C until use. Current recordings in MPG neurons were obtained under the whole-cell-ruptured configuration of the patch-clamp technique using an EPC-10 amplifier and pulse/pulsefit (v8.50) software (HEKA Electronik, Lambrecht, Germany) as previously described [10]. Cell types of the MPG neurons were identified according to previously established criteria: cell size as assessed by magnitude of the electrical capacitance, responses to GABA and 5-HT, presence or absence of the T-type Ca^{2+} channel-mediated anodal break rebound spike, and the firing patterns (i.e., tonic vs. phasic) in response to depolarizing current injection [13–15].

2.6. Statistical analysis

Data analysis was performed with the IGOR data analysis package (Wave-Metrics, Lake Oswego, OR, USA) or GraphPad Prism (ver 4.0, GraphPad Software Inc, La Jolla, CA, USA). Data are presented as the mean ± SEM. Student's *t* test or one-way ANOVA analyses with Fisher's PLSD were performed. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Release of sNRG1 from MPG by electrical stimulation of preganglionic nerves

Mature MPG neurons have been found to express type I and type III NRG1 [10]. In the present study, we focused on the release of sNRG1 cleaved from the type I isoform for two reasons. First, unlike type III NRG1, which is primarily bound to the pre-synaptic membrane, type I NRG1 is secreted as a soluble form after proteolytic cleavage [16]. Second, the recombinant type I NRG1 but not the type III NRG1 was found to up-regulate nAChR expression in adult MPG neurons [10]. To elicit sNRG1 release from MPG neurons, preganglionic nerves (i.e., HGN and PVN) in situ were electrically stimulated using a suction electrode. The frequency of stimulation

varied between 0 and 30 Hz with a fixed amplitude (1 V) and duration (10 s). Immediately after stimulation, MPG were isolated and incubated in serum-free MEM for 2 h. Then, the CM was subjected to western blotting analysis using the anti-neuregulin 1 (H-210) antibody directed against the N-terminal EGF-like domain. N-terminal fragments (NTFs) of NRG1 at 48~55 kDa are the sNRG1 cleaved from the type I NRG1 [12]. The appearance of two bands may be due to variable N-linked glycosylation of the extracellular domain [17]. Electrical stimulation of either the HGN or the PVN elicited sNRG1 release from MPG neurons in a frequency-dependent manner (Fig. 1A and B, n = 3). The nerve stimulation-induced sNRG1 release was saturated at a relatively low frequency (3–10 Hz).

3.2. Requirement of membrane depolarization for sNRG1 release from MPG neurons

Next, we tested whether the sNRG1 rlease from MPG neurons requires membrane depolarization. MPG neurons were incubated in serum-free culture media containing a low (25 mM) or high (50 mM) concentration of KCl for 1 h. Immunoblotting analysis of the CM showed that high concentration of KCl significantly increased sNRG1 release from MPG neurons (Fig. 2A). The high KCl elicited sNRG1 release approximately one order of magnitude higher than the low KCl. On average, the relative sNRG1 levels were 0.04 ± 0.02(n = 3), 0.45 ± 0.26(n = 3), and 5.3 ± 0. 6(n = 3) for control, low and high concentrations of KCl, respectively. Equivalent addition of sucrose (100 mM) instead of the high KCl did not affect sNRG1 release, indicating that the effects of KCl did not arise from osmotic changes.

3.3. Release of sNRG1 from MPG neurons by neurotrophic factors

Previous studies have shown that neurotrophic factors such as BDNF and NGF can induce cleavage and release of sNRG1 from precursors [18,19]. Thus, MPG neurons were exposed to NGF (100 ng/ml), CNTF (100 ng/ml), or BDNF (100 ng/ml) for 12 h and then the culture medium was immunoblotted for sNRG1. As a result, these neurotrophic factors significantly increased sNRG1 release from MPG neurons (Fig. 2B). On average, relative sNRG1 levels were 1.0 ± 0.4 (n = 3), 4.0 ± 0.5(n = 3), 4.6 ± 0.3(n = 3), and 4.0 ± 0.2(n = 3) for control, NGF, BDNF, and CNTF, respectively.

