

# A novel cysteine-rich neurotrophic factor in *Aplysia* facilitates growth, MAPK activation, and long-term synaptic facilitation

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Neurotrophins are critically involved in developmental processes such as neuronal cell survival, growth, and differentiation, as well as in adult synaptic plasticity contributing to learning and memory. Our previous studies examining neurotrophins and memory formation in *Aplysia* showed that a TrkB ligand is required for MAPK activation, long-term synaptic facilitation (LTF), and long-term memory (LTM) for sensitization. These studies indicate that neurotrophin-like molecules in *Aplysia* can act as key elements in a functionally conserved TrkB signaling pathway. Here we report that we have cloned and characterized a novel neurotrophic factor, *Aplysia* cysteine-rich neurotrophic factor (apCRNF), which shares classical structural and functional characteristics with mammalian neurotrophins. We show that apCRNF (1) is highly enriched in the CNS, (2) enhances neurite elongation and branching, (3) interacts with mammalian TrkB and p75<sup>NTR</sup>, (4) is released from *Aplysia* CNS in an activity-dependent fashion, (5) facilitates MAPK activation in a tyrosine kinase dependent manner in response to sensitizing stimuli, and (6) facilitates the induction of LTF. These results show that apCRNF is a native neurotrophic factor in *Aplysia* that can engage the molecular and synaptic mechanisms underlying memory formation.

[Supplemental material is available for this article.]

The processes of development and memory formation both involve growth and reorganization of synaptic connections. Thus it is often suggested that they share common molecular mechanisms (Carew et al. 1998; Bailey and Kandel 2008). Supporting this view, extensive evidence indicates that the neurotrophin family of growth factors regulates both neuronal cell survival and differentiation in development, as well as synaptic plasticity and learning and memory in adult animals (Poo 2001; Vicario-Abejon et al. 2002). In most species, neurotrophin signaling cascades are typically mediated by receptor tyrosine kinases (e.g., Trk A/B/C in mammals) and pan-neurotrophin receptor (p75<sup>NTR</sup>) (Bibel et al. 1999; Chao 2003).

Previous studies have demonstrated a critical role for neurotrophin signaling in *Aplysia californica* (Purcell et al. 2003; Ormond et al. 2004; Sharma et al. 2006; Kassabov et al. 2013), a powerful model system for mechanistic studies of learning and memory formation (Bailey et al. 1996). For example, the induction of both long-term memory (LTM) and one of its cellular correlates, long-term synaptic facilitation (LTF), is facilitated by the activation of a tyrosine kinase–MAPK signaling pathway (Purcell et al. 2003). MAPK can be viewed as a molecular node (Reissner et al. 2006) that is a broker for growth factor signaling, including TrkB signaling, and is critical in learning and memory in *Aplysia* and other animals (Atkins et al. 1998; Reissner et al. 2006). Moreover, the TrkB ligand BDNF facilitates serotonin (5HT)-induced LTF in a MAPK-dependent manner, suggesting the existence of an endogenous TrkB-like receptor in *Aplysia* (Purcell et al. 2003; Sharma et al. 2006). Supporting this idea, inhibition of tyrosine kinase ac-

tivity blocks induction of LTF (Purcell et al. 2003). Furthermore, CNS application of a TrkB-Fc chimera, which sequesters endogenous BDNF-like ligands, blocks the activation of MAPK, the induction of LTF, and the formation of LTM in response to training with either 5HT or tail shock (TS) (Sharma et al. 2006). In *Aplysia*, two Trk receptors with conserved tyrosine kinase domains, ApTrkI and ApTrk, and an *Aplysia* neurotrophin ortholog, ApNT, have recently been cloned and shown to contribute to LTF (Ormond et al. 2004; Kassabov et al. 2013). These results demonstrate the existence of endogenous neurotrophic factors and their receptors in invertebrates which contribute to synaptic plasticity and memory formation.

The analysis of neurotrophic factors provides an excellent platform to explore the hypothesis that development and learning share common molecular mechanisms. To support this hypothesis in *Aplysia*, two basic conditions must be met. First, the neurotrophic factors in *Aplysia* should be released in the CNS and behave like bona fide growth factors. For example, they should promote neuronal growth (Poo 2001). Second, these neurotrophic factors should engage the same molecular and cellular mechanisms that are known to subservise memory formation in *Aplysia*. In the present study we report the identification of apCRNF, a novel neurotrophic factor in *Aplysia*, which is expressed in the CNS of *Aplysia*, and enhances sensory neuron (SN) neurite elongation and branching. Moreover, we demonstrate that apCRNF interacts with mammalian neurotrophin receptors and is released by a training

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analog that induces activity-dependent memory for sensitization (Shobe et al. 2009). Additionally, apCRNF induces MAPK activation in conjunction with a single tail nerve shock (TNS, a sensitizing stimulus that releases 5HT in the CNS; Marinesco and Carew 2002), an effect which requires tyrosine kinase activity, and facilitates the induction of LTF in conjunction with a single pulse of 5HT. Taken together, these data support the hypothesis that apCRNF is a novel endogenous neurotrophic factor in *Aplysia* that contributes to the induction of synaptic plasticity underlying memory formation.

