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Cerebral Dopamine Neurotrophic Factor Is Essential for Enteric Neuronal Development, Maintenance and Regulation of Gastrointestinal Transit

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Abstract

Cerebral dopamine neurotrophic factor (CDNF) is expressed in the brain and is neuroprotective. We have previously shown that CDNF is also expressed in the bowel and that its absence leads to degeneration and autophagy in the enteric nervous system (ENS), particularly in the submucosal plexus. We now demonstrate that enteric CDNF immunoreactivity is restricted to neurons (submucosal > myenteric) and is not seen in glia, interstitial cells of Cajal, or smooth muscle. Expression of CDNF, moreover, is essential for the normal development and survival of enteric dopaminergic neurons; thus, expression of the dopaminergic neuronal markers, dopamine, tyrosine hydroxylase, and dopamine transporter are deficient in the ileum of *Cdnf* $^{-/-}$ mice. The normal age-related decline in proportions of submucosal dopaminergic neurons is exacerbated in *Cdnf* $^{-/-}$ animals. The defect in *Cdnf* $^{-/-}$ animals is not dopamine-restricted; proportions of other submucosal neurons (NOS-, GABA-, and CGRP-expressing), are also deficient. The deficits in submucosal neurons are reflected functionally in delayed gastric emptying, slowed colonic motility, and prolonged total gastrointestinal transit. CDNF is expressed selectively in isolated enteric neural crest-derived cells (ENCDC), which also express the dopamine-related transcription factor Foxa2. Addition of CDNF to ENCDC promotes development of dopaminergic neurons; moreover, survival or these neurons becomes CDNF-dependent after exposure to bone morphogenetic protein 4. The effects of neither glial cell-derived neurotrophic factor (GDNF) nor serotonin are additive with CDNF. We suggest that CDNF plays a critical role in development and long-term maintenance of dopaminergic and other sets of submucosal neurons.

Graphical Abstract

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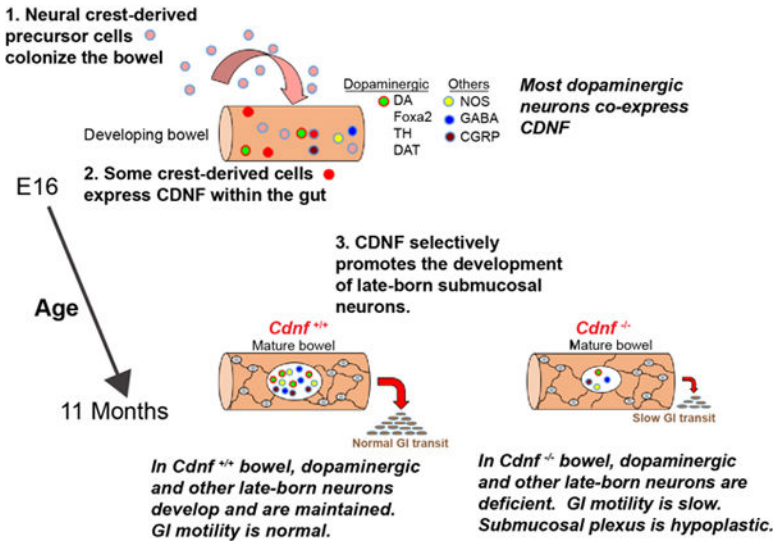
The authors declare no competing financial interests. MS and PL are inventors in the CDNF patent owned by Herantis Pharma Plc. All authors approved of the manuscript. All data will be made available upon request.



Cerebral dopamine neurotrophic factor (CDNF) is a member of a novel family of growth factors that is not only secreted but is highly retained in the rough endoplasmic reticulum and protective against endoplasmic reticulum stress. CDNF is expressed in the brain and bowel and is neuroprotective. We sought to determine the location of CDNF in the gut as well as its functions during development and maintenance of the enteric nervous system (ENS). The ENS is formed by precursor cells that migrate to the primordial bowel from the neural crest (1). These cells colonize the gut and some of the crest-derived émigrés express (CDNF) within the gut (2). CDNF selectively promotes the development of subsets of enteric neurons that are late-born (3). These include, but are not limited to enteric dopaminergic neurons. Most mature dopaminergic neurons co-express CDNF. Exposure to bone morphogenetic protein (BMP4) causes neurons isolated from the fetal gut to become CDNF-dependent. When the bowel contains CDNF, submucosal dopaminergic and other late-born neurons develop normally and are maintained; however, when CDNF is genetically deleted, these same neurons become deficient as a function of age. Although CDNF is expressed in subsets of neurons in both plexuses, the submucosal plexus selectively becomes hypoplastic in animals lacking CDNF, and parameters of gastrointestinal motility become abnormally slow. We conclude that enteric expression of CDNF is restricted to neurons and their precursors and that CDNF is essential for the development and maintenance of dopaminergic and other late-born submucosal neurons.

Keywords

Enteric nervous system; submucosal plexus; dopaminergic neurons; bone morphogenetic protein-4; serotonin; glial cell line derived neurotrophic factor

1) Introduction

Neurotrophic factors (NTFs) are small, secreted proteins that regulate neuronal development, survival, and maintenance. Members of three NTF families have been implicated in the development of the enteric nervous system (ENS). These include glial cell line-derived neurotrophic factor (GDNF) (Moore et al., 1996; Pichel et al., 1996; Sánchez et

al., 1996; Schuchardt, D'Agati, Larsson-Blomberg, Costantini, & Pachnis, 1994) and neurturin (Heuckeroth et al., 1999) of the GDNF family, neurotrophin-3 (NT-3) (Chalazonitis et al., 2001; Chalazonitis et al., 1994) and brain-derived neurotrophic factor (BDNF) (Levanti et al., 2009) of the neurotrophin family, and a ligand for the tripartite ciliary neurotrophic factor (CNTF) receptor (Chalazonitis, Rothman, Chen, Vinson, et al., 1998) of the neuropoietic cytokines. Recently, a novel family of growth factors, cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), has been discovered (Lindholm et al., 2007; Petrova et al., 2003). In contrast with other NTFs, CDNF and MANF are not only secreted, but are also retained in the endoplasmic reticulum (ER) of neurons, regulate the unfolded protein response (UPR), and protect cells against ER stress (Lindahl, Saarma, & Lindholm, 2017; Voutilainen et al., 2017). CDNF protects dopaminergic neurons of the substantia nigra from the neurotoxicity of both 6-hydroxydopamine (Lindholm et al., 2007(Voutilainen et al., 2011) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Airavaara et al., 2012). We have reported that CDNF is expressed in the developing and adult bowel and a null mutation of CDNF leads to neurodegeneration and autophagy, particularly in the submucosal plexus (Lindahl et al., 2020). These observations thus suggest that CDNF is an unusual factor in that it is essential for the development and/or long-term survival of neurons with particular relevance to only one of the two enteric plexuses.

In the present study, we sought to identify enteric neurons that are CDNF-responsive. Because the mammalian submucosal plexus, like the substantia nigra, contains dopaminergic neurons (Z. S. Li, Pham, Tamir, Chen, & Gershon, 2004), we also tested the hypotheses that enteric dopaminergic neurons require CDNF for their development and for their long-term survival. We first used immunocytochemistry to locate sources of CDNF within the developing and mature bowel. We then compared the ENS of *Cdnf* $^{+/+}$ mice to that of *Cdnf* $^{-/-}$ animals to evaluate the potential role of CDNF in maintaining dopaminergic and other subtypes of enteric neuron as a function of age. GI transit was also explored in *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice to determine the physiological consequences of CDNF deletion. Finally, we used cultures of isolated enteric crest-derived cells (ENCDC) to test the ideas that CDNF is able to promote neurogenesis, survival, and dopaminergic expression. These experiments included studies of interactions of CDNF with other neurotrophic factors, GDNF (Chalazonitis et al.,1998), serotonin (5-HT), and bone morphogenetic protein (BMP) 2 and 4, that are expressed in the fetal bowel and which have previously been demonstrated to affect enteric dopaminergic neuronal development (Chalazonitis et al., 2004; Chalazonitis et al., 2008; Chalazonitis, Rothman, Chen, & Gershon, 1998; Z. Li et al., 2011). Because BMPs induce dependence of developing enteric neurons on other factors, such as NT-3 (Chalazonitis et al., 2004), we also asked whether BMP exposure induces dependence of dopaminergic neurons on CDNF for survival.

Portions of this work have previously appeared in abstracts (Chalazonitis et al., 2013; Chalazonitis, Lindholm, & Gershon, 2010)

2) Materials and Methods

Animals:

Gravid female mice (CD-1; Charles River Laboratories) were anesthetized with CO_2 and euthanized with a thoracotomy; the fetuses were used for the immuno-isolation of ENCDC. Mice lacking CDNF (*Cdnf* $^{-/-}$) and their *WT* (*Cdnf* $^{+/+}$) littermates were derived from the crossing of heterozygous (*Cdnf* $^{+/-}$) animals either on a hybrid Ola129/C57BL/6JRcc/ICR (CD-1) mixed genetic background (Lindahl et al., 2020) *Cdnf* $^{-/-}$ and Cdnf$^{+/+}$ animals (male and female) were compared at 3 different ages, 1.5, 3, and 9–11 months. All cohorts were transported from the University of Helsinki to Columbia University. Mice (male) expressing enhanced green fluorescent protein (EGFP) under the control of the promoter for phospholipid protein 1 (PLP1-EGFP mice) (Mallon, Shick, Kidd, & Macklin, 2002; Rao et al., 2015) were used to identify enteric glia at P0, P28, and P56; when these animals were employed, FVB/N mice (the background of the PLP1-EGFP animals) were utilized as (*WT*) controls. PLP1 has recently been shown to be a marker for enteric glia when preparations of bowel are examined (Rao et al., 2015). The day at which a vaginal plug was found was called day 0 of gestation. The Animal Care and Use Committee of Columbia University approved all procedures.

Reverse transcription polymerase chain reaction (RT-PCR):

Total RNA was extracted from adult midbrain, positively immunoselected ENCDC isolated from CD-1 fetal gut at E15–16, negatively immunoselected non-ENCDC isolated at the same age, and adult female Ileum (Chalazonitis et al., 2001; Chalazonitis, Rothman, & Gershon, 1997) (see “tissue culture” section below). Following collection, cells were stored in RNAlater™ (Ambion Inc, Austin, TX) before transfer to Trizol™ (Invitrogen, Carlsbad, CA) for RNA extraction according to the manufacturer’s instructions. Extracted RNA was stored for further use at −80°C. Reverse transcription from 3 μg of total RNA was employed to prepare cDNA in a 30 μl reaction volume with 0.5 μg of random hexamer primers, 0.5 mM dNTPs, 40 units of RNAsin® (Promega, Cat# N2618, Madison, WI) and 400 units of Maloney murine leukemia virus reverse transcriptase (MMLV; Promega, Madison, WI).

PCR:

Pairs of oligonucleotide primers for amplification of cDNA encoding β- actin, $p75^{NTR}$, Hand1, CDNF and Foxa2 were designed from published mouse cDNA sequences and the programs employed for PCR amplifications with each primer pair are listed in Table 1.

Real-time qPCR using SYBR® Green:

The wall of the stomach, ileum and colon of P7 *Cdnf* $^{-/-}$ mice (4 animals) and of their WT littermates (4 animals) were opened and cleaned with phosphate-buffered saline (PBS; 0.9% NaCl in 0.01M sodium phosphate buffer, pH 7.4) that had been treated with 0.1% diethyl pyrocarbonate (DEPC-PBS). Total RNA was extracted with Trizol (Life Technologies) and treated with DNase to remove contaminating DNA (TURBO-DNA-free kit; Life Technologies). Complementary DNA (cDNA) syntheses were carried out using methods previously described (Xu, Cawthon, McCastlain, Slikker, & Ali, 2005). The sequences of

primers used for amplification of dopamine transporter (DAT) and GAPDH are described in Table 1. RT-qPCR was carried out using an ABI 7500 instrument and Power SYBR Green PCR Master Mix (Life Technologies). Quantitation of DAT transcripts was carried out using the threshold cycle (C_T), which was defined as the cycle at which the change of fluorescence in the reaction exceeds 10-fold the standard deviation above the background fluorescence. The background fluorescence was calculated as the mean fluorescence between cycles 3 and 15 of the reaction. The instrument software (7500 Fast System version 1.3.1) was used to calculate C_T. For each sample of stomach, ileum and colon, the expression of DAT was normalized to that of mouse GAPDH to account for differences in amounts of starting RNA and differences in cDNA synthesis efficiency. Data are expressed as the ratio of transcripts encoding DAT to those encoding GAPDH.

Immunocytochemistry in sections of gut:

Detection of antigens was carried out as previously described (Chalazonitis, D'Autreaux, Pham, Kessler, & Gershon, 2011). Gut was dissected from E17 or adult mice and fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer for 3 h. After rinsing, the gut was immersed in 30% sucrose for 24 h embedded in OCT (Tissue-Tek, Sakura Finetek, USA), and frozen with liquid N_2. Sections (10 μm thick) were cut in a cryostat-microtome and dried on Superfrost™ slides (Fischer). The primary antibodies that were used are listed in Table 2. Tissue was permeabilized for 30 min with 0.3%Triton X-100 (0.1% for PLP1-EGFP mice) and then incubated for 1 h in 0.1M PBS, 0.1%Triton X and 10% horse serum to neutralize non-specific IgGs. When primary antibodies were raised in chickens, sections were blocked with a fish serum-based immunoreagent (BlokHenII™, Aves Labs Inc. OR) and detected with affinity purified goat anti-chicken or donkey anti-chicken secondary antibodies coupled to Alexa 594 or 488. Sites of binding of antibodies raised in goat were detected using affinity purified donkey anti-goat coupled to Alexa 594. Bound antibodies to CDNF were detected with biotinylated goat anti-rabbit antibodies followed by streptavidin Alexa 488. Alternatively, sensitivity was enhanced with a ready-to-use kit (Vectafluor™ *Excel* DyLight™ 594, #DK-1594, Vector Labs Inc) that employs a goat anti-rabbit secondary antibody as an amplifier. The bound amplifying antibodies were detected with a horse anti-goat tertiary antibody coupled to a red fluorophore.

Immunocytochemistry of laminar preparations of gut wall:

Intestines were removed from *Cdnf*$^{+/+}$mice and *Cdnf*$^{-/-}$ littermates at 1.5-, 3- and 9–11 months of age. After perfusion and further fixation with 4% formaldehyde (from paraformaldehyde; pH 7.4), intestines were rinsed and stored in 0.1M phosphate buffer containing sodium azide. The walls of the ileum and colon were subsequently dissected to obtain separate laminar preparations containing the submucosal or myenteric plexus (Chalazonitis et al., 2008). Immunocytochemistry was used to identify cells as neurons as well as their chemically-defined subtypes. Bound primary antibodies (Table 2) were detected with species-specific secondary antibodies coupled to contrasting fluorophores as described above. Primary antibodies to CDNF were raised in rabbits (Lindholm et al., 2007) and were detected using an amplifying secondary antibody as described above. Controls included omission of primary antibodies, omission of primary and secondary antibodies, immunostaining of an antigen (gE protein of varicella zoster virus) that is not present in the

mouse gut, and the immunostaining of whole mounts of laminar preparations from *Cdnf*$^{-/-}$ mice. Nitrergic neurons were identified by a histochemical reaction to detect the NADPH diaphorase activity of nitric oxide synthase (Hope, Michael, Knigge, & Vincent, 1991).

Measurement of GI motility parameters:

Mice were weighed before transit was measured. Total gastrointestinal transit time (GITT), gastric emptying (GE), and colonic motility were determined as previously described (Z. Li et al., 2011). Briefly, to determine GITT, 300 μl of a non-absorbable dye, carmine red (6% in 0.5 % methylcellulose) was administered by gavage. GITT was taken as the time that elapsed between gavage and the first appearance of red dye in fecal pellets. Colonic motility was estimated as the time required to expel a glass bead (3 mm diameter) inserted into the rectum a distance of 2 cm from the anal verge. GE was measured after GITT and colonic motility. Mice were fasted overnight and water intake was suspended 3 hr prior to the procedure. Rhodamine B dextran (10 mg/ml in 100 μl of a solution with 2% methylcellulose) was administered by gavage and the animals were euthanized 15 minutes later. The stomach, small intestine, cecum, and colon were collected in 0.9% NaCl. The small intestine was divided into 10 segments of equal length and the colon (used to obtain total recovered rhodamine B fluorescence) was divided in half. Each piece of tissue was then transferred into a 14ml tube containing 4 ml of 0.9% NaCl, homogenized, and centrifuged (2000 × *g*) to obtain a clear supernatant. Rhodamine fluorescence was measured in 1 ml aliquots of the supernatant (VersaFluor Fluorometer; Bio-Rad Laboratories). The proportion of the rhodamine B dextran that emptied from the stomach was calculated as [(total recovered fluorescence − fluorescence remaining in the stomach) ÷ (total recovered fluorescence)] × 100.

