# Differential roles of calcitonin family peptides in the dendrite formation and spinogenesis of the cerebral cortex *in vitro*

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## ABSTRACT

We examined roles of calcitonin family peptides in the initial stages of dendrite formation and the maturation of dendritic spines in the rat cerebral cortex *in vitro*. Embryonic day 18 cortical neurons were dissociated and cultured for 2-3 days in the presence of calcitonin gene-related peptide (CGRP), calcitonin, amylin or adrenomedullin. The treatment of cortical neurons with CGRP promoted the formation of primary dendrites of non-GABAergic neurons. In contrast, the treatment with amylin and adrenomedullin for 3 days inhibited the dendritic elongation of non-GABAergic neurons. Calcitonin had no effect on the initial dendrite formation. Next, we examined roles of the peptides in the spine formation. Embryonic day 16 cortical neurons were cultured for 14 days and then treated acutely with CGRP, amylin or adrenomedullin for 24 hours. The density of filopodia, puncta /stubby spines and spines were increased by the CGRP treatment, whereas decreased by amylin. Therefore, CGRP and amylin showed opposite effects on the formation of dendritic filopodia, puncta and spines. Adrenomedullin had no effects on the spine formation. In conclusion, the present study showed that calcitonin family peptides have differential effects both in the dendrite formation during the initial stages and the spine formation of cortical neurons *in vitro*.

Key words: CGRP, amylin, neuropeptide, rat, culture, spine formation

## 1. Introduction

It has been shown that some neurotransmitters have neurotrophic activities during the brain development. Although the roles of classical neurotransmitters such as monoamines, glutamate and GABA have been extensively studied including the receptors mediating the neurotrophic activities (Lipton and Kater, 1989; Lujan et al., 2005; Represa and Ben-Ari, 2005), little is known about the neuropeptides. We have recently shown that neurotensin promotes the dendrite elongation and the maturation of dendritic spines of cultured cerebral cortical neurons (Gandou et al., 2010). It is possible that there are other neuropeptides that regulate the formation of dendrites and dendritic spines. In the present study, we examined the roles of calcitonin gene-related peptide (CGRP) and other calcitonin (CT) family peptides in the formation of dendrites and dendritic spines of cortical neurons *in vitro.*

CGRP is a 37-amino acid peptide that was originally identified as an alternate splicing product of CT gene (Amara et al., 1982; Rosenfeld et al., 1983). It is a member of the structurally conserved CT family peptides that include adrenomedullin (AM), amylin (AMY) and CT (Wimalawansa, 1996; van Rossum et al., 1997). CGRP is widely distributed in the brain and the spinal cord as well as various peripheral tissues (Amara et al., 1985; Kawai et al., 1985; Skofitsch and Jacobowitz, 1985; Ma et al., 2003), and it has been shown that CGRP has diverse biological actions including the vasodilatation and nociception (van Rossum et al., 1997; Ma, 2004). The mRNA and protein of CGRP appear early in the brain during the development (Terrado et al., 1997; 1999). Subsequently the expression level increases transiently during the postnatal days, followed by the decrease in the adult brain. These expression patterns suggest roles of CGRP in the brain development, but there are a few studies which examined the CGRP actions in the neural development. It has been shown that CGRP promotes differentiation of dopaminergic neurons in the olfactory bulb and midbrain (Denis-Donini, 1989; Bürvenich et al., 1998). A recent study reported that CGRP promotes the dendrite formation of cerebellar Purkinje cells (D'Antoni et al., 2010).

The receptors of CGRP, AM, AMY and CT are closely related (Poyner et al., 2002; Parameswaran & Spielman, 2006). The CGRP receptor is a heterodimer composed of

the calcitonin-like receptor (CLR) and the receptor activity modifying protein 1 (RAMP1) (McLatchie et al., 1998; Juaneda et al., 2000; Kuwasako et al., 2004). CLR is a seven trans-membrane G protein coupled receptor and RAMP1 is a chaperone/ligand specificity protein. The AM receptors are also heterodimers composed of CLR in combination with RAMP2 or RAMP3, while the AMY receptors are composed of the calcitonin receptor (CTR) and each of RAMP1-3. In contrast, the receptor of CT is a CTR monomer. These receptors are coupled with Gs and regulate the intracellular cAMP concentration, but mediate diverse physiological responses.

In the present study, we examined the actions of CGRP in the initial stages of dendrite formation and the maturation of dendritic spines by dissociation culture of cortical neurons and compared with other calcitonin family peptides. We showed that these peptides have differential effects on these developmental processes.

## 2. Material and methods

### *2.1. Dissociation culture of cortical neurons*

Rat embryos at embryonic day 16 (E16) and E18 were removed from the pregnant rats (Wistar/ST strain, Nihon SLC, Hamamatsu, Japan) under the deep anesthesia by ether. Embryos were quickly decapitated and the cerebral cortex was excised. After the removal of meninges, whole cerebral cortex was incubated in 0.05% trypsin-EDTA (Gibco, Carlsbad, CA) for 5 minutes at 37°C and cells were dissociated by trituration with a Pasteur pipette. After filtration with 70-μm nylon cell strainer (BD Falcon, San Jose, CA), dissociated cells were plated on 8-well chamber slides (Nunc, Rochester, NY) coated with 0.2% polyethyleneimine (Sigma, St. Louis, MO) at a density of 4 X $10^4$ cells/$cm^2$. The cells were cultured in the minimal essential medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 0.5 mM L-glutamine (Invitrogen, Carlsbad, CA), 25 μM glutamate (Wako, Osaka, Japan) and penicillin and streptomycin (25 U/25 μg/ml, Sigma) in a humidified atmosphere of 95%

air-5% $CO_2$ at 37°C. For the analysis of dendrite formation, E18 cortical neurons were cultured and the culture medium was replaced with the serum free Neurobasal medium (Gibco) with 2% B-27 supplement (Gibco), 0.5 mM L-glutamine, penicillin and streptomycin (25 U/25 μg/ml) 8 hours after plating. CGRP (Peptide Institute Inc., Osaka, Japan), AM (Peptide Institute Inc.), AMY (Peptide Institute Inc.) or CT (Sigma) were added in the culture medium at the concentration of 10 nM, 100 nM and 1000 nM. To remove proliferating glial cells and neuronal progenitors, 5 μM cytosine-β-d-arabinofuranoside (Ara-C, Sigma) was added 1 day after plating for 24 hours. The absence of astrocytes was confirmed by immunostaining using the antibody against glial fibrillary acidic protein (Hayashi et al. 2010). To analyze the dendritic protrusions, E16 cortical neurons were cultured in the serum-containing medium for 24 hours, and then the culture medium was replaced with the above-mentioned serum-free Neurobasal medium with 5 μM Ara-C for 24 hours. At 14 days *in vitro* (DIV), the peptides were added for 24 hours. The culture medium was changed every 2 days.

All the experiments followed the Guide for the Care and Use of laboratory Animals described by the National Institute of Health (USA), and were approved by the Animal Experimentation Committee of the University of Tsukuba.

