Brief Communication

Postsynaptic effects of *Aplysia* cysteine-rich neurotrophic factor in the induction of activity-dependent long-term facilitation in *Aplysia californica*

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The spatial and temporal coordination of growth factor signaling is critical for both presynaptic and postsynaptic plasticity underlying long-term memory formation. We investigated the spatiotemporal dynamics of *Aplysia* cysteine-rich neurotrophic factor (ApCRNF) signaling during the induction of activity-dependent long-term facilitation (AD-LTF) at sensory-to-motor neuron synapses that mediate defensive reflexes in *Aplysia*. We found that ApCRNF signaling is required for the induction of AD-LTF, and for training-induced early protein kinase activation and late forms of gene expression, exclusively in postsynaptic neurons. These results support the view that ApCRNF is critically involved in AD-LTF at least in part through postsynaptic mechanisms.

A prevailing model of the molecular mechanisms underlying long-term memory (LTM) formation involves a dynamic interaction between presynaptic and postsynaptic signaling occurring at critical synapses in response to learning-inducing experiences (Mirisis et al. 2016; Poo et al. 2016; Smolen et al. 2019). Growth factor (GF) signaling is a family of evolutionarily conserved molecular mechanisms with spatiotemporal dynamics that support the complex nature of this interaction (Kopec and Carew 2013; Edelmann et al. 2014). Following their pioneering discovery as molecules important for neural development (Levi-Montalcini 1987), GFs have been characterized as critical molecular components of multiple forms of plasticity underlying LTM (Park and Poo 2013; Poon et al. 2013). GFs are released extracellularly and bind to membrane-associated receptors to activate a variety of downstream intracellular signaling in different neurons, and have been shown to act, both in distinct brain regions, and at different time points following learning-inducing stimuli (Edelmann et al. 2014). Consequently, GF signaling constitutes a unique spatial and temporal molecular network that mediates both intercellular and intracellular signaling at synapses undergoing plasticity. Understanding the complexity of this network requires the delineation of the spatiotemporal dynamics of its individual molecular steps in a cell-specific manner, a challenging endeavor which has been difficult to achieve in the highly complex mammalian brain, but can be aided by studies of the simpler neural networks of invertebrate models. Indeed, two recent studies in mammals and invertebrates directly compared pre- and postsynaptic effects of GF signaling at synapses undergoing long-lasting plasticity, and found evidence for complex interactions between GF, neurotransmitter, and neuromodulator signaling, as well as between downstream intracellular events occurring in both compartments (Jin et al. 2018; Lin et al. 2018).

In the current work, we took a single-cell approach in studying the spatiotemporal dynamics of GF signaling during the initiation of long-term synaptic plasticity, by using the spatial resolution of the sensory-to-motor neuron (SN-MN) synapses mediating defensive withdrawal reflexes in *Aplysia californica*. This monosynaptic circuit constitutes a critical site of plasticity underlying behavioral sensitization of these reflexes (Pinsker et al. 1973; Cleary et al. 1998). To induce synaptic activity, which is known to regulate the synthesis, release, and signaling of GFs (Poo 2001), we used activity-dependent (AD) training, in which serotonin (5HT) neuromodulation is paired with neuronal activity, and which produces both LTM for sensitization (LTS; Walters 1987) and long-term facilitation (LTF) at SN-MN synapses (Schacher et al. 1997). We focused our analysis on *Aplysia* cysteine-rich neurotrophic factor (ApCRNF), a novel *Aplysia* GF which we previously identified and showed: (i) to be expressed in both SNs and MNs, (ii) to be released in the central nervous system in response to AD training, and (iii) to promote LTF at SN-MN synapses (Pu et al. 2014). In the present study, we investigated the spatiotemporal dynamics of ApCRNF signaling during the induction of AD plasticity and found that: (i) AD training induces LTF at SN-MN synapses, as well as activation of mitogen-activated protein kinase (MAPK) and increased expression of *CCAAT-enhancer binding protein* (*C/EBP*) mRNA in both SNs and MNs; (ii) ApCRNF signaling is required for the induction of AD-LTF; and (iii) ApCRNF signaling is required for AD training-induced activation of MAPK and mRNA expression of *C/EBP* and *ApCRNF*, exclusively in MNs. Collectively, our findings strengthen previous models of plasticity in *Aplysia* (Kandel 2012; Byrne and Hawkins 2015), and, in addition, reveal novel postsynaptic mechanisms of plasticity governed by GF signaling in *Aplysia*.