Tissue culture:

Two types of culture were used. To examine the development of enteric neurons in mixed preparations containing non-neuronal cells, the whole fetal bowel (E15–16) was dissociated and cultured (Chalazonitis et al., 2001). All cultures were maintained in a defined medium (Ziller, Dupin, Brazeau, Paulin, & Le Douarin, 1983). Gut from 14–17 fetuses was pooled for each experiment. The other type of culture was of isolated ENCDC. These cells were immunoselected with antibodies to $p75^{NTR}$ (donated by Dr. Moses Chao, Skirball Institute, NYU, NY), which is a marker for ENCDC in fetal bowel (Chalazonitis, Tennyson, Kibbey, Rothman, & Gershon, 1997). The secondary antibodies were coupled to magnetic beads. Suspensions of dissociated cells, in which ENCDC are decorated with antibodies were passed through a magnetized column (Miltenyi Biotec Inc, Auburn, CA). ENCDC are retained in the magnetic field, while the non-neuronal cells flow through; ENCDC are finally collected when the field is released. Suspended cells from the dissociated whole bowel and isolated ENCDC were plated at a density of 1.2×10^5 cells/ml on glass coverslips (12 mm diameter; #1001; RESY, Darmstadt, Germany). The glass coverslips were coated with Poly-D-lysine (Sigma# P7405), collagen (Roche Applied Sciences # 11179179001) and laminin (Invitrogen # 23017015). To insure proper adherence of the cells to the substrate, the defined medium, was supplemented with 20% horse serum (JRH Biosciences, Lenexa, KS) for 18 h after plating. Subsequently, cultures were fed only with defined medium, replaced fresh on the 4th day of culture and maintained until the 7th day. When the effects of growth

factors (human recombinant CDNF [donated by Dr. Päivi Lindholm, Institute of Biotechnology Univ. of Helsinki, Helsinki, Finland), rat recombinant GDNF (R&D Systems #512-GF), human recombinant BMP4 or BMP2 [R&D Systems #314-BP or #355-BM]), or 5-HT (Sigma USA #7752) on neuronal development were investigated, the factors were added to the medium at the time of plating and all, except the BMPs, were present throughout the culture period. BMPs were withdrawn on the 4th day of culture because cultured enteric neurons tend to die when exposed for longer periods to BMPs (Chalazonitis et al., 2004). In experiments involving exogenous 5-HT, parachlorophenylalanine (PCPA, Sigma Millipore USA #C6506) was added (1 μg/ml) 18 h after plating to inhibit tryptophan hydroxylase and thus prevent cells from synthesizing endogenous 5-HT in culture. Control cultures were maintained with the vehicle in which the growth factors were dissolved (defined medium supplemented with 0.5% bovine serum albumin).

Immunocytochemistry of cultures:

Previously described methods were used for the immunocytochemical detection of antigens in cultured ENCDC (Chalazonitis et al., 2004). Briefly, cultures were fixed for 30 min with 4% formaldehyde (from paraformaldehyde) on 0.1 M PBS. Fixed cultures were permeabilized for 30 min with 0.1%Triton X-100 (Sigma, St Louis, MO) and then incubated for 1 h in 0.1M PBS, 0.1%Triton X and 10% horse serum to neutralize non-specific IgGs. Primary antibodies are listed in Table 2. Preparations were incubated with these antibodies for 60–62 h at 4° C on a shaker. Sites of primary antibody binding were visualized with species-specific secondary antibodies coupled to contrasting fluorophores (Alexa, 488, 594, or 680) or, when primary antibodies were biotinylated, to streptavidin coupled to Alexa 594 (ThermoFisher, Invitrogen). Secondary antibodies were applied for 1–2 h at room temp. Cultures processed in the absence of primary antibodies were used as controls to verify specificity of immunostaining. Bisbenzimide (1 μg /ml for 2 min; Sigma, St Louis, MO) was used to stain DNA.

Quantitative determination of neuronal densities in culture and in whole mounts containing the myenteric or submucosal plexuses:

Effects of CDNF in cultured ENCDC isolated from fetal gut were quantified as described previously (Chalazonitis et al., 2004; Chalazonitis, Rothman, Chen, Vinson, et al., 1998; Chalazonitis, Tang, et al., 2011). Densities of neurons or subsets of neurons identified with specific markers were calculated either per culture, as % of vehicle treated cultures, or as proportions of total neurons or total cells from at least 4 to 16 replicate cultures. Neurons were quantified without knowledge of the genotype of the mice. Each neuronal density measurement (n) was made with a 40X objective and corresponded to the sum of neurons counted in 10 contiguous non-overlapping rectangular fields (1.254 mm^2) in a given whole mount.

Experimental Design and Statistics:

For *in vitro* experiments, 4–16 replicates were used for each condition. Numbers of mice for in vivo experiments were: (1.5 months) 6 *Cdnf*$^{+/+}$ and 6 *Cdnf*$^{-/-}$; (3 months) 5 *Cdnf*$^{+/+}$ and 5 *Cdnf*$^{-/-}$ mice; (11 months) 7 *Cdnf*$^{+/+}$ and 7*Cdnf*$^{-/-}$. GraphPad Prism software (version 7.0) was employed for calculations. Student's t-test with Welch's correction was used to

compare two means while multiple means were compared with analyses of variance (ANOVA). Tukey, Sidak, or Bonferroni-Dunn methods were employed for post hoc comparisons.

Imaging:

Images were captured with a digital CCD camera (Retiga Exi), mounted on a Leica DM 6000B microscope. Images were stored and visualized with computer assistance (Volocity 6.3 software; Improvision/PerkinElmer Life and Analytical Sciences). Adobe CS5 Photoshop software was used to assemble images.

3) Results

Identification of CDNF-immunoreactive cells in the gut of *Cdnf* $^{+/+}$ mice.

Immunocytochemistry was employed to identify sources of CDNF within the bowel wall. Whole mounts of longitudinal muscle with adherent myenteric plexus (LMMP) and submucosa (containing the submucosal plexus) were prepared from the small intestines of 11-month old *Cdnf*$^{+/+}$ and *Cdnf*$^{-/-}$ mice and immunostained with antibodies to CDNF (Lindholm et al., 2007). Neurons were identified simultaneously with ANNA-1, a human autoantibody that reacts mainly with HuC/D proteins, which is widely used as a neuronal marker (King, Redden, Palmgren, Nabors, & Lennon, 1999; Margolis et al., 2016). A subset of neurons was found to be CDNF-immunoreactive in each plexus of *Cdnf*$^{+/+}$ animals (Fig. 1a-c; g-l). In contrast, no neurons were immunostained with antibodies to CDNF in tissue from *Cdnf*$^{-/-}$ mice (Fig. 1d-e; j-l). This observation validates the immunocytochemical detection of CDNF. The proportion of neurons that were CDNF-immunoreactive in myenteric (Fig. 1a-c; 18.0 ± 1.5 %; n = 6) was less than that in submucosal ganglia (Fig. 1g-i; 23.1 ± 0.9 %; n = 6; p = 0.015). Further immunocytochemical studies were carried out with markers for enteric glia and interstitial cells of Cajal (ICC) to determine whether cells that interact with neurons in the gut are also immunoreactive for CDNF. To examine the full set of enteric glia comprehensively, we examined mice (ages P0-P56) that express EGFP under the control of the promoter for PLP-1 (Rao et al., 2015; Rao et al., 2017). Frozen sections were employed to permit regions of the bowel wall, such as the mucosa, that are devoid of neuronal perikarya to be investigated.

No PLP-1-EGFP expressing glial cells were found to be CDNF immunoreactive in any layer of the gut, although glial cells were frequently found enveloping CDNF-immunoreactive neurons within ganglia (Fig. 2a, Figure 2i,j). ICC were demonstrated with antibodies to the KIT proto-oncogene receptor tyrosine kinase at E17 (Fig. 2b) (Torihashi, Ward, & Sanders, 1997; J. J. Wu, Rothman, & Gershon, 2000). Although the subset of ICC that surround myenteric ganglia (ICC-my) were frequently observed in close proximity to CDNF-immunoreactive neurons, no ICC were ever found to express CDNF immunoreactivity (Fig. 2b). To determine whether the smooth muscle layers of the bowel exhibit CDNF-immunoreactivity, cross sections of gut from 1–2-month-old PLP-EGFP mice were immunostained with antibodies to γ smooth muscle actin, which is a smooth muscle marker together with antibodies to CDNF. Neurons were again found to be CDNF-immunoreactive, but no CDNF immunoreactivity was observed in smooth muscle (Fig. 2i). No

immunostaining was observed in the absence of antibodies to CDNF (Fig.2j). These observations suggest that CDNF expression in the bowel is restricted to neurons. Most dopaminergic neurons, which are primarily submucosal (Li et al., 2004), were CDNF-immunoreactive (Fig. 2c-e; 76.3 ±3.9%; n = 7); however, only a subset of CDNF-immunoreactive neurons in the SMP were dopaminergic (25.3 ± 1.6 %; n = 7). CDNF immunoreactivity in the gut, therefore, is not restricted to enteric dopaminergic neurons, even though the majority of dopaminergic neurons are CDNF-immunoreactive. Interestingly, even those dopaminergic neurons that lacked CDNF immunoreactivity (22.6± 4%, n=7) were often located in close proximity to CDNF-immunoreactive neurons (Fig. 2f-h). These data suggest that CDNF is located in subsets of enteric neurons of both plexuses.

CDNF is essential for normal development and maintenance of enteric dopaminergic neurons

Experiments were carried out to determine whether CDNF is essential for normal development of enteric dopaminergic neurons. Dopaminergic markers in the ENS of *Cdnf* $^{+/+}$ mice were compared to those of *Cdnf* $^{-/-}$ animals at 3 different ages. Because enteric dopaminergic neurons are born perinatally (Chalazonitis et al., 2008), we examined 3 dopaminergic neuronal markers in the bowel at P7 (Fig.3). These markers included the dopamine transporter (DAT), which all mature dopaminergic neurons express (Fig. 3a), dopamine itself (Fig. 3b), and the biosynthetic enzyme, TH (Fig. 3c). Although lymphocytes have been reported to express DAT (Mackie et al., 2018), enteric DAT immunoreactivity in the mouse has been found to be coincident with TH and dopamine in neurons and significant DAT immunoreactivity has not been observed in enteric lymphocytes (Li et al., 2004). The abundance of transcripts encoding DAT in the stomach and colon were compared to that in the Ileum (Fig. 3a), where dopaminergic neuronal density is highest (Li et al., 2004). The abundance of transcripts encoding DAT at P7 in the ileum of *Cdnf* $^{+/+}$ mice, was substantially higher than that in the stomach (by 83-fold) and colon (by 35-fold) (Fig. 3a). The abundance of transcripts encoding DAT in the ileum, moreover, was on average 3.5-fold lower in *Cdnf* $^{-/-}$ mice than in *Cdnf* $^{+/+}$ animals. Although no significant differences were found in the abundance of DAT transcripts in the stomach and colon of *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ animals, the transcript levels in these regions of the bowel were too low to make the comparisons meaningful. Because DAT transcripts are deficient in the ileum of mice lacking CDNF, these observations are consistent with the possibility that CDNF is essential for normal development or survival of enteric dopaminergic neurons.

If dopaminergic neuronal development were to be defective in the gut of postnatal *Cdnf* $^{-/-}$ mice, then it would also be anticipated that dopaminergic neuron numbers would be diminished in the adult ENS. Immunocytochemical studies verified the coincidence of dopamine and TH in neurons in 9-month-old mice. The two markers were fully coincident in all neurons detected (485 submucosal neurons of *Cdnf* $^{+/+}$ and 242 submucosal neurons of *Cdnf* $^{-/-}$ mice). Significantly, numbers of dopamine- and TH-immunoreactive neurons were deficient in *Cdnf* $^{-/-}$ mice (Fig. 3b-e). The density of dopaminergic neurons was 52% lower in *Cdnf* $^{-/-}$ animals (6.4 ± 0.4/mm^2) than in *Cdnf* $^{+/+}$ mice (13.3 ± 0.5/mm^2; p = 0.0001). In summary, enteric dopaminergic neurons were deficient by P7 in *Cdnf* $^{-/-}$ animals and this deficiency persisted as animals aged.

We analyzed the effect of CDNF deletion on the density and proportion of dopaminergic neurons in the ileal submucosal plexus as a function of age (Fig. 4, a-h). We used the TH-immunoreactivity of nerve cell bodies, which is coincident with that of dopamine (see above, Fig. 3 b-e), as a marker for dopaminergic neurons and HuC/D-immunoreactivity as a marker to detect total neurons. In both *Cdnf*$^{+/+}$ and *Cdnf*$^{-/-}$ mice the density of dopaminergic neurons increased between ages 1.5 and 3 months but then declined significantly between 3 and 11 months (Fig. 4a). The total number of enteric neurons has previously been demonstrated to increase through the first 4 months of life and to decline thereafter (Liu, Kuan, Wang, Hen, & Gershon, 2009). The density of dopaminergic neurons in *Cdnf*$^{-/-}$ mice, however, was significantly less than that in *Cdnf*$^{+/+}$animals at each age examined (Fig. 4a). These findings are consistent with the idea that CDNF plays a role early in development of dopaminergic neurons *in vivo* and that loss of such neurons in *Cdnf*$^{-/-}$ animals persists in adulthood. Because total neuron density changes as a function of age, dopaminergic neurons were also quantified as a proportion of total neurons (Fig. 4b). Strikingly, at each age examined, the proportions (Fig. 4b) were significantly lower in *Cdnf* $^{-/-}$ mice (Fig. 4d, f, h) than in *Cdnf* $^{+/+}$animals (Fig. 4c, c, c). These findings indicate that irrespective of the genotype, dopaminergic neurons are lost at a substantially faster rate than the non-dopaminergic neuronal population. Even though there is a relative increase in the proportion of dopaminergic neurons between 1.5 and 3 months in *Cdnf*$^{-/-}$ mice (Fig 4b), this change does not persist and the difference between *Cdnf* $^{+/+}$ and *Cdnf*$^{-/-}$mice widens again and is substantial by 11 months of age. To determine whether CDNF affects long-term maintenance of dopaminergic neurons, their proportions were compared between 3-month-old and 11-month-old animals. Between these ages, the decline in the proportion of dopaminergic neurons was significantly greater in *Cdnf* $^{-/-}$ (6.6 ± 0.4% n=69) than in *Cdnf* $^{+/+}$ mice (4.5 ± 0.6%; n = 60; p= 0.0057). Overall, these data suggest that even though enteric dopaminergic neurons are dependent on CDNF relatively early in development, they are also more vulnerable to age than the totality of enteric neurons and thus CDNF appears to protect them from aging.

To determine whether observations made in the ileum also pertain to other regions of the small intestine, dopaminergic neuronal density and proportion were also assessed in the jejunum. The number and proportion of dopaminergic neurons were each lower in *Cdnf* $^{-/-}$ than in *Cdnf* $^{+/+}$ mice at 1.5 months of age. The number of dopaminergic neurons in *Cdnf* $^{-/-}$ mice was 8.6 ± 0.7 per mm^2; n = 28, while that in *Cdnf* $^{+/+}$ animals was 13.9 ± 0.8 per mm^2; n =35 (p = 0.0001). The proportion of dopaminergic neurons in *Cdnf* $^{-/-}$ mice was 5.7 ± 0.5%; n = 27 while that in *Cdnf*$^{+/+}$ mice was 8.4 ± 0.5%; n = 27 (p = 0.0006). These observations confirm that the CDNF requirement of enteric dopaminergic neurons for normal development and/or survival is not restricted to the ileum.

Specificity of CDNF dependence in the ENS

Our findings establish that CDNF is essential for the normal development/survival of submucosal dopaminergic neurons and is relatively specific in this regard. The ENS however contains a large variety of neurotransmitter phenotypes. We therefore determined whether additional classes of neurons are affected by the loss of CDNF. Non-dopaminergic phenotypes of enteric neuron were examined in the myenteric and submucosal plexuses of

the ileum of 11-month-old mice. Enteric neurons of different phenotypes are born in a reproducible order (Bergner et al., 2014; Chalazonitis et al., 2008; Pham, Gershon, & Rothman, 1991). Serotonergic neurons are among the earliest born, while neurons of other phenotypes are born at later ages and their genesis is, in part, 5-HT-dependent (M. D. Gershon, 2013). Neurons in both early (5-HT)- and later-born categories (nNOS, GABA, CGRP) were selected for study to determine whether CDNF affects them differently. Three mice of each genotype were analyzed; ANNA-1 immunoreactivity was employed as a neuronal marker.