### *2.2. RNA isolation and reverse transcription PCR*

Dissociated cortical cells were plated at a density of 1 X $10^6$ cells/ml on 35-mm culture dish (Sumitomo Bakelite, Tokyo, Japan) pre-coated with 0.2% polyethyleneimine. They were cultured as described above. At 14 DIV, neurons were washed with 0.01 M phosphate-buffered saline (PBS) and the total RNA was extracted by TRIzol Reagent (Gibco). Isolated RNA (1 ng-5 μg) was reverse transcribed using the standard protocol with Oligo $(dT)_{12\text{-}18}$ (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen). PCR reactions were performed with 35 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, using TaKaRa EX Taq HS (Takara Bio, Shiga, Japan). Oligonucleotide primers for the PCR reactions were described in Table 1. The PCR products were resolved on a 2% agarose gel and

visualized using ethidium bromide. The size of the bands was confirmed by a 100 bp DNA ladder.

### *2.3. Immunohistochemistry*

After the culture, cortical neurons were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 minutes at room temperature (RT). They were washed with 0.1 M PBS containing 0.15% Triton X-100 (TPBS), and nonspecific antibody bindings were blocked by incubation with 5% normal goat serum (Gibco) and 0.1% Triton X-100 in 0.1 M PB for 30 minutes at RT. For the analysis of the dendrite formation, the neurons were incubated with chicken anti-microtubule-associated protein 2 (MAP2) antibody (1:4000 dilution, Chemicon, Temecula, CA) and mouse anti-glutamic acid decarboxylase 65 (GAD65) antibody (1:400 dilution, Sigma) overnight at 4°C. They were then incubated with biotinylated goat anti-chicken IgG antibody (1:500 dilution, Vector Laboratories, Burlingame, CA) and Alexa Flour 488-conjugated goat anti-mouse IgG antibody (1:500 dilution, Invitrogen, Eugene, OR) for 1 hour at RT, followed by the incubation with Streptavidin Pacific Blue (1:500 dilution, Invitrogen) for 1 hour at RT. For the analysis of dendritic protrusions, the neurons were incubated with rabbit anti-postsynaptic density 95 (PSD95) antibody (1:500 dilution, Chemicon) overnight at 4°C. They were then incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG antibody (1:500 dilution, Invitrogen) for 1 hour at RT, followed by the incubation with rhodamine-phalloidin (1:100 dilution, Invitrogen) for 30 minutes at RT.

For the analysis of the CGRP receptor distribution *in vitro*, the neurons were incubated with rabbit anti-CLR antibody (1:500 dilution, Acris Antibodies GambH, Herfod, Germany) or rabbit anti-RAMP1 antibody (1:400 dilution, sc-11379, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. They were incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG antibody (1:500 dilution) for 1 hour at RT. Thereafter, the neurons were stained with chicken anti-MAP2 antibody or rhodamine-phalloidin as described above. The specificity of the primary antibodies was

confirmed by the western blotting and the immunohistochemistry. The western blotting using anti-CLR and anti-RAMP1 antibodies showed a band with the prospective size (supplemental Fig. 1). The immunostaining without the anti-CLR and anti-RAMP1 antibodies yielded no positive reaction (data not shown) and the pre-adsorbed anti-RAMP1 antibody yielded no positive reaction in cryostat sections (Fig. 8I).

For the analysis of the expression of RAMP1 and CGRP in the developing cerebral cortex, rats at postnatal day 7 (P7) were perfused transcardially with a fixative containing 4% paraformaldehyde in PB under the deep anesthesia. The cerebra were excised and immersed in the same fixative overnight at 4˚C. They were immersed sequentially in 10%, 20% and 30% sucrose solutions in PB and frozen in Tissue Tek O.C.T. compound (Sakura Finetek Japan). Sixteen μm transverse sections were cut and collected onto MAS-coated glass slides (Matsunami Glass Ind., Japan). After treatment for 30 minutes at RT with 0.3% $H_2O_2$ in methanol, the sections were incubated for 1 hour at RT in a blocking solution containing 5% normal goat serum and 0.1% Triton X-100 in PBS. The sections were incubated overnight at 4˚C with rabbit anti-RAMP1 antibody or rabbit anti-CGRP antibody (1:8000, Chemicon) followed by the incubation with the biotinylated secondary antibody (Vector Laboratories; 1:500) for 1 hour at RT. The sections were incubated with the peroxidase-conjugated avidin–biotin complex (Vector Laboratories; 1:100) for 30 minutes at RT and the positive reactions were visualized with diaminobenzidine (DAB) using the ImmunoPure metal enhanced DAB substrate kit (Pierce).

### *2.4. Image acquisition and processing*

Images of cultured neurons were acquired using a LSM 510 META laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images of neurons immunostained by antibody against MAP2 in combination with antibodies against GAD65, RAMP1 or CLR were acquired using a 20x objective lens. Those labeled by anti-PSD95 antibody and rhodamine-phalloidin were acquired at 0.2 μm intervals in z-axis direction using a 63x aqua immersion objective lens and stacked. Those labeled

by antibodies against RAMP1 or CLR in combination with rhodamine-phalloidin were acquired using a 20x objective lens and a 63x aqua immersion objective lens. Those of the immunostained cryostat sections were acquired using Axioplan 2 (Carl Zeiss).

### *2.5. Morphometric analyses*

For the analysis of dendrite formation, the total dendritic length, number of primary dendrites which extend directly from the cell bodies, average dendritic length (total dendritic length/number of primary dendrites) and branching index (number of branch points/number of primary dendrites) were measured by an image analyzing software (Neurocyte Image Analyser Ver. 1.5, Kurabo, Osaka, Japan) according to Hayashi et al. (2010) and Gandou et al (2010). The MAP2-positive longest neurite and the other shorter neurites were classified as an axon and dendrites, respectively, as previously described (De Lima et al., 1997; Hayashi et al., 2010, Gandou et al., 2010, see also Discussion). For the analysis of dendritic protrusions, the protrusions were classified into three types: spines with a thin neck and a bulbous head, puncta (stubby spines) without neck and 0.5-1.5 μm long, and filopodia 1.5-5 μm long (Gandou et al., 2010). The length of dendrite was measured and the number of protrusions per 100 μm length of dendrite was calculated. Each experiment was repeated at least 3 times and all the analyses were performed blind to the treatment conditions.

### *2.6. Statistical analyses*

All the data were expressed as the mean ± SEM. Statistical analyses were performed by ANOVA followed by post hoc analysis (Fisher's protected least significant difference test). Differences were considered significant if the probability of error was less than 5%.

## 3. Results

### *3.1. Effects of CGRP treatment on dendrite formation of GAD-negative cortical neurons*

E18 cortical neurons were cultured for 2 days or 3 days in the presence of various concentrations (10-1000 nM) of CGRP. After the culture, neurons were immunostained by antibodies against MAP2 and GAD65 (Fig. 1), and the effects on GAD65-negative neurons were analyzed (Fig. 2). At 2DIV, compared to the control, CGRP (10 nM, 100 nM and 1000 nM) increased the total dendritic length by 35.5±6.7%, 26.1±5.8% and 24.8±6.1%, and the number of primary dendrites by 23.9±4.0%, 22.1±4.6% and 24.5±5.4%, respectively (Fig. 2A and B). In contrast, the average dendritic length and the branching index were not affected significantly by the CGRP treatment (Fig. 2C and D). At 3DIV, CGRP (10 nM, 100 nM and 1000 nM) increased the total dendritic length by 32.2±7.7 %, 45.4±6.8% and 23.3±6.7%, respectively (Fig. 2A). CGRP (10 nM, 100 nM) also increased the number of primary dendrites by 25.9±5.0% and 37.1±5.7%, respectively (Fig. 2B). In addition, 1000 nM CGRP increased the branching index by 71.6±24.4% (Fig. 2D).