3.4. Phosphorylation of ErbB2 receptors by the sNRG1 released from MPG neurons

MPG neurons of adult rats express ErbB2 and ErbB3 receptors [10]. ErbB2 has tyrosine kinase activity with no NRG1 binding sites; as such it functions as a co-receptor that forms a heterodimer with catalytically inactive ErbB3 receptors with NRG1 binding sites. To assess the biological activity of the sNRG1 released from MPG neurons, tyrosine phosphorylation (p185) of ErbB2 was examined by immunoblotting using an anti-phospho ErbB2 antibody. Freshly isolated MPGs were incubated in CM containing the sNRG1 released from MPG neurons by electrical stimulation of the PVN. As shown in Fig. 4A, phosphorylation of ErbB2 peaked within 10 min after incubation (Fig. 3A). The protein levels of pErbB2 normalized to the total ErbB2 are summarized in Fig. 3B. These results indicate that sNRG1 in the CM is capable of activating ErbB2/ErbB3 receptors expressed in MPG neurons.

3.5. Upregulation of nAChR protein levels by sNRG1 released from MPG neurons

The predominant composition of nAChR subunits in sympathetic and parasympathetic MPG neurons is α3β4* which mediates fast excitatory synaptic transmission [20]. Previously, recombinant NRG1 was found to upregulate the expression of nAChR α3 and β4 subunits in MPG neurons [10]. Thus, we examined whether CM exerts the same effects as recombinant NRG1. Western blotting analysis showed that the protein level of the nAChR α3 subunit was increased when MPG neurons were incubated for 6 h in CM (Fig. 4A and B). On average, the protein levels of the nAChR α3 subunit when normalized to β-actin were 0.48 ± 0.09 (n = 3) and 0.76 ± 0.07 (n = 3) in control and CM-treated neurons, respectively. However, the effects of CM on the nAChR β4 subunit expression were not significant.

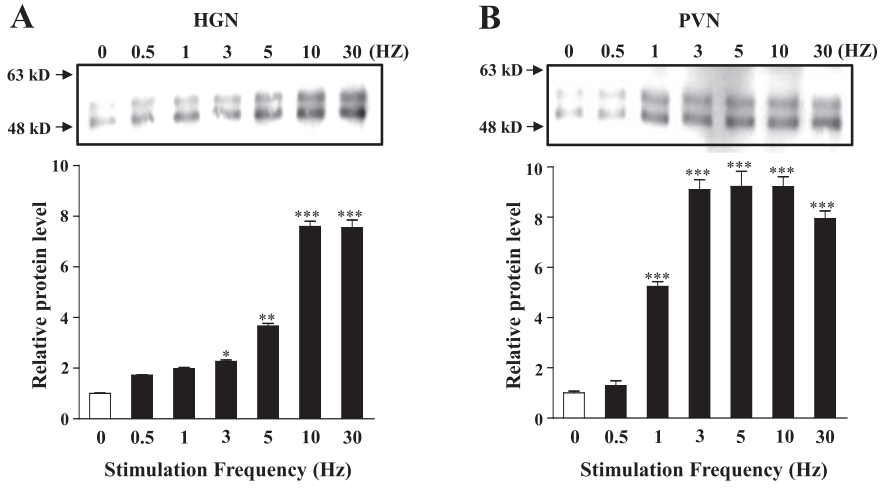

Fig. 1. Frequency-dependent release of sNRG1 from MPG neurons by preganglionic stimulation. (A) The hypogastric nerve (HGN) or (B) the pelvic nerve (PVN) were electrically stimulated using suction electrodes. The frequency of stimulation varied between 0 and 30 Hz with fixed amplitude (1 V) and duration (10 s). Immediately after stimulation, MPGs were isolated and incubated in serum-free MEM for 2 h. The MPG conditioned medium (CM) was then subjected to western blotting using an anti-neuregulin 1 (H-210) antibody directed against the N-terminal EGF-like domain. N-terminal fragments (NTFs) of NRG1 at 48~55 kDa are the sNRG1 cleaved from type I NRG1. The levels of sNRG1 proteins at different frequencies of stimulation were normalized to that acquired when no stimulation was applied (i.e., 0 Hz). Data are presented as the mean ± SEM. Three rats were used for each nerve stimulation experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

