Protein processing and releases of neuregulin-1 are regulated in an activity-dependent manner

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Abstract

Identification of the key molecules that bridge presynaptic neuronal events and long-term modification of the postsynaptic process is an important challenge which will have to be met in order to further our understanding of the mechanisms for learning and memory. This study is focused on neuregulin-1 (NRG-1), a neurotrophic factor, that is known to regulate the development of various tissues and/or the life/death of cells through activation of the ErbB family receptor tyrosine kinases. It was discovered that the soluble form of NRG-1 (sNRG-1) is produced from the transmembrane form of NRG through proteolytic cleavage during electrical stimulation of either cultured cerebellar granule cells (GCs) or pontine nucleus neurons (PNs) that are presynaptic to GCs. sNRG-1 was assayed by measuring the phosphorylation of both the ErbB receptors and cyclic AMP-responsive element-binding protein (CREB), and by means of antibodies to sNRG-1. The cleavage and release of NRG-1 depended on the frequency of electrical stimulation; the peak effect was at 50 Hz in both GCs and PNs. Activation of protein kinase C (PKC) mimicked this effect. The culture apparatus provided along with the mass-electrical stimulation that was employed proved to be a powerful tool for combining neuronal electrical events and chemical events. We conclude from the results that, in mossy fibre (PN axon)-GC synapses, electrical activity controls the proteolytic processing of NRG-1 in a frequency-dependent fashion and involves PKC. Furthermore, cleaved sNRG-1 plays an important functional role in regulating transmission across these synapses.

Keywords: activity-dependent, cyclic AMP-responsive element-binding protein (CREB), ErbB, neuregulin, proteolytic cleavage, synapses and cerebellum.


Activity-dependent changes in synaptic transmission are presumed neuronal correlates of the learning and memory mechanisms in the brain. These changes, at least in part, are mediated by a group of proteins collectively termed neurotrophic factors (Poo 2001; Tyler et al. 2002). The primary aim of the present study is to investigate the extent to which

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Abbreviations used: contlGCs, GC-conditioned media that were incubated with pre-immune serum IgG before immunization with peptides of the cleavage site; contlPNs, PN-conditioned media that were incubated with pre-immune serum IgG before immunization with peptides of the cleavage site; CREB, cyclic AMP-responsive element-binding protein; dep1GCs, GC-conditioned media that were incubated with antisNRG-1β1; dep1PNs, PN-conditioned media that were incubated with antisNRG-1β1; ΔGCs, GCs transfected with the deleted mNRG; E18, embryonic day 18; GABA_A, gamma-aminobutyric acid type A, GC(s), granule cell(s); H7, dihydrochloride; MF(s), mossy fibre(s); mNRG, transmembrane form of NRG; nGCs, non-transfected GCs; NMDA, N-methyl-D-aspartic acid; nPNs, non-transfected PNs; P7, post-natal day 7; pCREB, phosphorylated CREB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PN(s), pontine nucleus neuron(s); PSD-95, postsynaptic density-95; rNRG, recombinant NRG-1β1 overexpressed and purified by means of an Escherichia coli expression system; sNRG, soluble form of NRG; tGCs, mNRG-transfected GCs; t(K212G)GCs, GCs transfected with point-mutated mNRG; tPNs, mNRG-transfected PNs; TTX, tetradotoxin; vGCs, vector-only transfected GCs; vPNs, vector-only transfected PNs.
neuregulin (NRG) plays such a role in synapses. NRG is a family of proteins characterized by the inclusion of an epidermal growth factor (EGF)-like motif that activates membrane-associated EGF-receptor-related tyrosine kinases known as ErbB. ErbB receptors are similar to other receptor tyrosine kinases when activated by NRG, in that they are phosphorylated on their intracellular tyrosine residue (Fischbach and Rosen 1997). The involvement of NRG in the development and growth of neural tissues has been demonstrated (Buonanno and Fischbach 2001; Lemke 2001; Ozaki 2001; Falls 2003). More specifically, NRG-1 which is well characterized in terms of function, regulates the expression and activity of nicotinic acetylcholine receptors (Liu et al. 2001), N-methyl-D-aspartic acid receptors, gamma-aminobutyric acid type A (GABA$_A$) receptors and potassium channels in the hippocampal and cerebellar synapses (Ozaki 2001; Falls 2003). Such properties would be expected to influence the efficiency of synaptic activity. NRG also may activate the learning and memory-related transcription factor, CREB (Taberbero et al. 1998; Rieff et al. 1999), which is considered to act as a primary regulator of long-term synaptic change (Dash et al. 1990; Bourcichuladze et al. 1994; Mayford and Kandel 1999).

NRG, a subset of NRG1-4, has several alternative forms of modified molecular structure (Buonanno and Fischbach 2001; Falls 2003). Certain transmembrane forms of NRG (mNRG) consist of an extracellular segment containing an immunoglobulin (Ig)-like domain, an epidermal growth factor (EGF)-like domain required for receptor binding, a juxtamembrane region carrying protease-cleavage sites, a transmembrane domain and a long cytoplasmic tail. The soluble form (sNRG) contains an EGF-like domain, which is isolated from the transmembrane domain. sNRG is formed in two ways: (i) alternative splicing of mRNA and (ii) proteolytic cleavage of mNRG from the juxtamembrane region (the sNRG formed by the latter is hereafter referred to as ‘cleaved sNRG’). sNRG, on its own, mimics most of the biological effects of mNRG. Therefore, the cleavage action provides a way to supply functionally effective sNRG by freeing it from the membrane. It has been shown in such non-neuronal cell lines as the fibroblast cell lines and CHO cells that the cytoplasmic tail of mNRG is required for the cleavage and release of cleaved sNRG from cells (Liu et al. 1998a,b; Wang et al. 1998). We have previously reported that NRG-1 cleavage was induced by the activation of protein kinase C (PKC) (Ozaki et al. 2000) in a COS7 cell line expressing a recombinant full-length mNRG isoform (NRG-1 type I β1) cloned from the cerebellum. This effect of the mNRG cytoplasmic tail emerges from its interaction with the PKC-LIM kinase-1 cascade, LIM kinase-1 being a serine/threonine protein kinase known to regulate cytoskeletal organization (Liu et al. 1998a,b; Wang et al. 1998).

