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A Single *Aplysia* Neurotrophin Mediates Synaptic Facilitation via Differentially Processed Isoforms

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SUMMARY

Neurotrophins control the development and adult plasticity of the vertebrate nervous system. Failure to identify invertebrate neurotrophin orthologs, however, has precluded studies in invertebrate models, limiting our understanding of fundamental aspects of neurotrophin biology and function. We identified a neurotrophin (ApNT) and Trk receptor (ApTrk) in the mollusk *Aplysia* and found that they play a central role in learning-related synaptic plasticity. Blocking ApTrk signaling impairs long-term facilitation, whereas augmenting ApNT expression enhances it and induces the growth of new synaptic varicosities at the monosynaptic connection between sensory and motor neurons of the gill-withdrawal reflex. Unlike vertebrate neurotrophins, ApNT has multiple coding exons and exerts distinct synaptic effects through differentially processed and secreted splice isoforms. Our findings demonstrate the existence of bona fide neurotrophin signaling in invertebrates and reveal a posttranscriptional mechanism that regulates neurotrophin processing and the release of proneurotrophins and mature neurotrophins that differentially modulate synaptic plasticity.

INTRODUCTION

Vertebrate neurotrophins comprise a family of four closely related growth factors (NGF, BDNF, NT3, and NT4) that play central roles in the development and adult plasticity of the nervous system (Chao, 2003; Huang and Reichardt, 2003). Neurotrophins are synthesized as larger proneurotrophin precursors, which are processed to mature forms in the cell by furin-type endoproteases. Mature neurotrophins selectively bind three tropomyosin-related kinase receptors (TrkA, TrkB, and TrkC) as well as a common death-domain-containing receptor (p75NTR) that can either enhance their growth and survival functions or elicit apoptotic effects depending on the cellular context (Dechant and Barde, 1997; Hempstead et al., 1991). In addition to mature neurotrophins, unprocessed proneurotrophins were also recently found to be secreted in the CNS and to bind the p75NTR receptor with high affinity, eliciting apoptosis or synapse elimination (Lee et al., 2001; Teng et al., 2005; Woo et al., 2005). However, the physiological significance and function of proneurotrophin release in the adult CNS, and in particular its function in synaptic plasticity, remain poorly understood (Matsumoto et al., 2008; Yang et al., 2009).

The hope that the complex neurotrophin signaling system can be studied more effectively in simpler and more experimentally accessible invertebrate models, and the significant interest in the evolutionary origin of neurotrophin signaling have fueled the pursuit to identify invertebrate orthologs of the neurotrophins and their receptors (Bothwell, 2006; Chao, 2000; Jaaro et al., 2001).

Several lines of evidence have suggested that some form of neurotrophic signaling might be present in invertebrates. These include the identification of Trk-related receptors in mollusks (Ormond et al., 2004; van Kesteren et al., 1998) and the lancelet *Amphioxus* (AmphiTrk; Benito-Gutiérrez et al., 2005), the recent in silico detection of putative neurotrophin sequences in some invertebrate genomes (Bothwell, 2006; Hallböök et al., 2006; Wilson, 2009), and indirect evidence from physiological experiments with mammalian neurotrophins in *Aplysia* and *Lymnea* (M. Guistetto et al., 1999, Soc. Neurosci., abstract; Purcell et al., 2003; Ridgway et al., 1991). However, both the identity of endogenous factors implicated in physiological experiments and the functions of putative neurotrophins identified in silico remain unknown. Moreover, the absence of identified ligands for and important ligand-binding or signaling features in the Trk-related molluscan and *Amphioxus* receptors, respectively, leaves open the question about their functional conservation and has led to the suggestion that invertebrate neurotrophins might fulfill only some of the more "basic" developmental functions of their vertebrate counterparts, whereas higher-order functions such as control of calcium signaling and synaptic plasticity are likely innovations

of the vertebrate neurotrophin system (Benito-Gutiérrez et al., 2005; Hallböök et al., 2006).

Here we report the discovery and functional characterization of a bona fide invertebrate neurotrophin signaling system consisting of a fully conserved Trk receptor, ApTrk, and its neurotrophin ligand, ApNT, expressed in the CNS of *Aplysia*. This system mediates learning-related serotonin (5-HT)-induced long-term facilitation (LTF) and growth at the sensory-motor neuron synapse of the *Aplysia* gill-withdrawal reflex, a cellular model for long-term memory formation and storage. Moreover, in contrast to vertebrate neurotrophins, ApNT has multiple coding exons and generates functional diversity through alternatively spliced furin-cleavable or -resistant ApNT isoforms that are secreted as mature forms or proforms, respectively, and exert distinct enhancing effects on synaptic plasticity.

Our study clarifies the evolutionary origins of neurotrophin signaling and establishes its role in synaptic plasticity as one of its core ancestral functions. Moreover, our findings reveal that alternative splicing regulates the intracellular processing and ratio of secreted mature neurotrophins and proneurotrophins that differentially modulate synaptic function.

RESULTS

ApTrk Has Fully Conserved Structural and Signaling Features of Vertebrate Trk Receptors

Given previous evidence for the physiological effects of mammalian BDNF in the *Aplysia* culture system, we utilized a functional cloning approach to identify the endogenous receptor that mediates those effects. By screening a T7 phage display complementary DNA (cDNA) library from adult *Aplysia* CNS using human BDNF protein as bait, we isolated a partial sequence with homology to vertebrate Trks, which we then extended by rapid amplification of cDNA ends (RACE)-PCR to obtain the full-length 902 amino acid receptor ApTrk. ApTrk is highly conserved with vertebrate Trk receptors and possesses an identical domain architecture that includes two immunoglobulin G (IgG) domains in its ligand-binding extracellular region and the two main signaling sites for activating Erk and PLCγ in its signal-transducing cytoplasmic region (Figure 1A). Phylogenetic analysis using the tyrosine kinase (TK) domain confirms that ApTrk unequivocally belongs to the family of Trk receptors (Figures 1B and S1). Thus, so far, ApTrk is the only Trk receptor with all the structural and signaling features of vertebrate Trks that has been characterized in invertebrates (see Extended Discussion).

ApTrk Is Expressed in Sensory and Motor Neurons in the *Aplysia* CNS

RT-PCR analysis of *Aplysia* tissues revealed the strongest expression of ApTrk mRNA in the CNS and weaker expression in the heart and body wall muscle (Figure S2C). In the CNS, ApTrk is expressed in both the sensory and motor neurons of the gill-withdrawal reflex synapse, the locus for studies of learning-related memory processes in *Aplysia* (Figure 1C).

An antibody against a peptide from the extracellular region of ApTrk (ApTrk10) detected a major band of ~160 kDa in *Aplysia* CNS extracts, corresponding well with the expected size of ApTrk. (Figures 1D and S2A). A survey of ApTrk protein expression in various *Aplysia* tissues revealed a pattern similar to that of ApTrk mRNA expression (Figure S2D).

Immunocytochemistry of cultured *Aplysia* motor neurons with the ApTrk10 antibody detected strong expression of ApTrk in the cell soma, particularly along the initial segment (the receptive area for most of the functional synaptic contacts with sensory neurons), as well as along the distal processes (Figures 1E and S2B). We detected plasma membrane localization of the ApTrk as well as significant intracellular localization in punctuate structures, an expression pattern that is also observed with vertebrate Trk receptors (Meyer-Franke et al., 1998; Rajagopal et al., 2004).

ApNT Is a Canonical Invertebrate Neurotrophin Expressed in *Aplysia* Sensory Neurons

We next searched for the endogenous neurotrophin ligand of ApTrk by mining the genomic trace archive data publicly available through the *Aplysia* Genome Project and identified a 76 amino acid open reading frame (ORF) that had similarity to mammalian neurotrophins. Extending the sequence by 5′ and 3′ RACE-PCR yielded a 294 amino acid pre-proneurotrophin precursor with a putative signal peptide that we named ApNT (for *Aplysia* neurotrophin; Figure 2A).

ApNT is well conserved with vertebrate neurotrophins and is predicted to form a cysteine knot structure very similar to that of NGF (Figures 2B, 2C, and S3; McDonald and Blundell, 1991). ApNT unequivocally belongs to the neurotrophin family and is more closely related to vertebrate neurotrophins than it is to other putative protostome neurotrophin sequences identified so far in silico (Wilson, 2009) or the even more distant group of Spz growth factors characterized in flies (Zhu et al., 2008). ApNT is therefore the only bona fide neurotrophin thus far characterized in invertebrates (see Extended Discussion).

A survey of *Aplysia* tissues for ApNT mRNA expression revealed a high level of expression in the CNS, with a weaker signal detected in the ovotestis (Figure S4B). ApNT expression was restricted to the sensory neurons, which are presynaptic at the sensory-motor neuron synapse, and was undetectable in the postsynaptic motor neurons (Figure 2D). This finding is consistent with the axonal localization of BDNF in the mammalian CNS (Dieni et al., 2012) and with our earlier functional studies showing a presynaptic source of BDNF in BDNF-dependent forms of hippocampal synaptic plasticity (Zakharenko et al., 2003).

Antibodies raised against peptides derived from the mature region (M50) and pro region (P47) of ApNT detected a major 48 kDa band in *Aplysia* CNS extracts, indicating that this represents the unprocessed proApNT form (Figures 2E and S4B). The M50 and P47 antibodies also detected second, smaller bands at 22 and 25 kDa, respectively, consistent with the processed mature peptides and propeptides of ApNT. Neither of the antibodies against ApNT was capable of detecting the endogenous protein; therefore, the subcellular localization of the protein was visualized via imaging of GFP-fusion proteins (see Figure 4E).