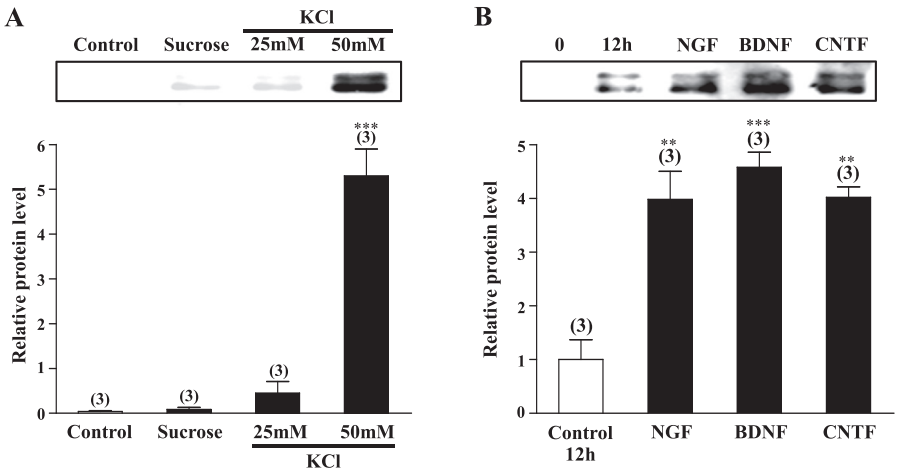

Fig. 2. sNRG1 release from MPG neurons by KCl-induced depolarization and neurotrophic factors. Freshly isolated MPG were incubated in the serum-free culture media containing (A) low (25 mM) or high (50 mM) KCl for 1 h, or (B) different neurotrophic factors for 12 h at 37 °C. Then, the MPG CM was subjected to western blotting using an anti-neuregulin 1 (H-210) antibody directed against the N-terminal EGF-like domain. The levels of sNRG1 proteins were normalized to control (no KCl for 1 h or no neurotrophic factor treatment for 12 h). Sucrose (100 mM) was used instead of high KCl to test osmotic effects. Data are presented as the mean ± SEM. Numbers of experiment are indicated in parentheses. $^{**}p < 0.01$; $^{***}p < 0.001$.

3.6. Increase in I_{ACh} density by sNRG1 released from MPG neurons

One of the unique features of the MPG is that sympathetic and parasympathetic neurons co-localize within the same ganglion capsule [21]. Thus, we evaluated nAChR channel activity in both types of MPG neurons which were incubated for 6 h in the CM containing sNRG1. Inward I_{ACh} was evoked by 100 μM ACh in the MPG neurons held at −60 mV under the whole cell-ruptured configuration of the patch-clamp technique. Consistent with the increased nAChR protein levels, the I_{ACh} was significantly increased when sympathetic and parasympathetic MPG neurons were incubated in the CM ($p < 0.01$) (Fig. 4C and D). On average, the I_{ACh} densities were 365 ± 14 pA/pF (n = 18) and 448 ± 17 pA/pF (n = 21) in control and CM-treated sympathetic MPG neurons, respectively, and 326 ± 11 pA/pF (n = 20) and 376 ± 9 pA/pF (n = 21) in control and CM-treated parasympathetic MPG neurons, respectively. The CM-induced changes in I_{ACh} were abolished by AG825(10 μM), a selective ErbB2 tyrosine kinase inhibitor, strongly suggesting that the sNRG1 in CM regulates nAChR expression and activity in MPG neurons (Fig. 4C and D). Genistein, a non-specific tyrosine kinase inhibitor (200 μM) mimicked AG825.