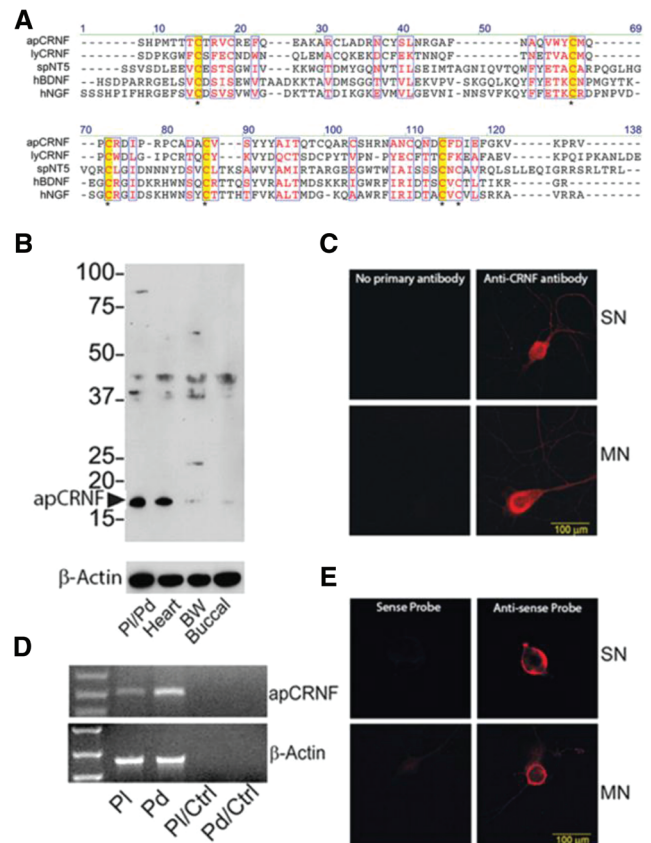
## Results

### Identification and characterization of apCRNF

Fainzilber et al. (1996) originally identified a novel molluscan neurotrophic factor, *Lymnaea* CRNF (lyCRNF) by its interaction with p75<sup>NTR</sup>. This neurotrophic factor is released into *Lymnaea* hemolymph and CNS conditioned medium, induces neurite outgrowth, and up-regulates voltage activated calcium currents in *Lymnaea* motor neurons (MNs) (Fainzilber et al. 1996). To determine if a similar neurotrophic factor is endogenous to *Aplysia*, we used the lyCRNF nucleotide sequence as a template for our search and cloned a novel CRNF gene from an *Aplysia* CNS cDNA library (GenBank accession no. EB259651), which shares 41.0% identity with lyCRNF. apCRNF protein consists of 123 amino acid residues, with a theoretical molecular weight of 18 kDa. The protein sequence alignment between full length apCRNF and lyCRNF exhibits 31.5% similarity and 22.3% identity, including 11 highly conserved cysteine residues (Supplemental Fig. 1A). Using the Clustal Omega multisequence alignment server, apCRNF was aligned against a panel of mammalian neurotrophins and invertebrate neurotrophic factors, which shows that apCRNF contains conserved cysteines essential for cystine-knot structure (Fig. 1A), a structural hallmark of neurotrophins (Wiesmann and de Vos 2001). Collectively, the sequence similarity of apCRNF to known invertebrate neurotrophic factors and mammalian neurotrophins and its potential containment of a cystine-knot structure raise the possibility that apCRNF may represent a bona fide neurotrophic factor in *Aplysia*.

### apCRNF is expressed in adult *Aplysia* CNS

Neurotrophins are critically important for nervous system development and neuronal growth and survival in mammals (Poo 2001). If apCRNF is acting as a bona fide neurotrophic factor, it should be expressed in the CNS of *Aplysia*. To further characterize apCRNF, recombinant GST- and His-apCRNF was expressed and purified (Supplemental Fig. 1B). We then raised a custom anti-apCRNF antibody, which specifically recognized GST- or His-apCRNF as major bands of ~43 kDa or ~20 kDa, respectively (Supplemental Fig. 1C); both are consistent with the molecular weights of the tagged recombinant proteins. Consistent with the predicted molecular weight of endogenous apCRNF, this antibody recognized a major band of ~18 kDa in *Aplysia* tissue extracts, indicating apCRNF is richly expressed in pleural-pedal ganglia and heart, and weakly expressed in body wall and buccal mass (Fig. 1B). To determine the expression of apCRNF in isolated SNs and MNs, we performed immunocytochemistry on cultured *Aplysia* neurons. Our results revealed that apCRNF is localized in the somata and neurites of both SNs and MNs (Fig. 1C). RT-PCR assay showed that apCRNF is expressed in both pleural and pedal ganglia (Fig. 1D). The pleural ganglia contain the somata and processes of the SNs, and the pedal ganglia contain the tail motor neuron (MN) somata and the SN synapses onto tail MNs (Sherff and Carew 2004). Moreover, fluorescence in situ hybridization



**Figure 1.** Cloning and characterization of *Aplysia* CRNF. (A) Structural alignment of cysteine-knot domains of *Aplysia* CRNF (apCRNF), *Lymnaea* CRNF (lyCRNF), *Strongylocentrotus purpuratus* (sea urchin) NT5 (spNT5), human BDNF (hBDNF), and human NGF (hNGF). The cysteines critical for the cystine-knot are highlighted in orange and labeled with a single asterisk (\*), and the conserved substitutions within the hydrophobic core are highlighted in boxes and shown in red. (B) Tissue distribution analysis reveals that apCRNF protein is expressed in CNS. (Top) apCRNF distribution determined by Western blot with anti-apCRNF antibody in the pleural-pedal ganglia (Pl/Pd), heart, body wall (BW), and buccal mass. (Bottom)  $\beta$ -actin is used as a loading control. (C) Immunocytochemical analysis reveals that apCRNF protein is expressed in SNs and MNs. (Top panels) SNs; (bottom panels) MNs. Controls (without primary antibody) are shown on the left. (D) RT-PCR shows apCRNF mRNA levels in pleural (Pl) and pedal (Pd) ganglia. Control experiments in the absence of reverse transcriptase show no signal in either pleural (Pl/Ctrl) or pedal ganglia (Pd/Ctrl). Actin is used as an internal control. (E) Fluorescent in situ hybridization shows apCRNF transcripts are expressed in SNs and MNs. (Top panels) SNs; (bottom panels) MNs. Controls with sense RNA probes are shown on the left.