ApNT is efficiently secreted from transfected HEK293 cells (Figure S5; see also Figure 4). Consistent with the presence of two N-glycosylation sites in it, the secreted mature ApNT was found to be glycosylated, which explains its higher than

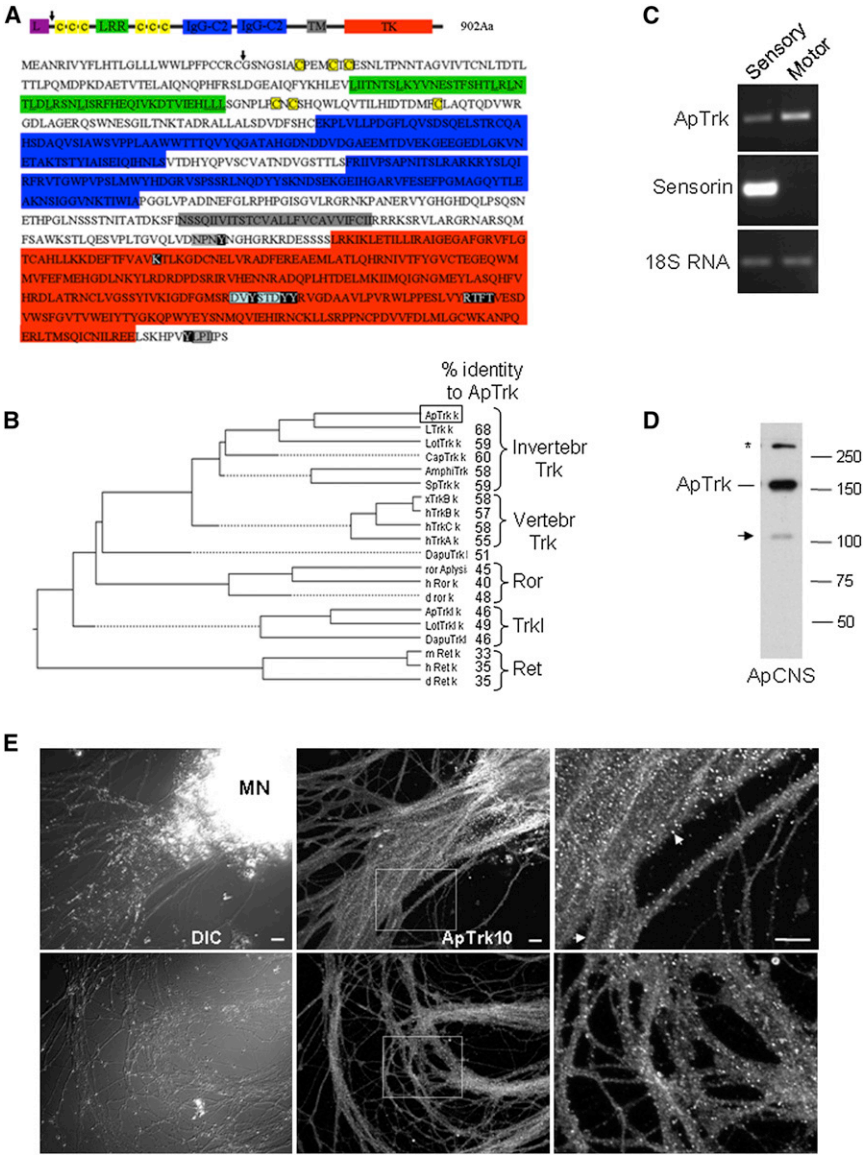

Figure 1. Cloning and Characterization of ApTrk

(A) Conserved domain architecture and sequence features of ApTrk. Top: Schematic representation of ApTrk structural domains. Bottom: Amino acid sequence of ApTrk. Arrow shows the position of cleavage of the signal peptide. Notable intracellular catalytic and tyrosine signaling residues are shown in black background with their surrounding consensus sequence highlighted in gray. Note the presence of the Shc site (NPNY) in the juxtamembrane region and the Sh2 site (YLPI) at the C terminus responsible in vertebrates for activating the Erk and PLCγ signaling pathways, respectively. L, leader peptide; C, cysteine; LRR, leucine rich repeat; IgG-C2, IgG-like; TM, transmembrane; TK, tyrosine kinase.

(B) ApTrk belongs to the Trk family of receptors. ClustalW analysis was done with the conserved TK domains of representative members of the Trk, Trkl, ROR, and RET families of RTKs. Percent identity to ApTrk is noted next to each sequence. See also Figure S1.

(C) RT-PCR analysis of ApTrk with cDNA from cultured *Aplysia* sensory or L7 motor neurons. Sensorin is a sensory neuron marker and 18S RNA is a loading control.

(D) Western blot analysis with ApTrk10 antibody against the ApTrk ectodomain on *Aplysia* CNS extract. A 300 kDa band representing an SDS-resistant receptor dimer is marked with an asterisk. A minor band at 110 kDa that is not consistently detected and might correspond to a truncated form of the receptor lacking a TK domain is marked with an arrow. See also Figure S2.

(E) Subcellular localization of ApTrk in cultured motor neurons. Immunostaining was done with the ApTrk10 antibody. Top: Cell body and initial segment; arrows point to ApTrk localization at the plasma membrane and concentration in puncta. Bottom: Distal processes of the same cell, showing a patchy/punctuate ApTrk appearance. Scale bar, 10 μm.

predicted observed molecular weight (Figure 2F). Similarly to vertebrate neurotrophins, ApNT exists as a homodimer in solution (Figure 2G).

ApNT Interacts with ApTrk, Activating Erk, Akt, and PLCγ Signaling and Inducing Neurite Outgrowth in PC12nnr5 Cells

To determine whether ApTrk and ApNT interact with each other and can activate downstream signaling pathways utilized by mammalian neurotrophins, we used PC12nnr5 cells, which do not express endogenous Trk receptors (Loeb and Greene, 1993). Stimulation of ApTrk-transfected PC12 nnr5 cells with purified ApNT led to phosphorylation of both Erk and Akt (Figure 3A). This effect was specific because cells that were transfected with GFP or mammalian Trks did not respond to the ApNT treatment (see below). Significantly, ApNT stimulation of ApTrk-transfected cells also activated the PLCγ pathway, consistent with the conservation in ApTrk of the YxxI site responsible for activating this pathway in mammals (Figure 3A). Thus, ApNT interacts with ApTrk and this interaction activates all three major neurotrophin signaling pathways (Erk, Akt, and PLCγ) in PC12 cells.

Moreover, we found that the ApTrk-transfected PC12nnr5 cells responded to ApNT with a robust outgrowth similar to that of TrkA-transfected, NGF-treated cells (Figures 3C and 3D). These results establish ApNT-ApTrk as a bona fide invertebrate neurotrophin signaling system.

ApTrk Cross-Interacts Specifically with BDNF

Prior functional results in *Aplysia* as well as the success of our functional cloning strategy suggested cross-reactivity between ApTrk and mammalian BDNF (M. Guistetto et al., 1999, Soc.

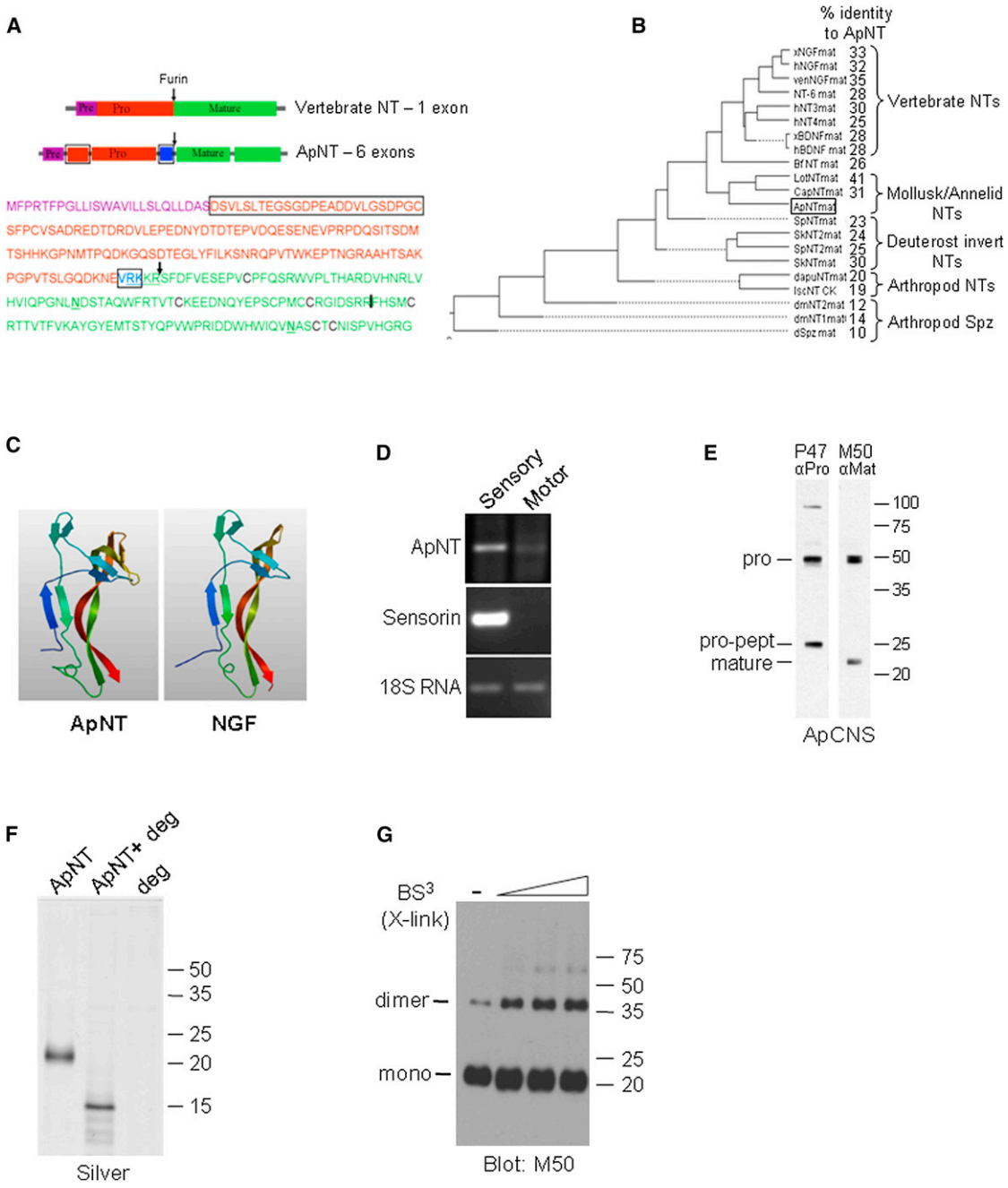

Figure 2. Cloning and Characterization of ApNT

(A) Multiple exon structure and sequence of ApNT. Top: Schematic representation of a single exon vertebrate neurotrophin and the multiexon structure and features of ApNT. Exon 1 (purple) encodes the cleavable signal peptide. Exons 2 and 3 (red) encode the pro region. Exon 4 (blue) is a short miniexon encoding the first half site of the consensus furin enzyme processing site (RKKR). Exons 5 and 6 (green) encode the conserved mature domain of ApNT. Bottom: ApNT amino acid sequence color-coded as above. Arrow points to the cleavage position of the furin processing site (underlined). Vertical bar shows the exon boundary. Boxed exons 2 and 4 are alternatively spliced. Cysteine residues forming the cysteine knot structure are in black. Asparagines predicted to be N-glycosylated in the mature domain are in bold and underlined.

(B) ClustalW phylogenetic analysis of the mature portions of ApNT with vertebrate and putative invertebrate neurotrophins. Percent identity to ApNT is noted next to each sequence. See also Figure S3.

(C) Three-dimensional structural models of ApNT and mouse NGF generated by the Swiss-Model comparative protein modeling server (http://swissmodel.expasy.org/workspace/).

(D) RT-PCR analysis of ApNT expression in sensory and L7 motor neurons as in Figure 1C.