We previously showed that ApCRNF is released extracellularly in an activity-dependent manner (Pu et al. 2014). In the present study, we tested the hypothesis that ApCRNF signaling is required

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for the induction of AD-LTF at cultured SN-MN synapses. To block the signaling of extracellularly released ApCRNF, we used an ApCRNF antibody, which we previously characterized as a function-blocking antibody. AD training in the presence of IgG control induced LTF, while application of anti-ApCRNF during and for 1 h after training blocked LTF. Moreover, treatment with anti-ApCRNF alone did not affect basal synaptic transmission (Fig. 1). These results support the hypothesis that ApCRNF signaling during and immediately after AD training is required for the induction of LTF at SN-MN synapses.

We next investigated the molecular mechanisms induced by ApCRNF during AD-LTF induction, focusing on the spatiotemporal dynamics of ApCRNF signaling. Long-lasting memory and plasticity require activation of protein kinases and gene expression across species (Mirisis et al. 2016; Smolen et al. 2019). Two well-established examples from the mammalian and *Aplysia* literature, are MAPK activation (Sharma and Carew 2004; Sweatt 2004) and cAMP response element binding protein (CREB)-dependent transcription (Alberini 2009). We first focused on activation of MAPK, a second messenger cascade that links extracellular events, such as GF release, to intracellular signaling, and is required for long-lasting forms of memory and synaptic plasticity in *Aplysia* and other species (English and Sweatt 1996; Atkins et al. 1998; Ota et al. 2008; Pagani et al. 2009; Michel et al. 2011a). In addition, in *Aplysia* SNs, an early phase of MAPK activation is reliably observed following a variety of LTF-inducing training paradigms, including AD training (Martin et al. 1997; Shobe et al. 2009; Philips et al. 2013; Liu et al. 2014). Hence we asked whether ApCRNF signaling is required for AD training-induced early MAPK activation in cocultured SNs and MNs. AD training in the presence of IgG induced a significant increase in 1-h MAPK activation in both SNs and MNs, when compared with controls. Interestingly, treatment with anti-ApCRNF blocked the increase in 1-h MAPK activation only in MNs (Fig. 2A1–B2). To further investigate the direct involvement of ApCRNF in postsynaptic MAPK activation, we asked whether ApCRNF can facilitate this process. We found that, although one pulse of 5HT in the presence of vehicle control was insufficient to induce a significant increase in 1-h MAPK activation in MNs, consistent with similar findings in SNs (Shobe et al. 2009; Ye et al. 2012), one pulse of 5HT paired with recombinant ApCRNF protein led to a significant increase in 1-h MAPK activation in MNs (Fig. 2B3,B4). These data are consistent with previous findings that one pulse of 5HT and ApCRNF (but not ApCRNF alone) induce LTF (Pu et al. 2014), suggesting that ApCRNF released in response to synaptic activity interacts with signaling downstream from 5HT receptors to induce plasticity. Thus, the combination of blocking and gain-of-function effects reported here supports the view that ApCRNF signaling during and immediately after AD training plays a significant role in MAPK activation exclusively in MNs.