In this study, the focus is on GCs in the cerebellar cortex and the pontine nuclear neurons (PNs), that are major presynaptic neurons to GCs as neuronal materials that retain electrical activity-related machinery. mNRG proteins in the adult mouse cerebellum are richly localized in both the presynaptic and postsynaptic regions of afferent mossy fibres (MFs), the major source of which is PN axons, and also in cell bodies and processes of GCs, as is known from the results of an immunohistological study (Ozaki et al. 2000). Two isoforms of the NRG receptor, namely ErbB2 and ErbB4, are also present in cerebellar tissues (Ozaki et al. 1997, 1998). We applied the mass stimulation method with multiple electrodes to simultaneously stimulate a great number of cultured GCs and/or PNs so that it was possible to examine activity-dependent phenomena in NRG-1. The main strategy used for detecting the cleaved sNRG released from the stimulated GCs and PNs was to collect and concentrate the conditioned media derived from these cells and to apply this media to further GC test culture. The presence of cleaved sNRG-1 in the concentrated conditioned media was biossayed by observing its effect on phosphorylating the ErbB receptors or CREB in the test GCs (Taberbero et al. 1998; Rieff et al. 1999), and by immunochemically detecting sNRG in the conditioned media. We thus obtained measurable chemical signals arising from GCs and PNs related to their electrical activity and demonstrated that the proteolytic cleavage of mNRG-1 in GCs and PNs was stimulated by the activity in a frequency-dependent manner.

Materials and methods

Cell preparation

Cerebellar GCs and PNs were, respectively, prepared by standard methods from post-natal day 7 (P7) (Levi et al. 1989) and embryonic day 18 (E18) in Balb/C mice following the protocol of the culture system MB-X9901 (Sumitomo Bakelite Co., Tokyo, Japan). Seven-day in vitro (DIV) cultures were used for all experiments. The culture dishes or coverslips were coated with poly-l-lysine (Sigma, St Louis, MO, USA). GCs were maintained in a Neurobasal™ medium (Gibco BRL) supplemented with B-27 (Gibco BRL) including 0.8 mM glutamine (Gibco BRL, Rockville, MD, USA) and 10 mM KCl. PNs were cultured for 1 or 2 days with Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) containing 10% horse serum, and then treated with 10 μM AraC (Sigma) for 2–3 days. PNs were subsequently cultured in the Neurobasal™ medium supplemented with B-27, 30 mM glucose and 0.8 mM glutamine. Two dishes were used per assay, each dish containing 5 × 10$^5$ cells, except for the direct detection of sNRG-1 cleaved by electrical stimulation, in which case 20 dishes were used per assay, each dish containing 1 × 10$^6$ GCs.

Plasmid constructs and transient transfection

The GFP gene excised from the green fluorescent protein vector, pEGFP (Clontech, Palo Alto, CA, USA), was inserted into full-length NRG-1 type I β1, which had been cloned from the cerebellum (Ozaki et al. 2000), between the Ig-like and EGF-like domains. The constructs of the full-length cDNA containing the GFP tag were
confirmed by DNA sequencing after plasmid construction. For the mutant plasmids, we constructed a deletion mutant lacking the region between amino acids 208 and 217 of NRG-1, and introduced a point mutation in which Lys212 was replaced by Gly. The GFP-tag was also inserted between the Ig-like and EGF-like domains. The plasmids (1 μg/dish) of full-length and mutant NRGs, including the GFP-tag and pEGFP vector (Clontech), were transfected with Lipofectamine™ 2000 (Gibco BRL). The transfection efficiency obtained was 1–3% in PNs and 5–10% in GCs.

**Electrical stimulation, PKC activation and collection of conditioned media**

GCs or PNs were subjected to mass electrical stimulation 24–36 h after transfection. The mass electrical stimulation system (custom-designed by the Unique Medical Co. Ltd, Japan) and a Panasonic MED system (Matsushita Electric Industrial Co. Ltd./Alpha MED Sciences Co. Ltd, Tokyo, Japan) were combined for this experiment. Twenty-five electrodes were assembled in an array of 5 × 5 at 5-mm intervals for stimulation (Fig. 1a), except for the measurement of the calcium signals for which an array at 1-mm intervals was preferred (Fig. 2a). Five electrodes in each row could be moved together in a longitudinal direction. A set of the 25 electrodes was adapted to a 35-mm culture dish and placed at the surface of the cultured cells under microscopic control. The reference electrode was a piece of platinum wire, which formed a ring encircling the stimulating electrodes along the diameter of the culture dish (Fig. 1a). Rectangular pulses of alternating polarity (0.2-ms width and 1-mA intensity) at various frequencies were applied between the 25 electrodes (connected in parallel) and the reference electrode. The positions of the electrodes were adjusted so that the impedance at each electrode tip for a 50-Hz pulse current of 1 mA remained 5–6 kΩ. The recording electrodes were made of platinum needles, were 150 μm in diameter, and covered with nickel (500-nm thick) and gold (50-nm thick), as described previously (Oka et al. 1999). The tip of each electrode had a square hole (50 × 50 μm square and 1 μm deep) for electroplating with platinum black. The electrical activities of the cultured cells were recorded through 64 planar electrodes embedded in the base of the culture dish (Figs 1a and b).