### *3.2. Effects of treatment of calcitonin family peptides on dendrite formation of GAD-negative cortical neurons*

We then examined the effects of other calcitonin family peptides (CT, AM and AMY) on the dendrite formation of GAD65-negative neurons. E18 cortical neurons were cultured for 3 days in the presence of CT, AM or AMY. As compared to the control, 100 nM AM decreased the total dendritic length by 20.6±2.6%, and 10 nM, 100 nM and 1000 nM AM decreased the average dendritic length by 11.7±3.8%, 15.6±2.7% and 15.0±3.2%, respectively (Fig. 3A and C). The number of primary dendrites and the branching index were not affected by AM (Fig. 3B and D). Similar to AM, AMY (100 nM, 1000 nM) decreased the total dendritic length by 10.7±3.8% and 14.4±4.0%, and

1000 nM AMY decreased the average dendritic length by 16.1±3.0% (Fig. 3A and C). The number of primary dendrites and the branching index were not affected by AMY. CT had no significant effect on any parameters of the dendrite formation (Fig. 3).

In summary, it was suggested that the treatment of AMY and AM inhibits the dendritic elongation of non-GABAergic neurons without effects on the formation of primary dendrites nor dendritic branchings.

### *3.3. Effects of acute treatment of calcitonin family peptides on spine formation*

Growing dendrites have small protrusions on the dendritic shafts, which are classified into filopodia, puncta and spines. Among these dendritic protrusions, dendritic spines are matured postsynaptic structures at excitatory synapse, and are formed from immature filopodia through puncta during the maturation of synapse (Sorra & Harris, 2000). To examine the effects of CT family peptides on the formation of dendritic spines, we cultured E16 cortical neurons for 14 days in the basal medium and then added various concentrations (10 nM-1000 nM) of CGRP, AM or AMY for 24 hours. At 15DIV, we fixed the cortical neurons and stained them by rhodamine-phalloidin and anti-PSD95 antibody (Fig. 4).

The acute treatment of CGRP (10 nM and 100 nM) increased the density of the total protrusions by 18.8±5.1% and 34.4±5.3%, respectively (Fig. 5A). In contrast, 10 nM and 100 nM AMY decreased it by 13.1±4.4% and 21.6±4.2%, respectively (Fig. 5G). Among the dendritic protrusions, 10 nM and 100 nM CGRP increased the filopodia density by 22.6±8.3% and 45.5±8.6%, respectively (Fig. 5B). CGRP (10 nM, 100 nM and 1000 nM) also increased the puncta density by 43.8±9.2%, 32.2±7.2% and 23.4±8.2%, and 100 nM CGRP increased the spine density by 31.4±5.2%. In contrast, 10 nM and 100 nM AMY decreased the filopodia density by 19.8±5.1% and 23.3±5.5%, respectively, and 100 nM AMY decreased the density of puncta and spines by 18.5±5.4% and 23.0±4.3%, respectively (Fig. 5H). 100 nM CGRP increased the density of PSD-95-immunoreactive protrusions by 33.6±6.7% (Fig. 5C), whereas 100 nM AMY decreased it by 18.3±4.3% (Fig. 5I). AM had a weak effect to decrease the density of

filopodia and PSD-95-immunoreactive protrusions (Fig. 5E, F).

### *3.4. Expression of CGRP receptor components in cultured cortical neurons*

To gain the insight into roles of the CGRP receptor in mediating the effects of CGRP, we examined the expression of the receptor components, CLR and RAMP1, in cultured neurons by immunohistochemistry. E18 cortical neurons at 3DIV and E16 cortical neurons at 14DIV were stained by antibodies against CLR or RAMP1 in combination with anti-MAP2 antibody or rhodamine-phalloidin (Fig. 6). At 3DIV, CLR was expressed in cell bodies of all neurons, while RAMP1 was expressed both in cell bodies and neurites of all neurons (Fig. 6A). At 14DIV, CLR and RAMP1 were expressed in the cell bodies of most cortical neurons (Fig. 6B). CLR and RAMP1 were also expressed weakly in the dendritic shafts, but no expression was detected in the dendritic protrusions (Fig. 6B).

### *3.5. Expression of receptors for calcitonin family peptides in cultured cortical neurons*

We examined the expression of mRNA of receptor components of calcitonin family peptides in E16 cortical neurons at 14DIV. RT-PCR showed that the mRNAs of CTR, CLR and RAMP1-3 were expressed in these neurons (Fig. 7).

### *3.6. Expression of CGRP receptor component and CGRP in developing cerebral cortex*

To find a clue to the role of CGRP *in vivo*, we localized RAMP1 and CGRP in P7 cerebral cortex. RAMP1 was expressed in cell bodies of all cortical layers (Fig. 8A). The pyramidal neurons in layer V expressed RAMP1 in the apical dendrites as well as cell bodies (Fig. 8F). In contrast to the widespread expression of RAMP1, limited number of CGRP-positive thin varicose fibers were observed throughout the cortical layers (Fig 8C, G, H).

## 4. Discussion

The present study examined roles of calcitonin family peptides in the initial stages of the dendrite formation and the maturation of dendritic spines by dissociation culture of embryonic cortical neurons. For the analysis of the dendrite formation, we immunostained the cortical neurons using the anti-MAP2 antibody which recognizes all the subtypes of MAP2. Although MAP2 is distributed abundantly in dendrites and often used as the marker of dendrite, MAP2C is also distributed in axons (for a review, see Tucker, 1990). A previous study reported that all the neurites (presumptive dendrites and axons) of cultured cerebral cortical neurons show MAP2-immunoreactivity at the initial stage of the neurite outgrowth, and subsequently the longest neurite loses the MAP2-immunoreactivity to differentiate into an axon (De Lima et al., 1997). Our previous study confirmed that the MAP2-immunoreactive longest neurite of cultured cortical neurons was concomitantly immunoreactive for SMI-31, an axonal marker (Hayashi et al., 2010). Therefore, we identified the MAP2-immunoreactive longest neurite as a presumptive axon and other shorter neurites as dendrites in the present analysis.

The present study showed that the chronic treatment of E18 cortical non-GABAergic neurons by 10 nM-1000 nM CGRP increased the total dendritic length and the number of primary dendrites at 2DIV, and the total dendritic length, the number of primary dendrites and the branching index at 3DIV. The increase of the total dendritic length may be caused by the increase of the number of primary dendrites, because the average dendritic length was not changed. These results suggest that CGRP promotes the formation of the primary dendrites (dendrite initiation) without effects on the average dendrite length (dendrite elongation). The mechanisms underlying the effects of CGRP to promote the dendrite initiation remain to be examined. A similar stimulatory effect of the dendrite formation was recently reported in the cerebellar Purkinje cells (D'Antoni et al., 2010). It was suggested that the effects on Purkinje cell dendrite may be mediated by astrocytes. However, the observed effect on the cortical neurons in the present study

seems to be direct, because Ara-C treatment removed astrocytes (Hayashi et al., 2010) and the cortical neurons expressed components of the CGRP receptor.