MAPK is a major signaling pathway through which GFs regulate gene expression, and its activation is required for CREB-dependent transcription in *Aplysia* and other systems (Finkbeiner et al. 1997; Roberson et al. 1999; Chin et al. 2006; Rajasethupathy et al. 2009). One of the primary downstream targets of CREB is C/EBP, an immediate-early gene and transcription factor, which is induced following LTM-producing training across species (Guan et al. 2002; Hatakeyama et al. 2006; Levitan et al. 2008; Arguello et al. 2013) and is required for LTM (Taubenfeld et al. 2001) and LTF expression (Alberini et al. 1994; Lee et al. 2001). Consequently, we hypothesized that ApCRNF signaling is required for AD training-induced transcription of C/EBP. To examine the presynaptic sensory compartment, we conducted qPCR on SN clusters collected from Pleural-Pedal ganglia that received either AD or control training ex vivo. The postsynaptic motor compartment was comprised of cocultured MNs that received AD or control training in vitro and were analyzed using single-cell qPCR. Importantly, the relative expression levels of *C/EBP* mRNAs to those of the housekeeping gene GAPDH were similar in both compartments, indicating that results from the two preparations are comparable. In SNs, we found that *C/EBP* mRNA levels were significantly increased at 1 and 3 h after AD training, when compared with controls. However, there was no significant effect of anti-ApCRNF treatment on the AD training-induced increase in *C/EBP* mRNA expression in SNs at either time point (Fig. 3A1). In MNs, *C/EBP* mRNA levels were also significantly increased at 1 and 3 h after AD training. Interestingly, application of anti-ApCRNF blocked the increase in *C/EBP* expression in MNs at 3 h (but not at 1 h) posttraining (Fig. 3B1). These results suggest that AD training induces early and late increased expression of *C/EBP* in both SNs and MNs, but that only the late expression in MNs is dependent on ApCRNF signaling.

Synaptic activity induces the release and transcription of GFs (Poo 2001), and, interestingly, GF transcription has been reported downstream from both GF and C/EBP signaling (Bambah-Mukku et al. 2014). Thus, in a final set of experiments, we examined pre- and postsynaptic ApCRNF gene expression and its possible dependence on ApCRNF signaling after AD training. In SNs, *ApCRNF* mRNA levels were not significantly regulated by AD training in the presence of IgG or anti-ApCRNF at either 1 or 3 h after training (Fig. 3A2). In MNs however, the pattern of ApCRNF gene expression was different. AD training in the presence of IgG or anti-ApCRNF did not significantly regulate *ApCRNF* expression at 1 h

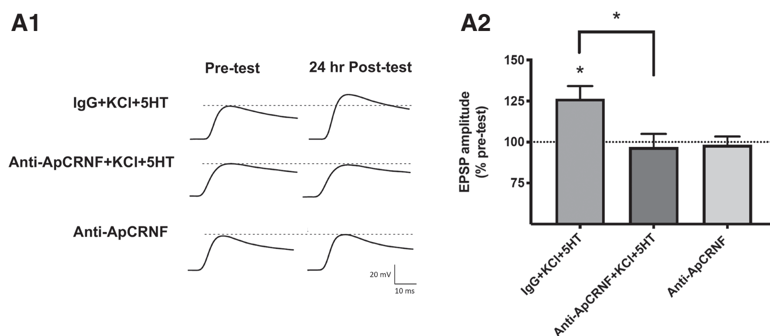

Figure 1. ApCRNF signaling is required for AD-LTF. Pleural SNs and abdominal L7 MNs were cocultured according to an established protocol (Zhao et al. 2009) and kept in culture for 5 d, prior to the start of experiments. LTF was induced by a molecular analog of AD training: a single 5 min pulse of 5HT (10 µM) combined with high-KCl (100 mM) artificial sea water (ASW), which depolarizes neurons (Shobe et al. 2009). A custom-made blocking ApCRNF polyclonal antibody (AnaSpec, raised against the epitope CSHRNANCQNDCFDIEFGKVKPR, 5 µg/mL) was used to block ApCRNF signaling 30 min before, during, and for 1 h after training. Intracellular recordings were performed in SN-MN cocultures as described in Liu et al. (2014). The amplitude of excitatory postsynaptic potentials (EPSPs) was recorded before (pretest) and 24 h after AD training (posttest). LTF is reflected by a significant increase in EPSP amplitude at posttest, and is presented as a percentage of pretest values. Representative traces (*A1*) and summary data (*A2*) show that AD training induced LTF in the presence of IgG (126.3 + 7.9%, $n = 7$, $t_6 = 2.926$), but not in the presence of anti-ApCRNF (96.8 + 8.1%, $n = 6$, $t_5 = 0.518$, NS). Anti-ApCRNF treatment did not affect basal synaptic transmission (98.2 + 5.1%, $n = 6$, $t_5 = 0.277$, NS). Mean + SEM, two-tailed, paired *t*-tests for within-group comparisons, one-way ANOVA ($F_{(2,16)} = 5.454$, $P < 0.05$) followed by Tukey's multiple comparisons tests for between-group comparisons (IgG + KCl + 5HT vs. Anti-ApCRNF + KCl + 5HT: $P < 0.05$, IgG + KCl + 5HT vs. Anti-ApCRNF: $P < 0.05$), (*) $P < 0.05$.