(E) Western blot analysis of ApNT in *Aplysia* CNS extract (soluble fraction) using pro-region-specific (P47) and mature-region-specific (M50) antibodies. Positions of intact precursor and processed propeptides and mature peptides are shown with arrows. See also Figure S4.

(legend continued on next page)

Neurosci., abstract; Purcell et al., 2003; Sharma et al., 2006). Indeed, we found that BDNF was able to cross-react with and activate ApTrk, particularly at higher concentrations (Figure 3B). Interestingly, NGF (as well as NT3 and NT4) failed to activate ApTrk even at high concentrations (Figure 3B and data not shown). These results demonstrate that the functional effects observed in our earlier *Aplysia* studies were indeed due to the activation of ApTrk by BDNF.

ApNT did not activate TrkB or the other two vertebrate Trk receptors even at high concentrations (Figure 3B and data not shown). However, we cannot exclude the possibility that nonproductive binding in the absence of receptor activation could still exist between NT3, NT4, or NGF and ApTrk, or between ApNT and mammalian Trks as was previously shown for LTrk and NT3 (van Kesteren et al., 1998).

Multiexon Gene Structure and Alternative Splicing of ApNT

Sequencing of multiple independent RACE-PCR- and RT-PCR-derived clones revealed that unlike vertebrate neurotrophins, which have a single coding exon, ApNT is encoded by six separate exons, at least two of which (exons 2 and 4) are subject to alternative splicing (Figure 2A). Strikingly, exon 4 is a 9 nt miniexon encoding the amino acids (VRK) that comprise the first half of the (RKKR) recognition site for furin-like endoproteases, the major enzymes responsible for intracellular processing of neurotrophins. Its alternative splicing would therefore be expected to create a furin-cleavable isoform we denominated as ApNT(+) (+ furin site) and a furin-resistant isoform denominated as ApNT(−) (− furin site; Figure 4A). A third alternatively spliced ApNT transcript of lower abundance, lacking exons 2 and 4 and encoding an isoform with a 27 amino acid shorter proregion, was also detected and denominated ApNTS(−) (for Short).

RT-PCR analysis of *Aplysia* CNS cDNA with primers closely flanking the furin site miniexon revealed that the (+) and (−) furin site transcripts are present at roughly similar levels in the *Aplysia* CNS (Figure 4B).

ApNT Isoforms Are Differentially Processed and Secreted as Mature Forms and Proforms in HEK293 Cells

To study the processing and secretion of the three ApNT isoforms, we expressed them as C-terminal GFP fusion proteins in HEK293 cells. Consistent with the predicted differential processing, cells expressing the furin-cleavable ApNT(+)GFP isoform efficiently secreted only the mature ApNT-GFP fusion without any detectable intact precursor, whereas cells expressing the furin-resistant short prodomain ApNTS(−) isoform secreted only the intact unprocessed precursor without detectable processed mature ApNT (Figure 4C). Media from cells expressing the other furin-resistant long ApNT(−)GFP isoform also contained no detectable mature ApNT, but had only trace amounts of the ApNT(−)GFP precursor because the long ApNT prodomain is strongly acidic and adheres strongly to the culture dish surface, and therefore was not recoverable in the cell media (Figure 4D; also see Figure 4E and text below). Indeed, a single amino acid substitution at position P1 in the processing site of ApNT(+) (RKKR to RKKG), creating an uncleavable ApNT mutant, rendered a secretion pattern identical to that of ApNT(−), confirming that ApNT processing does takes place at this specific site and that the lack of such processing is responsible for the altered secretion of the ApNT(−) isoform (Figure S5).

These results demonstrate that the splicing of the 9 nt furin site miniexon determines the outcome of intracellular processing of the respective ApNT splice isoforms and their secretion as mature forms or proforms.

ApNT Isoforms Are Differentially Processed, Localized, and Secreted as Mature Forms and Proforms in *Aplysia* Sensory Neurons

To determine whether ApNT splice forms are differentially processed and secreted in *Aplysia* neurons, we expressed the C-terminal ApNT-GFP fusion constructs in sensory neurons and immunostained them with the P47 prodomain-specific antibody to independently visualize the mature domain (GFP signal) and the prodomain (P47 signal). We found that the furin-uncleavable ApNT(−)GFP and ApNTS(−)GFP isoforms showed extensive colocalization between the GFP fluorescence and the P47 prodomain signal in both the soma and processes, as would be expected if these isoform precursors are trafficked unprocessed (Figure 4E, middle and bottom panels). Although we cannot exclude the possibility that some processing occurs after storage in immature vesicles, the striking observation that most of the ApNT(−)GFP/p47 signal formed a halo emanating from the cell body indicates secretion of the unprocessed acidic full-length precursor and its adherence to the positively charged substrate surface, consistent with the results in HEK293 cells.

In stark contrast, cells expressing the furin-cleavable ApNT(+) GFP isoform showed extensive antilocalization between the mature domain and the prodomain in both the soma and processes, as would be expected if the protein is cleaved efficiently intracellularly and the resulting cleaved propeptides and mature peptides are trafficked independently (Figure 4E, top panel). Notably, such cleavage must precede storage of the mature ApNT domain in the puncta/vesicles. Interestingly, the cleaved prodomain immunoreactivity pattern in the ApNT(+)GFP-expressing cells was very similar to the intact precursor staining pattern of ApNT(−)GFP expressing cells, indicating that the cleaved prodomain is trafficked and secreted similarly to the intact precursor protein and, consistent with its comparably high negative charge, also adheres strongly to the substrate.

In addition to splicing of exon 4 dictating differential processing, we found that alternative splicing of exon 2 (encoding the highly acidic N-terminal portion of the pro-domain) dictates a differential expression pattern. Thus, the mature protein and

(F) C-terminally hexahistidine tagged ApNT purified from conditioned medium of ApNT(+) expressing HEK293 cells was mock treated or treated with a mix of deglycosylating enzymes for 3 hr at 37°C and analyzed on SDS-PAGE and silver stained.
(G) ApNT exists as dimer in solution. Purified ApNT (50 μg/ml) was crosslinked with 0.5, 1, or 2 mM bis(sulfosuccinimidyl)suberate (BS^3) for 30 min at room temperature or mock treated, and the reactions were analyzed by SDS-PAGE and western blotting with M50 antibody.

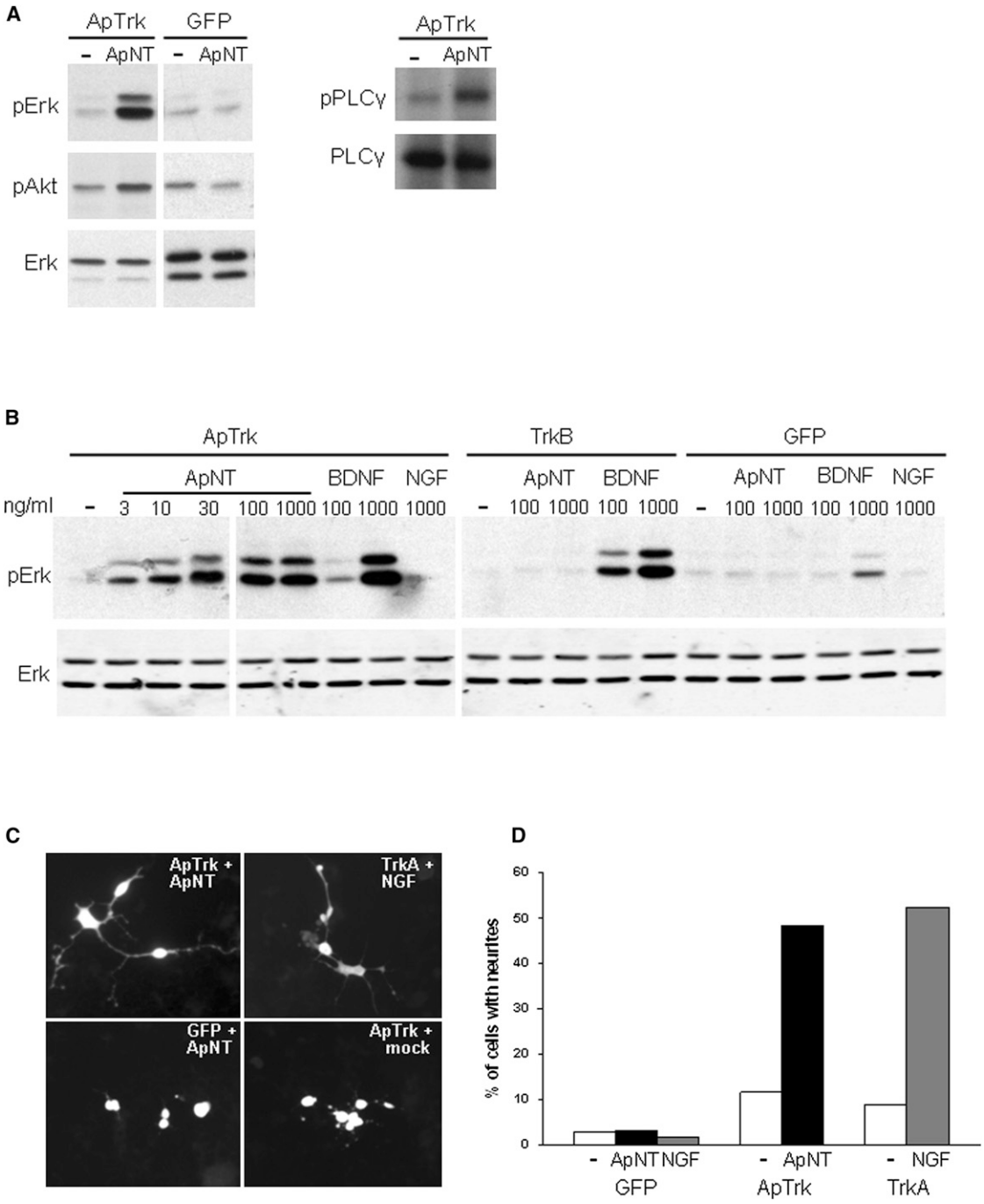

Figure 3. Functional Interaction between ApNT and ApTrk in PC12 Cells

(A) ApNT interacts functionally with ApTrk and activates Erk, Akt, and PLCγ. PC12nnr5 cells were transfected with ApTrkGFP or GFP and treated for 5 min with 100 ng/ml of purified ApNT or with control vector transfected purified media (–) and then lysed and analyzed by western blotting with the indicated antibodies.

(B) BDNF cross-reacts with ApTrk. Cells were transfected with Trk receptors or GFP as indicated and treated for 5 min with the indicated concentrations of purified ApNT or recombinant BDNF or NGF lysed and analyzed as in Figure 3A.