Total density of submucosal neurons was found to be significantly lower ($p \leq 0.0001$) in 11-month-old *Cdnf* $^{-/-}$ mice than in *Cdnf* $^{+/+}$ animals of the same age (Fig. 5a). In contrast, no difference was seen as a function of genotype in numbers of myenteric neurons. Within the submucosal plexus, significant differences between *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice were found in the proportion of neurons of each phenotype examined. *Cdnf* $^{-/-}$ animals had significantly lower proportions of nNOS-, GABA-, and CGRP-expressing neurons, as well as the expected lower proportion of dopaminergic neurons (Fig. 5b; e-g). These observations indicated that different phenotypes of submucosal neuron differ in their dependence on CDNF. Although the proportions of the subtypes examined were all less in *Cdnf* $^{-/-}$ mice than in *Cdnf* $^{+/+}$ animals, the proportion of other submucosal neurons, which were not individually identified, was actually a greater proportion of the total in *Cdnf* $^{-/-}$ than in *Cdnf* $^{+/+}$ mice (Fig. 5c). Interestingly, dopaminergic neurons had the greatest relative dependence on CDNF and thus demonstrated the largest difference between *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice. In contrast to the submucosal plexus, which was highly sensitive to CDNF, the myenteric plexus was relatively CDNF-insensitive; the proportions of each phenotype of myenteric neuron that was studied were not significantly different in *Cdnf+/+* and *Cdnf−/−* mice (Fig. 5d; h-j). Even the relatively rare myenteric dopaminergic neurons were not significantly different in density in *Cdnf* $^{+/+}$ (3.5 ± 0.6, n = 35) and *Cdnf* $^{-/-}$ mice (2.9 ± 0.3; n=20; $p = 0.4145$). Similarly, neurons marked by nNOS and GABA immunoreactivities were CDNF-sensitive in the submucosal plexus and CDNF-insensitive in the myenteric plexus. These data support the idea that the effects of CDNF are restricted to the submucosal plexus but are not limited to dopaminergic neurons; moreover, neurons that express the same neurotransmitter differ in their sensitivity to CDNF depending on whether they are submucosal or myenteric. Interestingly, each of the phenotypes of enteric neuron that were found to be CDNF-dependent were in the later born category. Early born serotonergic neurons were CDNF-resistant, but these neurons are all myenteric.

Gastrointestinal (GI) transit is abnormal in *11-month-old Cdnf* $^{-/-}$ mice

GI transit was investigated as a function of age in *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice to determine whether measurable functional abnormalities accompany the submucosal abnormalities found in *Cdnf* $^{-/-}$ animals, including smaller than WT numbers of neurons as well as the neuronal degeneration and autophagy reported previously (Lindahl et al., 2020). Gastrointestinal transit time (GITT), gastric emptying, and colonic bead expulsion time, were evaluated. GITT tends to increase in mice as the gut slows as a function of age and, in fact, we found that GITT of 3-month-old *Cdnf* $^{+/+}$ mice (234 ± 48 min, $n = 6$) was significantly shorter than that of 11-month-old *Cdnf* $^{+/+}$ animals (407 ± 55 min, n = 6). The

effect of age on GITT, however, was significantly greater in 11-month-old *Cdnf* $^{-/-}$ mice (647 ± 35 min, $n = 6$; $p = 0.0056$) than in the 3-month-old *Cdnf* $^{-/-}$ animals (264 ± 48, $n = 6$) (Fig. 6a). To account for the age-related slowing of intestinal transit, parameters for *Cdnf* $^{-/-}$ mice were normalized to those of *Cdnf*$^{+/+}$ animals of the same age. Normalized GITT did not differ between *Cdnf*$^{+/+}$ and *Cdnf*$^{-/-}$ in 3-month-old mice; however, the normalized GITT was significantly longer in older, 11-month-old *Cdnf* $^{-/-}$ than in *Cdnf*$^{+/+}$ mice of the same age (Fig. 6b). Colonic motility was estimated from the time required to expel a glass bead inserted into the rectum. Again, data from *Cdnf*$^{-/-}$ mice were compared to those from simultaneously analyzed *Cdnf*$^{+/+}$ animals of the same age. Bead expulsion in young *Cdnf*$^{+/+}$ and *Cdnf* $^{-/-}$ mice did not differ significantly; however, beads were expelled significantly more slowly in 11-month-old *Cdnf* $^{-/-}$ than in *Cdnf*$^{+/+}$ mice of the same age (Fig. 6c; $p = 0.0251$). Finally, the rate of gastric emptying did not differ significantly between 3-month-old *Cdnf*$^{+/+}$and *Cdnf* $^{-/-}$ mice; nevertheless, the rate of gastric emptying was significantly slower in 11-month-old *Cdnf*$^{-/-}$mice than in *Cdnf*$^{+/+}$mice of the same age (Fig. 6d). Despite the difference in parameters of GI motility body weight in 11-month-old in *Cdnf* $^{-/-}$ mice (35.13 ± 2.2g, n=7) was not significantly different (p = 0.6606) from that of their *Cdnf* $^{+/+}$ littermates (36.51 ± 2.14, n = 7).

Enteric neural crest-derived cells (ENCDC) express CDNF and Foxa2 at E16

We have found that transcripts encoding CDNF are expressed postnatally in the human and murine bowel (Lindahl et al., 2020; Lindholm et al., 2007). Because enteric dopaminergic neurons are already deficient in 1.5-month-old *Cdnf* $^{-/-}$ mice (see Fig. 4), it is possible that CDNF is not only neuroprotective for enteric dopaminergic neurons, but also trophic for their development. We utilized *in vitro* experiments to test the hypotheses that exogenous CDNF enhances the development and survival of enteric dopaminergic neurons. The peak birthdate for enteric dopaminergic neurons occurs at E16 in mice but enteric dopaminergic neurogenesis continues to be active at least through P3 (Bergner et al., 2014; Chalazonitis et al., 2008). We therefore first determined whether CDNF is produced in the fetal gut (E16) and whether the ENCDC that give rise to enteric neurons express it. Positive and negative selection with antibodies to p75^{NTR} were used to separate ENCDC and non-crest-derived cells from fetal bowel at E16 (Fig. 7a). RT-PCR was employed to identify transcripts encoding markers for neural crest (p75^{NTR}) (Huber & Chao, 1995), non-crest (Hand1) (D'Autreaux, Morikawa, Cserjesi, & Gershon, 2007), dopaminergic neurons (Foxa2 [forkhead box A2]) (Ferri et al., 2007; Kittappa, Chang, Awatramani, & McKay, 2007; Stott et al., 2013), and CDNF. Transcripts encoding CDNF were detected in isolated ENCDC, along with transcripts encoding p75^{NTR} and Foxa2 (Fig. 7a). In contrast, transcripts encoding CDNF were not detected in non-ENCDC, which contained transcripts encoding Hand1, but not those encoding either p75NTR or Foxa2 (Fig. 7a). In comparison, transcripts encoding each of these markers were found in cDNA prepared from whole organs (adult midbrain and ileum), which were examined as positive controls.

To investigate the time-action relationship for the effect of CDNF, cultures were maintained for up to 8 days (4–15 replicates per condition) and were examined at 2-day intervals (Fig 7b-f). The numbers of total and dopaminergic neurons were assessed as a function of time in culture with or without CDNF treatment. Antibodies to HuC/D, were used to define cells as

neurons, while dopaminergic neurons were identified as cells that were triply labeled with antibodies to HuC/D, Foxa2, and TH (Fig. 7d-f). Some cells were observed that expressed Foxa2 but not TH. Such cells were not considered to be fully differentiated dopaminergic neurons. The abundance of developing total neurons treated with vehicle increased significantly through day 8 ($p < 0.0001$) although the increase was rapid through day 4 and remained relatively stable thereafter. The time-dependent increase in the number of total neurons was not significantly different in cultures exposed to CDNF from that in cultures exposed only to vehicle (Fig. 7b). In contrast to total neurons, the addition of CDNF enhanced the development/survival of dopaminergic neurons; far more of these neurons were present in cultures exposed to CDNF at all time points than in cultures exposed to vehicle (Fig. 7c; $p < 0.0001$ on day 8). Dopaminergic neuronal development was rapid and evident by the second day in vitro. A modest but significant increase over vehicle ($p < 0.03$) occurred in the presence of CDNF between days 2 and 8. Interestingly, a significant decline in numbers of dopaminergic neurons occurred between days 4 and 8 in cultures exposed to vehicle ($p < 0.05$) that did not occur in cultures exposed to CDNF (Fig 7c). The ability of CDNF to enhance dopaminergic neuronal development and also to prevent their time-related decline in culture suggests that CDNF promotes both the development and the survival of enteric dopaminergic neurons.

CDNF selectively promotes *in vitro* development/survival of dopaminergic neurons from ENCDC

Enteric neurons develop in a complex environment where ENCDC interact with non-ENCDC. We therefore compared the concentration-effect relationship for CDNF promotion of the differentiation/survival of total and dopaminergic neurons in cultures of isolated ENCDC (Fig 8a, b and f-k) with those in cultures of mixed cells from dissociated whole gut (Fig. 8c and d, e). This strategy allows the influence of the non-ENCDC on the differentiation/survival of the ENCDC to be assessed. HuC/D immunoreactivity was again used to identify total neurons (Fig. 8b, j, k) and TH immunoreactivity was used as a marker for dopaminergic neurons (Fig. 8a, d, e, g). Coincident labeling of TH-immunoreactive cells with antibodies to DAT confirmed that these cells were dopaminergic (Fig. 8f-i). The numbers of dopaminergic neurons developing in the presence of CDNF (8 replicate cultures at each concentration) were normalized to those developing in the presence of vehicle for both types of culture. The development of dopaminergic neurons was a concentration-dependent function of CDNF in cultures of isolated ENCDC ($p < 0.05$; Fig. 8a). In contrast to the dopaminergic subset, the development of total neurons was independent of the concentration of CDNF (Fig. 8b and j, k). At maximum efficacy, which was reached at 100 ng/ml, the proportion of dopaminergic neurons was 15 ± 2.3 % (n = 26, pooled data) and 2.5-fold greater than that found in cultures exposed only to vehicle ($p = 0.0011$). At this concentration of CDNF, the total number of neurons/culture in the presence (12,634 ± 2021) or absence (11,009 ± 1651) of CDNF did not differ significantly ($p = 0.2406$). When mixed cultures containing non-ENCDC were analyzed instead of $p75^{NTR}$-selected crest-derived cells, CDNF again promoted development of dopaminergic neurons (Fig. 8c); however, the effect of CDNF saturated at 1 ng/ml, the lowest concentration tested. Higher concentrations of CDNF did not significantly enhance its effect. The difference between immunoselected ENCDC and mixed cultures in the concentration-effect relationship of CDNF promotion of

the development/survival of enteric dopaminergic neurons suggests that the non-crest-derived cells in the mixed cultures interact with and alter the action of CDNF. These observations emphasize the importance of isolating ENCDC to study the effects of CDNF or other molecules on their development/survival.

Ability of GDNF and 5-HT to alter the actions of CDNF

Because the ability of CDNF to promote development of enteric dopaminergic neurons is altered by the presence of other types of cell, experiments were carried out to compare the actions of CDNF with specific growth factors and neurotransmitters that such cells might secrete, both alone and in combination. GDNF and 5-HT were studied initially as examples of factors secreted, respectively by the mesenchyme and neurons. GDNF, which is expressed by enteric mesenchymal cells (Natarajan, Marcos-Gutierrez, Pachnis, & de Graaff, 2002), is essential for ENS development (Pichel et al., 1996; Schuchardt et al., 1994) and stimulates proliferation of precursor cells as well as development and survival of neurons (Chalazonitis, Rothman, Chen, & Gershon, 1998; Gianino, Grider, Cresswell, Enomoto, & Heuckeroth, 2003; Hearn, Murphy, & Newgreen, 1998; Heuckeroth, Lampe, Johnson, & Milbrandt, 1998). GDNF also interacts strongly with other growth factors, such as NT-3 (Chalazonitis, Rothman, Chen, & Gershon, 1998) and endothelin-3 (Barlow, de Graaff, & Pachnis, 2003; J.J. Wu, Chen, Rothman, & Gershon, 1999). Serotonergic neurons are among the first to arise in the mammalian ENS (Pham et al., 1991) and the 5-HT they secrete is essential for the development of many of the neurons that follow them in the sequence of enteric neurogenesis (Z. Li et al., 2011; Margolis et al., 2016). 5-HT is already known to be a neurotransmitter that stimulates development of enteric dopaminergic neurons.

Experiments were carried out in culture to evaluate the promotion of total neurons, dopaminergic neurons, and the proportion of dopaminergic neurons. ENCDC were isolated from the gut at E15 and 8–16 replicate cultures were exposed for 6 days either to vehicle, CDNF (100 ng/ml), GDNF (50 ng/ml), CDNF+GDNF, 5-HT (1.0 μM) or 5-HT+CDNF. Whenever 5-HT was studied, PCPA was added to the culture medium to prevent endogenous 5-HT from interfering with the experiments. CDNF again failed to affect the total number of neurons in cultures of isolated ENCDC. In contrast, GDNF increased the total number of neurons significantly (> 2-fold compared to vehicle or CDNF) and, as anticipated, the effect of co-treatment with CDNF and GDNF (Figs. 9a, j) was not significantly different from that of GDNF alone. (Figs. 9a, h, i). The numbers of dopaminergic neurons (as % vehicle) were significantly greater in the presence of CDNF (3 - fold increase) (Fig. 9b, h) or GDNF (12-fold increase) (Figs. 9b, i) than in vehicle (Fig. 9g), demonstrating that the impact of GDNF on dopaminergic neurons was significantly greater than that of CDNF (Fig. 9b). Again, including both factors together significantly increased dopaminergic development but this effect was not significantly different from that of GDNF alone (Figs 9b, j). The CDNF- and GDNF-induced increases in the numbers of dopaminergic neurons could reflect either a selective effect on such neurons or an expansion of the whole population. To distinguish between these two possibilities, the proportion of dopaminergic neurons was also expressed as a percent of total neurons (Fig. 9c). The proportion of neurons that were dopaminergic was greater in cultures exposed either to CDNF or GDNF than in vehicle (GDNF>CDNF); however, the proportion of dopaminergic neurons in cultures exposed to the combination of

GDNF and CDNF was not significantly different from that in cultures exposed to GDNF alone. These observations demonstrate that the actions both of CDNF and GDNF on dopaminergic neuronal development/survival are relatively selective; however, the effects of the two factors are not additive.

5-HT, like GDNF, powerfully increased the total number of neurons (as % of vehicle) developing in culture (Fig. 9d). Interestingly, a small but significant additional increase in total neurons was found in cultures exposed both to 5-HT and CDNF. The proportions of dopaminergic neurons (as % vehicle) were again greater in cultures exposed to CDNF or 5-HT (Fig. 9e). Although more dopaminergic neurons were found in cultures exposed to the combination of 5-HT and CDNF than in cultures exposed to either factor alone, this apparent enhancement of dopaminergic development/survival did not reach statistical significance. The proportion of neurons that were dopaminergic (Fig. 9f), moreover, was greatest when CDNF was present in the culture medium. The combination of CDNF with 5-HT produced a significantly higher proportion of dopaminergic neurons than vehicle, but not a proportion that was significantly different from that found in cultures exposed only to CDNF. In contrast to GDNF, 5-HT, therefore, did not selectively enhance dopaminergic neuronal development. Instead, 5-HT stimulated the development of the entire population of neurons, not only that of the dopaminergic subset (Fig. 9f).