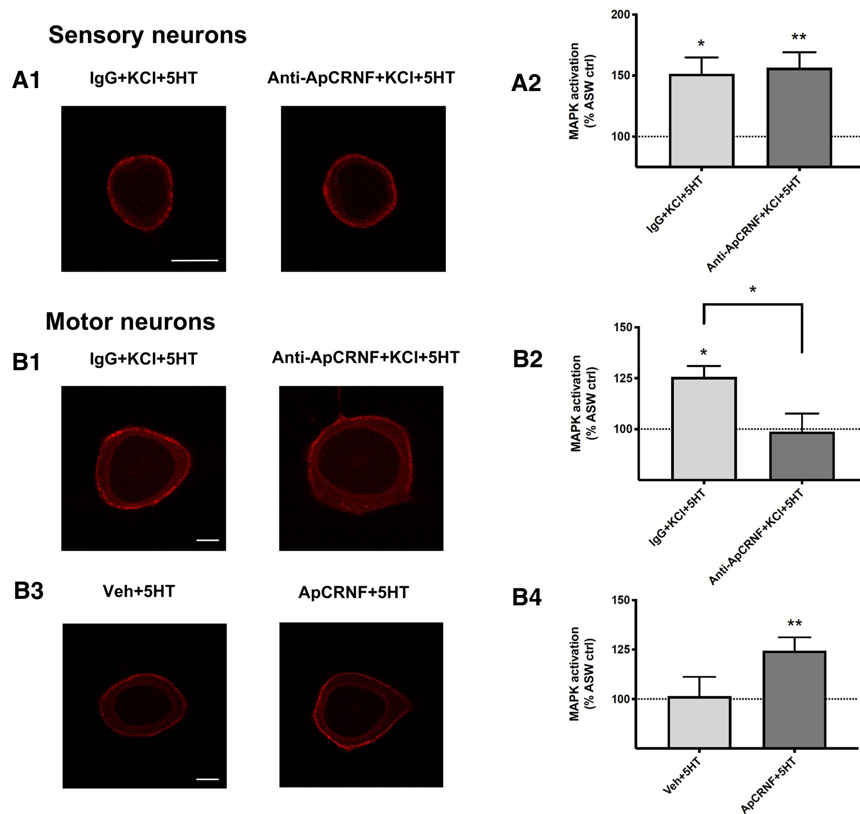

Figure 2. ApCRNF signaling during AD training is required for MAPK activation exclusively in MNs. Immunofluorescence analysis (as described in Liu et al. 2014) was performed on SN-MN cocultures at 1 h after AD training, or after treatment with a single 5 min pulse of 5HT (10 µM) and recombinant ApCRNF protein (500 ng/mL), added 30 min before, during, and for 1 h after the 5HT pulse. MAPK activation was assessed as mean fluorescence intensity of phosphorylated cytoplasmic MAPK in the cell body (primary antibody: P-MAPK, 1:200 dilution, Cell Signaling Technology; secondary antibody: Cy5, Abcam, 1:500 dilution). The trained samples were compared to and represented as a percentage of ASW-treated controls. Representative images (*A1*) and summary data (*A2*) show that, in SNs, AD training induced a significant increase in MAPK activation in the presence of IgG (151.4 ± 13.5%, $n = 6$, $t_{13} = 2.817$) and anti-ApCRNF (156.3 ± 12.8%, $n = 6$, $t_{13} = 3.143$). (*B1*,*B2*) In MNs, AD training induced a significant increase in MAPK activation in the presence of IgG (125.7 ± 5.4%, $n = 11$, $t_{21} = 2.206$), but not in the presence of anti-ApCRNF (98.8 ± 8.9%, $n = 12$, $t_{22} = 0.092$, NS; between-group comparison $t_{21} = 2.533$). (*B3*,*B4*) In MNs, one pulse of 5HT and vehicle (0.1% BSA in ASW) did not significantly regulate MAPK activation (101.5 ± 9.7%, $n = 17$, $t_{25} = 0.132$, NS), but one pulse of 5HT and recombinant ApCRNF induced a significant increase in MAPK activation (124.4 ± 6.7%, $n = 18$, $t_{29} = 2.810$). Mean + SEM, two-tailed, unpaired *t*-tests for within- and between-group comparisons, (*) $P < 0.05$, (**) $P < 0.01$. Scale bar 25 µm.

posttraining. But, at 3 h posttraining, *ApCRNF* levels were significantly increased, and anti-ApCRNF treatment blocked this increase (Fig. 3B2). Taken together, these findings suggest that AD training increases late *ApCRNF* expression exclusively in MNs, and that this postsynaptic induction requires ApCRNF signaling during and immediately after training.

In the present study we investigated the effects of AD training in the *Aplysia* SN-MN microcircuit, and found novel pre- and postsynaptic spatiotemporal dynamics of evolutionarily conserved molecular mechanism such as GF signaling, protein kinase activation, and CREB-dependent transcription. In addition, our data show that signaling of the *Aplysia* GF ApCRNF is required during AD training for the induction of LTF and for postsynaptic molecular events. While presynaptic MAPK activation and *C/EBP* induction have been reported previously in *Aplysia* in response to a variety of training paradigms (Alberini et al. 1994; Michael et al. 1998; Lyons et al. 2006; Philips et al. 2013), we found that these molecular events occur at similar timepoints in postsynaptic MNs, a novel finding adding to the existing literature on postsynaptic effects of LTF induction (Li et al. 2005; Hu et al. 2015; Jin et al. 2018). Interestingly, only the postsynaptic (not presynaptic) events we described are regulated by ApCRNF signaling, suggesting that comparable molecular events can have different requirements and possibly different roles in distinct cells. Moreover, these findings suggest that a GF expressed both pre- and postsynaptically, can exert cell-specific effects during the initiation of long-term plasticity. It would be interesting to examine whether and how these cellular specific effects of GF signaling are affected by different learning-inducing patterns (e.g., activity-dependent vs. activity-independent training, LTF vs. long-term depression inducing training).

An important question raised by our data is how the different molecular events induced by AD training interact to support LTF. Our experiments, in which we disrupted ApCRNF signaling with extracellular application of a blocking antibody during and for 1 h following AD training, suggest that ApCRNF is released and binds to receptors to induce postsynaptic intracellular signaling during the induction and/or the first hour of consolidation of LTF. This is in agreement with GFs being both rapidly released in response to AD stimulation (Kuczewski et al. 2009), and required for memory during or shortly after behavioral training (Park and Poo 2013). Furthermore, GF signaling has been shown to activate the MAPK pathway in presynaptic neurons following LTF-inducing training in *Aplysia* (Hu et al. 2004; Chin et al. 2006; Kopec et al. 2015). Our results add to this literature by showing that GF signaling also regulates postsynaptic MAPK activation. While the endogenous ApCRNF receptor and its signaling mechanisms are not yet known, one way in which GF signaling can activate MAPK is through receptor tyrosine kinase phosphorylation of Ras GTPases (Chao 2003). In addition, GF signaling and MAPK activation have been reported upstream of C/EBP expression (Finkbeiner et al. 1997; Lyons et al. 2006; Kopec et al. 2015). Interestingly, our data show that ApCRNF signaling is required for 1-h MAPK activation and for 3-h (but not 1-h) *C/EBP* expression in MNs, suggesting a possible mechanistic link between these two molecular events. Although our data do not provide evidence for a causal interaction between early MAPK activation and late *C/EBP* expression, other studies in *Aplysia* have reported that MAPK can positively regulate CREB-dependent transcription through multiple pathways in response to learning-inducing stimuli (Michael et al. 1998; Yamamoto et al. 1999; Lyons et al. 2006; Philips et al. 2013). Alternatively, activation of kinases other than MAPK could be induced downstream from ApCRNF and/or 5HT signaling and could contribute to late *C/EBP* expression. Intriguingly, in this and our previous study (Pu et al. 2014), we found that ApCRNF requires additional signaling downstream from 5HT receptors to induce its