(C) ApNT-ApTrk induced neurite outgrowth in transfected PC12nnr5 cells. Cells were transfected with GFP alone or cotransfected with GFP and the indicated receptor, stimulated with 20 ng/ml of the indicated ligand, and then imaged after 4 days.

(D) Quantification of the percentage of cells exhibiting neurite outgrowth in Figure 3C.

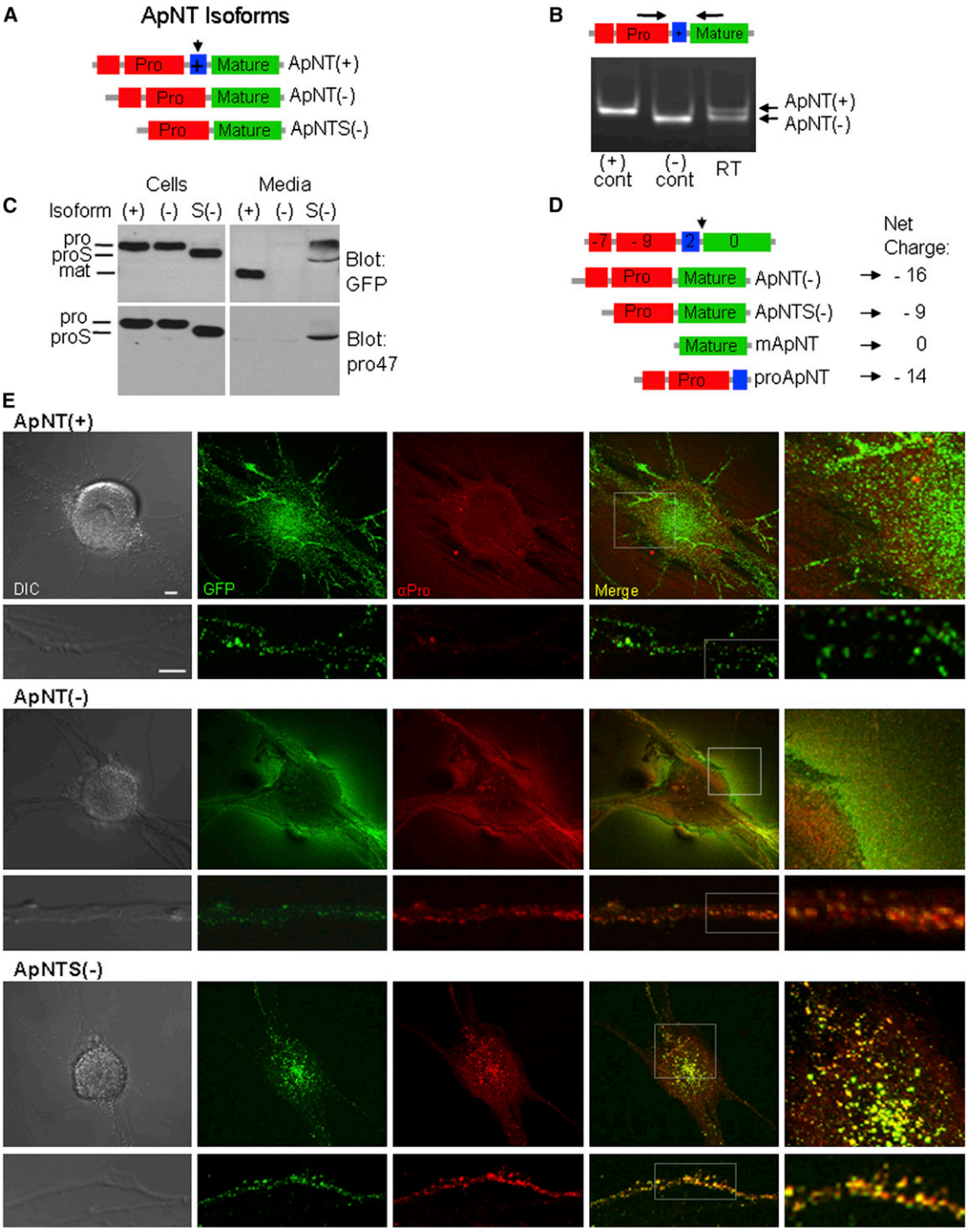

Figure 4. Differential Processing, Trafficking, and Localization of ApNT Splice Isoforms

(A) Schematic representation of ApNT splice isoforms.

(B) Denaturing PAGE analysis of RT-PCR reactions of *Aplysia* CNS cDNA with primers flanking the 9 nt miniexon. Plasmids containing (+) or lacking (−) the miniexon were used for control reactions with the same primer set.

(C) Processing and secretion of ApNT-GFP isoforms from transfected HEK293 cells. Western blot analysis of cell extracts and media collected 48 hr after transfection. See also Figure S5.

(D) Net charge distribution across ApNT functional domains (top) and net charge of the ApNT processing products.

(E) Differential processing expression and secretion pattern of ApNT isoforms in sensory neurons. Confocal imaging of ApNT(+)GFP (top panel), ApNT(−)GFP (middle panel), and ApNTS(−) (bottom panel) expressing sensory neurons. Top row: cell bodies; bottom row: distal neurites. Columns as labeled from left: differential interference contrast (DIC), GFP fluorescence (green), P47 staining (red), merged, and zoomed detail of boxed area from merged. Scale bar, 5 μm.

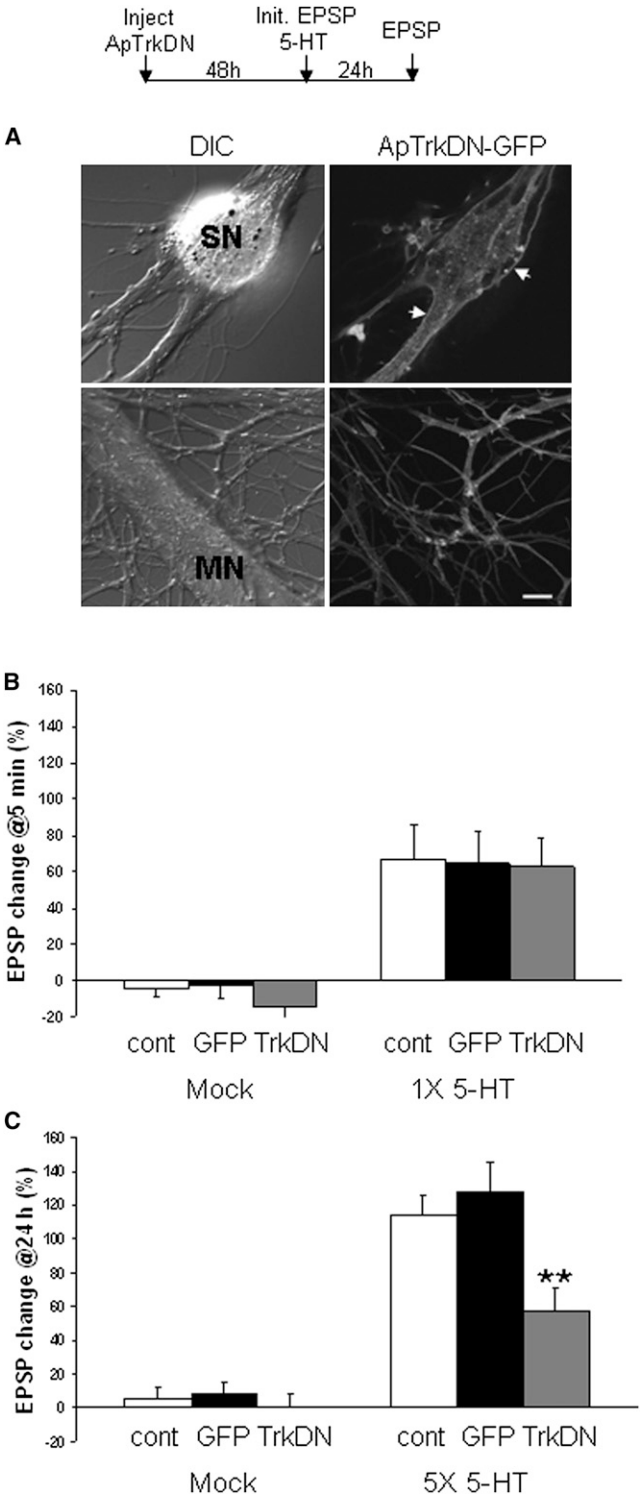

Figure 5. Endogenous ApNT-ApTrk Signaling Mediates LTF

(A) ApTrkDN-GFP fusion protein is properly expressed, trafficked, and targeted in sensory neurons cocultured with motor neurons. Top: Localization in soma and initial axon segment; arrows point to plasma membrane localization. Bottom: Localization in distal SN processes in contact with the motor neuron initial segment (synaptic area). Scale bar, 10 μm.

short prodomain precursor generated by the ApNT(+)GFP and ApNTS(−)GFP isoforms, respectively, have mostly punctate expression, whereas the long prodomain precursor produced by ApNT(−)GFP has a mostly diffuse expression pattern.

Taken together, the above results demonstrate that alternative splicing of the ApNT isoforms determines their differential processing, sorting and expression patterns, and secretion as mature forms and proforms in *Aplysia* sensory neurons.

ApNT-ApTrk Signaling Mediates 5-HT-Induced LTF

We next explored the function of the ApNT-ApTrk system in modulating the strength of the sensory-motor neuron synapse of the *Aplysia* gill-withdrawal reflex reconstituted in culture. In this system, application of 5-HT mimics 5-HT release induced by tail shocks in the intact animal, which produces behavioral sensitization of the reflex. Application of a single 5-HT pulse induces protein-synthesis-independent short-term facilitation (STF) of the synapse, whereas application of five spaced 5-HT pulses induces protein-synthesis-dependent LTF that lasts at least 24 hr (Bailey et al., 2000).

To block ApNT signaling, we expressed in the sensory neuron an ApTrk deletion construct (ApTrkDN) that lacks the intracellular signaling portion of the receptor but can bind and sequester the ApNT ligand. A similar approach has been used in mammalian systems in which truncated, catalytically inactive Trk receptors are naturally occurring and are thought to spatially limit the extent of NT signaling (Carim-Todd et al., 2009; Haapasalo et al., 2001). The overexpressed ApTrkDN-GFP had clear membrane localization in the sensory neuron, indicating that the truncated receptor is properly trafficked and targeted to the cell membrane (Figure 5A).