BMPs synergistically enhance the action of CDNF and induce CDNF-dependence

In addition to receptors for GDNF and 5-HT, ENCDCs express subtypes of BMP receptor (IA, IB, and II) that respond to BMP2 and BMP4. The effects of these BMPs on in vitro enteric neuronal development are strikingly concentration-dependent. At optimal concentrations (~20ng/ml), BMPs enhance dopaminergic neuronal development and cause the precocious acquisition of dependence on other growth factors. Consequently, survival of BMP-treated neurons declines unless these factors are supplied (Chalazonitis et al., 2004). We therefore tested the hypotheses that exposure of ENCDCs to BMP2 or 4 enhance the response of ENCDCs to CDNF and that these BMPs induce ENCDCs to become CDNF-dependent. Because continuous long-term exposure of neuronal progenitors to BMPs is capable of provoking apoptosis (Chalazonitis et al., 2004; Mabie, Mehler, & Kessler, 1999), the experimental paradigm was to expose ENCDCs to vehicle (not illustrated), CDNF (100 ng/ml), BMPs alone (1 or 20 ng/ml), or BMPs plus CDNF (100 ng/ml), for the initial 4 days of a total of 7 days of culture, (Fig. 10a, b). Dopaminergic neurons were identified by their coincident expression of DAT and TH (Fig. 10c-k) while total neurons were visualized with antibodies to HuC/D (not illustrated). As in previous experiments, the total number of neurons was not significantly different in cultures exposed to CDNF or vehicle. In contrast, transient exposure to BMP4 alone (20 ng/ml, but not 1.0 ng/ml) significantly increased the total number of neurons (Fig.10a). Addition of CDNF to cultures exposed to the higher concentration of BMP4 (20 ng/ml) did not further enhance the development of total neurons (Fig. 10a). The proportion of dopaminergic neurons (as % vehicle) was enhanced in the presence of BMP4 alone (Fig. 10b; 20 ng/ml, but not 1.0 ng/ml) for the first 4 days. The effect of BMP4 on dopaminergic neuronal development/survival was greater than that of CDNF alone (Fig. 10b); however, an initial exposure to BMP4, when combined with CDNF, acted synergistically to produce a much greater enhancement of dopaminergic neuronal

development than the sum of the individual effect of BMP4 and CDNF (Fig. 10b, i-k compare with c-h). When effects on dopaminergic neurons were expressed as a proportion of total neurons, the BMP4 synergism with CDNF was evident as an increase that was 16-fold that seen in cultures exposed only to BMP4 (20 ng/ml; 6.2-fold) or CDNF (3.2-fold). Again, the effects of the combination of CDNF with BMP4 were greater than the sum of the effects of the individual factors (Fig. 10b, i-k compare with c-h). The extension of dopaminergic neurites was also greater in cultures exposed to BMP4 (20 ng/ml) or to the combination of CDNF with BMP4 (Fig. 10j, k) than in those exposed to CDNF alone (Fig. 10d, e). These observations support the idea that transient BMP4 exposure enhances the ability of CDNF to promote dopaminergic neuronal development. Similar results were obtained with BMP2 at the same concentrations (data not illustrated).

To determine whether BMP4 exposure causes dopaminergic neurons to become dependent on CDNF, we investigated the effects of the withdrawal of CDNF from cells that had been transiently exposed to it. Cultures of ENCDC were incubated either with vehicle or with CDNF for 7 days, or with CDNF ± BMP4 (20 ng/ml) for the first 4 days in culture, as described above, and then incubated for days 5–7 either with continued CDNF or with vehicle (withdrawal). Cells undergoing apoptosis were identified immunocytochemically with antibodies to phospho-histone 2A.X (pH2A.X) (Holubec et al., 2005; Lu et al., 2006; Rogakou, Nieves-Neira, Boon, Pommier, & Bonner, 2000) and dopaminergic neurons were identified with antibodies to TH. The proportion of the dopaminergic neuronal population undergoing apoptosis was significantly lower in cultures exposed continuously for 7 days to CDNF than in cultures exposed continuously to vehicle (Fig. 11a). This observation suggests that CDNF alone enhances survival of dopaminergic neurons. When cultures were transiently exposed to BMP4 (20 ng/ml) but continuously incubated in the presence of CDNF, the proportion of dopaminergic neurons undergoing apoptosis was significantly lower ($p < 0.0311$) than in cultures exposed continuously only to CDNF, suggesting that BMP4 exposure increases the survival effect of CDNF (Fig. 11a-c). Again, the enhanced neurite outgrowth seen in cultures exposed to BMP4 was evident (Fig. 11b). When CDNF was withdrawn for the final 3 days in culture, the proportion of dopaminergic neurons undergoing apoptosis increased significantly over that seen in the continued presence of CDNF. These findings indicate that dopaminergic neurons that had been exposed to BMP4 and CDNF became dependent on the continued presence of CDNF for survival (Fig. 11a, d, e).

4) Discussion

We tested and confirmed the hypothesis that the newly discovered neurotrophic factor, CDNF, plays an essential role in the development of the ENS. In a previous study, we have shown that CDNF is expressed in the bowel and that its deletion is associated with neurodegeneration and a decreased density of neurons in the submucosal plexus, thus providing support for this idea (Lindahl et al., 2020). We now report that enteric CDNF is found in both myenteric and submucosal plexuses but is restricted to subsets of neurons within each. The immunoreactivity of CDNF, moreover, is lacking in *Cdnf* $^{-/-}$ mice confirming the specificity of the antibodies used for immunostaining. Neither enteric glia, ICCs, nor enteric smooth muscle, express CDNF. The lack of expression of CDNF in enteric

glia is consistent with our previous observation that the density of enteric glia in the ENS of *Cdnf*- $^{-/-}$ is not significantly different from that in *Cdnf*$^{+/+}$ mice (Lindahl et al., 2020) and with reported results of RNA sequencing of cells from the ENS (Zeisel et al., 2018). A role for CDNF, in the expression of dopaminergic neurons, is indicated in the observations that transcripts encoding DAT as well as dopaminergic neurons are significantly less abundant in the ileum of *Cdnf* $^{-/-}$ than in that of *Cdnf* $^{+/+}$mice. The deficiency in density of dopaminergic neurons is age-dependent. Although the numbers of these neurons decline as a function of age, even in *Cdnf* $^{+/+}$ animals, dopaminergic neurons are less abundant in *Cdnf* $^{-/-}$ than in *Cdnf* $^{+/+}$ mice as early as 1.5-months of age and the relative *Cdnf*$^{-/-}$ to *Cdnf* $^{+/+}$ deficit in dopaminergic neurons persists as animals age and is quite severe in mice at 11 months of age. Despite the presence of CDNF-expressing neurons in both plexuses, the dopaminergic neuronal deficiency associated with the deletion of CDNF occurs only in the submucosal plexus where dopaminergic neurons are most numerous; moreover, the defect is not restricted to the dopaminergic neuronal phenotype. Other submucosal neurons are affected as well, and abnormally low numbers of nitrergic, gabaergic, and CGRP-expressing neurons are seen in *Cdnf* $^{-/-}$ mice. It is of interest that transcripts encoding CDNF have been reported to be expressed in some nitrergic and cholinergic neurons of the mouse gut (http://mousebrain.org) and that most submucosal CGRP-containing neurons co-express choline acetyltransferase and thus are cholinergic (Mongardi Fantaguzzi, Thacker, Chiocchetti, & Furness, 2009). It is thus possible that these non-dopaminergic types of submucosal neuron also contain and respond to CDNF, at least for their maintenance. Neurons that express CDNF might respond to it as a trophic factor or as a survival factor that protects them, for example, from endoplasmic reticulum stress (Lindahl et al., 2017; Voutilainen et al., 2017). Neurons that do not express CDNF might respond to it as a trophic factor. Each of the CDNF-dependent neuronal phenotypes, including dopaminergic neurons, have in common that they are born after the generation of serotonergic neurons (Bergner et al., 2014; Chalazonitis et al., 2008; Pham et al., 1991) and that their normal development depends on neuronal 5-HT (Z. Li et al., 2011; Margolis et al., 2016). *In vitro* studies, however, suggest that the effects of CDNF are independent of 5-HT and, in contrast to 5-HT, submucosa-specific. Because the submucosal plexus also contains neurons that are not-CDNF-dependent, the relative proportions of the CDNF-insensitive neurons actually increase in the hypoplastic submucosal plexus of *Cdnf* $^{-/-}$ mice. The defect in *Cdnf* $^{-/-}$ animals is not dependent on the phenotype of affected neurons; for example, myenteric neurons that express nNOS and GABA are present in equivalent numbers in *Cdnf* $^{-/-}$ and *Cdnf* $^{+/+}$mice even though they are deficient only in the *Cdnf* $^{-/-}$ submucosal plexus. It is conceivable that submucosal neurons are CDNF-dependent because they are more subject to stress than myenteric neurons. The proximity of the projections of most submucosal neurons to the luminal barrier epithelium and the absence of a blood-tissue barrier around the submucosal plexus analogous to that which protects myenteric neurons (M.D. Gershon & Bursztajn, 1978) might each expose submucosal neurons to stress. In fact, the enteric microbiome has been found to attract the migration of enteric glial cells to the mucosa (Kabouridis et al., 2015), supporting the idea that the submucosal plexus is more subject to luminal influences than the myenteric. The need for neuroprotection might thus account for the evolution of the abundance of submucosal CDNF-immunoreactive neurons as well as for the expression of CDNF within enteric dopaminergic neurons, which are primarily submucosal.

Importantly, the CDNF-dependence of the development/survival of subsets of submucosal neurons is reflected functionally in the transit of the bowel. The deletion of CDNF results in an age-dependent slowing of total gastrointestinal transit, gastric emptying, and colonic motility. It is interesting that transit should be affected by the absence of CDNF even though myenteric neurons, which directly innervate the intestinal smooth muscle, are spared in *Cdnf* $^{-/-}$animals. The submucosal plexus is thought to be principally concerned with the regulation of secretion, mucosal blood flow, and epithelial proliferation (Cooke & Christofi, 2006; Furness, Callaghan, Rivera, & Cho, 2014; Gross, Gershon, Margolis, Gertsberg, & Cowles, 2012; Vanner, Jiang, & Surprenant, 1993). It is possible that the age-related deficiency of submucosal neurons in *Cdnf* $^{-/-}$ mice interferes with secretory mechanisms and thus indirectly affects transit. On the other hand, the submucosal plexus contains intrinsic primary afferent neurons (IPANs) that are crucial to peristaltic reflexes (Bülbring, Lin, & Schofield, 1958; Jiang, Kirchgessner, Gershon, & Surprenant, 1993; Pan & Gershon, 2000). Some of these cells project to the myenteric plexus (Kirchgessner & Gershon, 1988) and CGRP, which is affected in *Cdnf* $^{-/-}$animals, is a transmitter of IPANs that have been linked to the peristaltic reflex (J.R Grider, 1994; J.R. Grider, 2003; Pan & Gershon, 2000). The neurodegeneration and excessive autophagy that occurs in *Cdnf* $^{-/-}$ mice (Lindahl et al., 2020) might also negatively affect transit (Hernandez et al., 2012); autophagy in dopaminergic neurons has been shown to affect transmitter release, which if significant in these and other neurons, could interfere with the neuronal coordination necessary for propulsive GI transit. Interestingly, the age-dependence of the abnormally slow GI transit in aged mice lacking CDNF supports the idea that the neuroprotective effect of CDNF helps to maintain the ENS..

Experiments carried out with ENCDC isolated from E16 murine bowel implied that these cells selectively express CDNF. Transcripts encoding CDNF were found in positively selected ENCDC, together with transcripts expressing $p75^{NTR}$ and the dopaminergic marker, Foxa2, while each 3 of these transcripts were absent from negatively selected non-ENCDC. These observations are compatible with the idea that precursor committed to the dopaminergic neuronal phenotype are already present in the gut at E16, the age when the ENCDC were first isolated. The expression of transcripts encoding Foxa2 by ENCDC has not previously been reported. Foxa2 plays major roles in development of midbrain dopaminergic neurons, including specification, fate-determination, and maturation (Gale & Li, 2008; Kittappa et al., 2007; Stott et al., 2013; Veenvliet & Smidt, 2014). The expression of Foxa2 in ENCDC suggests that dopaminergic neurons of the ENS and CNS may share common regulatory pathways despite their very different locations and functions.

Exogenous CDNF concentration-dependently enhanced *in vitro* development of enteric dopaminergic neurons, which were both TH and Foxa2 co-immunoreactive, although CDNF was without effect on the total number of neurons developing from ENCDC *in vitro*. These observations suggest that CDNF can act as a soluble NTF and that it affects expression of the dopaminergic neural phenotype but not the total universe of enteric neurons. Results are thus compatible with *in vivo* observations showing that the absence of CDNF affects restricted sets of enteric neuron and that most dopaminergic neurons in the submucosal plexus express CDNF.

The concentration-effect relationship for the ability of CDNF to promote dopaminergic neuronal development was different in mixed cultures containing non-neuronal cells than in isolated ENCDC, suggesting that a cross-talk occurs between CDNF and factors derived from non-ENCDC. In fact, GDNF, which is secreted in the developing bowel by mesenchymal cells, 5-HT, which is released from early-born enteric neurons, and BMPs, which are expressed both by neurons and by non-neuronal cells, all cooperated with CDNF to enhance dopaminergic neuronal development. 5-HT powerfully enhanced the genesis of total neurons although it did not selectively enhance dopaminergic development. GDNF strongly promoted dopaminergic neuronal development and did so to an extent that surpassed that of CDNF. The effects of CDNF were additive neither with those of 5-HT, nor GDNF. GDNF and CDNF can both activate survival-promoting AKT pathways (Airaksinen & Saarma, 2002; Voutilainen et al., 2017), while 5-HT also signals via G-protein coupled receptors (McCorvy & Roth, 2015). In contrast to CDNF, both GDNF and 5-HT (more so than GDNF under the conditions tested) increased total as well as dopaminergic neurons. When combined with 5-HT, however, CDNF significantly increased the development of total neurons over that seen with either 5-HT or CDNF alone. This interaction with 5-HT, which was not seen with GDNF, might be due to the ability of CDNF to enhance survival of neurons, which might be stressed by the high level of 5-HT-driven proliferation.

Transient exposure to BMP4 strikingly and selectively potentiated the efficacy of CDNF in promoting dopaminergic development; moreover, exposure to BMP4 induced dopaminergic neurons to become dependent on CDNF for survival. This observation could be explained if BMPs were to promote development of a receptor mediating the survival-promoting effect of CDNF. BMPs, for example, have been shown to enhance expression of the NT-3 receptor, TrkC, and also to induce NT3-dependence (Chalazonitis et al., 2004). Because enteric neurons are necessarily exposed to BMPs during in vivo development, it is likely that the late-born subsets of neurons that respond to CDNF are also CDNF-dependent. This likelihood raises the possibility that an acquired loss of CDNF contributes to the pathophysiology of functional GI disorders during adult life. BMPs continue to enhance development of enteric dopaminergic neurons during postnatal life, an effect that is regulated by the homeodomain interacting protein kinase-2 (Chalazonitis, Tang, et al., 2011). A plasmalemmal receptor responsible for the neurotrophic effects of CDNF has not yet been identified.

It is important to note that the effects of exogenous CDNF on cultured ENCDC that we observed in the current study are different from those reported for sensory, sympathetic, hippocampal, motor neurons, and fetal midbrain dopaminergic neurons, which do not respond *in vitro* to the addition of CDNF (Lindholm et al., 2007). It has therefore been argued that although CDNF is a secreted protein, it is primarily a resident protein of the endoplasmic reticulum (ER) that regulates the unfolded protein response and protects cells from ER stress (Sun et al., 2011; Voutilainen, Arumae, Airavaara, & Saarma, 2015; Voutilainen et al., 2017). Extracellular CDNF has been found to protect cultured cells other than ENCDC, but only after induction of ER stress or injury short of a physiological death stimulus (Latge et al., 2015; Zhou et al., 2016). In contrast, cultured ENCDC appear to respond to CDNF in a manner analogous to a classical NTF, exerting effects that are both trophic and protective. Uptake of exogenous CDNF has been reported following its infusion

into the striatum with retrograde transport to the substantia nigra (Matlik et al., 2017); therefore, uptake of CDNF might have contributed to its ability to promote dopaminergic neuronal development from ENCDC. Certainly, protection from ER stress could also contribute to the protective effects of CDNF on enteric neurons *in vitro* and *in vivo*. The neurons of the ENS might well be more stressed than their counterparts in the CNS and in other peripheral ganglia. Enteric neurons are constantly subjected to mechanical deformation due to the motility of the bowel and, particularly those of the submucosal plexus, are also subjected to the possible collateral effects of immune responses enlisted in defense against invasion from the enteric luminal microbiome.

Relatively little is known about the selection of neuronal phenotypes. Factors, such as GDNF, promote the development of all neurons, but other molecules, which act relatively late in development, such as NT-3 (Chalazonitis et al., 2008) and 5-HT (Li et al., 2011; Margolis et al., 2016), promote restricted late-born subtypes. Signaling through the 5-HT_4 receptor enables not only enteric neurons (Li et al., 2011; Margolis et al., 2016), but also enterochromaffin (EC) cells, to promote the development of enteric neuronal subsets (De Vadder et al., 2018; Margolis et al., 2016). The role of CDNF is unusual, not only because of its trophic and protective effects, but also because of its submucosal selectivity. CDNF is likely to be an important molecule in sculpting the nature of the ENS, in determining the proportions of various neuronal types and in the long-term maintenance of vulnerable neurons. Parkinson disease is one of several neurodegenerative disorders of the ENS (Braak & Braak, 2000; Braak, de Vos, Bohl, & Del Tredici, 2006; Chalazonitis & Rao, 2018; Rao & Gershon, 2016) and is one that affects, but is not limited, to dopaminergic neurons (Kuo et al., 2010). In fact the submucosal plexus is a site of pathological alpha-synuclein accumulation in Parkinson patients (Beach et al., 2016). Future studies are needed to determine whether a defect in CDNF (Choi et al., 2011) contributes to the pathogenesis of the enteric manifestations of Parkinson disease and to the possibility, now in phase 1–2 clinical trial, that CDNF can be used to treat it (Huttunen & Saarma, 2019; Latge et al., 2015; Voutilainen et al., 2015).