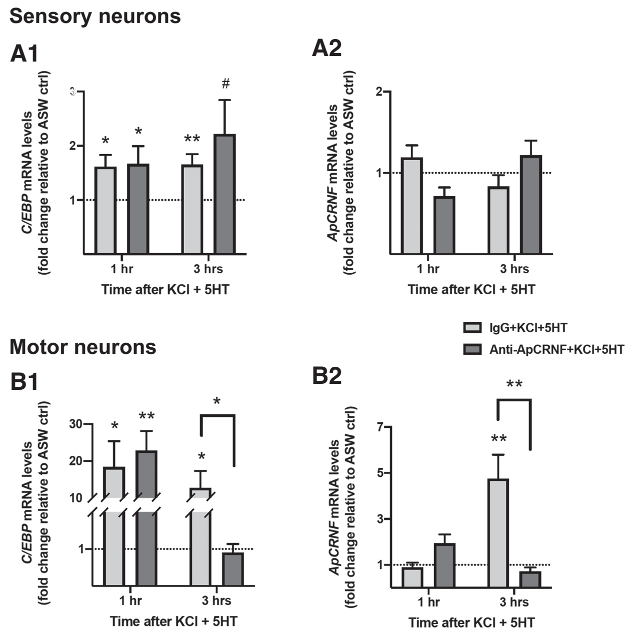

Figure 3. ApCRNF signaling during AD training is required for C/EBP and ApCRNF gene expression exclusively in MNs. Total RNA was isolated from single Pleural SN clusters and cocultured L7 MNs and quantified by qRT-PCR using the RNAqueous-Micro Total RNA Isolation Kit (Invitrogen), the SuperScript IV Reverse Transcriptase Kit (Invitrogen), and LightCycler 480 Sybr Green I Master (Roche), according to the manufacturers' instructions. The following primers were used: ApGAPDH (F-5′-ctctgagggtgctttgaagg-3′; R-5′-gttgtcgttgagggcaattc-3′), ApC/EBP (F-5′-tacgtggataagaggg ccaga-3′; R-5′-gacttcacacgaccctctgtt-3′), ApCRNF (F-5′-cgacgcgtgtgtg tcatact-3′; R-5′-agcagtcgttctggcagttt-3′). The amount of each gene was normalized to that of ApGAPDH within the same sample using the ΔΔCt method. Data are displayed as fold induction relative to ASW-treated controls (for SN clusters within-animal controls were used). (*A1*) In SNs, AD training caused a significant increase in *C/EBP* expression in the presence of IgG or anti-ApCRNF at 1 h (1.62 + 0.22, $n = 8$, $t_7 = 2.839$; 1.66 + 0.32, $n = 7$, $t_6 = 2.460$), and 3 h posttraining (1.65 + 0.19, $n = 8$, $t_7 = 3.781$; 2.21 + 0.62, $n = 7$, $t_6 = 1.933$). (*A2*) In SNs, AD training did not regulate *ApCRNF* levels in the presence of IgG or anti-ApCRNF at either 1 h (1.19 + 0.15, $n = 8$, $t_8 = 0.957$, NS; 0.71 + 0.10, $n = 8$, $t_5 = 2.006$, NS) or 3 h posttraining (0.83 + 0.14, $n = 8$, $t_6 = 0.846$, NS; 1.22 + 0.17, $n = 8$, $t_7 = 1.468$, NS). (*B1*) In MNs, AD training significantly increased *C/EBP* expression in the presence of IgG (18.41 + 6.94, $n = 7$, $t_6 = 2.508$) or anti-ApCRNF (22.82 + 5.28, $n = 7$, $t_6 = 4.134$) at 1 h posttraining. At 3 h posttraining, AD training significantly increased *C/EBP* expression in the presence of Ig G (12.73 + 4.56, $n = 10$, $t_9 = 2.569$), but not in the presence of anti-ApCRNF (0.90 + 0.23, $n = 5$, $t_{10} = 0.299$; between-group comparison $t_9 = 2.590$). (*B2*) In MNs, AD training did not regulate *ApCRNF* levels in the presence of IgG (0.89 + 0.20, $n = 6$, $t_8 = 0.302$, NS) or anti-ApCRNF (1.94 + 0.38, $n = 5$, $t_8 = 1.403$, NS) at 1 h posttraining. At 3 h posttraining, AD training significantly increased *ApCRNF* expression in the presence of IgG (4.75 + 1.04, $n = 9$, $t_8 = 3.563$), but not in the presence of anti-ApCRNF (0.72 + 0.17, $n = 5$, $t_{10} = 1.332$, NS; between-group comparison $t_8 = 3.816$). Mean + SEM, paired and unpaired *t*-tests for within-group comparisons in SNs and MNs, respectively, unpaired *t*-tests for between-g roup comparisons, (#) $P = 0.05$, one-tailed, (*) $P < 0.05$, (**) $P < 0.01$, two-tailed.