In contrast to CGRP, the treatment of AM and AMY decreased the average dendrite length without effects on the number of primary dendrites and the branching index. It was recently reported that AM and proadrenomedullin (PAMP, N-terminal 20 peptide of AM) bind to the cytoskeleton and that reduction of these peptides *in vitro* leads to the hyperpolymerization of tubulin and an increase of the immunoreactivity of detyrosinated tubulin (Sackett et al., 2008). In addition, brain-specific knockout of *adm* which leads to the deletion of both AM and PAMP results in the hyperpolymerized tubulin in the cerebral cortex (Fernández et al., 2008). These findings suggest that AM inhibits the tubulin polymerization, and thus it is likely that AM may inhibit the dendrite elongation of cortical neurons by the depolymerization of tubulin.

The present study revealed that CGRP promotes the dendrite initiation, whereas AM and AMY inhibits the dendrite elongation. The CGRP receptor consists of CLR and RAMP1, whereas the AM receptors consist of CLR and RAMP2 or RAMP3, and AMY receptors consist of CTR and each of RAMP1-3 (Poyner et al., 2002; Parameswaran and Spielman, 2006). Therefore, the different effects of CGRP and AM on the dendrite formation may be dependent on RAMP1-3, but not CLR. In addition, the receptors for CGRP, AM and AMY are all coupled with Gs which stimulates adenylate cyclase to increase the intracellular cAMP concentration. Therefore, the diverse effects of these neuropeptides on the dendrite formation cannot be explained simply by the changes of the intracellular cAMP. We have recently shown that neurotensin, the receptor of which is also coupled with Gs, promotes the dendrite elongation without effects of the number of primary dendrites of cortical neurons (Gandoh et al., 2010). These results suggest that the neuropeptides regulate various aspects of the dendrite formation through multiple signaling pathways.

The present study showed that the acute treatment of cortical neurons with CGRP at 14DIV increased the density of all the dendritic protrusions (filopodia, puncta and spines) and PSD95-positive dendritic protrusions. In contrast, the acute treatment with AMY had opposite effects by decreasing the density of all the dendritic protrusions and

PSD-95 positive protrusions. The mechanisms by the CT family peptides to regulate the formation of dendritic protrusions are not known. It was recently reported that serotonin 2A receptor is distributed in subsets of dendritic spines of cortical neurons and the activation of the receptor increases the spine size, suggesting the local action of serotonin on spines (Jones et al. 2009). The present immunohistochemical study revealed that the receptor components of CGRP (CLR and RAMP1) were localized in dendritic shafts as well as cell bodies at 14DIV. However, neither CLR nor RAMP1 was detected in the dendritic protrusions. Therefore, the effects of CGRP on the formation of dendritic protrusions may be mediated through cell bodies and/or dendritic shafts.

The present study showed that CGRP affects the maturation of post-synaptic structures. It was previously shown that CGRP increases the synthesis of acetylcholine receptors in the neuromuscular junction (New & Mudge, 1986; Fontaine et al., 1986; 1987; Rossi et al., 2003). Therefore, it is possible that CGRP may be involved in the formation of post-synaptic structures both in the central and peripheral nervous systems. It may be interesting to determine the effects of CGRP in the development of glutamate receptors in the cortical neurons.

In conclusion, the present study revealed differential effects of calcitonin family peptides in the dendrite formation and spinogenesis of non-GABAergic neurons the cerebral cortex *in vitro*. CGRP promoted both initial dendrite formation and spine formation, whereas amylin inhibited both developmental processes and adrenomedullin inhibited dendrite formation. It may be possible that CGRP plays a similar role in the cortex *in vivo*, because developing cortical neurons showed a similar expression of the CGRP receptor component. In contrast to the ubiquitous expression of the expression of the CGRP receptor, CGRP fibers were distributed sparsely, as previously shown in the mismatch between the peptide and the receptor for many neuropeptides (Henkenham, 1987; Kruger et al., 1988; Agnati et al., 1995). The actions of CGRP *in vivo* may be explained by the non-synaptic (volume) transmission through CGRP derived from remote axon terminals through extracellular space, cerebrospinal fluid and/or blood (Kruger et al., 1988; Agnati et al., 1995).

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## Figure legends

**Figure 1** Effects of the CGRP treatment on the dendrite formation of cortical neurons.
E18 cortical neurons were cultured for 2 days (2DIV) or 3 days (3DIV) in the presence of various concentrations (10 nM-1000 nM) of CGRP. After the culture, neurons were double-stained with antibodies against MAP2 (red) and GAD65 (green). Arrows indicate GAD65-positive neurons. The lower panels at 2 DIV and 3DIV are higher magnification views of the areas surrounded by the white square in each upper panel. The total dendritic length (TL), the number of primary dendrites (PD), average dendritic length (AL) and the branching index (BI) of the typical GAD-negative neurons are shown. Scale bars: 100 μm (upper panels of 2 DIV and 3 DIV), 50 μm (lower panels of 2 DIV and 3 DIV),

**Figure 2** Effects of the CGRP treatment on the dendrite formation of non-GABAergic neurons.
E18 cortical neurons were cultured for 2 days (2DIV) or 3 days (3DIV) in the presence of CGRP and GAD-negative neurons were analyzed. We identified the MAP2-positive longest neurite as a presumptive axon, and examined all the other shorter neurites as dendrites. The total dendritic length was increased by CGRP (10, 100 and 1000 nM) at 2DIV and 3DIV (A). The number of primary dendrites was increased by CGRP (10, 100 and 1000 nM) at 2DIV and by CGRP (10 and 100 nM) at 3DIV (B). The average dendritic length was not changed by CGRP (C). The branching index was increased by CGRP (1000 nM) at 3DIV (D). The experiment was repeated at 3-4 times, and the number of neurons examined in each experimental group is indicated in bars of A. Mean ± SEM. *$P$<0.05, **$P$<0.01, ***$P$<0.001

**Figure 3** Effects of the treatment of calcitonin family peptides on the dendrite formation of non-GABAergic neurons.
E18 cortical neurons were cultured for 3 days in the presence of various concentrations (10 nM-1000 nM) of calcitonin (CT), AM (adrenomedullin) or AMY (amylin), and

GAD-negative neurons were analyzed. We identified the MAP2-positive longest neurite as a presumptive axon, and examined all the other shorter neurites as dendrites. The total dendritic length was decreased by 100 nM AM, and 100 nM and 1000 nM AMY (A). The average dendritic length was decreased by AM (10 nM, 100 nM and 1000 nM), and 1000 nM AMY (C). The number of primary dendrites and the branching index were not changed by any treatment (B, D). The experiment was repeated at 3 times, and the neuronal numbers examined in each experimental group are indicated in bars of A. Mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001

**Figure 4** Effects of the acute treatment of calcitonin family peptides on the spine formation

E16 cortical neurons were cultured for 14 days in the basal medium and then treated with CGRP, AM (adrenomedullin) or AMY (amylin) for 24 h. Neurons were double-stained with anti-PSD95 antibody (green) and rhodamine-phalloidin (red). f: filopodia, p: puncta, s: spines. Scale bars: 5 μm.