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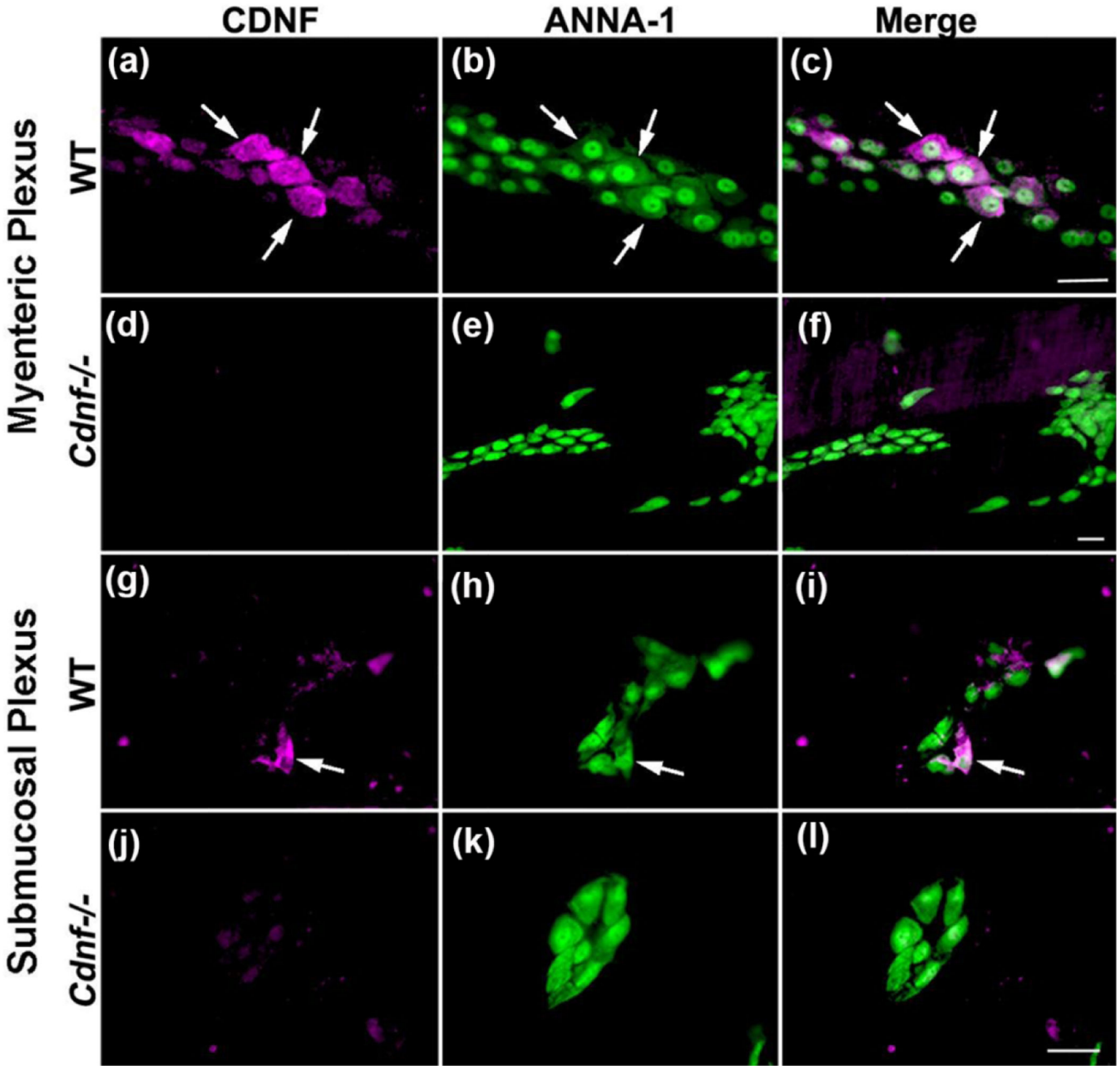

Figure 1. CDNF immunoreactivity is found in neurons of each enteric plexus.

Laminar preparations of bowel wall from the Ileum containing the myenteric (a-f) or submucosal plexuses (g-l) were dissected, immunostained to demonstrate the immunoreactivities of CDNF and the neuronal marker, ANNA-1, and viewed as whole mounts. Tissues from *Cdnf*$^{+/+}$ (WT) and *Cdnf*$^{-/-}$ mice (11-month-old) were compared to validate the antibodies to CDNF. **a-c**. A ganglion of the myenteric plexus of a *Cdnf*$^{+/+}$ mouse illuminated to reveal CDNF-immunoreactive cells (a, magenta), ANNA-1-immunoreactive cells (b, green), and the merged image (c). The arrows show the location of the same cells in each panel. The bar = 35 μm (a-c). **d-f**. A ganglion of the myenteric plexus of a *Cdnf*$^{-/-}$ mouse illuminated to reveal CDNF-immunoreactivity (d, magenta; no cells are

immunoreactive), ANNA-1-immunoreactive cells (e, green), and the merged image (f). The bar = 35 μm (d-f). **g-i**. A ganglion of the submucosal plexus of a *Cdnf*$^{+/+}$ mouse illuminated to reveal CDNF-immunoreactive cells (g, magenta), ANNA-1-immunoreactive cells (H, green), and the merged image (I). The arrow shows the location of the same cell in each panel. **j-l**. A ganglion of the submucosal plexus of a *Cdnf*$^{-/-}$ mouse illuminated to reveal CDNF-immunoreactive cells (j, magenta; no cells are immunoreactive), ANNA-1-immunoreactive cells (k, green), and the merged image (l). The bar = 35 μm (g-l).

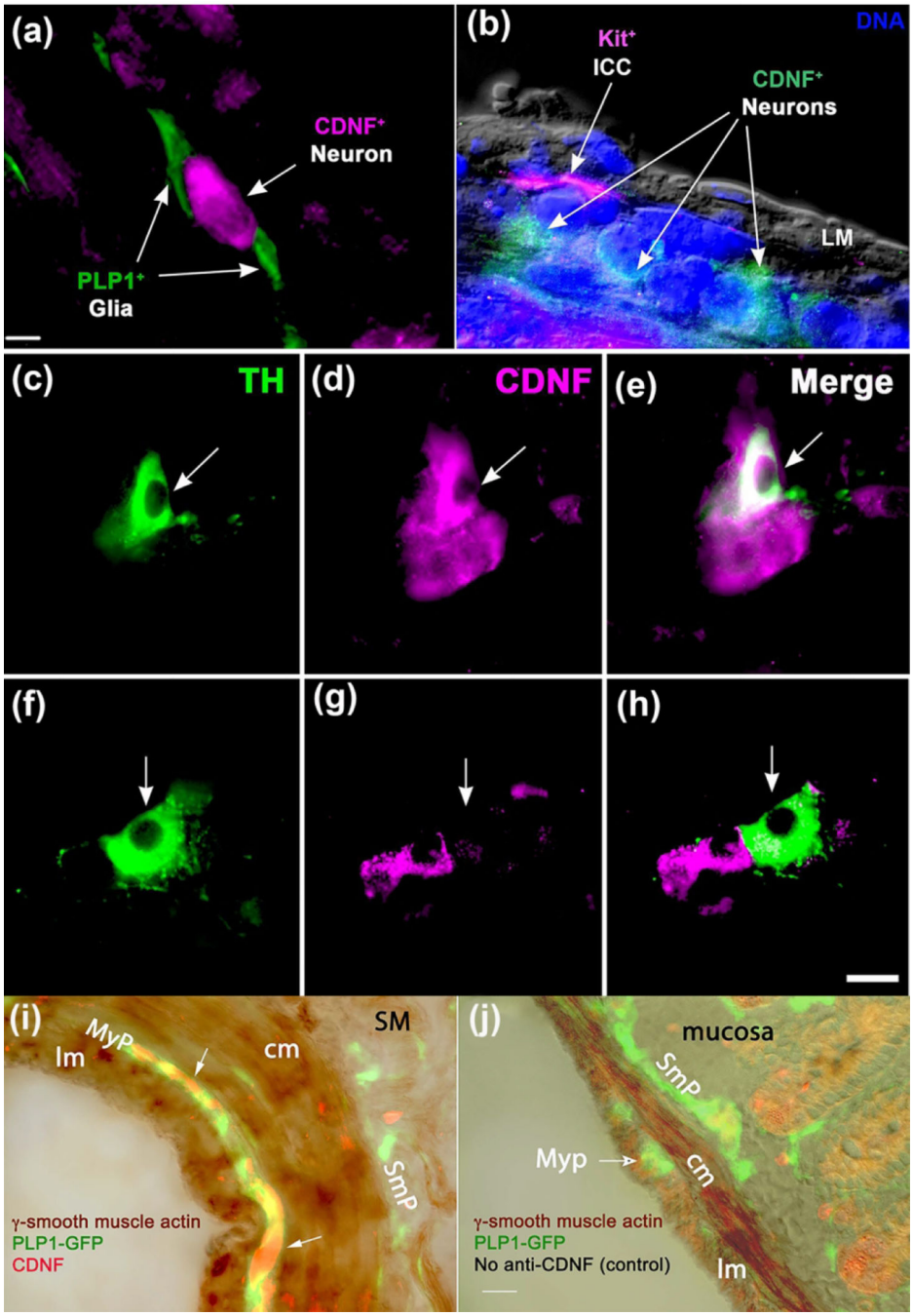

Figure 2. Enteric CDNF immunoreactivity is found in subsets of dopaminergic and other neurons but not in enteric glia, interstitial cells of Cajal, or smooth muscle.

a. Section of bowel wall cut through the myenteric plexus of a PLP-1-GFP mouse immunostained to demonstrate CDNF. PLP-1 marks enteric glia. GFP fluorescent processes (green) of enteric glial cells closely interact with a CDNF-immunoreactive neuron (magenta). Note absence of CDNF immunoreactivity from glia. The bar = 10 μm (a, b). **b.** Bowel wall cut through the myenteric plexus of a *Cdnf*$^{+/+}$ mouse immunostained to demonstrate immunoreactivities of Kit (magenta) and CDNF (green). Kit marks ICC. DNA

was demonstrated with bisbenzimide (blue) and the field was also viewed in differential interference contrast. The plexus of ICC that surround myenteric ganglia lack CDNF immunoreactivity. No CDNF immunoreactivity can be seen in the longitudinal muscle (LM) layer outside of the ganglion. c-h. Whole mounts of submucosa containing ganglia immunostained for TH (green) to mark dopaminergic neurons and CDNF (magenta). **c-e.** The illustrated ganglion contains a TH-immunoreactive neuron that displays coincident CDNF immunoreactivity. Note in D and E that not all CDNF-immunoreactive neurons are TH-immunoreactive. The arrow locates a dopaminergic neuron in each panel. **f-i**. The illustrated ganglion contains a TH-immunoreactive neuron that is not CDNF-immunoreactive. Note in g and h that several CDNF-immunoreactive neurons lack TH-immunoreactivity. The arrow shows the location of the dopaminergic neuron in each panel. The bar = 16 μm (c-h). **i-j**. Enteric glia in PLP-1-EGFP mice (bowel sections). Smooth muscle was immunostained with antibodies to γ–smooth muscle actin and CDNF. In a control section, antibodies to CDNF were omitted. **(i)** Animal at about 2 months of age (P58). γ–smooth muscle actin antibodies directly coupled to horseradish peroxidase (HRP) label smooth muscle cells (brown). Triple labeling demonstrates CDNF (red fluorescence) and GFP (glia; green fluorescence). HRP reaction product photographed in white light and merged with the fluorescent images of EGFP and CDNF. Myenteric plexus = MyP, submucosal plexus = SmP, and submucosa = SM. Arrows locate CDNF-immunoreactive neurons. Antibodies to CDNF fail to label smooth longitudinal (lm) or circular (cm) muscle or glia. **(j)** Control. Antibodies to CDNF were omitted. Section is PLP-1-EGFP bowel from P21 mouse. Mucosa is indicated. Bar (i and j) = 16 μm.

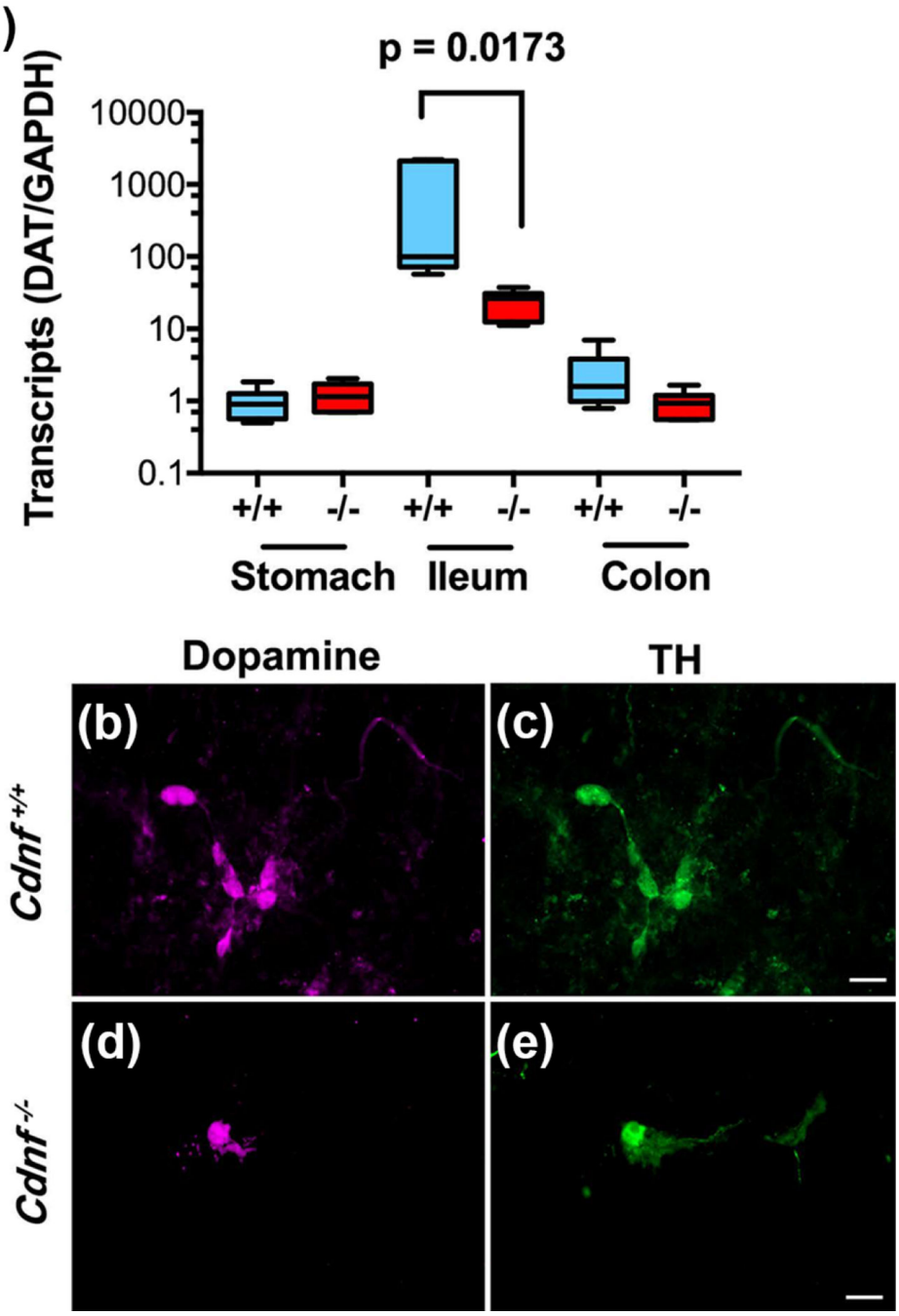

Figure 3. Markers of dopaminergic neuronal development are less abundant in the ileum of *Cdnf* $^{-/-}$ mice than in *Cdnf* $^{+/+}$ animals.

a. Transcripts encoding DAT were employed as a marker for the development of dopaminergic neurons in the murine stomach, ileum, and colon at P7. Transcripts encoding DAT were far more abundant in the ileum than in either of the other regions of the bowel (note that the ordinate is logarithmic) [one way Anova, $F(5, 30) = 3.088$ $p = 0.0230$]. Transcripts encoding DAT in the ileum were significantly more abundant in the *Cdnf* $^{+/+}$ mice (blue) than in *Cdnf* $^{-/-}$ animals (red); [Sidak's multiple comparison test $p = 0.0173$].