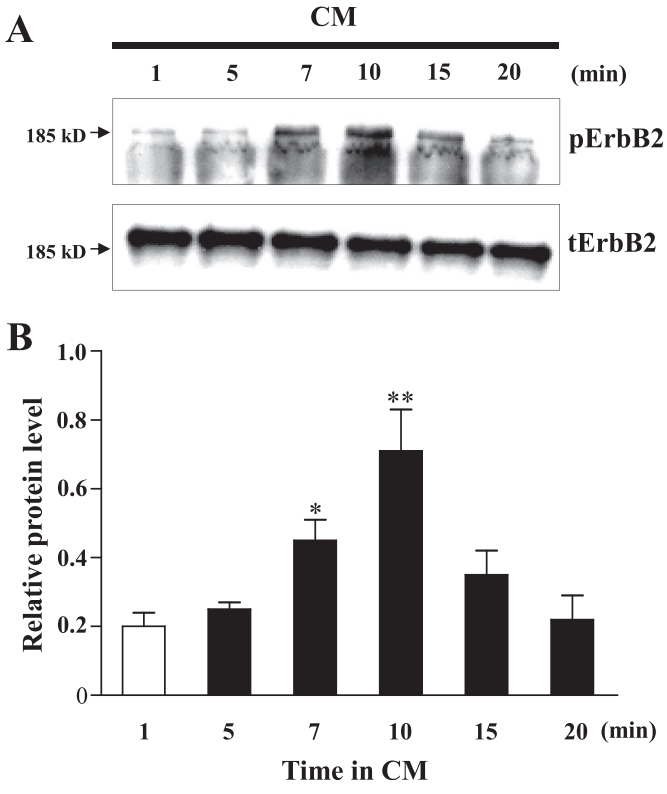

Fig. 3. Time course of tyrosine (p185) phosphorylation of ErbB2 receptors by sNRG1 in MPG neurons. (A) Freshly isolated MPGs were incubated for different time durations (1–20 min) at 37 °C in CM containing sNRG1 released by electrical stimulation of the PVN. MPG lysates were then subjected to 1.0% SDS-PAGE. Tyrosine (p185) phosphorylation of the ErbB2 receptors peaked within 10 min after incubation. (B) The relative protein levels at different incubation time points were summarized after normalizing phosphorylated ERbB2 (pErbB2) to total ErbB2 (tErbB2). Data are presented as the mean ± SEM. Numbers of experiments are indicated in parentheses. $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

The key observations in the present study are that (i) endogenous NRG1 can be released as a soluble form (sNRG1) from MPG neurons of adult rats by preganglionic nerve stimulation, chemical depolarization, and neurotrophic factors, and (ii) the sNRG1 is capable of upregulating nAChR proteins and currents through activation of ErbB receptors in sympathetic and parasympathetic MPG neurons.

The presence of sNRG1 in the CM was detected using the anti-neuregulin 1 (H-210) antibody directed against the N-terminal EGF-like domain. Biological activity of the sNRG1 in the CM could be assayed by immunoblotting for tyrosine (p185) phosphorylation of the ErbB2 receptors which form heterodimers with ErbB3 receptors in MPG neurons [10]. The CM containing sNRG1 significantly increased nAChR α3 protein levels. The nAChR β4 subunit appears to be less sensitive to the CM when compared with the nAChR α3 subunit in MPG neurons. A study using transgenic mice has suggested that the α3 subunit is the most important component in ganglionic nAChRs [22]. Accordingly, the CM-induced change in nAChR α3 proteins is considered to be sufficient to increase I_{ACh} in MPG neurons. More importantly, we found that the CM-induced increases in I_{ACh} density are due to the sNRG1.