(FISH) showed that apCRNF transcripts are expressed in both SN and MN cell bodies (Fig. 1E). These results are consistent with the pre- and postsynaptic localization of neurotrophins at mammalian synapses that exhibit activity-dependent plasticity (Poo 2001). Interestingly, ApNT, a recently characterized neurotrophin ortholog endogenous to *Aplysia*, is primarily localized to the sensory neurons (Kassabov et al. 2013), suggesting that ApNT and apCRNF may serve overlapping but distinct functional roles in the *Aplysia* CNS (see Discussion).

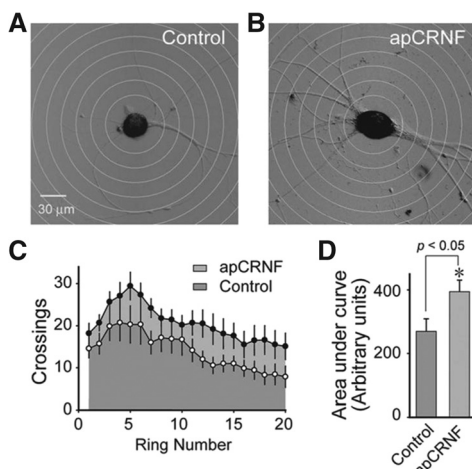
### apCRNF enhances SN neurite growth and branching

In vertebrates, neurotrophins are critical for neurite growth, including neurite elongation, axonal arborization, dendritic

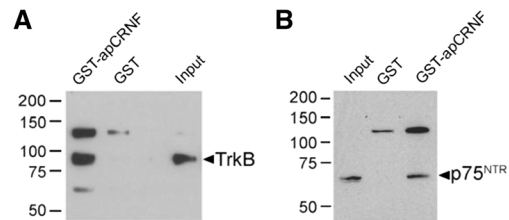
branching, and spine formation (Levi-Montalcini and Angeletti 1968; Cohen-Cory et al. 2010). To examine the effect of apCRNF on neurite growth, we treated isolated *Aplysia* SNs in culture with GST-apCRNF (250 ng/mL) or vehicle (0.1% BSA in PBS) for 3 d. Images of the SNs were then taken and subjected to Sholl analysis (see Materials and Methods). The number of intersections between neurites and each ring (Fig. 2A,B) was independently counted using a blind procedure. The numbers of neurite crossings after apCRNF treatment ( $n = 12$ ) was consistently greater than those of control ( $n = 7$ ) (Fig. 2C). To quantify the aggregate data, we determined the areas under curves in Figure 2C (Gensel et al. 2010), and found that the area for apCRNF treatment was significantly larger than the area for the control treatment (two-tailed  $t$ -test,  $t_{(17)} = 2.214$ ,  $P < 0.05$ ) (Fig. 2D). These data indicate that apCRNF enhances neurite growth and branching of SNs and suggest the existence of an endogenous receptor for apCRNF.

### apCRNF interacts with rat TrkB and p75<sup>NTR</sup>

Given that apCRNF shares sequence similarity with neurotrophins, we next asked if apCRNF can bind neurotrophin receptors, which may mediate the facilitatory effects on neurite outgrowth seen in Figure 2. As part of a Trk-p75<sup>NTR</sup> receptor complex, Trk receptor tyrosine kinases can be activated and phosphorylated by the binding of neurotrophins, which leads to the transduction of a variety of downstream signaling events, including MAPK phosphorylation (Chao 2003). In addition, the presence of p75<sup>NTR</sup> can enhance the neurotrophin-Trk affinity (Bibel et al. 1999). In the present study, we explored the possible interaction of apCRNF with mammalian neurotrophin receptors using a GST pull-down assay with recombinant GST-apCRNF protein (Supplemental Fig. 1B). Specifically, we asked if GST-apCRNF interacts with rat TrkB and p75<sup>NTR</sup>, since (1) TrkB signaling is critical for learning and memory in *Aplysia* (Purcell et al. 2003; Sharma et al. 2006) and (2) all neurotrophins bind to p75<sup>NTR</sup> (Chao 2003). As shown in Figure 3, GST-apCRNF, but not GST alone, binds to both rat TrkB (Fig. 3A) and p75<sup>NTR</sup> (Fig. 3B). These data implicate activation of neurotrophin receptor orthologs in *Aplysia* as a possible mechanism for mediating apCRNF's neurotrophic effects.



**Figure 2.** apCRNF increases neurite branching of *Aplysia* sensory neurons. Representative images of SNs after 3 d of vehicle (0.1% BSA in PBS (A) or apCRNF (250 ng/mL) treatment (B). A set of 20 rings of increasing radii was generated and superimposed on the bright field images. (C) Sholl analysis indicates that apCRNF treatment increases the number of SN neurite crossings compared to the control treatment. (D) The area beneath the curve in C was significantly larger for apCRNF treatment ( $n = 12$ ) compared to control ( $n = 7$ ).