Each bar represents the mean values derived from analyses of 6 samples pooled from 2 animals of each genotype. **b.** Visualization of dopamine and TH immunoreactivities in the submucosal plexus of the murine ileum (9-month-old). In both *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice, these markers are fully coincident; however, dopamine- and TH-immunoreactive cells are more numerous in *Cdnf* $^{+/+}$ than in *Cdnf* $^{-/-}$ mice. The bar = 32 μm.

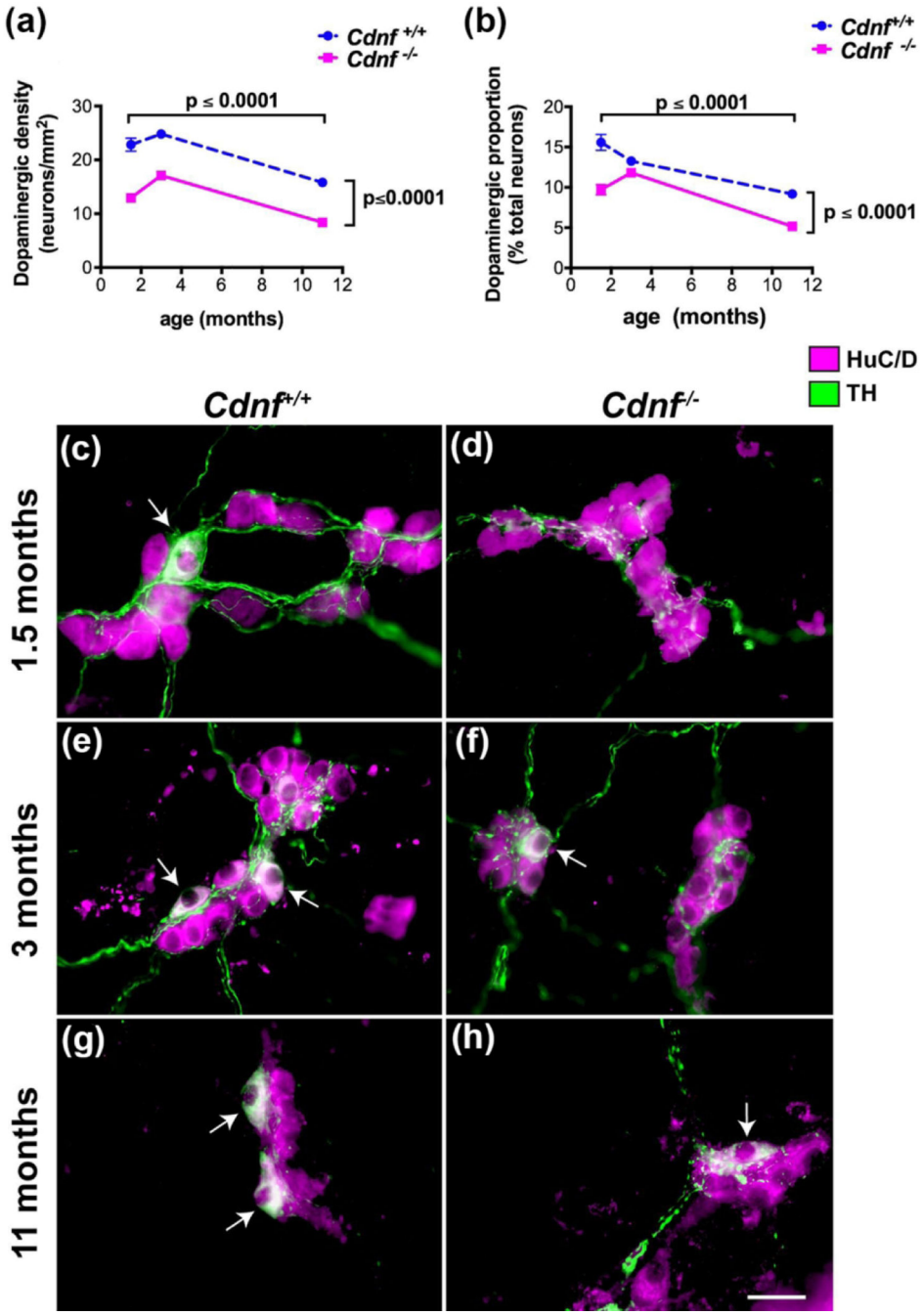

Figure 4. Both the packing density and the proportion of dopaminergic neurons are decreased as a function of age in *Cdnf* $^{-/-}$ compared to *Cdnf* $^{+/+}$ mice.

a. Packing density (number/unit area) of dopaminergic neurons is plotted as a function of age in *Cdnf* $^{+/+}$ (blue) and *Cdnf* $^{-/-}$ mice (magenta). Data were obtained from 60–102 measurements from 3–6 mice at each age and genotype. The decline from 1.5-month-old to 11-month-old is significant in mice of both genotypes; however, at each age examined the density is significantly greater in *Cdnf* $^{+/+}$ than in *Cdnf* $^{-/-}$ animals [two way Anova $F(2, 476) = 73.63$]. Coincident immunoreactivity of TH and HuC/D was used to identify

dopaminergic neurons. **b.** The proportion of total neurons (HuC/D-immunoreactive) that were dopaminergic (TH-immunoreactive) is plotted as a function of age. The proportion of dopaminergic neurons declines significantly as a function of age in both *Cdnf* $^{+/+}$ and *Cdnf* $^{-/-}$ mice; moreover, the proportion of dopaminergic neurons is always greater in *Cdnf* $^{+/+}$ than in *Cdnf* $^{-/-}$ animals [two way Anova $F(2, 403) = 61.62$]. **c-h.** Representative images of TH (green) and HuC/D immunoreactivities (magenta) in the ileal submucosal plexus of *Cdnf* $^{+/+}$ (c, e, g) and *Cdnf* $^{-/-}$ mice (d, f, h) at 1.5 (c, d), 3 (e, f), and 11 months of age (g, h). The bar = 35 μm.

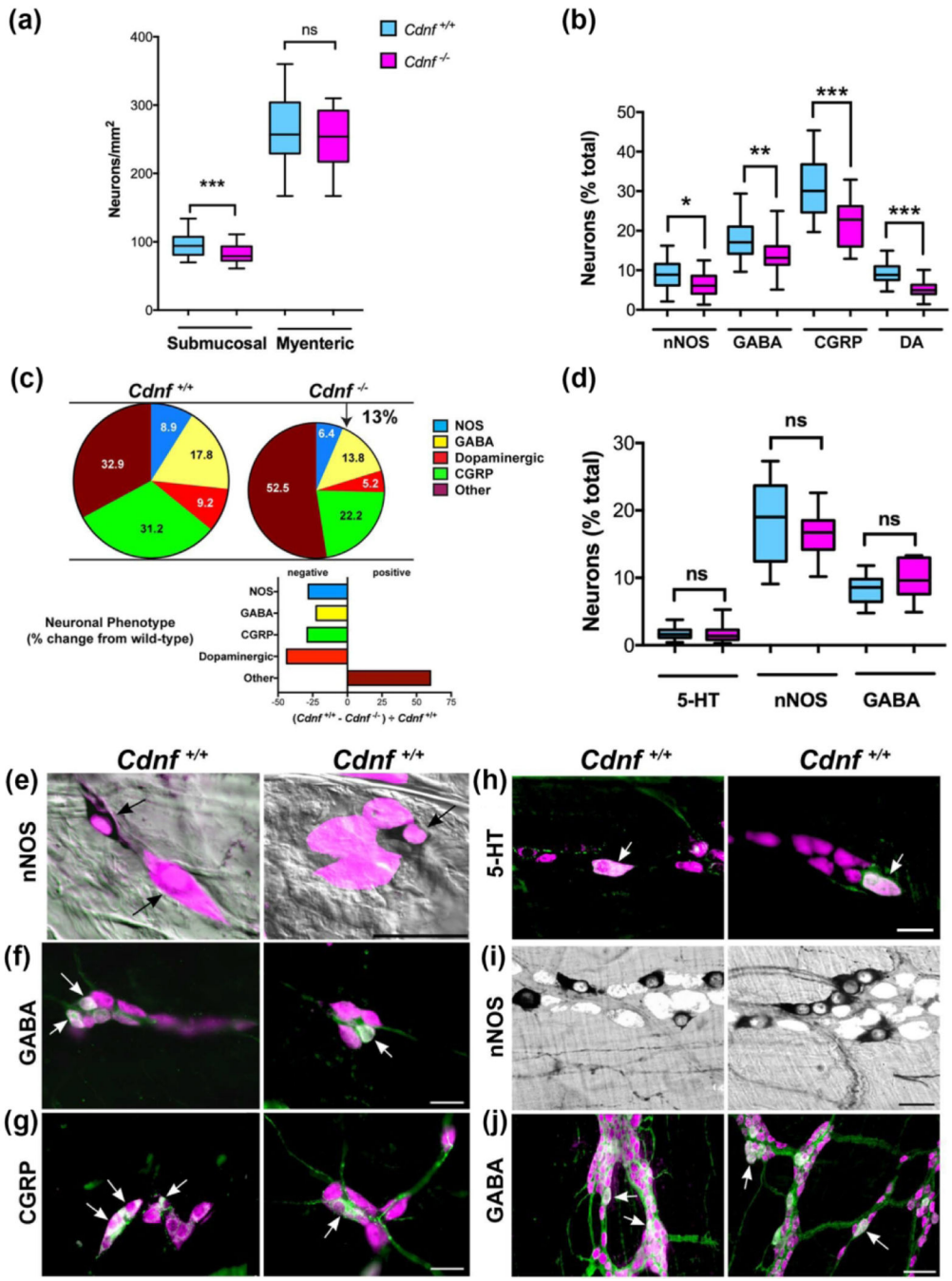

Figure 5. Specific subsets of submucosal neurons are CDNF-dependent.

a. The density of submucosal ANNA-1-immunoreactive neurons is significantly lower in 10–12-month–old *Cdnf* $^{-/-}$ (n = 42) than in *Cdnf* $^{+/+}$ mice (n = 40 measurements) (two-tailed unpaired t test p = 0.0003; t = 3.775 df = 80). The density of myenteric ANNA-1-immunoreactive neurons in the *Cdnf* $^{-/-}$ animals (n = 31), however, is not significantly different from that of *Cdnf* $^{+/+}$ (n = 37) mice (p = 0.2434; t = 1.177 df = 66 p = 0.2434). **b.** The proportions of submucosal nNOS-, GABA-, CGRP-, and DA/TH- containing neurons are all lower in *Cdnf* $^{-/-}$ mice than in *Cdnf* $^{+/+}$ animals [two-tailed unpaired t tests]. The

numbers of measurements were nNOS (50 in *Cdnf* $^{+/+}$; 30 in *Cdnf* $^{-/-}$; p = 0.0012; t = 3.375 df = 77), GABA (26 in *Cdnf* $^{+/+}$; 20 in *Cdnf* $^{-/-}$; p = 0.0075; t = 2.804 df = 44), CGRP (20 in *Cdnf* $^{+/+}$; 20 in *Cdnf* $^{-/-}$; p = 0.0004; t = 3.898 df = 38), DA/TH (69 in *Cdnf*; 70 in *Cdnf* $^{-/-}$; p ≤ 0.0001; t = 11.55 df = 137). **c.** The proportions of identified and unidentified submucosal neurons as parts of the whole submucosal population. Note that the whole population is 13% lower in *Cdnf* $^{-/-}$ than in *Cdnf* $^{+/+}$ animals; moreover, the proportions of unidentified neurons are greater in *Cdnf* $^{-/-}$ than in *Cdnf* $^{+/+}$ mice because of the corresponding reductions in the proportions of nNOS-, GABA-, CGRP-, and DA/TH-containing neurons in the *Cdnf* $^{-/-}$ mice. The % change for each phenotype is shown below the pie charts. The largest numerical decline is seen in dopaminergic neurons. **d.** The proportions of myenteric 5-HT- nNOS-, and GABA-containing neurons in *Cdnf* $^{-/-}$ mice are not significantly different from those than in *Cdnf* $^{+/+}$ animals [two-tailed unpaired t tests]. The numbers of measurements were 5-HT (39 in *Cdnf* $^{+/+}$; 37 in *Cdnf* $^{-/-}$; p = 0.5518, t=0.5978 df = 74), nNOS (26 in *Cdnf* $^{+/+}$; 20 in *Cdnf* $^{-/-}$; p = 0.2032, t = 1.292 df = 44), GABA (10 in *Cdnf* $^{+/+}$; 16 in *Cdnf* $^{-/-}$ p = 0.1031, t = 1.695 df = 24). **e.** Submucosal nNOS-expressing neurons. NADPH diaphorase activity was used to identify nNOS-containing neurons (dark purple, differential interference microscopy; arrows) and ANNA-1 immunoreactivity was used to identify neurons (magenta, merged immunofluorescence). **f.** Submucosal GABA-expressing neurons (green immunofluorescence; arrows) and ANNA-1 immunoreactivity (magenta, merged immunofluorescence). **g.** Submucosal CGRP-expressing neurons (green immunofluorescence; arrows) and ANNA-1 immunoreactivity (magenta, merged immunofluorescence). **h.** Myenteric 5-HT-expressing neurons (green immunofluorescence; arrows) and ANNA-1 immunoreactivity (magenta, merged immunofluorescence). **i.** Black and white gray scale pictures, superimposed differential interference and fluorescence microscopic images. Myenteric nNOS-expressing neurons (black; NADPH diaphorase activity) and ANNA-1 immunoreactivity (white). **j.** Myenteric GABA-expressing neurons (green immunofluorescence; arrows) i = 35 μm; j = 70 μm

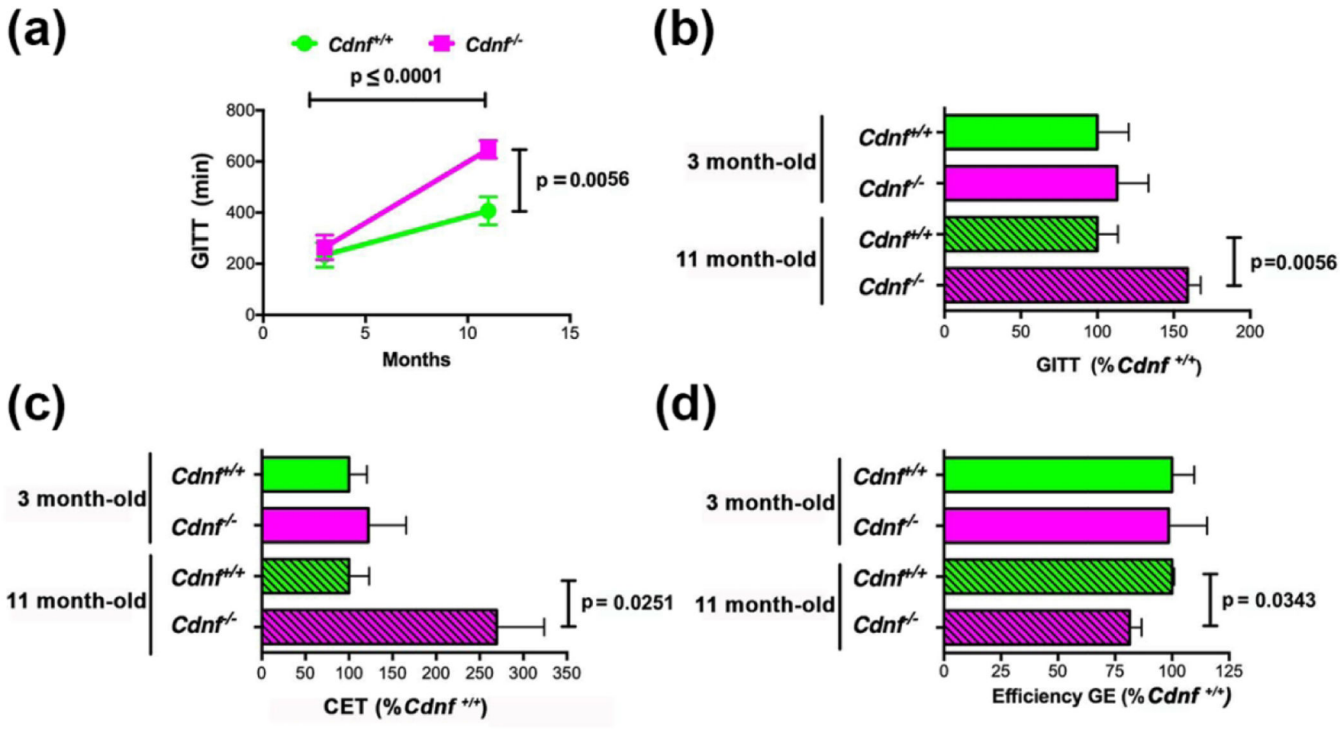

Figure 6. All measured parameters of GI motility are abnormal in 11-month-old *Cdnf*$^{-/-}$ mice.