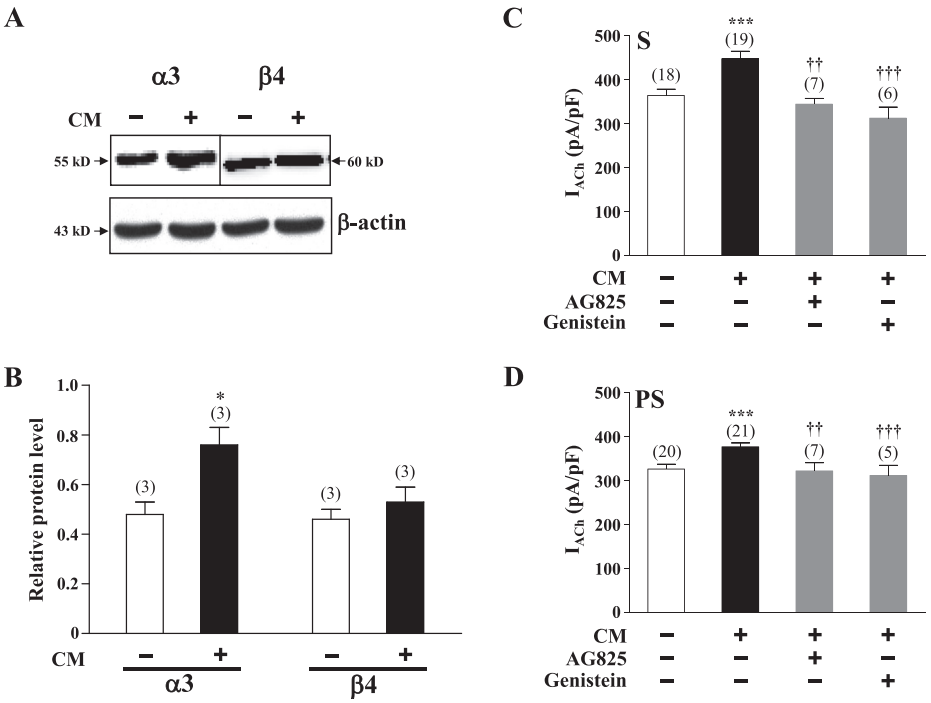

Fig. 4. Upregulation of nAChR expression by sNRG1 in MPG neurons. (A) Western blots of nAChR α3 and β4 subunits in MPG neurons with (+) and without (−) CM. (B) Summary of the relative protein levels of each nAChR subunit normalized to β-actin. (C & D) Effects of CM on I_{ACh} densities in sympathetic (S) and parasympathetic (PS) MPG neurons with (+) and without (−) either AG825(10 μM) or genistein (200 μM). For western blotting, the freshly isolated MPGs were incubated for 6 h at 37 °C in the CM containing sNRG1 released by electrical stimulation of the PVN. Western blotting was conducted using primary antibodies against nAChR-α3 (H-100) (1:200), nAChR-β4 (1:200), and β-actin (1:2000). Inward I_{ACh} was evoked by 100 μM ACh in the MPG neurons held at −60 mV under the whole cell-ruptured configuration of the patch-clamp technique. The cell type was identified as described in the methods for electrophysiological recording. For electrophysiology, enzymatically dissociated MPG neurons were treated with vehicle (control), CM, and CM plus inhibitors for 6 h. Data are presented as the mean ± SEM. Numbers of experiments are indicated in parentheses. $^{***}p < 0.001$ between control and CM-treated neurons. $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$ between CM- and CM plus inhibitor-treated neurons.

Similar to the findings in the central nervous system neurons [23], electrical stimulation is require for cleavage and release of sNRG1 from MPG neurons. The sympathetic and parasympathetic neurons within the MPG appear to release sNRG1 independently in response to the corresponding preganglionic stimulation, given that no postganglionic neurons receive synaptic inputs from both preganglionic nerves [24]. The amount of sNRG1 released from MPG neurons is dependent on the frequency of nerve stimulation. The release of sNRG1 from MPG neurons reached its maximal level with tonic low-frequency (3–10 Hz) stimulation. It is reasonable to presume that the sNRG1 release is physiologically relevant because preganglionic neurons discharge at the same rate as the stimulation frequency during resting and reflex activation [25]. Tonic preganglionic stimulation could be replaced with continuous KCl-induced depolarization of MPG neurons for sNRG1 release. As in the cerebellar synaptosome [26], strong depolarization (as predicted by the Nernst equation) is critical to evoke noticeable sNRG1 release from MPG neurons. In addition to cleavage and release, expression of NRG1 is regulated by increased neural activity [27]. A recent study has shown that type I but not type III NRG1 is upregulated by KCl-induced depolarization as well as kinate-induced epileptic seizure activity leading to neural plasticity in the cerebral cortex [28]. Overall, it is likely that both expression and release of endogenous NRG1 are regulated by presynaptic input-induced depolarization of MPG neurons. In addition to presynaptic activity, retrograde signals from the target tissues also promote maintenance of nAChR levels in mature autonomic neurons [29]. The target-derived signals include neurotrophic factors such as NGF, BDNF, and CNTF. Neurotrophic factors have been found to regulate expression and release of NRG1 in sensory and spinal motor neurons [19,30]. We showed for the first time that neurotrophic factors are capable of inducing the release of sNRG1 in adult autonomic neurons.

Rat MPG neurons appear to provide primarily relay functions because they have a relatively high safety factor for ganglionic transmission [2,24]. The nAChR density on neuronal membranes is one of the factors determining synaptic strength [31]. A previous study has shown that the increased arrival of synaptic inputs enhances expression of nAChR proteins in parasympathetic ganglia [32]. Thus, synaptic input-dependent release of endogenous NRG1 (especially type I) from MPG neurons may facilitate the transmission of central command signals to target tissues through upregulation of nAChR levels.

In conclusion, NRG1 is endogenously released by neural activity and neurotrophic factors and upregulates nAChR expression through activation of ErbB receptors in autonomic MPG neurons of adult rats.

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