**Figure 5** Differential effects of the acute treatment of calcitonin family peptides on the spine formation

E16 cortical neurons were cultured for 14 days in the basal medium and then treated with CGRP (A-C), AM (adrenomedullin; D-F) or AMY (amylin; G-I) for 24 h. CGRP increased the density of total protrusions (A), the density of filopodia, puncta and spines (B) and the PSD95-immunoreactive protrusions (C). AM decreased the filopodia density (E), while increased the PSD95-immunoreactive protrusions (F). AMY decreased the density of total protrusions (G), the density of filopodia, puncta and spines (H) and the PSD95-immunoreactive protrusions (I). The experiment was repeated 3 times, and the numbers of dendrites examined in each experimental group are indicated in bars of A, D and G. Mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

**Figure 6** Expression of CLR and RAMP1 in the cultured cortical neurons.

(A) E18 cortical neurons were cultured for 3 days and double-stained with antibodies

against MAP2 (red) in combination with CLR (green) or RAMP1 (green). All neurons expressed CLR and RAMP1. (B) E16 cortical neurons were cultured for 14 days and double-stained with rhodamine-phalloidin to label F-actin (red) in combination with the antibodies against CLR (green) or RAMP1 (green). CLR and RAMP1 were expressed in the cell bodies and dendritic shafts of most neurons. Scale bars: 50 μm (A, upper panels in B and C), 15 μm (lower panel in B and C).

**Figure 7** Expression of CT family peptide receptors mRNA in the cultured cortical neurons.

E16 cortical neurons cultured for 14 days and the total RNA was isolated from them. RT-PCR was performed for CTR, CLR and RAMP1-3. The mRNAs of all the receptor components were detected. Two bands of the CTR were splicing variants. M: 100 bp DNA ladder, (+) and (-) show the reaction with and without the reverse transcriptase (RT), respectively.

**Figure 8** Expression of RAMP1 and CGRP in the postnatal cerebral cortex.

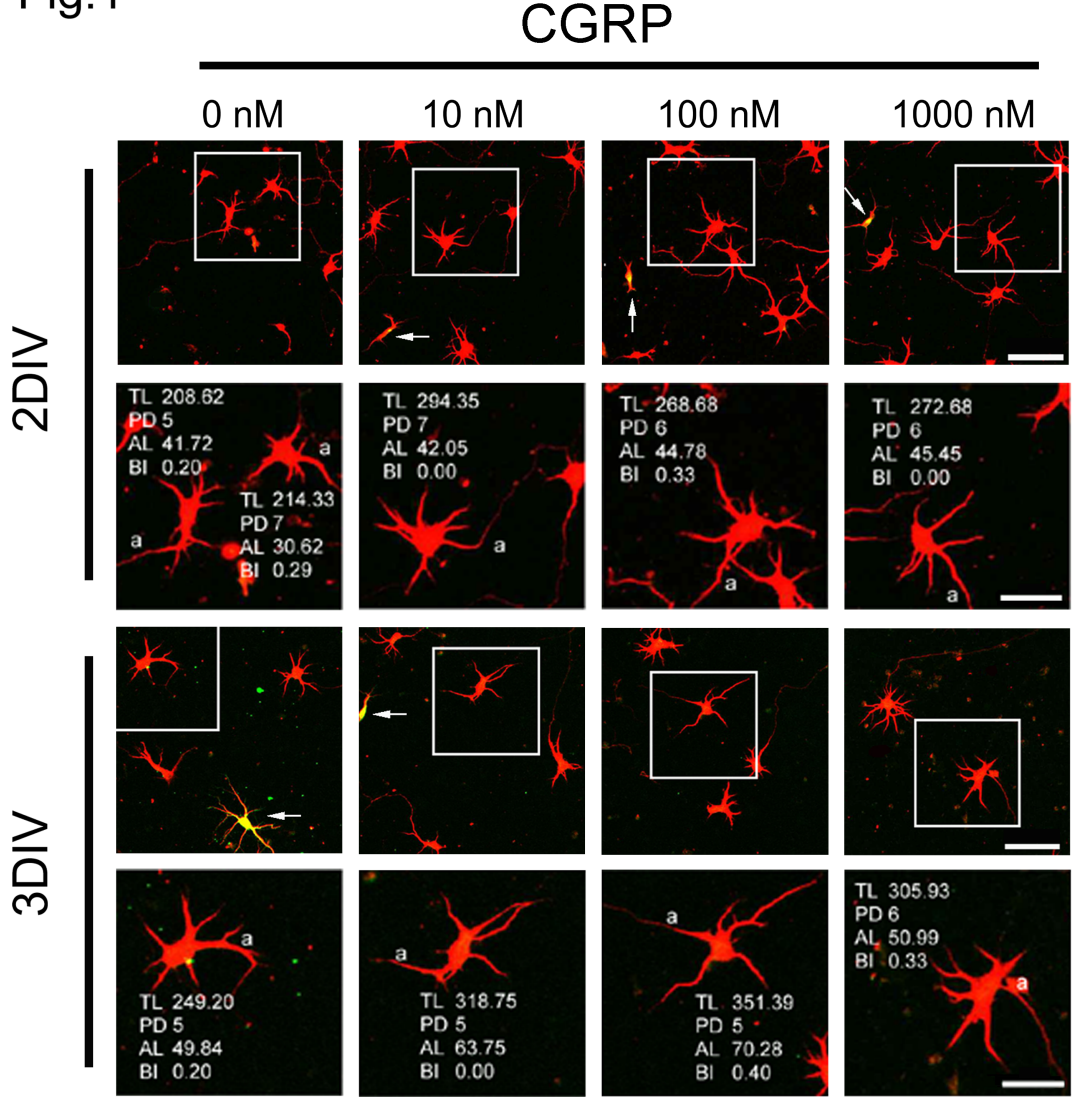
Cryostat sections of the cerebral cortex at postnatal day 7 were immunostained by the anti-RAMP1 antibody (A, F), anti-CGRP antibody (C, G, H) or the antibody pre-adsorbed with RAMP1 (I). (A, F) RAMP1 was expressed in cell bodies throughout the cortical layers of the parietal cortex, including the apical dendrites of the pyramidal neurons (arrowheads in F). F is a higher magnification view of B. (C, G, H) CGRP-immunoreactive thin varicose fibers were sparsely distributed in the cortex. G and H are higher magnification views of D and E, respectively. (I) The incubation with the pre-adsorbed antibody showed no staining. Scale bars: 100 μm (A, C, I), 20 μm (F-H).

## Supplemental figure legend

**Figure 1** Western blot analyses of anti-CLR and anti-RAMP1 antibody.

The western blot of cerebral cortex at postnatal day 7 by anti-CLR and anti-RAMP1 antibodies showed a single positive band with a prospective size.