GITT, gastric emptying, and colonic expulsion time, were evaluated.All values shown are means determined from measurements made with *Cdnf*$^{+/+}$ (*n* =6) and *Cdnf*$^{-/-}$ (*n* =6) mice at each age. **(a)** GITT increases as mice age in both *Cdnf*$^{+/+}$ (green) and *Cdnf*$^{-/-}$ (magenta) mice; however, the degree to which transit slows as mice mature was significantly greater in *Cdnf*$^{-/-}$ than in *Cdnf*$^{+/+}$ animals (2 way Anova F (1, 20) =34.9). **(b)** GITT (expressed as % of *Cdnf*$^{+/+}$ GITT) in 3-month-old *Cdnf*$^{+/+}$ mice was not significantly different from that of their *Cdnf*$^{-/-}$ littermates (two tailed t test t = 0.4463 df = 10 p = 0.6649); however, GITT in 11-month-old *Cdnf*$^{-/-}$ mice was significantly longer than in their *Cdnf*$^{+/+}$ littermates (two-tailed t test t = 3.682 df = 8.491). **(c)** The time to expel a bead inserted into the rectum (colonic expulsion time, CET) in 3-month-old *Cdnf*$^{+/+}$ mice (expressed as CET % *Cdnf*$^{+/+}$) was not significantly different from that of their *Cdnf*$^{-/-}$ littermates (two-tailed t test t = 0.4594; df = 7.144 p = 0.6596); however, CET in 11-month-old *Cdnf*$^{-/-}$ mice was significantly greater than in their *Cdnf*$^{+/+}$ littermates (two-tailed t test t = 2.866 df = 6.748). **(d)** The efficiency of gastric emptying (expressed as % *Cdnf*$^{+/+}$ GE) in 3-month-old *Cdnf*$^{+/+}$ mice was not significantly different from that in their *Cdnf*$^{-/-}$ littermates (two tailed t test t = 0.075587 df = 7.998 p = 0.9414); however, the efficiency of gastric emptyping in 11-month-old *Cdnf*$^{-/-}$ mice was substantially less than that in their *Cdnf*$^{+/+}$ littermates (two-tailed t test t = 3.537; df = 3.221), that is, gastric emptying was delayed in the aged *Cdnf*$^{-/-}$ mice.

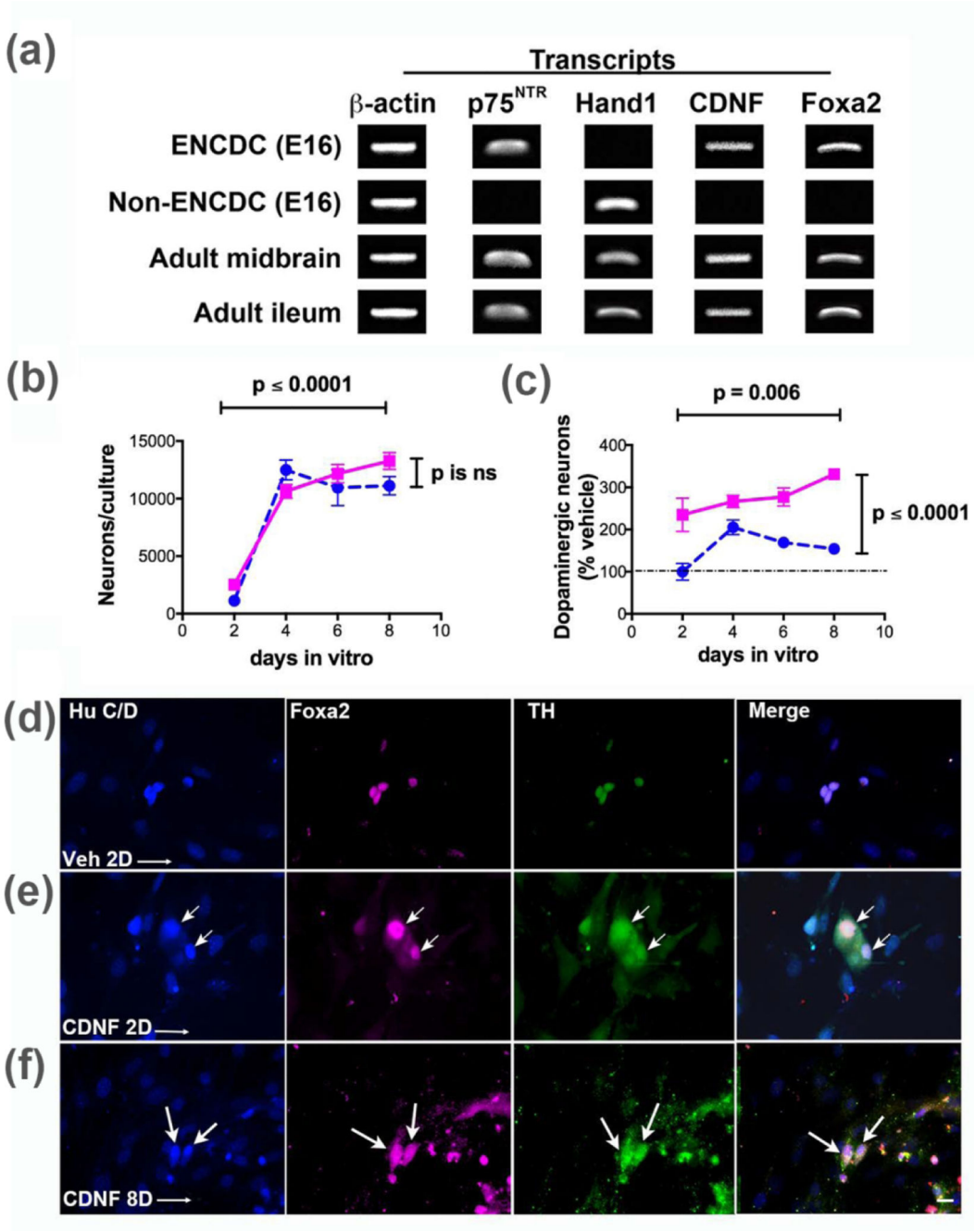

Figure 7. CDNF selectively promotes the *in vitro* development/survival of dopaminergic neurons from ENCDC isolated from fetal gut at E16.

a.(ENCDC) Transcripts encoding the crest-derived cell marker, $p75^{NTR}$, but not those encoding the mesenchymal cell marker Hand1, were found in a positively immunoselected fraction of fetal cells. Transcripts encoding CDNF and the dopaminergic neuron marker, Foxa2, were also found in this fraction. (Non-ENCDC) Transcripts encoding Hand1, but not those encoding $p75^{NTR}$, CDNF or Foxa2 were found in the negatively immunoselected cell fraction. Transcripts encoding all markers were detected in the homogenates of adult midbrain and ileum, which were investigated as positive controls. **b-c.** The time-action

relationship is shown for the effect of CDNF on development/survival from (b) isolated ENCDC of total neurons and (c) dopaminergic neurons as % of vehicle. Results obtained after exposure to CDNF (magenta) are compared to vehicle (blue) from 4–8 cultures: Numbers of total neurons increase significantly between 2 and 8 days of culture [2way Anova $F(3, 24) = 40.95$] but are not affected by CDNF [$F(1, 24) = 0.8654$ $p=0.3615$]. c: Number of differentiated dopaminergic neurons are significantly enhanced with CDNF [2way Anova $F(1, 30) = 39.18$] and with time in culture [$F(3, 30)=3.314$]. CDNF rapidly, stably, and selectively promotes development/survival of the dopaminergic subset of the neuronal population. **d-f.** The immunoreactivity of HuC/D (blue) was used as a neuronal marker while those of Foxa2 (magenta) and TH (green) were used to identify dopaminergic neurons. d = exposure for the first 2 days in vehicle. e = exposure to CDNF for the first 2 days. f = exposure to CDNF for the full 8 days. Cells in which the immunoreactivities of all 3 markers were coincident (arrows) were identified as dopaminergic neurons. The numbers of dopaminergic neurons were greater in the presence of CDNF than in vehicle and, in the presence of CDNF, increased as a function of time in culture. The bar = 16 μm.

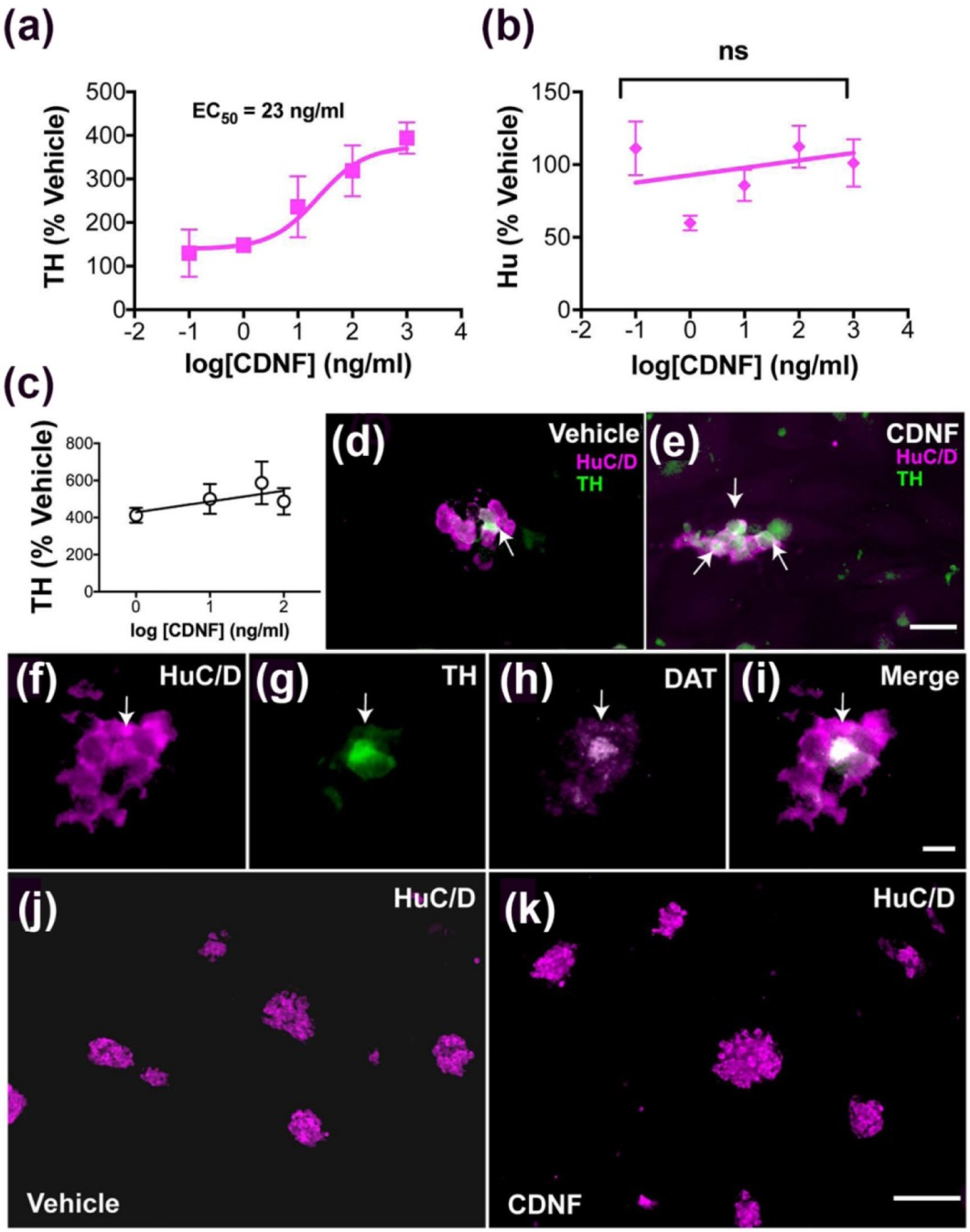

Figure 8. CDNF promotion of dopaminergic neuronal development/survival *in vitro* is concentration-dependent and altered by the presence of non-neuronal cells.

a. TH immunoreactivity was used as a dopaminergic neuronal marker and plotted as a function of the log of the concentration of CDNF. Non-linear regression analysis revealed that the data for isolated ENCDC. were best fit by a sigmoidal concentration-effect curve (3-parameter; Hill coefficient ~1). **b.** CDNF did not enhance the development of total neurons at any concentration tested [One way Anova $F(4, 23) = 1.515$] in immunoselected ENCDC. **c.** Although CDNF enhanced the development/survival of dopaminergic neurons in mixed

cultures, the maximal effect was reached at 1 ng/ml, the lowest concentration tested, and a sigmoidal concentration-effect curve could not be obtained. **d-e.** The immunoreactivity of neurons (HuC/D; magenta) and TH (green) developing in the presence of vehicle (d) and CDNF (e) is illustrated. Coincidence of HuC/D and TH immunoreactivities marks the presence of dopaminergic neuron (arrows). **f-i.** Cultures of immunoselected ENCDC. The immunoreactivities of the neuronal marker, HuC/D (f), TH (g) and DAT (h) are coincident (l). **j-k**. Similar numbers of total neurons (HuC/D; magenta) develop from isolated ENCDC in the presence of vehicle (j) or CDNF (k). All bars = 25 μm.

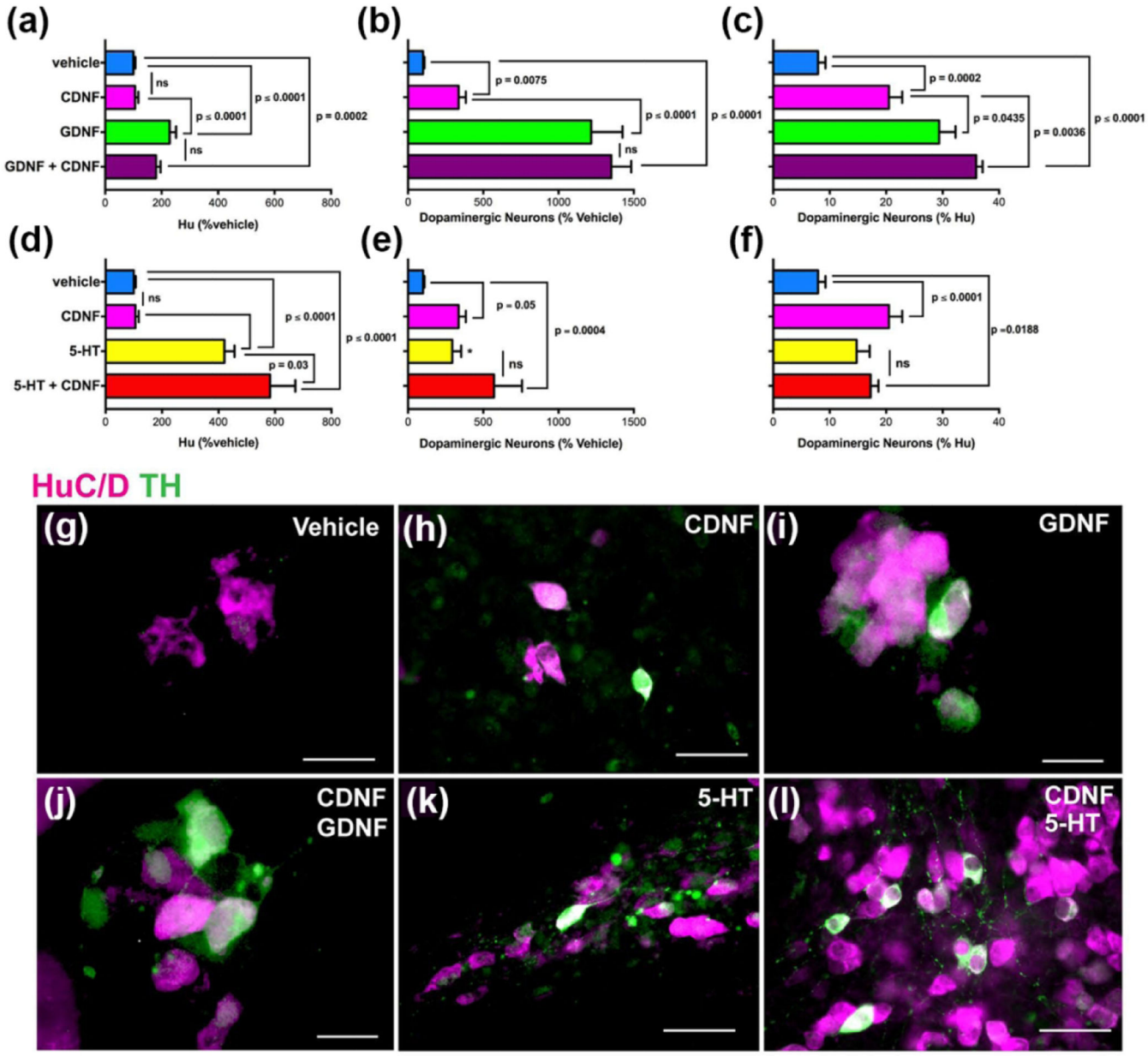

Figure 9. CDNF and GDNF but not 5-HT selectively promote dopaminergic neuronal development.