GCs were stimulated in the Neurobasal™ medium supplemented with B-27, 0.8 mM glutamine and 10 mM KCl, and PNs in the Neurobasal™ medium supplemented with B-27, 30 mM glucose and 0.8 mM glutamine. Tetradotoxin (1 μM, TTX, Tocris Cookson, Ballwin, MO, USA) was used for suppressing the spike activity in the cultured cells. Phorbol 12-myristate 13-acetate (1 μM, PMA, Tocris) was used as an activator of PKC, and H7 dihydrochloride (20 μM, Tocris) as a selective inhibitor of PKC. In analyzing the conditioned media, proteins of approximately 30–60 kDa molecular weight were concentrated with a centricron 10 and 100 (Millipore Corporation, Bedford, MA, USA) to remove the high- (> 100 kDa) and low- (< 10 kDa) molecular-weight proteins and other unknown factors to the greatest extent possible. In order to show the effect on ErbB and CREB phosphorylation by NRG, the extracellular domain of the NRGβ1 protein overexpressed in *Escherichia coli* (Neo-Markers) was used.

**Measurement of intracellular Ca²⁺ levels**

The intracellular free Ca²⁺ level was quantified by fluorescence imaging using fura-2 calcium indicator dye. GCs and PNs were

**Fig. 1** Effects of mass electrical stimulation on electrical activity in GCs. (a) Arrangement of the multielectrode array (composed of 25 platinum wires) and the planar recording planar microelectrodes (64 wires) in a poly-L-lysine-coated culture dish with mounted GCs. The platinum wire reference electrode forms a ring enclosing the multi-electrodes. The measuring unit contains eight sets of an alternating current amplifier consisting of an 8/64 switch-box (SH-MED8, ·10 head amplifier, Panasonic) and an 8-channel main amplifier (SU-MED8, ·10–1000 main amplifier, Panasonic). (b) Sixty-four planar microelectrodes arrayed in an 8 × 8 array with an interpolar distance of 150 μm (the detail is described by Oka et al. 1999). (c) Electrical activities recorded from cultured GCs. To remove artifact signals, five signal traces were superimposed under the condition of an absence of neurons and the background was subtracted from each recording trace. Upper: hardly any spontaneous spike discharges were apparent in the absence of stimulation. Lower: responses of GCs during the application of 50-Hz mass stimulation (indicated by horizontal bars).
plated within a circle 1 cm across on 1-mm-thick glass coverslips that had been pre-coated with poly-L-lysine (1%). The cells were incubated in Locke’s solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM glucose, and 15 mM HEPES at pH 7.4) in the presence of 2 mM fura-2 (fura-2AM; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C. Immediately before imaging, each dish was washed twice with Locke’s buffer. The cells were observed under a Zeiss Axiolab microscope (20 × objective) coupled to an Attofluor imaging system (Fig. 2a). The calcium signal was expressed as the ratio between the fluorescence emission recorded at 510 nm following sequential excitation of a culture preparation with 340- and 380-nm light. The average [Ca²⁺]-value (in nM) in 30 neuronal somata in each culture was quantified and was averaged over three or four separate cultures for each stimulating condition (Fig. 2c).

**Phosphorylation assay for ErbB**
Both ErbB2 and ErbB4 are expressed in MF-GC synapses (Ozaki et al., 1997, 1998) but, as ErbB4 expression yields a dominant signal over that of ErbB2 in GCs, both in vitro and in vivo, being particularly dominant in the latter (Ozaki et al., 1998), ErbB4 phosphorylation in GCs was examined. ErbB4 was collected by immunoprecipitation with a polyclonal anti-ErbB4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A western blot analysis was performed by the standard method, using a mouse monoclonal anti-phosphotyrosine antibody (anti-TYK, 1 : 1000 dilution; 4G10, Upstate Biotechnology, Lake Placid, NY, USA). For immunoprecipitation, the lysates of GCs after treating the conditioned medium stimulated with PMA or electrically, were incubated with an immunoprecipitating antibody at an appropriate dilution (ErbB4; 0.1 μg/μL) for 1 h at 4°C, followed by another 1 h of incubation at 4°C with protein A-Sepharose (Sigma). The lysates were then centrifuged for 3 min at 12 000 g and the supernatant was discarded. The resulting pellets were then washed twice in a lysis buffer and then re-suspended in a gel-loading buffer. The samples were boiled for 3 min, and proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The blots were probed with an anti-ErbB4 antibody and an anti-TYK antibody that was able to recognize the phosphorylated ErbB receptors. The chemiluminescent signals of ErbB4 and phosphorylated ErbB4 were detected by using ECL Plus™, a western-blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Images of the blots were captured with a CanoScan D2400U scanner and a densitometric analysis was performed with NIH Image software.

**Phosphorylation assay for CREB**
Cultured GCs were fixed with 4% paraformaldehyde for 10 min and stained with a polyclonal anti-pCREB antibody (1 : 100 dilution) that could recognize phosphorylated Ser133 (BioLabs, Beverly, MA, USA). Alexa™ dyes (Molecular Probes) were used as a secondary antibody (1 : 1000 dilution). The stained GCs were examined under a confocal laser microscope (Carl Zeiss). Nuclear localization was verified by scanning z-series sections under this microscope. Phosphorylated CREB (pCREB)-positive cells within a 20 × objective field of the microscope were counted in five to six different randomly selected parts of the dish. The total cell number was counted after staining with pyronin-metylgreen. The number of pCREB-positive cells in a culture was normalized to the total cell number in that culture. The measurement was repeated independently by using different cultures prepared from different animals (n = 3–15). Statistical analyses were performed with StatView software (Abacus, Cary, NC, USA), the unpaired two-tailed t-test was used unless otherwise stated.