We first tested whether ApNT-ApTrk signaling is involved in STF. We expressed ApTrk-DN-GFP or GFP alone in sensory neurons for 48 hr and then recorded excitatory postsynaptic potentials (EPSPs) before and 5 min after cultures were mock treated or treated with a single pulse of 5-HT. ApTrkDN-GFP or GFP expression had no effect on synaptic transmission with mock treatment (% change in EPSP of mock-treated group: uninjected: −4.5 ± 4.93, n = 6 versus GFP expressing: −3.14 ± 6.92, n = 7; ApTrkDNGFP-expressing: −14.33 ± 9.76, n = 6, $p > 0.05$ for both; Figure 5B, left set). Similarly, ApTrk-DN-GFP or GFP expression had no effect on the amplitude of the evoked EPSPs measured 5 min after a single 5-HT pulse, demonstrating that ApNT-ApTrk signaling is not involved in STF (% change in EPSP of 5-HT treated group: uninjected: 66.83 ± 18.52, n = 5 versus GFP expressing: 64.5 ± 17.11, n = 6, versus ApTrkDNGFP-expressing: 63.18 ± 15.28, n = 11, $p > 0.05$ for both; Figure 5B, right set).

In contrast, we found that expression in the sensory neuron of ApTrk-DN-GFP, but not GFP alone, led to a significant reduction

(B) Presynaptic ApTrk signaling is not required for STF. Overexpression of ApTrkDN in sensory neurons did not inhibit the EPSP increase measured 5 min after a single pulse of 5-HT (10 μM, 5 min). Data are presented as mean ± SEM. (C) ApTrk signaling is required for LTF induction. Overexpression of the ApTrkDN in sensory neurons significantly reduced the EPSP increase measured 24 hr after induction by five pulses of 5-HT (10 μM, 5 min). Data are presented as mean ± SEM.

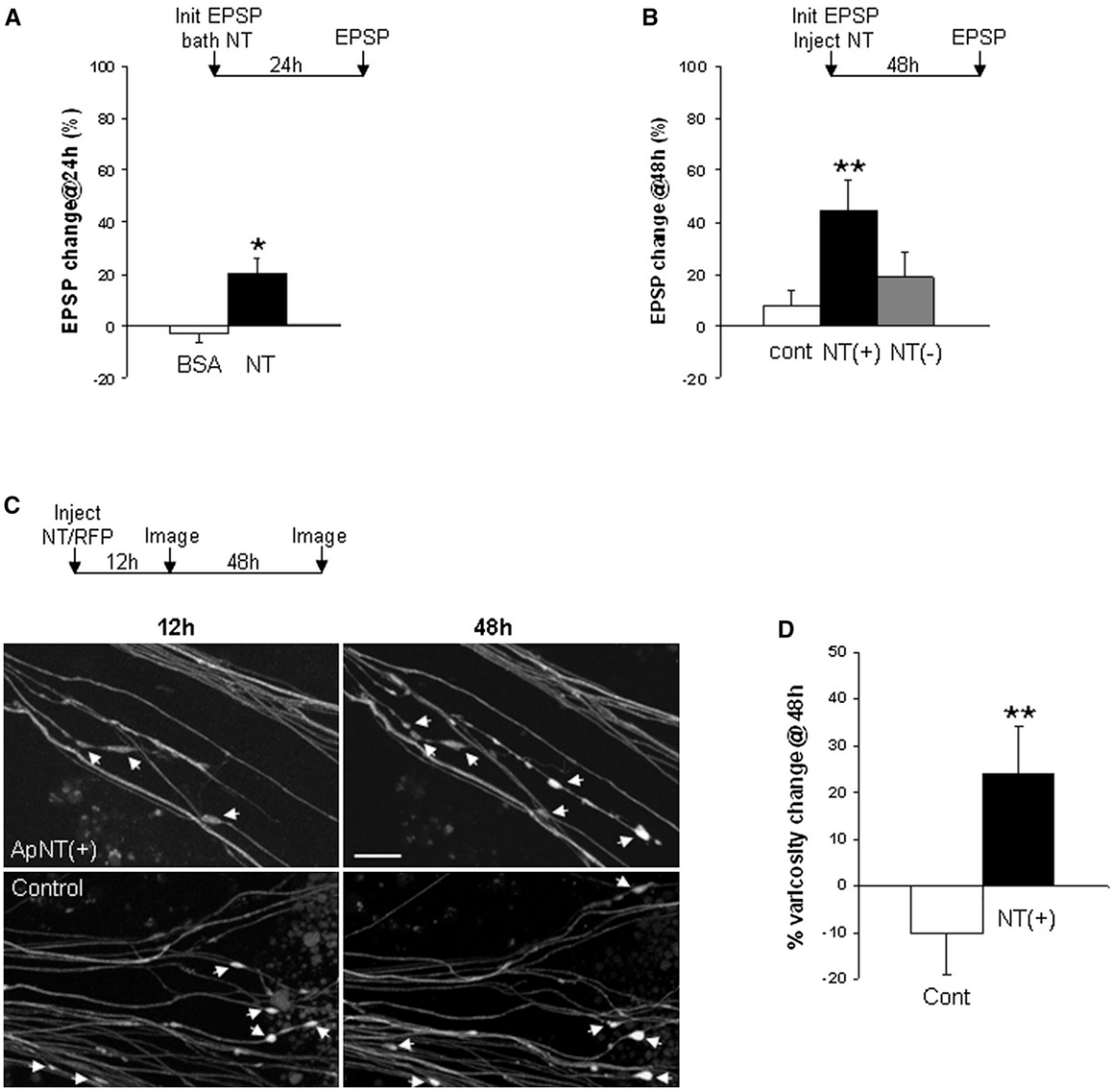

Figure 6. ApNT Signaling Induces Synaptic Enhancement and Growth in Sensory-Motor Neuron Cocultures

(A) Recombinant ApNT induces modest synaptic enhancement. EPSPs in sensory-motor neuron cocultures were recorded before or 24 hr after purified ApNT (50 ng/ml) or control BSA protein was added to the media. Data are presented as mean ± SEM.

(B) ApNT overexpression in sensory neurons induces significant synaptic enhancement. EPSPs were recorded before and 48 hr after injection of ApNT isoforms as labeled. Data are presented as mean ± SEM.

(C) ApNT overexpression in sensory neurons of sensory-motor neuron cocultures induces growth of new synaptic varicosities. ApNT(+)GFP and RFP (top) or RFP alone (bottom) were injected in sensory neurons and confocal images collected 12 and 48 hr after injection. Arrows point to synaptic varicosities in apposition to the motor neuron receptive surface. Scale bar represents 10 μm.

(D) Quantification of percent varicosity change in sensory cells injected as described in Figure 6C at 48 hr. Data are presented as mean ± SEM.

in LTF induced with five pulses of 5-HT measured at 24 hr (% change in EPSP of 5-HT-treated group: uninjected: 114.28 ± 11.99, n = 25; GFP-expressing: 127.83 ± 17.75, n = 12, $p > 0.05$; ApTrk-DN-GFP-expressing: 57.92 ± 12.74, n = 13, $p < 0.001$; Figure 5C, right set). The reduction of LTF with ApTrk-DN-GFP expression was not due to a reduction of basal synaptic transmission, because it had no effect on mock-treated cells over the same period (% change in EPSP for mock-treated group: uninjected: 5.12 ± 6.55, n = 25 versus GFP-expressing: 8.2 ± 6.73, n = 10, versus ApTrkDNGFP-expressing: 0.69 ± 7.03, n = 13, $p > 0.05$ for both). These results demonstrate that ApNT-ApTrk signaling is necessary for 5-HT-induced LTF.

Mature ApNT Release Is Sufficient to Enhance Synaptic Transmission

We next bath-applied recombinant mature ApNT and found that this treatment induced a modest increase in the amplitude of the evoked EPSPs of the sensory-motor neuron synapses measured 24 hr later (Figure 6A; % change in

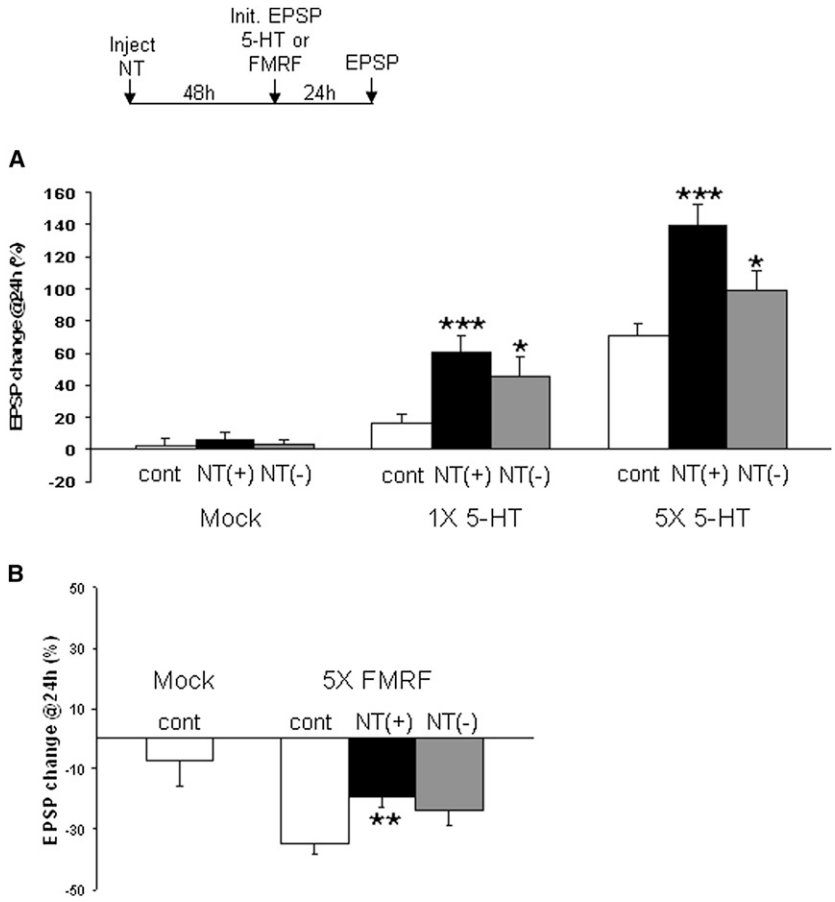

Figure 7. ApNT Overexpression Facilitates 5-HT Induced LTF and Counteracts FMRFamide-Induced LTI

(A) ApNT expression facilitates LTF. Forty-eight hours after injection of ApNTGFP, initial EPSPs were recorded and cells were treated with one (STF) or five (LTF) 5-HT pulses (10 μM, 5 min) or mock treated, and EPSPs were recorded again after 5 min (STF) or 24 hr (LTF). Data are presented as mean ± SEM.

(B) Same as (A) except that cultures were treated with five FMRFamide pulses (1 μM, 5 min) or mock treated and EPSPs were recorded again after 24 hr. Data are presented as mean ± SEM.

EPSP: ApNT-treated: 20.09 ± 6.08, n = 22 versus bovine serum albumin [BSA]-treated controls: −2.74 ± 3.84, n = 38 $p < 0.05$).