**Figure 3.** apCRNF interacts with mammalian TrkB and p75<sup>NTR</sup>. Rat brain extract was incubated with recombinant GST or GST-apCRNF, and then TrkB (A) or p75<sup>NTR</sup> (B) was detected by Western blotting.

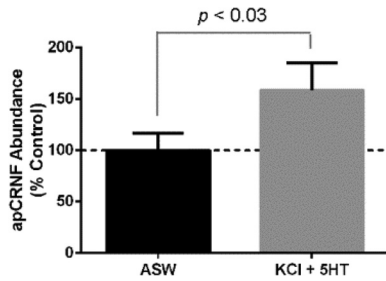
### apCRNF is released in the CNS in an activity-dependent fashion

Because many neurotrophic factors play dual roles in development and adult plasticity, we next asked if apCRNF is engaged during the induction of plasticity in *Aplysia*. For a neurotrophic factor to contribute to learning and memory formation, it must (1) be released in response to plasticity-inducing stimuli, and (2) engage molecular and cellular substrates required for learning and memory formation. In previous studies, we found that the combination of a pulse of KCl (which depolarizes *Aplysia* SN membranes  $\sim 20$  mV and induces calcium influx) and a pulse of 5HT can be used as a molecular analog of associative learning to induce MAPK activation and activity-dependent intermediate-term memory (Ye et al. 2008; Shobe et al. 2009). Using an enzyme-linked immunosorbent assay (ELISA) for apCRNF, we observed a significant increase of apCRNF protein level in the conditioned medium of *Aplysia* pleural-pedal ganglia that received a pulse of KCl and 5HT ( $158.6 \pm 26.1\%$ ,  $n = 10$ ), compared to the control ganglia receiving ASW treatment ( $100.0 \pm 16.3\%$ ; one-tailed  $t$ -test,  $t_{(18)} = 1.903$ ,  $P < 0.04$ ) (Fig. 4). The release of apCRNF triggered by the combination of KCl and 5HT is consistent with related observations showing regulated secretion of NGF, BDNF, and NT3 (Poo 2001).

### apCRNF facilitates MAPK activation

A single 5HT exposure or a single tail shock (TS) induces only short-term plasticity, but not LTF/LTM in *Aplysia* (Sutton et al. 2002). In contrast, multiple TSs induce LTM in a MAPK dependent manner (Sharma et al. 2003). In addition, Purcell et al. (2003) showed that subthreshold training with 5HT or TS, when paired with up-regulation of tyrosine kinase signaling by bpV (a tyrosine kinase phosphatase inhibitor), is able to induce MAPK activation and LTM. If apCRNF is released by sensitizing stimuli in *Aplysia* and engages a receptor tyrosine kinase cascade (potentially mediated by a TrkB-like receptor), one would predict that sensitizing stimuli such as tail nerve shocks (TNSs) would give rise to activation of signaling molecules canonically downstream of tyrosine kinase activity, including MAPK activation (Huang and Reichardt 2003). To test this prediction, we examined MAPK activation induced by (1) apCRNF alone, (2) a single TNS, and (3) a combined treatment of apCRNF and a single TNS (CRNF + 1  $\times$  TNS). Phospho-MAPK levels were normalized to total MAPK within the same sample, and then the relative amount of phospho-MAPK in the experimental group was compared to the within-animal control group. Neither apCRNF alone (500 ng/mL) nor a single TNS alone ( $100.1 \pm 16.3\%$ ,  $n = 6$ ;  $118.5 \pm 13.2\%$ ,  $n = 7$ ; NS) induced MAPK activation 1 h after the shock above within-animal control levels (Fig. 5A), a time point at which there should be no MAPK activation by a single sensitizing trial alone (Philips et al. 2007, 2013). In contrast, the combined treatment of apCRNF (500 ng/mL) and a single TNS induced significant





**Figure 4.** apCRNF is released by activity-inducing stimuli in the CNS. ELISA analysis shows that apCRNF protein level in conditioned medium of *Aplysia* pleural-pedal ganglia which received KCl + 5HT treatment is significantly higher than that in control ( $n = 10$ ).

MAPK activation above within-animal control levels ( $181.0 \pm 10.1\%$ ,  $n = 12$ ,  $P < 0.01$ ) (see Materials and Methods). A one-way ANOVA revealed a significant difference among the three groups ( $F_{(2,22)} = 12.38$ ,  $P < 0.001$ ). Subsequent planned comparisons indicated a significant difference between the apCRNF +  $1 \times$  TNS group and the apCRNF alone (Fischer's LSD,  $t_{(22)} = 4.451$ ,  $P < 0.001$ ), and a single TNS alone (Fischer's LSD,  $t_{(22)} = 3.645$ ,  $P < 0.01$ ), respectively. Finally, we found that the facilitation of MAPK activation by apCRNF +  $1 \times$  TNS requires tyrosine kinase activity: apCRNF +  $1 \times$  TNS did not significantly increase MAPK activation above within-animal control levels (see Materials and Methods) in the presence of genistein ( $100 \mu\text{M}$ ), a general tyrosine kinase inhibitor (Wilcoxon matched-pairs signed rank test,  $n = 8$ ,  $W = 0$ , NS), but MAPK was significantly increased above within-animal control levels in the presence of vehicle (0.33% DMSO/0.43% EtOH,  $n = 6$ ; Wilcoxon matched-pairs test,  $W = 21$ ,  $P < 0.04$ ) (Fig. 5B). Collectively, these data show that apCRNF facilitates the induction of a tyrosine kinase-MAPK cascade in response to a subthreshold sensitizing stimulus.