**Immunodepletion experiments on the cleaved form of NRG-1**
After the PMA treatment of transfected GCs, the supernatant was immunoprecipitated for 1 h at 4°C with anti-sNRG (1.5 μg/μL) that recognized only the C-terminus of sNRG-1 at the cleaved site to
deplete the sNRG. The polyclonal antibody (anti-sNRG) for the cleaved form of NRG-1 was made into tandem peptides of EELYQ or EELYQK to make the antibody for the cleaved protein and then purified by means of an affinity column (Saido et al. 1995) before the quality of the antibody was checked by the method of current protocols in molecular biology (Ausubel et al. 1999). A non-specific antibody, that is pre-immune serum IgG (1.5 μg/μL), was used as a control for the depletion experiments.

Direct assay of cleaved sNRG-1 under electrical stimulation
In each trial of this assay, conditioned media were collected from 20 dishes, each dish containing 1 × 10⁶ tGCs (as compared with the two dishes, each containing 5 × 10⁵ tGCs, that were used for the other assays already described). tGCs were electrically stimulated for 30 min, maintaining the steady-state [Ca²⁺]ᵢ level at each frequency, and the conditioned media were collected and concentrated by Centricon as already described. The concentrated conditioned media were immunoprecipitated with an anti-sNRG-1 antibody (1.5 μg/μL) by the same immunoprecipitation method as that with ErbB4. Western blot analyses were then performed with an antibody (1 : 1000 dilution) that was able to recognize part of the EGF-like domain (β1 specific region) by a method of current protocols in molecular biology (Ausubel et al. 1999). The signals of NRG were detected by using ECL Plus™ (Amersham).

Results

Effects of mass electrical stimulation
The effectiveness of the adopted mass stimulation method was examined by using the electric stimulation system (Fig. 1) in three ways. First, the field potential generated by cultured neurons was recorded. Figure 1(a) shows a diagram of the experimental set-up, and Fig. 1(b), a microarray on which the neurons were cultured to record their neuronal activity (see Materials and methods). Any signals unrelated to the membrane response were omitted by amplitude calibration (see legend to Fig. 1). In the absence of stimulation, dissociated GCs did not exhibit any obvious spontaneous signals related to membrane responses (Fig. 1c, upper trace). During the 5-s 50-Hz stimulation procedure, the spike discharges did appreciably increase in rate (Fig. 1c, lower trace).

Second, the intracellular free calcium [Ca²⁺]ᵢ level was measured in GCs that had been cultured, using fura-2 as the calcium indicator. Figure 2(b) is an example of the recorded data showing that the strongest fura-2 fluorescence appeared in many GCs stimulated at 50 Hz. As quantified in Fig. 2(c), stimulation at 50 and 100 Hz induced the quickest calcium response, which reached a peak within 15 s during stimulation. The elevated intracellular calcium concentration was prolonged following both 10 and 100 Hz stimulation, but decreased faster after 50 Hz stimulation. These results suggest that intracellular calcium homeostasis varied depending on the stimulus frequency. In this series of calcium imaging experiments, it was noted that the stimulus-induced increase in [Ca²⁺]ᵢ occurred uniformly among the 30 neuronal somata in the same dish. It was also noted that the temporal pattern of [Ca²⁺]ᵢ change also remained fairly uniform among different dishes stimulated at the same frequency (see the relatively small SD values in Fig. 2c).

Third, labelling with an antibody against Ser133-pCREB revealed that stimulation enhanced CREB phosphorylation in GCs (Fig. 3a). Without stimulation, there were few pCREB-positive GCs, but their number increased under continuous mass stimulation for 5 min. The stimulation effect increased with increasing stimulus frequency up to 50 Hz and then declined at 100 Hz (Fig. 3b, p < 0.01, ANOVA). The possibility that the decrease in number of pCREB-labelled GCs at 100 Hz represents their having been damaged due to excessive stimulation is unlikely, because cells stained with trypan blue by the standard method exhibited no abnormality in cell viability (data not shown). We also confirmed that stimulation at any frequency within the range of 1–100 Hz did not influence the pH value or temperature of the culture medium. When stimulated at 1 Hz, pCREB labelling developed maximally within 10 min, but as the stimulating frequency was increased to 50 and 100 Hz, the time needed to attain maximal pCREB labelling was, respectively, reduced to 5 and 2 min (Fig. 3c). The application of 1 μM TTX, a selective inhibitor of Na⁺ channel conductance, to the 50-Hz-stimulated GCs significantly reduced the pCREB expression level (Fig. 4), consistent with the view that the expression was induced activity dependently (p < 0.01, t-test in Fig. 4b). In this experiment, TTX was used to confirm the effect of the electrical stimulation apparatus by showing that CREB had been phosphorylated activity dependently. Approximately 50% was blocked by TTX in GCs. This value is reasonable because TTX blocks only TTX sensitive-Na⁺ channels. The obtained data depicted in Figs 3 and 4 show that the stimulus frequency determined the extent and speed of CREB phosphorylation in GCs, confirming the reported stimulus dependence of CREB phosphorylation (Bito et al. 1996; Bading 2000; Fields et al. 2001; Hardingham et al. 2001).