We then expressed the furin-cleavable ApNT(+)GFP and furin-resistant ApNT(−)GFP isoforms in the sensory neurons of sensory-motor neuron cocultures and found that ApNT(+)GFP expression produced significant long-term enhancement of synaptic strength measured 48 hr after injection (Figure 6B; % change in EPSP: ApNT(+) expressing: 44.63 ± 11.53, n = 18 versus uninjected controls: 7.65 ± 6.20, n = 16, $p = 0.01$). Similar results were obtained 24 and 72 hr after injection (not shown). Expression of the ApNT(−)GFP isoform did not cause a statistically significant increase in synaptic strength, although it showed a positive trend (ApNT(−) expressing: 18.82 ± 9.69, n = 6 versus uninjected controls: 7.65 ± 6.20, n = 16, $p > 0.05$). These results demonstrate that the secreted mature form of ApNT can induce long-term synaptic enhancement in the absence of additional physiological stimuli.

ApNT Signaling Induces Growth of New Sensory Neuron Synaptic Varicosities

To determine whether the synaptic enhancement induced by expression of the ApNT(+) isoform is accompanied by the growth of new synaptic connections, we imaged cells expressing ApNT(+)GFP and found this led to a significant increase of the number of presynaptic varicosities, structures that harbor transmitter release sites in *Aplysia* sensory-motor neuron cocultures (Kim et al., 2003; % change in varicosity number: ApNT(+) GFP + RFP-expressing: 23.54 ± 8.53, n = 10 versus RFP-expressing controls: −10.64 ± 7.13, n = 11, $p < 0.01$; Figures 6C and 6D).

Release of Both Mature and Pro-ApNT Facilitates the Induction of LTF by 5-HT

We next asked whether ApNT expression could also enhance LTF at *Aplysia* sensory-motor neuron synapses induced with five pulses of 5-HT. We first expressed the ApNT(+) and ApNT(−) isoforms in the sensory neurons for 48 hr, allowing the effect of ApNT expression on basal synaptic transmission to plateau, as confirmed by the fact that evoked EPSPs measured at 48 hr post-injection had not changed significantly when tested 24 hr later (% change in EPSP of uninjected controls: 2.86 ± 4.35, n = 33 versus ApNT(+)GFP-expressing: 6.31 ± 4.39, n = 25, $p > 0.05$ versus ApNT(−)GFP-expressing: 3.16 ± 3.10, n = 8, $p > 0.05$; Figure 7A, left set). We then found that LTF induced by five pulses of 5-HT was doubled in cells expressing ApNT(+)GFP and was also enhanced, though less robustly, in cells expressing the ApNT(−) GFP isoform compared with nonexpressing controls (Figure 7A, right set; % change in EPSP of the 5 × 5-HT-treated group: uninjected controls: 70.92 ± 7.89, n = 35 versus ApNT(+)GFP-expressing: 139.12 ± 13.69, n = 28, $p < 0.0001$ versus ApNT(−) GFP-expressing: 99.52 ± 11.59, n = 14, $p = 0.05$).

We next tested whether expression of ApNT could lower the threshold of 5-HT induction of LTF. We found that a single pulse of 5-HT had little effect on control nonexpressing cells, but produced a robust LTF in cells expressing ApNT(+)GFP and a significant though lower-magnitude LTF in cells expressing ApNT(−) GFP (% change in EPSP of the 1 × 5-HT-treated group: uninjected controls: 16.96 ± 4.77, n = 16 versus ApNT(+)GFP-expressing: 60.85 ± 10.01, n = 17, $p < 0.001$ versus ApNT(−) GFP-expressing: 46.05 ± 11.63, n = 9, $p < 0.05$; Figure 7A, middle set). Thus, augmenting ApNT signaling with expression of either the furin-cleavable or furin-resistant ApNT isoform lowers the threshold for induction of LTF so that a single 5-HT pulse that normally produces only STF now produces LTF lasting at least 24 hr.

Taken together, these results indicate that expression of the ApNT(+) isoform, which generates mature ApNT, can enhance synaptic transmission in the absence of an external stimulus and can then further enhance it in combination with a 5-HT modulatory stimulus. In contrast, expression of ApNT(−)GFP, which generates pro-ApNT, has a more moderate enhancing effect on synaptic transmission, which requires a 5-HT modulatory stimulus for full expression.

Release of Both Mature and Pro-ApNT Counteracts FMRFamide-Induced Long-Term Synaptic Inhibition

Given the proposed function of pro-BDNF in LTD induction, we next asked whether expression of the ApNT(−)GFP isoform, which generates pro-ApNT, could enhance the induction of synaptic depression when combined with an inhibitory stimulus. We treated sensory neurons expressing ApNT(+)GFP and ApNT(−)GFP with five pulses of FMRFamide, which induces long-term synaptic inhibition (LTI) in *Aplysia* sensory-motor neuron synapses. We found that expression of both isoforms reduced the magnitude of synaptic depression induced by FMRFamide. Similarly to the pattern we observed in the LTF experiments, this effect was more pronounced with ApNT(+)GFP (Figure 7B; % change in EPSP of FMRFamide-treated: uninjected controls: −34.64 ± 3.72, n = 23 versus ApNT(+)GFP-expressing: −19 ± 3.52, n = 15, $p < 0.01$ versus ApNT(−)GFP-expressing: −23.84 ± 11.59, n = 18, p = 0.08 versus mock-treated, uninjected controls: −7.51 ± 8.14, n = 8, $p < 0.01$). These results further support a role of proApNT release in synaptic enhancement, but not in FMRFamide-induced synaptic inhibition at the *Aplysia* sensory-motor neuron synapse.

DISCUSSION

ApNT and ApTrk Mediate LTF and Synaptic Growth

The ability of vertebrate neurotrophins to control diverse functions both during development and in the mature brain is thought to result from the expansion of a single ancestral neurotrophin ligand-receptor pair at the stem of vertebrates into a family of four related neurotrophins and three Trk receptors, allowing new functions, such as control of synaptic plasticity, to be acquired by the new family members (Hallböök et al., 2006). This idea is supported by the absence in invertebrate Trk receptors of signaling elements for PLCγ activation, which is required for the control of calcium signaling and synaptic plasticity by vertebrate neurotrophins (Benito-Gutiérrez et al., 2005; Hallböök et al., 2006).

Here we identify a fully structurally and functionally conserved neurotrophin system consisting of a Trk receptor (ApTrk) and ligand (ApNT) expressed in the sensory and motor neurons of the gill-withdrawal reflex in the marine mollusk *Aplysia californica.* We find that ApNT-ApTrk signaling plays a central role in the control of learning-related synaptic plasticity in *Aplysia*. Expression of ApNT in the sensory neurons induces a long-lasting increase in the basal strength of *Aplysia* sensory-motor neuron synapses, and in addition increases the magnitude and lowers the threshold for induction of LTF elicited in these synapses by 5-HT. Conversely, dominant negative inhibition of ApTrk signaling in the sensory neuron impairs 5-HT-induced LTF. We further find that ApNT induces structural synaptic growth, providing a mechanistic basis for its ability to elicit long-lasting enhancement of synaptic strength. Thus, the ability to control synaptic plasticity is not an innovation of vertebrate neurotrophins, but rather is a core ancestral function of neurotrophin signaling that could be lost in some divergent lineages (see Extended Discussion).

Alternative Splicing Directs Differential Processing and Secretion of ApNT Isoforms as Mature Forms and Proforms

Although recent studies have found that proneurotrophins can be secreted from neurons and could have separate and possibly opposite functions compared with those of mature neurotrophins, the physiological roles of proneurotrophin release and many aspects of proneurotrophin function remain poorly understood. Particularly unclear are the mechanisms that control the intracellular processing of neurotrophins determining the ratio of mature forms to proforms secreted in the CNS (Matsumoto et al., 2008; Yang et al., 2009). Neurotrophin processing is carried out primarily by constitutively and ubiquitously expressed furin-type and proconvertase proteases, but how this critical enzymatic conversion is regulated is not known (Hosaka et al., 1991; Mowla et al., 1999; Rouillé et al., 1995).

We have uncovered a mechanism in *Aplysia* whereby alternative splicing of a 9 nt miniexon generates ApNT isoforms that have either (1) an intact furin site and are fully processed inside the cell and secreted only as mature forms or (2) a deficient furin site and consequently escape intracellular cleavage and are secreted solely as precursor forms. Thus, rather than the efficiency of proteolytic cleavage alone, as found in vertebrates, a fundamentally different posttranscriptional regulatory mechanism controls ApNT processing and determines the relative ratios of secreted mature and proneurotrophin forms in *Aplysia*. This indicates a regulatory role for intracellular neurotrophin processing and the functional significance of the secretion of both neurotrophin forms in *Aplysia*.

Distinct Enhancing Effects of Mature and Pro-ApNT Secretion on Synaptic Facilitation

Recent studies have suggested that although the well-established positive functions of BDNF in synaptic plasticity are carried out by its mature form interacting with Trk receptors, pro-BDNF interacts preferentially with p75NTR to elicit synaptic depression and elimination (Nagappan et al., 2009; Woo et al., 2005). These conclusions, however, have been challenged by the finding that LTP, but not LTD, is compromised by BDNF deletion (Matsumoto et al., 2008).

We find that secretion of each of the two ApNT processing forms exerts an enhancing effect on synaptic facilitation at *Aplysia* sensory-motor neuron synapses, but these effects differ in their magnitude and requirement for additional stimuli. The mature form secreted by the furin-cleavable ApNT(+) isoform has a stronger enhancing effect observed with or without a 5-HT neuromodulatory stimulus, whereas proApNT secreted by the furin-resistant ApNT(−) isoform has a weaker enhancing effect that requires 5-HT for full expression. Moreover, proApNT secretion does not enhance the best-characterized form of

synaptic depression in *Aplysia* mediated by the inhibitory peptide FMRFamide. Thus, rather than bidirectional control of plasticity, as has been proposed for mammalian mature and proneurotrophins, our findings indicate a primary role for the alternative splicing of ApNT and independent release of mature and proneurotrophin ApNT forms in differential positive modulation of synaptic plasticity at the *Aplysia* sensory-motor neuron synapse.

Our findings are consistent with evidence that mammalian proneurotrophins engage the p75NTR-Trk receptor system to produce similarly positive yet mechanistically distinct signaling and physiological outcomes compared with mature neurotrophins (Boutilier et al., 2008; D'Onofrio et al., 2011), and with reports that an *Aplysia* homolog of the vertebrate p75NTR receptor is expressed in the sensory neurons of the gill-withdrawal reflex (L. Pu et al., 2010, Soc. Neurosci., abstract).

We did not detect extracellular processing of proApNT secreted by the ApNT(−) isoform in HEK cells or in *Aplysia* neurons with our assays, but it is also possible that plasmin- or metalloprotease-mediated cleavage at the remaining dibasic site of secreted proApNT(−) takes place specifically at synapses, as has been shown for secreted mammalian pro-BDNF and pro-NGF (Bruno and Cuello, 2006; Pang et al., 2004).