### apCRNF facilitates the induction of LTF

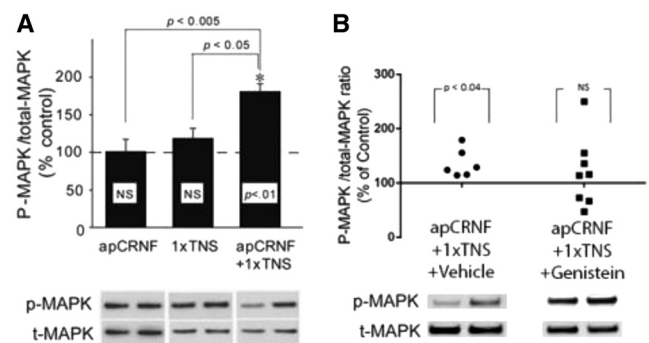
To explore the effect of apCRNF on synaptic plasticity, we examined LTF induction at cultured SN–MN synapses. EPSPs at SN–MN synapses were measured before and 18–24 h after apCRNF treatment. Neither apCRNF alone (median  $\pm$  IQR,  $125 \pm 118$ ,  $n = 5$ ; Wilcoxon matched-pairs test,  $W = 9$ ,  $P = 0.31$ ) nor a single pulse of 5HT (median  $\pm$  IQR,  $86 \pm 62$ ,  $n = 12$ ; Wilcoxon matched-pairs test,  $W = -10$ ,  $P = 0.73$ ) induced significant facilitation from pretreatment baseline measurements 18–24 h after treatment (Fig. 6). However, following apCRNF–5HT pairing, significant LTF was induced relative to pretreatment baseline levels (median  $\pm$  IQR,  $158 \pm 149$ ,  $n = 7$ ; Wilcoxon matched-pairs test,  $W = 28$ ,  $P < 0.02$ ). Furthermore, Kruskal–Wallis ANOVA revealed that there was an overall significant difference among the groups ( $\chi^2(2) = 7.258$ ,  $P < 0.03$ ) (Fig. 6B). Subsequent planned comparisons identified a significant difference between 5HT alone and apCRNF–5HT paired groups (Dunn's multiple comparisons test, Mean rank diff. =  $-8.881$ ,  $P < 0.05$ ). Intriguingly, there was not a significant difference between the apCRNF alone and apCRNF–5HT groups (Dunn's multiple comparisons test, Mean rank diff. =  $-3.714$ ), although only the apCRNF–5HT group (and not the apCRNF alone group) showed significant facilitation from baseline. Additionally, there appeared to be a trend toward synaptic facilitation with apCRNF alone treatment ( $P = 0.31$ ), an observation reminiscent of a finding by Kassabov et al. (2013) who reported that ApNT alone can cause synaptic facilitation in *Aplysia* SN–MN coculture. In conclusion, consistent with our previous findings examining the facilitating effects of mam-

malian BDNF (Purcell et al. 2003; Sharma et al. 2006), we here show that apCRNF also facilitates the induction of LTF at SN–MN synapses.

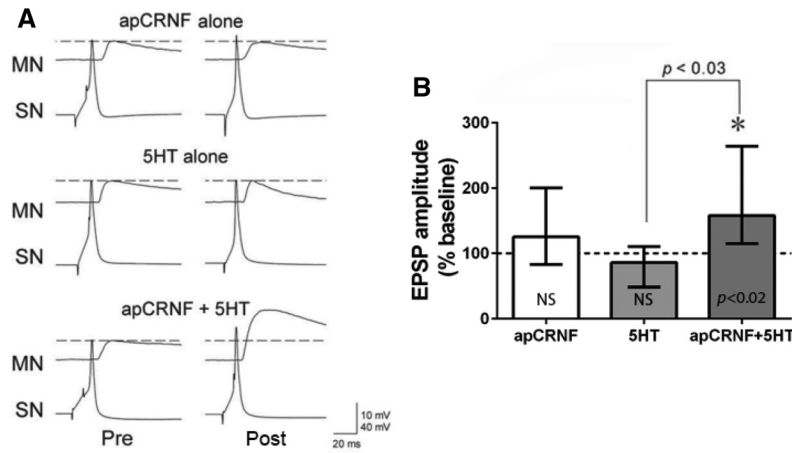
## Discussion

### Endogenous *Aplysia* growth factors

In the present study, we report the cloning and characterization of a novel *Aplysia* neurotrophic factor, apCRNF. In addition to the functional similarities between apCRNF and known neurotrophins, Clustal Omega multi-sequence alignment shows that apCRNF possesses cysteines essential for a cystine-knot (Fig. 1A). A cystine-knot usually consists of six highly conserved cysteines forming three intramolecular disulfide bonds; it is characteristically found in neurotrophins such as NGF, BDNF, NT-3, and NT-4, and is critical for stabilizing their molecular conformation (Wiesmann and de Vos 2001). In addition, the tertiary structures of apCRNF and canonical neurotrophins also share considerable similarity. During evolution, orthologous proteins may have diverged significantly in primary sequences, yet their three-dimensional structures may remain conserved. FUGUE, a structure-based search algorithm, was developed to identify the structural similarity in three dimensions (Shi et al. 2001). Recently, novel *Drosophila* neurotrophins with structural conservation but sequence divergence from mammalian neurotrophins were identified using a FUGUE-based search (Zhu et al. 2008). We used FUGUE to compare apCRNF with mammalian neurotrophins with known crystal structures, including BDNF and NGF. FUGUE predicts that apCRNF belongs to the mammalian neurotrophin family with a confidence level that is  $>99\%$  (ZSCORE = 15.54). Although we do not have a definite structure for apCRNF, the FUGUE prediction supports the structural similarity between apCRNF and mammalian neurotrophins. Hence, despite the divergence of the apCRNF primary sequence, its structural and functional similarities to vertebrate neurotrophins, as well as its ability to interact with the TrkB and p75<sup>NTR</sup> receptors, strongly indicate that apCRNF is a bona fide *Aplysia* neurotrophin.