Induction of the NRG receptor, and of ErbB and CREB phosphorylation by NRG-1
ErbB4 was dominantly expressed and CREB phosphorylation occurred further downstream of the ErbB signal transduction pathway in GCs. In order to confirm the phosphorylation effect was induced by NRG, the responses of ErbB4 and CREB phosphorylation were examined by using purified recombinant NRG-1 [β1 (rNRG)]. As shown in Fig. 5, P7, GCs at 7 days-in vitro (DIV) were prepared and treated for 10 min with rNRG in a range of 1–100 nM. ErbB and CREB phosphorylation were, respectively, detected from the concentration of between 5 and 10 nM rNRG and at 1 nM. The effect of phosphorylation in ErbB increased as the concentration increased up to 100 nM. In contrast, the effect
on CREB phosphorylation was saturated with a 10 nM concentration. The dose-dependence of NRG-1 in the induction of phosphorylation is pointed out here. The results from these experiments, as well as from data in the literature (Tabernero et al. 1998; Rieff et al. 1999), prompted us to use this phosphorylation step as an assay system to detect the cleaved and released NRG.

Proteolytic cleavage of NRG-1 induced by PKC activation

E18, PNs and P7, GCs transfected at 7DIV with GFP-tagged full-length NRGβ1 (tGCs and tPNs), were incubated with the PKC activator, PMA, for 60 min. The conditioned media from these cells were then collected, concentrated and applied to test GCs for 10 min. GC lysates were then immunoprecipitated with the anti-ErbB4 antibody and blotted with the anti-phosphotyrosine (anti-TYK) antibody. As controls, the conditioned media were collected from non-transfected PNs and GCs (nPNs and nGCs), and from vector-only transfected PNs (vPNs) and GCs (vGCs) (Fig. 6).

The conditioned media derived from either tPNs or tGCs evidenced a strong phosphorylation effect on ErbB4 in GCs, as compared with the media derived from nGCs, nPNs, vGCs or vPNs (see the clear bands of tyrosine-phosphorylated ErbB4 (180 kDa) in Fig. 6(b). As shown in Fig. 6(c and d), the conditioned media of tPNs and tGCs indicated significant values (vPNs vs. tPNs, n = 5; vGCs vs. tGCs, n = 3; p < 0.01, t-test) in terms of ErbB phosphorylation efficiency. The phosphorylation effect was suppressed when tPNs and tGCs were incubated with the PKC inhibitor, H7, for 10 min before PMA stimulation, and then co-incubated with H7 and PMA (tPNs vs. tPN/H7, n = 5; tGCs vs. tGCs/H7, n = 3; p < 0.01, t-test). These results indicate that the activation of PKC played a crucial role in the cleavage and release of sNRG.

Next, test GCs with distinct CREB-phosphorylation patterns were immunocytochemically labelled 5 min, or even later, after being treated with the conditioned media collected from either the PMA-treated tPNs or tGCs [Fig. 6(e(iii) and e(vi), respectively]. The efficiency of

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**Fig. 3** Effects of mass stimulation on CREB phosphorylation in GCs.

(a) Photomicrographs showing antibody-labelled pCREB in GCs taken after 5 min of mass stimulation at the frequencies indicated. Scale bar: 20 μm. (b) Number of pCREB-positive GCs plotted against stimulus frequency. **p < 0.01 (ANOVA). (c) Number of pCREB-positive GCs plotted against time after starting the continuous mass stimulation with a 1-mA pulse current. Three sets of plots are presented for 1, 50 and 100 Hz stimulation. An error bar in (b) and (c) indicates the mean ± SD.
phosphorylation in the conditioned media of tGCs was significantly higher than that of nGCs or vGCs \((p < 0.01, t\text{-test in Fig. 6f})\). These results indicate that the sNRG cleaved from the transfected recombinant mNRG-1 under PKC activation effectively phosphorylated CREB. Far fewer pCREB-immunoreactive GCs were observed after being treated with the conditioned media from vGCs. CREB-phosphorylation in these cells might have been caused by a soluble type of the non-cleaved form of NRG or by some other, unidentified endogenous factor(s).

To verify the present assumption that the ability of a conditioned medium to facilitate ErbB4 and CREB phosphorylation depended on the amount of sNRG cleaved and released from the transfected recombinant NRGs, we deleted or point mutated the amino acid sequence of the mNRG-1 region required for proteolytic cleavage (tGCs vs. ΔGCs and tGCs vs. t(K212G) GCs, \(p < 0.01, t\text{-test in Fig. 6(f)}\). Based on the reported molecular weight of the cleaved form of sNRG \((\text{Ozaki et al. 2000; Ozaki 2001})\) and the general knowledge that the cleavage site is frequently in the proximity of lysine, we focused on amino acids 208–216 of NRG-1 (see Fig. 6g). A mutant with a deletion of ELYQKRVLT from the region of amino acids 208–216 did not exhibit significant phosphorylation of ErbB and CREB. This means that this region is required for a protease to recognize the cleavage site. The point mutation from Lys212 to Gly within the region also reduced the degree of ErbB and CREB phosphorylation, although modestly. Apparently, the wide region of mNRG-1, including Lys212, is required for the PKC-activated cleavage of mNRG-1 in GCs.