A major caveat of studies in the mammalian system that reported inhibitory synaptic effects of pro-BDNF is that they relied primarily on the use of noncleavable mutants to completely block conversion to the mature form. A unique advantage of the *Aplysia* system for investigating the differential synaptic functions of mature neurotrophins and proneurotrophins is that these forms are separately produced and secreted in the *Aplysia* CNS from different isoforms of a single neurotrophin gene with a single cognate Trk receptor. Future experiments in which the ratio of the endogenous ApNT isoforms is manipulated by selective knockdown or by targeting of their alternative splicing efficiency should provide a detailed understanding of the role of mature neurotrophin and proneurotrophin release in synaptic plasticity.

Differential Sorting and Localization of ApNT Isoforms

In addition to determining which form of the neurotrophin is secreted, alternative splicing also directs the differential sorting and trafficking of ApNT isoforms in sensory neurons. Our findings indicate that determinants in the mature region of ApNT and in the alternatively spliced N-terminal exon of the proApNT domain are necessary and sufficient to direct a punctate/vesicular or diffuse expression pattern, respectively. Moreover, the finding that the cleaved propeptide of ApNT has a subcellular localization that is distinct from that of mature ApNT and that it is secreted from sensory neurons indicates it has independent physiological functions.

Finally, alternative splicing of the ApNT isoforms could also determine the range at which they exert their effects, because the large difference in the net charge of the strongly acidic prodomain and the neutral mature domain of ApNT dictates distinct adherence to substrates. Localized actions upon secretion of the strongly basic and adherent mammalian neurotrophins have been shown to be crucial for their effects in synapse-specific, activity-dependent plasticity (Blöchl and Thoenen, 1996; Tanaka et al., 2008).

Thus, alternative splicing determines not only the differential processing and secretion of ApNT isoforms but also a differential expression pattern and, potentially, the spatial range of their effects after secretion, thereby greatly expanding the functionality of the single ApNT gene.

Alternative Splicing of ApNT and Gene Duplication of Vertebrate Neurotrophins as Parallel Evolutionary Strategies for Increasing Functional Capacity

Vertebrate neurotrophins are highly related, but their processing, localization, and secretion patterns exhibit large differences. As with the ApNT isoforms, these are due to differences in the more-divergent prodomains of the four neurotrophins, such as the strength of their furin-processing sites or the binding sites for sorting proteins such as sortilin (Chen et al., 2005; Mowla et al., 1999). Thus, the expansion of the vertebrate neurotrophin gene family and the alternative splicing of the single ApNT gene could be viewed as parallel evolutionary mechanisms for increasing functional capacity. BDNF and NT4 both bind to the same TrkB receptor but have different effects on synaptic plasticity: BDNF is required for both the induction and late phase of LTP, whereas NT4 is selectively required only for the late maintenance phase (Xie et al., 2000). These distinct effects are proposed to be due to differences in the processing and secretion patterns of the two neurotrophins (Chen et al., 2005), suggesting that ApNT isoforms could similarly be differentially involved in distinct stages of memory formation and storage in *Aplysia*.

EXPERIMENTAL PROCEDURES

Cloning of ApTrk

A T7 phage display cDNA library was constructed from *Aplysia* CNS-derived mRNA using the T7 select system (Novagen) according to the manufacturer's instructions. This creates fusions of *Aplysia* CNS proteins and the coat protein of phage T7, which are expressed on the phage surface. Briefly, the cDNA was generated using the Orient express cDNA synthesis random primer kit (Novagen), digested with EcoRI and HindIII, and ligated in the T7 Select 10-3B vector (Novagen). The library contained 6×10^6 independent clones, with >96% of the phage containing inserts with sizes between 0.3 and 1.5 kb. The library was screened by biopanning against recombinant hBDNF immobilized onto ELISA plates according to the T7 select system protocols (Novagen). Briefly, 50 μl library aliquots were incubated overnight at 4°C in BDNF-coated wells, unbound phage was washed out, and bound particles were eluted and amplified in Rosetta-gami 5615 cells (Novagen). The resulting amplified phage lysate was incubated again with the BDNF bait and the iterative biopanning process was repeated five times. The inserts from 20 independent phage clones from the last round were PCR amplified and sequenced. Eleven of these clones encoded a sequence related to the extracellular domain of Trk receptors.

The full-length ApTrk receptor sequence was then obtained by extending this sequence in both directions by 5′ and 3′ RACE-PCR using a SMART RACE kit (Clontech) according to the manufacturer's instructions.

Cloning of ApNT

The *Aplysia* partial genome database available at NCBI was queried using BDNF and NGF sequences from multiple species with TBLASTN. A contig containing a 78 amino acid ORF with homology to the mature portion of NGF was identified and the full-length sequence of the ApNT was obtained by extending this ORF using the SMART RACE-PCR technique as described above for ApTrk.

Constructs and Mutagenesis

pcDNA plasmids encoding full-length TrkA, TrkB, and TrkC were a kind gift from Dr. Moses Chao at New York University. All other constructs in this study were generated using the Gateway cloning system (Invitrogen). Full-length ApTrk and ApNT were PCR amplified and directionally TOPO cloned into pENTR D-TOPO (Invitrogen). After complete sequencing of the inserts, they were transferred into Gateway system-adapted destination vectors for mammalian expression (pcDNA 6.2 EmGFP-DEST; Invitrogen). For expression in *Aplysia* neurons, pNEX3EGFP was adapted by insertion of a gateway reading frame cassette at the BamHI site using the Gateway vector conversion system (Invitrogen). For the dominant-negative construct ApTrkDN, the N-terminal 564 amino acid portion of the ApTrk receptor, including its transmembrane (TM) region, was PCR amplified and cloned into the appropriate expression vectors as described above. The uncleavable ApNT mutant was generated by converting arginine at position 167 in ApNT(+) to glycine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Antibody Production, Western Blotting, and Immunocytochemistry

Rabbit polyclonal antibodies were raised against an extracellular ApTrk peptide (MDPKDAETVTELAIQNQPH; ApTrk10 antibody), and against a peptide in the mature region of ApNT (IQPGNLNDSTAQWFRT; ApNTM50 antibody) and the pro region of ApNT (IDFNFRPSLPKDLDSPFFRV; ApNTP47 antibody; 21st Century Biochemicals). N-terminal cysteines were added to all peptides for coupling to ovalbumin and injection into rabbits. All antibodies were affinity purified onto columns conjugated with the corresponding immunizing peptides. *Aplysia* CNS and pleural ganglia protein extracts were prepared and analyzed by western blotting as previously described (Puthanveettil et al., 2008). Commercial antibodies used against phospho Akt (1:3,000 dilution), total and phospho Erk (1:5,000 dilution), and total and phospho PLCγ (1:1,000 dilution) were obtained from Cell Signaling Technologies.

Immunocytochemistry was performed essentially as previously described (Puthanveettil et al., 2008) and all primary antibodies were used at 1:300 dilution.

RT-PCR

RNA from whole *Aplysia* CNS or pleural ganglia was isolated with Trizol (Invitrogen). For isolation of RNA from sensory or L7 motor neurons, ~30 sensory or 10 motor neurons were plated per dish, and after 5 days they were lysed and processed with 1 ml Tryzol per dish according to the manufacturer's instructions for isolating small amounts of RNA. cDNA was synthesized using SuperscriptII (Invitrogen), and PfuTurbo Cx (Stratagene) was used for PCR amplification.

ApNT Purification and Functional Assays

For purification of ApNT, a hexahistidine tag was added to the C terminus of ApNT by PCR and the amplified insert was cloned into pcDNA 6.2 DEST. The ApNT-His construct or a CATGFP control were transiently transfected in HEK293 cells by the calcium phosphate method. The next day, the media were changed with reduced serum medium (GIBCO). Conditioned medium was collected after 24 hr and ApNT was purified in parallel with control conditioned medium over Ni-NTA-coupled magnetic beads (QIAGEN) according to the manufacturer's instructions.

For biochemical functional experiments, Trk receptors or GFP DNA constructs (4 μg) were transiently transfected into PC12nnr5 cells (a kind gift from Dr. Philip Barker, McGill University) maintained in DMEM supplemented with 10% horse serum and 5% FBS (Loeb and Greene, 1993) using Lipofectamine LTX (Invitrogen), and the cells were left overnight. After the cells were serum deprived for 12 hr, they were stimulated with different amounts of purified ApNT or human BDNF, NGF, NT3, or NT4 (Peprotech) for 5 min. Cultures were then rinsed with ice-cold PBS, solubilized in SDS lysis buffer, and western blotted. For neurite outgrowth assays, PC12nnr5 cells were plated onto collagen-coated plates and 24 hr later were cotransfected with GFP and the respective Trk receptors. Following overnight incubation, the medium was changed and supplemented with 50 ng/ml of purified ApNT or NGF, or control purified medium, and renewed after 3 days. After 5 days, the cells were imaged and those having processes at least twice as long as the cell body diameter were counted as differentiated.

Aplysia Cell Culture, Treatment, and Electrophysiology

Aplysia sensory-motor neuron cocultures were prepared as previously described (Rayport and Schacher, 1986). For electrophysiological analysis, we measured the strength of the sensory-motor neuron synapse by obtaining intracellular recordings of the EPSPs from the motor neuron that were evoked by extracellular stimulation of the sensory neuron as previously described (Montarolo et al., 1986). We used bath application of 5-HT (10 μM), delivering five pulses (5 min each), separated by intervals of 15 min to induce LTF. We assessed 5-HT-induced LTF or strengthening of the synapse by overexpression by recording the EPSPs at 24 hr or 48 hr after 5-HT treatment or ApNT expression. We applied a single (5 min) pulse of 5-HT to induce STF, which we assessed by measuring EPSPs again 5 min after 5-HT treatment. LTI was induced by bath application of five pulses (5 min each) of FMRFamide (1 μM). All data are presented as mean percentage changes ± SEM in EPSP amplitudes measured at 5 min (STF) or 24–48 hr (LTF or LTI), as compared with the initial EPSP amplitudes. Two-tailed Student's t test was performed to calculate p values as indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Sensory Neuron Microinjection

We dissolved various DNA constructs (0.5 μg/μl) in sterile water containing 0.1% fast green and injected them under visual guidance into the nucleus of *Aplysia* neurons by applying positive air pressure through a picospritzer.

Cell Imaging and Quantification of the Structural Changes that Accompany ApNT Overexpression

We acquired images of *Aplysia* neurons using an Olympus Fluoview 1000 laser confocal scanning microscopes. Images were taken with a 60×, NA 1.4 objective, and the gains were adjusted to prevent saturation of the detection threshold. A z-series consisting of 30–40 optical sections was collected throughout the entire volume of the sensory neuron varicosities, and maximum projections were made offline for analysis with ImageJ (NIH). We assessed the long-term structural changes by comparing the images of each sensory neuron 12 hr and 48 hr after ApNT(+) and/or RFP overexpression. Sensory neuron varicosities were identified as elongated spheres (≥3 μm in diameter) in apposition to the initial segment and major neurites of L7 motor neurons (Kim et al., 2003). Quantitative analysis followed a blind procedure.