**Figure 5.** apCRNF facilitates the induction of a tyrosine kinase-MAPK cascade in SN clusters. (A) Summary data of MAPK activation induced by apCRNF ( $500 \text{ ng/mL}$ ,  $n = 6$ ), a single TNS ( $n = 7$ ), and apCRNF in combination with a single TNS ( $n = 12$ ). Representative Western blots are presented below the summary data: phospho-MAPK (top) is normalized to total-MAPK (bottom), and then experimental phospho-total MAPK ratios (left) are compared to control phospho-total MAPK ratios (right). (B) Tyrosine kinase activity is required for MAPK activation induced by apCRNF +  $1 \times$  TNS. Each circle (apCRNF [ $500 \text{ ng/mL}$ ] +  $1 \times$  TNS + Vehicle [0.33% DMSO/0.43% EtOH]) or square (apCRNF +  $1 \times$  TNS + Genistein [ $100 \mu\text{M}$ ]) represents an individual experiment. There was significant MAPK activation in the vehicle group ( $P < 0.04$ ), but not in the genistein group ( $P > 0.99$ ). Representative Western blots are presented below the summary data as in A.



**Figure 6.** apCRNF facilitates the induction of LTF at SN–MN synapses. (A) Representative traces from independent experiments before (Pre) and 18–24 h after (Post) apCRNF alone ( $n = 5$ ), a 5-min pulse of 5HT alone ( $n = 12$ ), and a 5-min pulse of 5HT in combination with apCRNF (apCRNF + 5HT,  $n = 7$ ). (B) Summary data indicating the normalized EPSP amplitude at the long-term test point (18–24 h after treatment). apCRNF or 5HT alone do not induce LTF, but in conjunction they induce significant LTF. Data are expressed as median EPSP  $\pm$  IQR.

In addition to our previous findings that BDNF-like molecules are critical in learning and memory in *Aplysia* (Purcell et al. 2003; Sharma et al. 2006), two additional *Aplysia* growth factors have been identified. In a recent seminal study, Kassabov et al. (2013) identified ApNT, an invertebrate neurotrophin ortholog belonging to the canonical vertebrate neurotrophin family. ApNT is expressed as both a pro- and mature neurotrophin based on different splice variants. Both forms of ApNT are secreted and participate in serotonin-induced LTF in *Aplysia*, although interfering with ApNT signaling alone does not appear to be sufficient to completely disrupt LTF (Kassabov et al. 2013). Interestingly, a clustal alignment indicates apCRNF and ApNT both contain conserved cysteines similar to mammalian neurotrophins (Supplemental Fig. 2). However, apCRNF and ApNT have distinct sequences, suggesting these neurotrophic factors could serve different functional roles in *Aplysia*. In earlier work, Hu et al. (2004) found that another growth factor-like molecule, sensorin, is located in SNs and is secreted in response to repeated pulses of 5HT. Similar to neurotrophins, the released sensorin is required for synapse formation and LTF, indicating its critical role in synaptic plasticity in *Aplysia*. However, with only two cysteines, sensorin cannot form the cystine-knot typical of neurotrophin family members. Consistent with this view, a FUGUE analysis predicts that the structure of sensorin is not similar to that shared between apCRNF and mammalian neurotrophins.

Other growth factor families have also been found to be important for plasticity in *Aplysia*. Zhang et al. (1997) discovered that transforming growth factor  $\beta$ -1 (TGF $\beta$ -1), a member of the TGF $\beta$  superfamily of growth factors, is required for LTF in *Aplysia*. Interestingly, TGF $\beta$ -1 can rapidly activate MAPK (Chin et al. 2002), indicating that different growth factor families may contribute to synaptic plasticity through converging molecular mechanisms. Collectively, these results support the conclusion that there are multiple endogenous growth factor families in *Aplysia* that can participate in learning-related synaptic plasticity. Many growth factors employ distinct, but overlapping, signaling cascades to mediate similar functional outcomes (Kopeck and Carew 2013). Thus it will now be particularly interesting to utilize *Aplysia*'s well-characterized CNS to determine both the shared and perhaps unique contributions of different growth factor families to learning and memory formation.

## Endogenous *Aplysia* neurotrophin receptors

The identification of different invertebrate neurotrophic factors naturally gives rise to a search for endogenous invertebrate neurotrophin receptors. In mammalian systems, there are three neurotrophin Trk receptors: NGF binds to TrkA, BDNF and NT4/5 to TrkB, and NT3 to TrkC (Chao 2003). Similarly, multiple neurotrophins and receptor tyrosine kinases may be involved in LTF and LTM in *Aplysia* and other invertebrate systems.