Furthermore, on phosphorylation of ErbB and CREB (Figs 6c, d and f), depleted conditioned media of PN cultures (deplPNs) and GC cultures (deplGCs), depleted by an anti-sNRG antibody, did not yield evidence of phosphorylation of ErbB and CREB. This means that this region is required for a protease to recognize the cleavage site. The point mutation from Lys212 to Gly within the region also reduced the degree of ErbB and CREB phosphorylation, although modestly. Apparently, the wide region of mNRG-1, including Lys212, is required for the PKC-activated cleavage of mNRG-1 in GCs.
Proteolytic cleavage of NRG-1 induced by electrical stimulation

After treating test GCs with the conditioned media derived from electrically stimulated tGCs or tPNs, the GC lysate immunoprecipitated with the anti-ErbB antibody was immunoblotted with the anti-phosphotyrosine (anti-TYK) antibody. The bands of a tyrosine-phosphorylated product of 180 kDa (ErbB4) were not clear for nPNs, nGCs, vPNs or vGCs, but are prominent for tPNs and tGCs. The blots for tPNs/H7 and tGCs/H7 show the suppression of ErbB4 phosphorylation following treatment of these cells with H7. (c, d) Relative density of the bands on PNs and GCs, respectively. The density captured with the scanner was analyzed (see Materials and methods). The degree of tyrosine phosphorylation was normalized to the signal of ErbB4 antibody. The supernatant was incubated with anti-sNRG (deplPNs and deplGCs) and pre-immune serum IgG (contlPNs and contlGCs). The NRG-depleted media (deplPNs and deplGCs) did not show the phosphorylation effects in either ErbB or CREB. Each column represents the average of five experiments using PNs, and of three using GCs. An error bar indicates the mean ± SE. **p < 0.01 (t-test). (e) Test GCs treated for 5–10 min with the conditioned media collected from PMA-activated PNs (i, ii and iii) and GCs (iv, v and vi). These test GCs were stained with a pCREB antibody after fixation. The conditioned media were collected from nPNs (i), vPNs (ii) tPNs (iii), nGCs (iv), vGCs (v) and tGCs (vi). By assuming that CREB phosphorylation in (ii) and (iii) arose from the sNRG cleaved and released from endogenous mNRG or other factors, whereas that in (iii) and (vi) arose from the sNRG cleaved and released from both the endogenous and transfected mNRGs, the difference in CREB phosphorylation between (ii) and (iii), or between (v) and (vi), represents the effect of sNRG cleaved and released from transfected mNRG. Scale bar: 20 μm. (f) Plot of the percentage of pCREB-positive cells relative to the total number of GCs. The graph depicts a plot indicating the effect of the conditioned media derived from GCs transfected with the deleted (DGCs) or point-mutated [t(K212G)] GCs mNRG (**p < 0.01, t-test). ΔGCs do not exhibit a significant value as compared with vGC (n = 6, p > 0.6, t-test), whereas t(K212G) GCs exhibit an intermediate effect between that of tGCs and vGC. The conditioned media were incubated with anti-sNRG (deplGCs) and pre-immune serum (contlGCs). The effect of depletion by antibodies is evident (contlGCs vs. deplGCs; n = 6, **p < 0.01, t-test). An error bar shows the mean ± SE. (g) Amino acid sequence of the NRG cleavage site and the results by PMA stimulation are summarized.
indicate that sNRG-1 was cleaved and released from the transfected cells in both an activity-dependent manner, particularly so, and frequency-dependent manner.

The effect of the conditioned media from 50-Hz-stimulated tGCs or tPNs in facilitating ErbB phosphorylation was blocked in the presence of 20 μM H7 (p < 0.01, t-test in Fig. 8b). Similarly, the effect of the conditioned media from 50-Hz-stimulated tGCs or tPNs (data not shown) in phosphorylating CREB was partially suppressed in the presence of 20 μM H7 (n = 7, p < 0.01, t-test to 50 Hz stimulation). These data suggest that the signal pathway from electrical stimulation to NRG cleavage was mediated or modulated by PKC (see Discussion). When GCs (ΔGCs) transfected with the deletion mutant of mNRG-1 were stimulated at 50 Hz, the conditioned media derived from them induced a significantly smaller number (46.3 ± 2.20%, n = 5, p < 0.01, t-test to 50 Hz stimulation) of pCREB-positive GCs. This result indicates that the CREB phosphorylation observed in GCs was induced by cleaved sNRG derived from electrically stimulated tGCs or tPNs. 1- and 100-Hz stimulation produced a lower effect on ErbB phosphorylation than 50-Hz stimulation, although still resulting in a relatively high CREB phosphorylation. The reason for this may lie in the experimental sensitivity and antibody specificity in CREB phosphorylation. Immunodepletion experiments were performed to investigate further the direct effect of NRG (see Fig. 8b). The supernatant from 50-Hz stimulation was incubated with the anti-sNRG (deplGCs) and pre-immune serum IgG (contlGCs). The effect of phosphorylation on contlGCs was similar to that on tGCs; in contrast, the effect on NRG-depleted media (deplGCs) dropped to the level of that of ΔGCs or H7 treatment. The difference between contlGCs and deplGCs (contlGCs vs. deplGCs, n = 5, p < 0.01, t-test) evidently came from the effect of sNRG.

For the next step, we detected cleaved sNRG-1 more directly by using an anti-sNRG antibody able to recognize only the C-terminal portion of cleaved sNRG-1 (Fig. 9). The conditioned media collected and concentrated from electrically stimulated GCs were immunoprecipitated with the anti-sNRG antibody and immunoblotted with the anti-NRGb1 antibody (Fig. 9b). Signals of the cleaved sNRG-1 were observed at approximately 30–40 kDa. No appreciable signal of cleaved sNRG was detected in the conditioned media derived from GCs stimulated at 50 Hz in the presence of H7. We conclude from these data that sNRG-1 was produced from mNRG-1 via proteolytic cleavage initiated and controlled in a frequency-dependent manner. This experiment was conducted three times and identical results were obtained in each instance.

A high KCl solution induces sustained membrane depolarization in neurons and is often used as a replacement for electrical stimulation. However, we were unable to detect an appreciable release of cleaved sNRG to induce ErbB and CREB phosphorylation in test GCs when we applied a 25–35 mM KCl solution to tGCs (data not shown). This concentration is significantly higher than that of the KCl (10 mM) required in culture media to maintain Balb/C mouse GCs of high neuronal viability.