Fig.1


CGRP
0 nM
10 nM
100 nM
1000 nM
2DIV
3DIV
TL 208.62
PD 5
AL 41.72
BI 0.20
TL 214.33
PD 7
AL 30.62
BI 0.29
TL 294.35
PD 7
AL 42.05
BI 0.00
TL 268.68
PD 6
AL 44.78
BI 0.33
TL 272.68
PD 6
AL 45.45
BI 0.00
TL 249.20
PD 5
AL 49.84
BI 0.20
TL 318.75
PD 5
AL 63.75
BI 0.00
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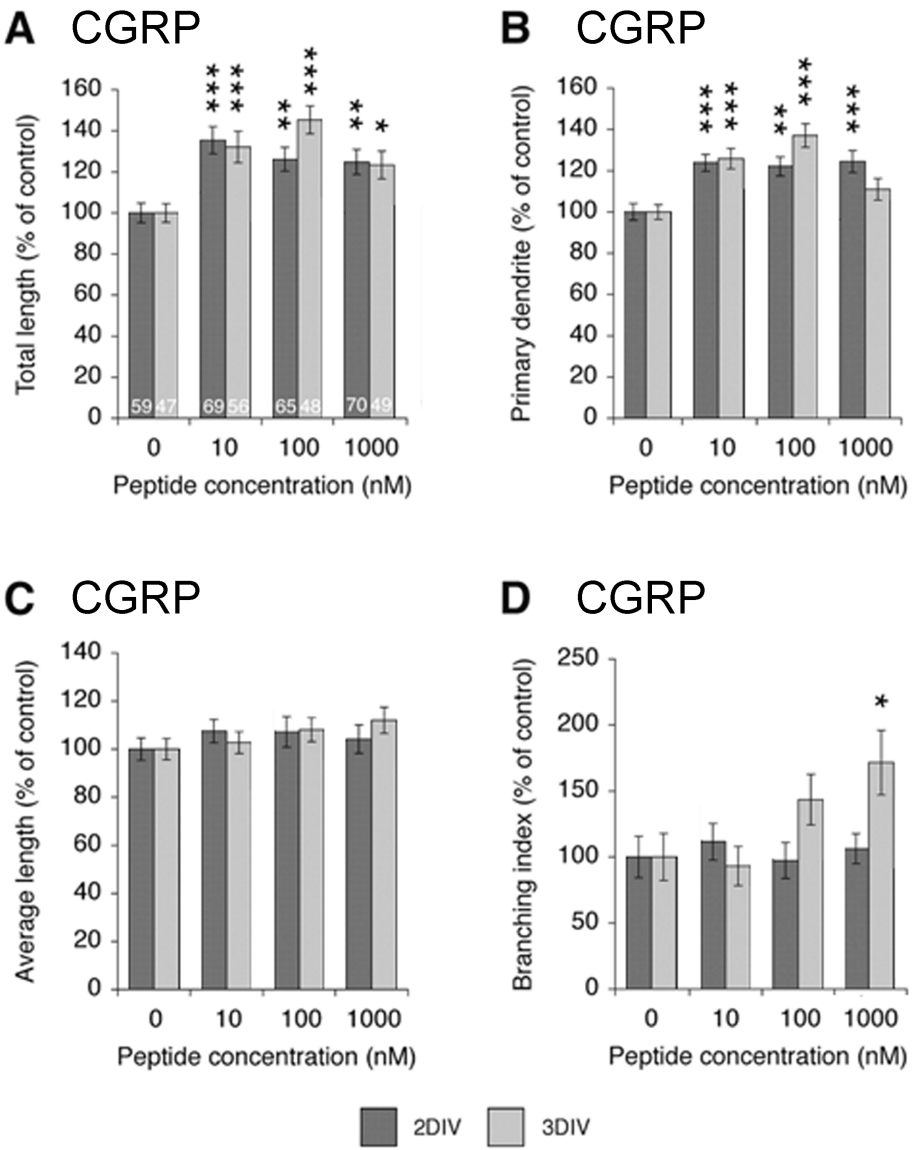
PD 5
AL 70.28
BI 0.40
TL 305.93
PD 6
AL 50.99
BI 0.33
a

# Fig.2


A CGRP
Total length (% of control)
160
140
120
100
80
60
40
20
0
59 47
69 56
65 48
70 49
0
10
100
1000
Peptide concentration (nM)
B CGRP
Primary dendrite (% of control)
Peptide concentration (nM)
C CGRP
Average length (% of control)
Peptide concentration (nM)
D CGRP
Branching index (% of control)
250
200
150
100
50
Peptide concentration (nM)
2DIV
3DIV

# Fig.3

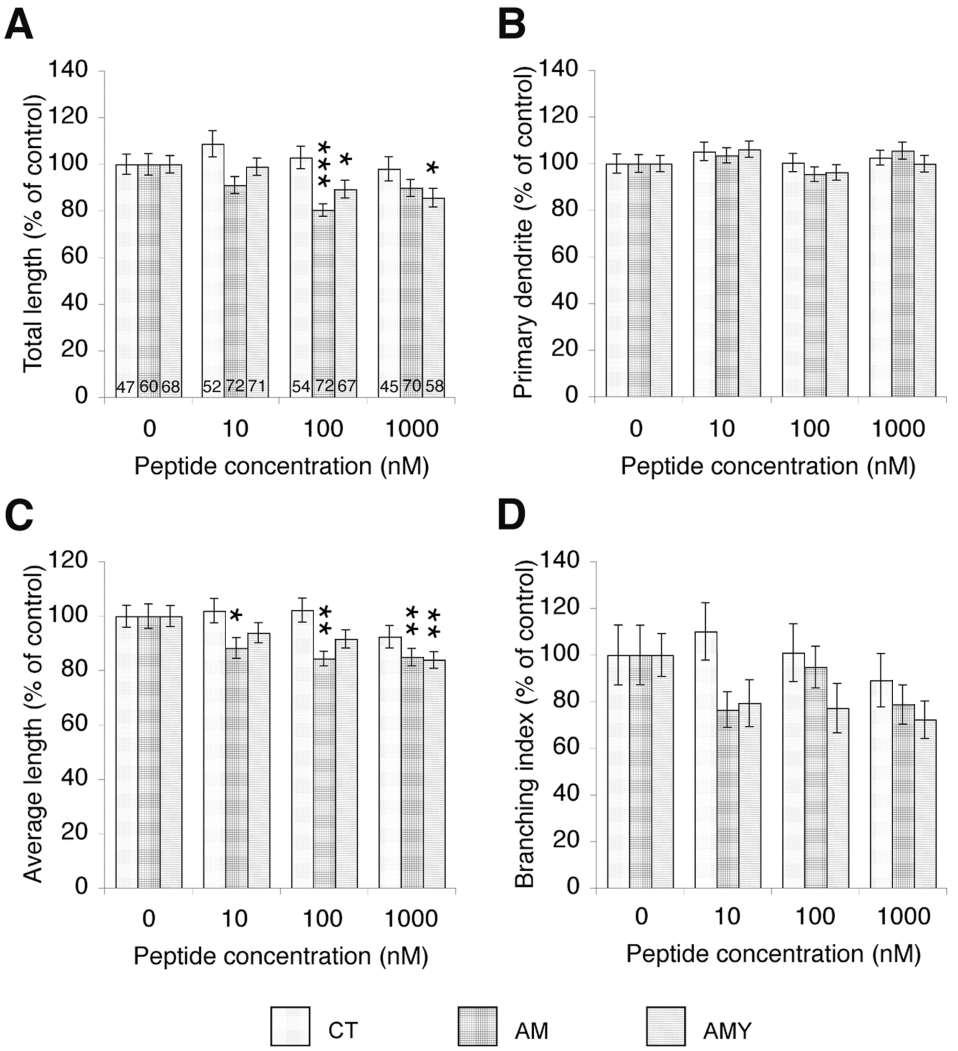
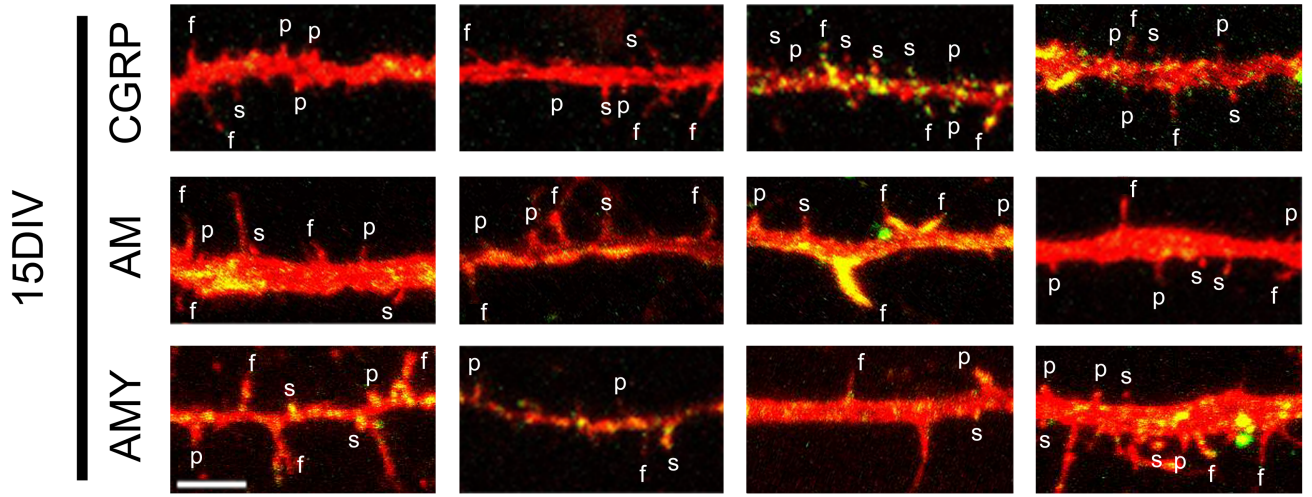