Although to date no Trk receptors have been reported in *Drosophila* or *C. elegans*, several Trk or Trk-like receptors have been identified in mollusks (Beck et al. 2004; Ormond et al. 2004; Kassabov et al. 2013). LTrk, a prototypical Trk receptor in *Lymnaea*, is required in neuronal survival and outgrowth (Beck et al. 2004). In *Aplysia*, in addition to the two aforementioned neurotrophins, ApNT (Kassabov et al. 2013) and apCRNF (described here), two Trk receptors, ApTrkl and ApTrk, have been identified (Ormond et al. 2004; Kassabov et al. 2013). ApTrkl is localized in *Aplysia* sensory neurons and is required for 5HT-induced MAPK activation and LTF. It has a highly conserved intracellular tyrosine kinase domain and a novel extracellular domain not homologous to the sequences of any known Trk receptors (Ormond et al. 2004). The mechanism of 5HT activation of ApTrkl, which might require the transactivation via G-protein coupled receptors, remains unknown (Ormond et al. 2004). Moreover, another Trk receptor, ApTrk, is localized to both SNs and MNs (and is particularly enriched in MNs), is capable of activating MAPK, AKT, and PLC $\gamma$  in PC12nnr5 cells upon stimulation with ApNT, and plays an important role in LTF in *Aplysia* SN–MN coculture (Kassabov et al. 2013). Collectively, an intriguing possibility for future exploration is that, in response to activity-inducing stimuli, apCRNF is released and interacts with one of the *Aplysia* neurotrophin receptors to elicit the canonical Trk–MAPK signaling cascades.

In an important recent study, McIlroy et al. (2013) showed that *Drosophila* neurotrophins DNT1 and DNT2 bind to Toll-6 and Toll-7 of the Toll receptor superfamily, critically important for mediating innate immunity responses. These data raise the interesting possibility that invertebrate neurotrophins may promiscuously bind to several different types of receptors to induce their trophic effects. It now will be important to determine if apCRNF is, indeed, activating one of the identified Trk receptors in *Aplysia*, or if it is perhaps acting through novel receptors such as Toll receptors, or as yet unknown receptors, to mediate plasticity.

## Mechanisms of *Aplysia* neurotrophin signaling in learning and memory

More than a century ago, Ramón y Cajal raised the intriguing possibility that development and learning may have shared mechanisms (Carew et al. 1998; Bailey and Kandel 2008). Both neuronal development and memory formation are highly complex. Nevertheless, several processes have been proposed as critical points of contact for possible shared mechanisms of development and memory formation: (1) structural modifications of the synapse mediated by both target-oriented neuronal growth and synapse formation, (2) activity-dependent synaptic refinement in

neural pathways via coincident activity, and (3) regulation of synaptic efficacy, including LTP- and LTD-like mechanisms (Carew et al. 1998; McKay et al. 1999). Interestingly, neurotrophin signaling is involved in all of these processes and is likely to be highly conserved in both vertebrates and invertebrates (Carew et al. 1998; Bailey and Kandel 2008). Considering these several principles, in the present study we present evidence consistent with the view that apCRNF is a functional *Aplysia* neurotrophic factor and may be a secreted TrkB ligand essential to both synaptic refinement and memory formation in *Aplysia*. Thus, apCRNF may serve as a strategic focal point for studying the shared mechanisms of development and learning and memory in *Aplysia*.

## Materials and Methods

### Animals

Wild-caught *A. californica* (150–250 g) were obtained from Marinus Scientific (Long Beach, CA), Charles Hollahan (Santa Barbara, CA), and South Coast Bio-Marine, LLC (Los Angeles, CA) and were dissected as described in Reissner et al. (2010).

### Cloning and recombinant protein expression

apCRNF cDNA was isolated from an *Aplysia* CNS cDNA library by PCR, using primers 5'-CACCATGGCGGCAGAGTCCCA-3' and 5'-TGTTTACGAGTACATGAAGGGC-3'. For expression in bacteria, apCRNF was cloned into pDEST-15 (GST-tagged) or pDEST-17 (His-tagged) vectors. All constructs were verified by sequence analysis. GST- or His-apCRNF was expressed in Rosetta2 (DE3) pLys cells and purified using glutathione-agarose or His-binding resins as described in Reissner et al. (2008). Glutathione or imidazole was then removed using either dialysis or gel filtration. The purity of GST-CRNF was examined using SDS-PAGE with Coomassie Blue staining.

### Structure-based alignment

Multiple sequence alignment of mature neurotrophins (pre and/or pro sequences were removed) was performed using Clustal Omega (Sievers et al. 2011; Clustalo Version 1.1.0, <http://www.ebi.ac.uk/Tools/msa/clustalo/>). The multiple sequence alignment result was subsequently submitted to the sequence-structure homology recognition (FUGUE) server (Shi et al. 2001; FUGUE version 2.0 Profile Library Search Against HOMSTRAD, <http://tardis.nibio.go.jp/fugue/prfsearch.html>). Results with ZSCORE  $\geq 6$  have confidence levels  $\geq 99\%$ .

### Antibody preparation, tissue distribution, and GST pull-down assay

Custom polyclonal anti-CRNF antibodies were made against the epitope CSHRNANCQNDICFDIEFGKVKPR. For apCRNF tissue distribution, homogenates from *Aplysia* pleural-pedal ganglia, heart, body wall, and buccal mass were subjected to Western blot analysis using anti-apCRNF antibody (1:5000). GST pull-down assays were performed as described in Reissner et al. (2008).

### Cell culture, apCRNF treatment, and assay for neurite growth

Cell culture was performed as described in Zhao et al. (2009) and Reissner et al. (2010). Sensory neurons from pleural ganglia and LFS motor neurons from abdominal ganglia were used. Control (vehicle alone) and apCRNF treatment were always performed on SNs from the same animal in parallel. Recombinant apCRNF (250 ng/mL) or vehicle (0.1% BSA in PBS) was added on cultured SNs at Day 3 in vitro and incubated for 3 d. Bright field images were captured with a Zeiss Axiovert 200 microscope. Twenty evenly spaced concentric circles with increasing radii from 30 to 315  $\mu\text{m}$  were overlaid onto the center of a cell soma; neurite elonga-

tion and branchings were measured by Sholl analysis (Sholl 1953; Gensel et al. 2010).