**Discussion**

**Involvement of NRG in MF-GC transmission**

The relationship between synaptic activity, intracellular signalling and lasting downstream physiological effects is central to understanding the neuronal mechanisms for learning and memory. Presynaptic events lead to the release of neurotransmitters, neuropeptides and neurotrophic factors such as NRG and the brain-derived neurotrophic factor,
BDNF, which in turn activate specific signal transduction systems in postsynaptic neurons, eventually leading to long-term effects such as the formation of new synapses and the strengthening of existing connections (Poo 2001; Tyler et al. 2002). These cellular events are thought to play a role in short-term memory formation and may be fairly characterized as a crucial first step in learning and long-term acquisition.

However, the chemical signals induced by electric signals in synapses are generally too faint to be experimentally detected by the best, currently available methods. To overcome this difficulty, we adopted a mass stimulation technique by which numerous GCs or PNs in a culture dish are simultaneously stimulated and chemical signals of a measurable magnitude are generated (Figs 1a and b). This stimulation system enabled us to analyze the relationships between the patterns of electrical neuronal activity, intracellular calcium homeostasis and the processing of NRG. We discovered that electrical stimulation effecting membrane depolarization and spike discharges in GCs or PNs induced cleavage and release of sNRG, which resulted in the phosphorylation the CREB and ErbB receptors in GCs. We also discovered that the cleavage and release of sNRG from GCs or PNs were regulated in a stimulus-frequency-dependent manner (Figs 7 and 8). A stimulus frequency dependence has been reported in the release of BDNF (Balkowiec and Katz 2002), in the expression of certain adhesion molecules (Fields and Itoh 1996; Itoh et al. 1997) and in the regulation of second messengers (Buonanno and Fields 1999; Fields et al. 2001). In addition to this growing body of evidence, proteolytic cleavage and release controlled by frequency are reported here for the first time.

Fig. 8 Proteolytic cleavage of NRG-1 detected by CREB phosphorylation under electrical stimulation. (a) Photograph of granule cells (GCs) culture. The percentage of GCs in that culture was over 93%. (b) CREB phosphorylation in test GCs treated for 15 min with the conditioned media derived from GCs that had been mass-stimulated at the frequencies indicated. In 50Hz/H7, mass-stimulated GCs had been transfected with full-length NRG-1 in the presence of H7. In 50Hz/ΔGCs, the 208–216 segment of the amino acids of NRG-1 had been deleted. (c) Plot of the number of pCREB-positive GCs to the total number of GCs counted under the various conditions examined in (b). In deplGCs, after electrical stimulation of tGCs, the supernatant was incubated with anti-sNRG; contlGCs, after electrical stimulation of tGCs, the supernatant was incubated with pre-immune serum. Further details can be found in the results. **p < 0.01 (t-test and ANOVA).

Fig. 9 Proteolytic cleavage of NRG-1 detected directly by the immunoprecipitation method under electrical stimulation. (a) Procedure for direct detection of cleaved sNRG-1. The lower picture of (b) illustrates the structures of mNRG-1 and sNRG-1, and the affinity sites for the anti-sNRG and anti-NRGβ1 antibodies. The arrowhead indicates the position of the cleavage site. (b) Immunoblot of sNRGβ1 after immunoprecipitation of sNRG. Signals of cleaved sNRG were observed at a position of approximately 30–40 kDa.
These observations lead us to postulate that, when impulses of MFs from PNs reach GCs, the proteolytic cleavage of NRG occurs on the presynaptic side of MF-GC synapses in an impulse-activity-dependent manner in MFs, and subsequently on the postsynaptic side as well (Fig. 10).

The results of this present study also provide evidence that cleaved NRG in turn induces a series of subsequent events in GCs, including the phosphorylation of ErbB receptors and CREB. These observations jointly suggest an important role for NRG in the function of MF-GC synapses.

**Induction of NRG cleavage by electrical stimulation**

The results of the present study provide evidence to show that, while electrical pulse stimulation of GCs or PNs is able to induce the cleavage and release of NRG-1, stimulation of these cells with a KCl solution of 25–35 mM is ineffective for doing so. In certain similar such cases as NRG cleavage and adhesion molecule L1 expression (Fields and Itoh 1996; Itoh et al. 1997), membrane depolarization by KCl or dose-dependent depolarization by KCl has been shown to not work. The persistent membrane depolarization induced by KCl is thus distinct from the membrane depolarization and spiking induced by electrical stimulation in leading to NRG-1 cleavage and release. This observation may suggest that spiking, but not mere membrane depolarization, is essential for inducing NRG cleavage. The present results also reveal that intracellular signalling leading to NRG cleavage was induced by either the activation of PKC with PMA or electrical stimulation. Because electrically induced NRG-1 cleavage was able to be blocked by a PKC inhibitor, it is likely that PKC mediates the signalling pathway for NRG cleavage, or that PKC at least modulates the signalling pathway at a step amenable to phosphorylation.

To induce cleavage, the protease-sensitive area of NRG-1 (Fig. 6g) was required for both the cleavage induced by PKC activation and that induced by electrical stimulation. It has recently been reported that NRG was a substrate of the metalloprotease (ADAMs) family and that NRG cleavage by metalloproteases occurs mainly in the Golgi-apparatus (Shirakabe et al. 2001; Yan et al. 2002). However, the NRG isoform that we used does not have a catalytic site consensus sequence for metalloproteases. Certain types of proteolytic cleavage of nNRG have been reported to occur on the cell surface (Loeb et al. 1998; Wang et al. 2001). It is possible that the proteases cleaving NRG may have a variety of different types, depending on the kinds of cell, localization of NRG and the proteases, and the timing of protease activation relative to the reaction stage of NRG molecules necessary for cleavage.