effects. Two attractive candidates for such parallel pathways are protein kinases A and C, which are known to be activated following long-lasting memory-inducing training (Sutton and Carew 2000; Shobe et al. 2009; Michel et al. 2011b) and to be required for C/EBP activity (Kaang et al. 1993; Yamamoto et al. 1999). Additionally, our data do not address whether the observed 3-h *C/EBP* expression represents a wave of *de novo* gene expression, or stabilization of previously (1-h) transcribed *C/EBP* mRNA, a posttranscriptional regulation required for LTF (Yim et al. 2006). Lastly, our data show that ApCRNF signaling is also required for *ApCRNF* induction in MNs. Autoregulation of GF transcription has been previously described and proposed to produce positive feedback loops that support long-lasting plasticity and memory (Zhang et al. 2016; Jin et al. 2018). In addition, in one of these proposed feedback loops, early GF signaling is required for C/EBP expression, which in turn is necessary for late GF gene expression (Bambah-Mukku et al. 2014). This raises the intriguing possibility that the 1-h *C/EBP* induction phase we observed in MNs is required for the 3-h *ApCRNF* induction in the same neurons. Given our present results, it would be interesting for future research to investigate the causal links between postsynaptic MAPK activation, C/EBP and ApCRNF gene expression, and their functional requirement in the induction of LTF.

Our findings suggest that, during and immediately after AD training, ApCRNF specifically activates receptors on MNs. However, it is possible that there are SNs effects that were not captured by the time points and signaling cascades we investigated here. Moreover, our current results do not reveal whether ApCRNF is released from pre- and/or postsynaptic sites, or whether it acts in an autocrine or paracrine manner. Future studies can readily address these important questions by studying signaling in isolated cultured neurons. Finally, ApCRNF is one of several *Aplysia* GFs critically involved in memory-related plasticity (Zhang et al. 1997; Kassabov et al. 2013; Kukushkin et al. 2019), raising interesting questions regarding how different GFs coordinate their unique and shared signaling across time and space to contribute to the complex molecular interactions between neighboring neurons at critical synapses that support long-lasting plasticity and memory.

Competing interest statement

The authors declare no competing interests.

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