### Immunocytochemistry

Immunocytochemistry of cultured SNs or MNs was performed as described by Lyles et al. (2006) using anti-apCRNF antibody (1:1000) and Alexa-633 goat anti-rabbit antibody (1:1000). Images were acquired with an Olympus Fluoview confocal laser-scanning microscope.

### Semi-quantitative RT-PCR and fluorescence in situ hybridization (FISH)

Pleural and pedal ganglia cDNA was prepared and semi-quantitative PCR was performed as described in Reissner et al. (2008). To ensure that amplification was kept within linear range, the number of cycles was optimized for each primer pair. apCRNF sense or antisense riboprobes were generated by in vitro transcription from SP6 or T7 promoters. FISH of cultured SNs or MNs was performed as described in Lyles et al. (2006). Images were acquired with an Olympus Fluoview laser-scanning microscope.

### apCRNF immunosorbent assay (ELISA)

Isolated pleural-pedal ganglia were desheathed to expose the somata of SNs/MNs. For each animal, one pair of pleural-pedal ganglia was assigned to the experimental group and the other pair to control. Ganglia were incubated with KCl + 5HT (KCl, 150 mM; 5HT, 50  $\mu\text{g}/\text{mL}$ ) or ASW for 15 min. Plates were coated with the conditioned medium samples, blocked with 0.5% BSA, and washed. Subsequently, the primary antibody (anti-apCRNF antibody, 1:5,000) was added and incubated overnight at 4°C. After washing, the secondary antibody (alkaline phosphatase-conjugated secondary anti-rabbit, 1:4,000) was added and incubated for 1.5 h at room temperature. The ELISA samples were then developed with *para*-nitrophenylphosphate and the absorbance was obtained with ThermoLabSystems Multiskan ascent spectrophotometer.

### MAPK activation

Isolated pleural-pedal ganglia were desheathed to expose the somata of SNs/MNs. For each animal, one pair of pleural-pedal ganglia was assigned to the experimental group and the other pair to control. Ganglia were incubated with apCRNF (500 ng/mL) or vehicle (0.1% BSA in PBS) for 2.5 h, a single TNS (30 V, 40 Hz, 5-msec shock duration, 1-sec train duration) was delivered to one pair of pleural-pedal ganglia in the experimental group, followed by another 1-h treatment of either apCRNF or ASW. For genistein experiments, bilateral pleural-pedal ganglia from the same animal were incubated with 100  $\mu\text{M}$  genistein (Purcell et al. 2003) or vehicle (0.33% DMSO/0.43% ethanol) for 30 min, followed by application of apCRNF (500 ng/mL) for an additional 30 min. A single TNS (30 V, 40 Hz, 5-msec shock duration, 1-sec train duration) was delivered to one pair of pleural-pedal ganglia, followed by another 1-h treatment of apCRNF and either genistein or vehicle. Immediately after the treatment, SN clusters were excised, lysed, and subjected to Western blot. Blots were probed with a total p44/42 MAPK antibody and a phospho p44/p42 MAPK antibody, and developed using chemiluminescence or Li-cor Odyssey imaging systems.

### Synaptic facilitation

Intracellular recordings were made from SN–MN coculture as described in Reissner et al. (2010). Baseline EPSPs were recorded before treatment. Coculture was then exposed to a 2.5-h treatment of either vehicle or apCRNF (500 ng/mL), coterminated with a 5-min 5HT (10 mM) pulse, followed by another 1-h treatment of either vehicle or apCRNF (500 ng/mL), and incubated in culture medium overnight at 18°C. LTF at the SN–MN synapses was tested 18–24 h later.



## Data analysis

Unless indicated, all the data were presented as mean  $\pm$  SEM. All *t*-tests and post hoc tests used are two-tailed. Nonparametric statistics were used in Figures 5B and 6 due to the non-normality of the data. When  $P < 0.05$ , the differences were considered significant.

### Statistical analysis of neurite growth

For individual cells, a curve was generated by plotting crossings versus ring numbers (as in Fig. 2C). The area under the curve was then obtained by integration. The data in Figure 2D were summarized as the average areas of the control group versus apCRNF-treated group. Between-group comparisons were performed using unpaired *t*-tests.

### Statistical analysis of ELISA

The data were normalized to the mean value of baseline measurement (ASW), and one-tailed paired *t*-test was performed for between-group comparisons (ASW vs. KCl+5HT).

### Statistical analysis of MAPK activation

The experimental ganglia phospho-MAPK versus total-MAPK ratio was normalized to the within-animal control ganglia phospho versus total MAPK ratio. Paired *t*-tests or a nonparametric Wilcoxon matched-pairs signed rank test (due to the non-normality of the data for the genistein experiments [Fig. 5B]) was used for within-group comparisons between experimental ganglia and the corresponding control. A one-way ANOVA followed by Fischer's LSD procedure were used for between-group comparisons.

### Statistical analysis of physiology

EPSP amplitudes were normalized to the baseline measurement. Data are presented as median  $\pm$  IQR. A Wilcoxon Signed Rank test was used for within-group comparisons due to non-normality of the data. Kruskal–Wallis test followed by Dunn's multiple comparison procedure were used for between-group comparisons.

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