ACCESSION NUMBERS

The genes identified in this study have been deposited in GenBank under the following accession numbers: ApTrk, FJ861323; ApNT, FJ861324; and ApLRRTK, FJ969839.

SUPPLEMENTAL INFORMATION

Supplemental Information includes an Extended Discussion and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.008.

LICENSING INFORMATION

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REFERENCES

Bailey, C.H., Giustetto, M., Zhu, H., Chen, M., and Kandel, E.R. (2000). A novel function for serotonin-mediated short-term facilitation in aplysia: conversion of a transient, cell-wide homosynaptic hebbian plasticity into a persistent, protein synthesis-independent synapse-specific enhancement. Proc. Natl. Acad. Sci. USA *97*, 11581–11586.

Benito-Gutiérrez, E., Nake, C., Llovera, M., Comella, J.X., and Garcia-Fernàndez, J. (2005). The single AmphiTrk receptor highlights increased complexity of neurotrophin signalling in vertebrates and suggests an early role in developing sensory neuroepidermal cells. Development *132*, 2191–2202.

Blöchl, A., and Thoenen, H. (1996). Localization of cellular storage compartments and sites of constitutive and activity-dependent release of nerve growth factor (NGF) in primary cultures of hippocampal neurons. Mol. Cell. Neurosci. *7*, 173–190.

Bothwell, M. (2006). Evolution of the neurotrophin signaling system in invertebrates. Brain Behav. Evol. *68*, 124–132.

Boutilier, J., Ceni, C., Pagdala, P.C., Forgie, A., Neet, K.E., and Barker, P.A. (2008). Proneurotrophins require endocytosis and intracellular proteolysis to induce TrkA activation. J. Biol. Chem. *283*, 12709–12716.

Bruno, M.A., and Cuello, A.C. (2006). Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. Proc. Natl. Acad. Sci. USA *103*, 6735–6740.

Carim-Todd, L., Bath, K.G., Fulgenzi, G., Yanpallewar, S., Jing, D., Barrick, C.A., Becker, J., Buckley, H., Dorsey, S.G., Lee, F.S., and Tessarollo, L. (2009). Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo. J. Neurosci. *29*, 678–685.

Chao, M.V. (2000). Trophic factors: An evolutionary cul-de-sac or door into higher neuronal function? J. Neurosci. Res. *59*, 353–355.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. *4*, 299–309.

Chen, Z.Y., Ieraci, A., Teng, H., Dall, H., Meng, C.X., Herrera, D.G., Nykjaer, A., Hempstead, B.L., and Lee, F.S. (2005). Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. J. Neurosci. *25*, 6156–6166.

D'Onofrio, M., Paoletti, F., Arisi, I., Brandi, R., Malerba, F., Fasulo, L., and Cattaneo, A. (2011). NGF and proNGF regulate functionally distinct mRNAs in PC12 cells: an early gene expression profiling. PLoS One *6*, e20839.

Dechant, G., and Barde, Y.A. (1997). Signalling through the neurotrophin receptor p75NTR. Curr. Opin. Neurobiol. *7*, 413–418.

Dieni, S., Matsumoto, T., Dekkers, M., Rauskolb, S., Ionescu, M.S., Deogracias, R., Gundelfinger, E.D., Kojima, M., Nestel, S., Frotscher, M., and Barde, Y.A. (2012). BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. J. Cell Biol. *196*, 775–788.

Haapasalo, A., Koponen, E., Hoppe, E., Wong, G., and Castrén, E. (2001). Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival. Biochem. Biophys. Res. Commun. *280*, 1352–1358.

Hallböök, F., Wilson, K., Thorndyke, M., and Olinski, R.P. (2006). Formation and evolution of the chordate neurotrophin and Trk receptor genes. Brain Behav. Evol. *68*, 133–144.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature *350*, 678–683.

Hosaka, M., Nagahama, M., Kim, W.S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991). Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J. Biol. Chem. *266*, 12127–12130.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. *72*, 609–642.

Jaaro, H., Beck, G., Conticello, S.G., and Fainzilber, M. (2001). Evolving better brains: a need for neurotrophins? Trends Neurosci. *24*, 79–85.

Kim, J.H., Udo, H., Li, H.L., Youn, T.Y., Chen, M., Kandel, E.R., and Bailey, C.H. (2003). Presynaptic activation of silent synapses and growth of new synapses contribute to intermediate and long-term facilitation in Aplysia. Neuron *40*, 151–165.

Lee, R., Kermani, P., Teng, K.K., and Hempstead, B.L. (2001). Regulation of cell survival by secreted proneurotrophins. Science *294*, 1945–1948.

Loeb, D.M., and Greene, L.A. (1993). Transfection with trk restores "slow" NGF binding, efficient NGF uptake, and multiple NGF responses to NGF-nonresponsive PC12 cell mutants. J. Neurosci. *13*, 2919–2929.

Matsumoto, T., Rauskolb, S., Polack, M., Klose, J., Kolbeck, R., Korte, M., and Barde, Y.A. (2008). Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat. Neurosci. *11*, 131–133.

McDonald, N.Q., and Blundell, T.L. (1991). Crystallization and characterization of the high molecular weight form of nerve growth factor (7 S NGF). J. Mol. Biol. *219*, 595–601.

Meyer-Franke, A., Wilkinson, G.A., Kruttgen, A., Hu, M., Munro, E., Hanson, M.G., Jr., Reichardt, L.F., and Barres, B.A. (1998). Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron *21*, 681–693.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R., and Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. Science *234*, 1249–1254.

Mowla, S.J., Pareek, S., Farhadi, H.F., Petrecca, K., Fawcett, J.P., Seidah, N.G., Morris, S.J., Sossin, W.S., and Murphy, R.A. (1999). Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J. Neurosci. *19*, 2069–2080.

Nagappan, G., Zaitsev, E., Senatorov, V.V., Jr., Yang, J., Hempstead, B.L., and Lu, B. (2009). Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. Proc. Natl. Acad. Sci. USA *106*, 1267–1272.

Ormond, J., Hislop, J., Zhao, Y., Webb, N., Vaillaincourt, F., Dyer, J.R., Ferraro, G., Barker, P., Martin, K.C., and Sossin, W.S. (2004). ApTrkl, a Trk-like receptor, mediates serotonin- dependent ERK activation and long-term facilitation in Aplysia sensory neurons. Neuron *44*, 715–728.

Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L., and Lu, B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science *306*, 487–491.

Purcell, A.L., Sharma, S.K., Bagnall, M.W., Sutton, M.A., and Carew, T.J. (2003). Activation of a tyrosine kinase-MAPK cascade enhances the induction of long-term synaptic facilitation and long-term memory in Aplysia. Neuron *37*, 473–484.

Puthanveettil, S.V., Monje, F.J., Miniaci, M.C., Choi, Y.B., Karl, K.A., Khandros, E., Gawinowicz, M.A., Sheetz, M.P., and Kandel, E.R. (2008). A new component in synaptic plasticity: upregulation of kinesin in the neurons of the gill-withdrawal reflex. Cell *135*, 960–973.

Rajagopal, R., Chen, Z.Y., Lee, F.S., and Chao, M.V. (2004). Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. J. Neurosci. *24*, 6650–6658.

Rayport, S.G., and Schacher, S. (1986). Synaptic plasticity in vitro: cell culture of identified Aplysia neurons mediating short-term habituation and sensitization. J. Neurosci. *6*, 759–763.

Ridgway, R.L., Syed, N.I., Lukowiak, K., and Bulloch, A.G. (1991). Nerve growth factor (NGF) induces sprouting of specific neurons of the snail, Lymnaea stagnalis. J. Neurobiol. *22*, 377–390.

Rouillé, Y., Duguay, S.J., Lund, K., Furuta, M., Gong, Q., Lipkind, G., Oliva, A.A., Jr., Chan, S.J., and Steiner, D.F. (1995). Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. Front. Neuroendocrinol. *16*, 322–361.

Sharma, S.K., Sherff, C.M., Stough, S., Hsuan, V., and Carew, T.J. (2006). A tropomyosin-related kinase B ligand is required for ERK activation, long-term synaptic facilitation, and long-term memory in Aplysia. Proc. Natl. Acad. Sci. USA *103*, 14206–14210.

Tanaka, J., Horiike, Y., Matsuzaki, M., Miyazaki, T., Ellis-Davies, G.C., and Kasai, H. (2008). Protein synthesis and neurotrophin-dependent structural plasticity of single dendritic spines. Science *319*, 1683–1687.

Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.D., Kermani, P., Torkin, R., Chen, Z.Y., Lee, F.S., et al. (2005). ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J. Neurosci. *25*, 5455–5463.

van Kesteren, R.E., Fainzilber, M., Hauser, G., van Minnen, J., Vreugdenhil, E., Smit, A.B., Ibáñez, C.F., Geraerts, W.P., and Bulloch, A.G. (1998). Early evolutionary origin of the neurotrophin receptor family. EMBO J. *17*, 2534–2542.

Wilson, K.H. (2009). The genome sequence of the protostome Daphnia pulex encodes respective orthologues of a neurotrophin, a Trk and a p75NTR: evolution of neurotrophin signaling components and related proteins in the bilateria. BMC Evol. Biol. *9*, 243.

Woo, N.H., Teng, H.K., Siao, C.J., Chiaruttini, C., Pang, P.T., Milner, T.A., Hempstead, B.L., and Lu, B. (2005). Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat. Neurosci. *8*, 1069–1077.

Xie, C.W., Sayah, D., Chen, Q.S., Wei, W.Z., Smith, D., and Liu, X. (2000). Deficient long-term memory and long-lasting long-term potentiation in mice with a targeted deletion of neurotrophin-4 gene. Proc. Natl. Acad. Sci. USA *97*, 8116–8121.

Yang, J., Siao, C.J., Nagappan, G., Marinic, T., Jing, D., McGrath, K., Chen, Z.Y., Mark, W., Tessarollo, L., Lee, F.S., et al. (2009). Neuronal release of proBDNF. Nat. Neurosci. *12*, 113–115.

Zakharenko, S.S., Patterson, S.L., Dragatsis, I., Zeitlin, S.O., Siegelbaum, S.A., Kandel, E.R., and Morozov, A. (2003). Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron *39*, 975–990.

Zhu, B., Pennack, J.A., McQuilton, P., Forero, M.G., Mizuguchi, K., Sutcliffe, B., Gu, C.J., Fenton, J.C., and Hidalgo, A. (2008). Drosophila neurotrophins reveal a common mechanism for nervous system formation. PLoS Biol. *6*, e284.