**Signalling triggered by cleaved and released sNRG**

Cleaved and released sNRG-1 facilitates ErbB receptor activity, which in turn may lead to intracellular signalling involving CREB phosphorylation. Certain ionotropic receptors may also be a target for the signalling triggered by cleaved sNRG. NRG has been shown to regulate the expression of NMDA receptor subunit NR2C in cerebellar slice cultures and GABA<sub>A</sub> β2 in a dissociated GC culture (Rieff et al. 1999; Ozaki 2001, 2002a,b). NMDA receptors have been strongly implicated in learning and memory in numerous studies (Huerta et al. 2000; Rondi-Reig et al. 2001; Nakazawa et al. 2002). In fact, NMDA receptors are involved in LTP in MF-GC transmission (D’Angelo et al. 1999; Maffei et al. 2002). In MF and GC synapses, NR2A/NR2C subunits and particularly their C-termini, regulate synaptic NMDA receptor activation and function by enhancing channel-opening probability, which is critical for LTP (Rossi et al. 2002). Furthermore, signals from Golgi cells, which are neurons inhibitory to GCs, control GC excitation via GABA<sub>A</sub> receptor-mediated hyperpolarizing potentials (Maffei et al. 2002).

In contrast, a consensus-binding site in the C-terminus of ErbB4 directly interacts with the PDZ domains of membrane-associated guanylate kinase PSD95 (postsynaptic density-95) in vivo and in vitro (Buonanno and Fischbach 2001). NMDA receptors interact with PSD95, and NMDA and ErbB receptors co-localize at postsynaptic density in hippocampal neurons (Garcia et al. 2000; Huang et al. 2000; Rondi-Reig et al. 2002) and in GCs (Ozaki, M., unpublished observation). A variety of these C-terminal interactions may be involved in the regulation of intracellular signalling, including the kinases implicated in LTP (Buonanno and Fischbach 2001). Thus, NRG cleavage is unusually well positioned to bridge the presynaptic impulse activity and the biochemical regulation of NMDA and other types of ionotropic receptors that mediate the transmission of synaptic signalling.

**The frequency-dependence of NRG cleavage and release**

The mechanism for the stimulus frequency dependence of NRG cleavage and release is as yet unclear, but it is likely to involve frequency-dependent calcium homeostasis (Fig. 2).
A discrepancy, however, is noted in Figs 2, 7 and 8, in that the proteolytic cleavage and release of sNRG-1 optimally occurred at 50 Hz, whereas the maximal intracellular calcium level was attained by stimulation at 100 Hz rather than at 50 Hz. This discrepancy may be accounted for if it is postulated that, while a high calcium level accelerates NRG cleavage, an excess of cleaved sNRG inhibits NRG cleavage by means of metabolic product inhibition or, alternatively, an excess of calcium triggers another, as yet unknown, process that inhibits NRG cleavage. In any event, further investigation is obviously required to clarify the chemical mechanism underlying the frequency-dependent control of NRG cleavage.

The frequency–dependence of NRG cleavage and release is consistent with the frequency–dependence noted in various synaptic events, such as long-term potentiation and depression (LTP and LTD; Rick and Milgram 1996; Ito 2001). A variable threshold for LTP and LTD induction has been hypothesized from the observation that LTP and LTD are, respectively, induced with high- and low-frequency stimulation trains. In respect of MF-GC synapses, intermittent 100-Hz stimulation of MFs paired with membrane depolarization induced LTP (D’Angelo et al. 1999; Armano et al. 2000). It has also been reported that LTP is inhibited by short-term treatment with NRG in hippocampal neurons (Huang et al. 2000). These data favour the view that frequency-dependent NRG cleavage plays a role in the cellular and molecular processes underlying synaptic plasticity as a neuronal correlate of learning and memory.

Possible roles of NRG in synaptic transmission

Based on both previous and the present results, we hypothesize that presynaptic electrical signals, particularly those occurring at approximately 50 Hz, bring about cleavage of mNRG in the presynaptic membrane, thereby resulting in sNRG release into the synaptic cleft (Fig. 10). We further hypothesize that postsynaptic electrical activity induced by synaptic transmission at approximately 50 Hz also causes cleavage of mNRG in the postsynaptic membrane and release of sNRG into the synaptic cleft. These sNRG molecules released into the synaptic cleft activate ErbB receptors located in both the presynaptic and postsynaptic membranes. Thus, sNRG mediates bidirectional intercellular signal transduction between the presynaptic terminal and postsynaptic membrane. sNRG also mediates autocrine signals from the postsynaptic back to the postsynaptic membrane, and from the presynaptic membrane back to the presynaptic membrane. Based on Fig. 10, it may be suggested that if presynaptic impulses effectively activate the postsynaptic cell, the consequent synchronized activities on the pre- and postsynaptic sides would result in synergism between these bidirectional and autocrine sNRG signals. It is an interesting possibility that the Hebbian rule, that is, that synchronized pre- and postsynaptic activities trigger synaptic plasticity, might be undergirded by a synergistic presynaptic and postsynaptic cleavage and release of sNRG that would in turn modulate synaptic transmission.

In this model, the intracellular signal transduction triggered through activation of the ErbB receptors by sNRG is followed by CREB phosphorylation, the regulation of certain receptors, and other cellular and molecular processes in synapses, eventually leading to an activity-dependent regulation of transmission efficacy across the synapse. NRG thus links electrical signals to chemical signals in MF-GC synapses and it may play a role in regulating transmission across these synapses. Identification of the exact mechanism by which NRG cleavage takes place, and rigorous analyses of the signalling pathways mediated by the cleaved and released sNRG, will shed some new and much-needed light on the neuronal mechanisms for learning and memory.

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