A
Total length (% of control)
140
120
100
80
60
40
20
0
47 60 68
52 72 71
54 72 67
45 70 58
0
10
100
1000
Peptide concentration (nM)
B
Primary dendrite (% of control)
140
120
100
80
60
40
20
0
0
10
100
1000
Peptide concentration (nM)
C
Average length (% of control)
120
100
80
60
40
20
0
0
10
100
1000
Peptide concentration (nM)
D
Branching index (% of control)
140
120
100
80
60
40
20
0
0
10
100
1000
Peptide concentration (nM)
CT
AM
AMY

Fig.4



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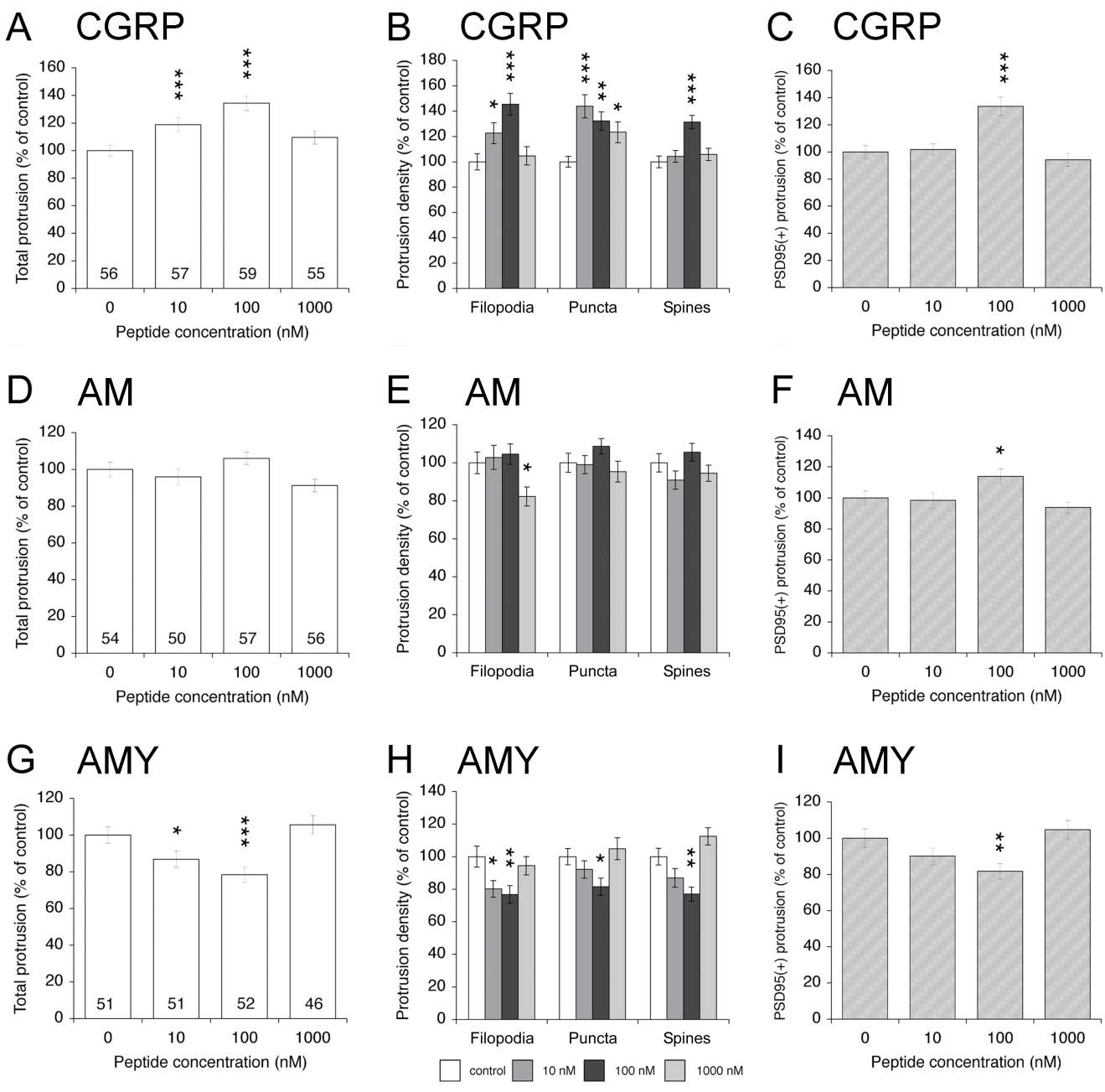
A CGRP
Total protrusion (% of control)
Peptide concentration (nM)
0
10
100
1000
56
57
59
55
B CGRP
Protrusion density (% of control)
Filopodia
Puncta
Spines
C CGRP
PSD95(+) protrusion (% of control)
Peptide concentration (nM)
D AM
Total protrusion (% of control)
54
50
57
56
Peptide concentration (nM)
E AM
Protrusion density (% of control)
Filopodia
Puncta
Spines
F AM
PSD95(+) protrusion (% of control)
Peptide concentration (nM)
G AMY
Total protrusion (% of control)
51
51
52
46
Peptide concentration (nM)
H AMY
Protrusion density (% of control)
Filopodia
Puncta
Spines
control
10 nM
100 nM
1000 nM
I AMY
PSD95(+) protrusion (% of control)
Peptide concentration (nM)

Fig.6

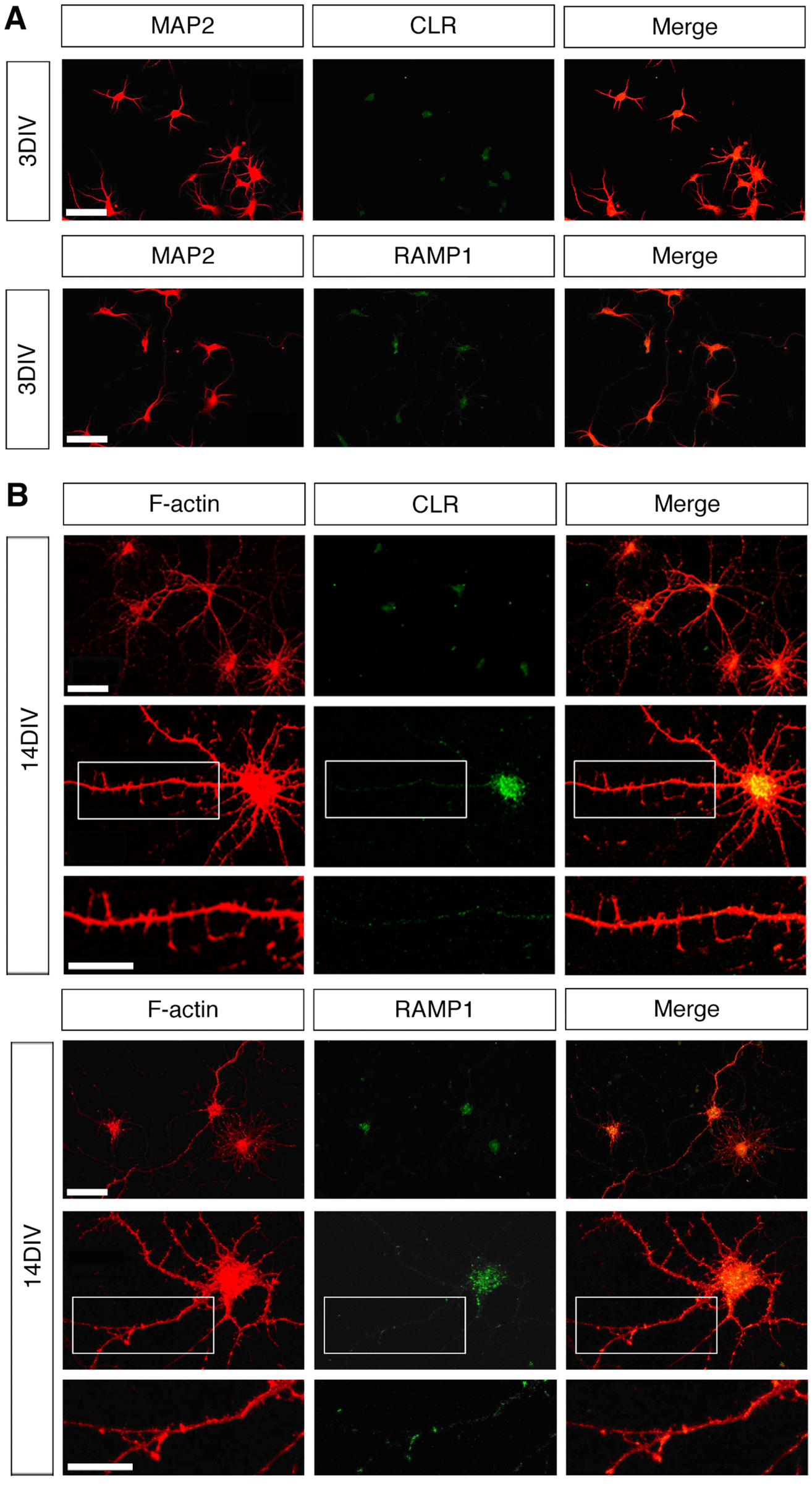
A
MAP2
CLR
Merge
3DIV
MAP2
RAMP1
Merge
3DIV
B
F-actin
CLR
Merge
14DIV
F-actin
RAMP1
Merge
14DIV

Fig.7

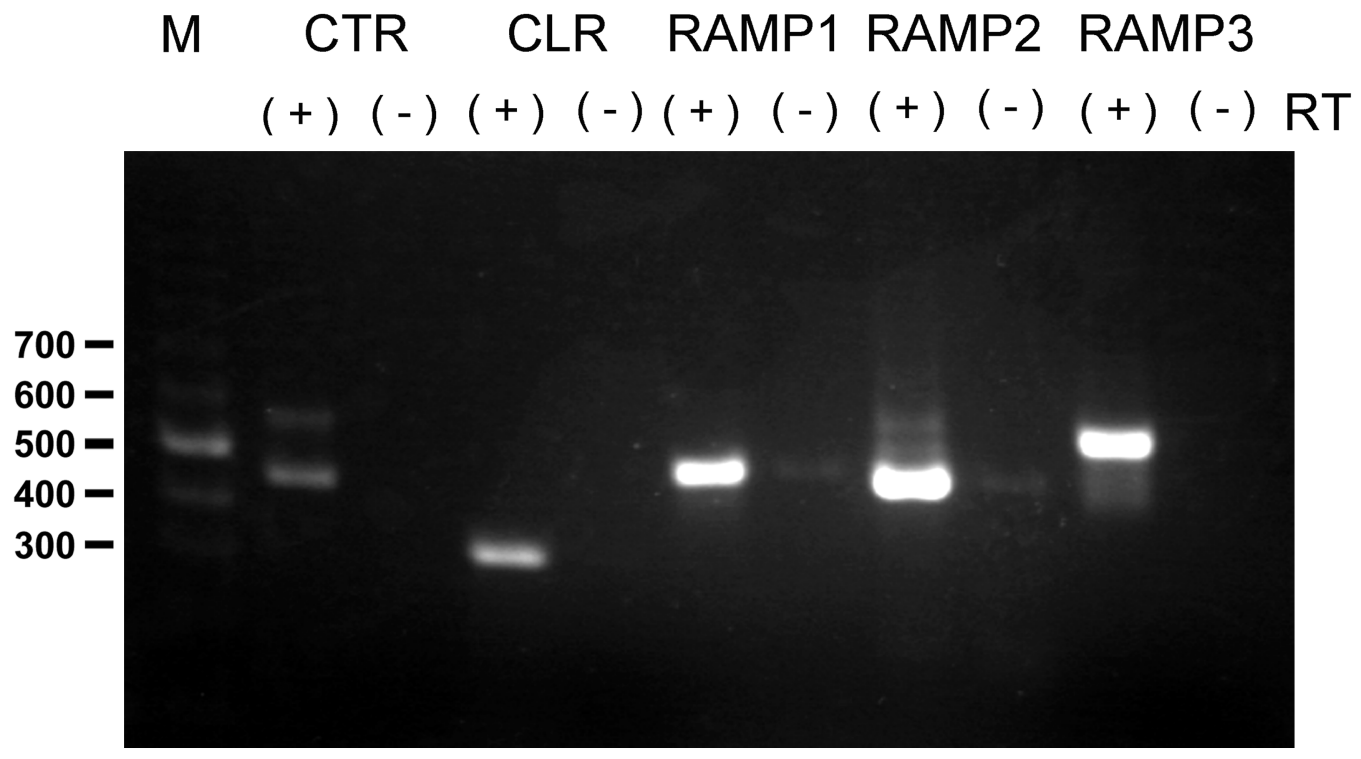

Fig.8