a-c. Immunoselected ENCDC were cultured in the presence of vehicle (blue), CDNF (100 ng/ml; magenta), GDNF (50 ng/ml; green), or a combination CDNF and GDNF (plum) for 6 days. 8 replicate cultures were studied for each condition. a. Total neuron density (HuC/D immunoreactivity). b. Dopaminergic neuron density (TH immunoreactivity). c. Proportion of the neuronal population that was dopaminergic. GDNF, but not CDNF, promoted the development of total neurons [a: one-way ANOVA $F(3, 40) = 17.92$]. Both GDNF and CDNF enhanced dopaminergic neuronal development/survival measured either as % vehicle [b: $F(3, 36) = 69.41$] or as a proportion of total Hu-immunoreactive neurons [c: $F(3, 40) = 23.83$]; however, their effects were not additive. **d-f**. Immunoselected ENCDC were cultured

in the presence of vehicle (blue), CDNF (100 ng/ml; magenta), 5-HT (1.0 μM; yellow), or a combination CDNF and 5-HT (red) for 7 days. 8 replicate cultures were studied for each condition. 5-HT, but not CDNF, promoted the development of total neurons [d: $F(3, 44) = 47.49$]. Both 5-HT and CDNF enhanced dopaminergic neuronal development/survival; however, the effects of 5-HT and CDNF were not additive [e: $F(3, 44) = 6.66$]. **f**. 5-HT did not, by itself, affect the proportion of total neurons that were dopaminergic; however, the addition of 5-HT did not affect the ability of CDNF to enhance this proportion [F: $F(3, 44) = 8.95$]. **g-l.** Representative merged images of HuC/D (magenta) and TH (green) in cultures grown in the presence of the indicated factors. **g**. Neurons in a vehicle-treated culture. **h**. A dopaminergic neuron displaying coincident HuC/D and TH immunoreactivities in a CDNF-treated culture. **i**. Dopaminergic neurons displaying coincident HuC/D and TH immunoreactivities in a GDNF-treated culture. **j**. Dopaminergic neurons displaying coincident Hu/CD and TH immunoreactivities in a culture exposed to the combination of CDNF and GDNF. **k**. Dopaminergic neurons displaying coincident HuC/D and TH immunoreactivities in a 5-HT-treated culture. **l**. Dopaminergic neurons displaying coincident HuC/D and TH immunoreactivities in a culture exposed to the combination of CDNF and 5-HT. Note the relatively great abundance of both total and dopaminergic neurons. All bars = 35 μm

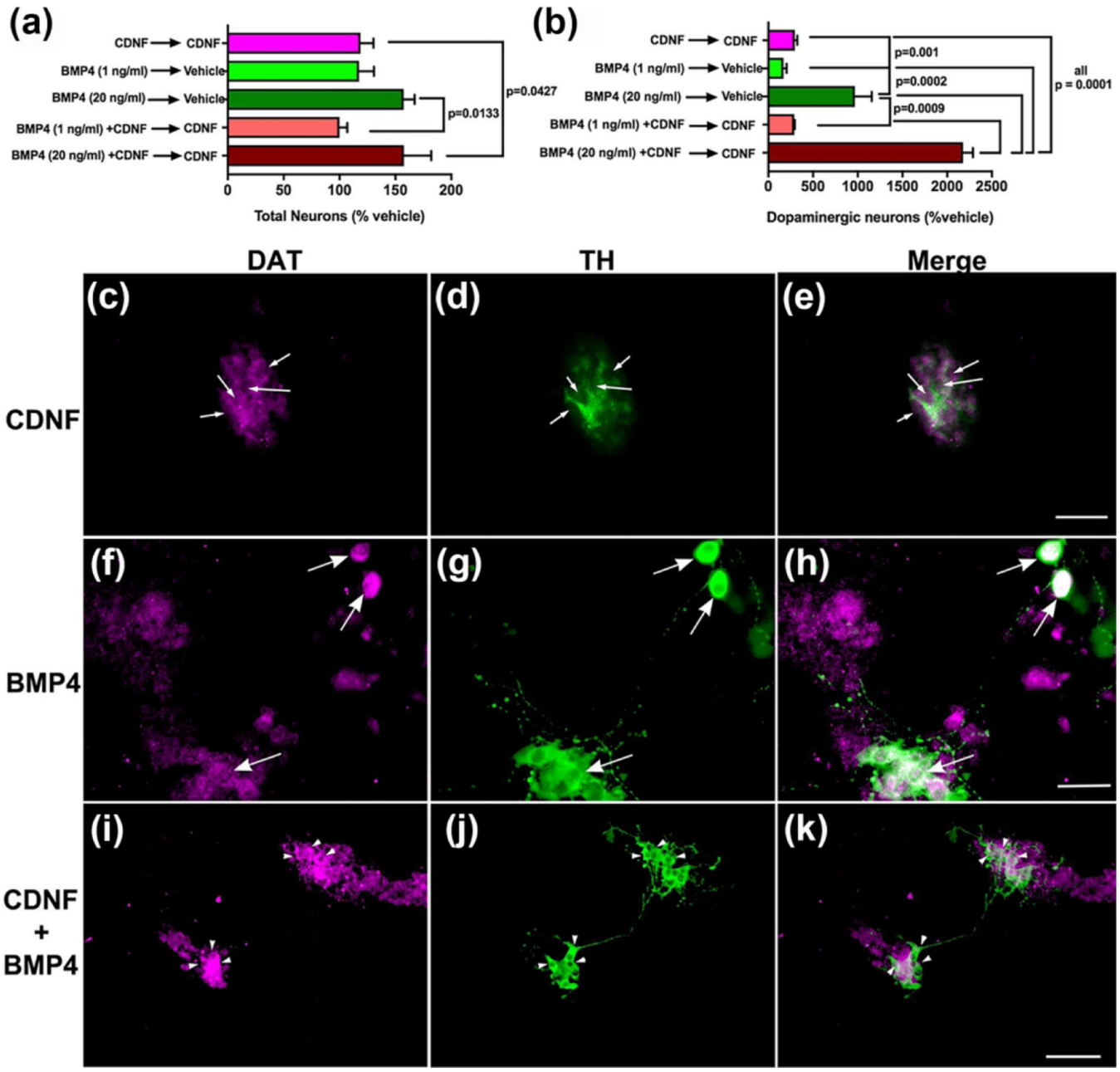

Figure 10. BMP4 enhancement of CDNF-induced development of dopaminergic neurons from ENCDC.

a-b. ENCDC were cultured for a total of 7 days. The culture time was divided into initial (days 1 to 4) and subsequent (days 5 to 7) periods. Four replicate cultures were exposed initially to media containing CDNF (100 ng/ml), BMP4 (1 ng/ml), BMP4 (20 ng/ml), or BMP4 (1 or 20 ng/ml) plus CDNF. During the subsequent culture period, cells were exposed either to vehicle or to CDNF. **a**. Total neurons developing/surviving under each of the listed conditions. Initial exposure to 20 ng/ml, but not 1 ng/ml, BMP4 enhances neurogenesis. Addition of CDNF does not alter the response to BMP4 [one way Anova $F(4,15) = 3.22$]. **b**. Dopaminergic neurons developing/surviving under the same sets of conditions. Initial

exposure to 20 ng/ml, but not 1 ng/ml, BMP4 enhances development of dopaminergic neurons. Addition of CDNF to 20 ng/ml BMP4 now induces a synergistic increase in the development/survival of dopaminergic neurons [one way Anova $F(4,15) = 69.97$]. Representative fields illustrating dopaminergic neuronal development in cultures exposed continuously to CDNF **(c-e)**, 20 ng/ml BMP4 **(f-h)**, and the combination of 20 ng/ml BMP4 and CDNF **(i-k)**. Dopaminergic neurons (arrows) are marked by their coincident expression of the immunoreactivities of DAT (magenta) and TH (green). The bars (c-h) = 35 μm; the bar (i-k) = 70 μm. The ability of BMP4 to enhance neurite outgrowth is evident.

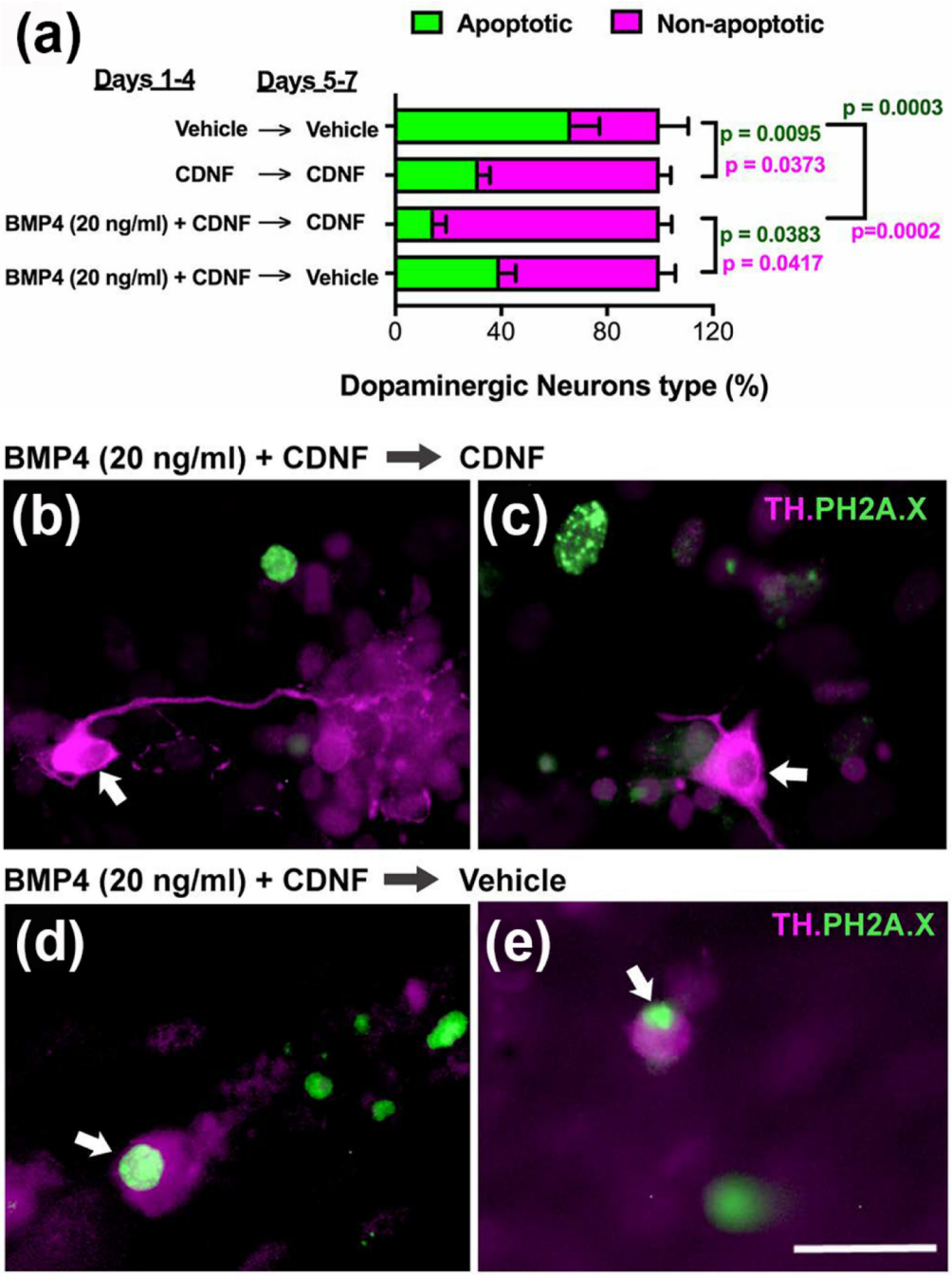

Figure 11. Exposure to BMP4 enhances the development of dependence of dopaminergic neurons on CDNF for survival.

a. Isolated ENCDC were cultured for 7 days. As in Fig. 4, the full culture time was divided into an initial period of 4 days and a subsequent period of 3 days during which the cells were either maintained in vehicle or in CDNF (100 ng/ml). Cells were exposed during the initial period to vehicle, CDNF (100 ng/ml; n = 4 replicates), or to 20 ng/ml BMP4 plus CDNF (5 replicates) in order to evaluate the effects of CDNF withdrawal. Expression of TH immunoreactivity was used to identify dopaminergic neurons and that of PH2A.X was

employed to identify cells undergoing apoptosis. The proportion of the population of dopaminergic neurons that was apoptotic (green) or non-apoptotic (magenta) was quantified for each of the tested conditions. A significantly smaller proportion of dopaminergic neurons was apoptotic in cultures continuously exposed to CDNF than in cultures exposed continuously to vehicle. Initial exposure of cultures to BMP4 (20 ng/ml) resulted in a significant further reduction of the proportion of apoptotic dopaminergic neurons as long as CDNF was maintained during the subsequent 3 days in vitro; however, a significant increase in the proportion of dopaminergic neurons that was apoptotic occurred when CDNF was withdrawn and cells were exposed only to vehicle for the final 3 days in culture [live dopaminergic as %total dopaminergic, one way Anova $F(4, 19) = 5.435$ $p = 0.0043$; apoptotic dopaminergic as % total dopaminergic, one way Anova $F(3, 14) = 10.6$ $p=0.0007$]. **b, c**. Illustration of cultures initially exposed to BMP4 (20 ng/ml) plus CDNF and then maintained in the presence of CDNF. Note that although some cells in the cultures have PH2A.X-immunoreactive nuclei and thus are undergoing apoptosis, the dopaminergic neurons in the field (arrows) are healthy and exhibit abundant neurite outgrowth. **d. e.** Illustration of cultures initially exposed to BMP4 (20 ng/ml) plus CDNF but then subjected to CDNF withdrawal. Note that dopaminergic neurons now have PH2A.X-immunoreactive nuclei and thus are undergoing apoptosis; the apoptotic dopaminergic neurons (arrows) do not exhibit abundant neurite outgrowth. The bar = 35 μm.

Table 1:

PCR Primers, Conditions Used for Amplification, Expected Sizes of Products

Primers/Gen Bank Accession Number	Sense (5’−3’) Nucleotides	Antisense (5’−3’) Nucleotides	Cycles (n)	Annealing Temp (°C)	Product (bp)
p75^{NTR}/*NM_033217*	CCGATACGGTGACCACTGTGATG	AGCAGCCAAGATGGAGCAATAGAC	35	58	98
	835–857	932–909			
Hand1/*NM_008213*	TTGAAGGCTCGACTCAAGGT	AGCGACAAGAAGGAAAACCA	39	63	269
	1266–1283	1534–1513			
CDNF/*NM_177647*	ATGCGGTGCATCAGTCCAACTGC	GAGCTCCGTTTGGGGGTATATC	35	60	561
	63–85	623–602			
Foxa2/HNF-3β/	GTATGCTGGGAGCCGTGAA	GGAGGTTTGGCGTGTGTGTAG	39	63	463
NM_010446	110–128	573–593			
β-actin/ *NM_007393*	TGTTTGAGACCTTCAACACC	CAGTAATCTCCTTCTGCATCC	30	57	588
	448–467	1035–1015			
DAT/*NM_010020*	CTTCTCCTCTGGCTTCGTTGT	CAGGGTAGATGATGAAGATCAACC	qPCR	qPCR	116
	1225–1245	1340–1317			
GAPDH/*NM_008084.2*	TGATGGGTGTGAACCACGAG	GGCATGGACTGTGGTCATGAG			
	439–458	584–564	qPCR	qPCR	146

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Table 2:

Antibodies used in culture and in whole mounts

Antigen/Species	Host type	Dilution	Source (RRID)	Reference
Antibodies used in vitro				
DAT/rat (aa 42–59 near N-terminal)	Rabbit polyclonal	1:130	Millipore # AB1591P (AB_90808)	(Chalazonitis et al., 2008)
Foxa2/rat (Recombinant aa 7–86 no labeling in Foxa2 mice)	Rabbit polyclonal	1:1000	Abcam # ab40874 (AB_732411)	(Viotti, Niu, Shi, & Hadjantonakis, 2012) (Besnard et al., 2004)
HuC/D/human (Full length protein)	Mouse monoclonal	1:50	Molecular Probes #A-21272 (AB_221448)	(Chalazonitis, D'Autreaux, et al., 2011)
PhosphoHistone-H2A.X (peptide PSer13; 20E3)	Rabbit monoclonal	1:180	Cell Signaling # 9718 (AB_2118009)	(Rogakou et al., 2000)
Human $_{p75}^{NTR}$ (extracellular)/mouse (peptide aa 43–161;no labeling in bowel wall of $p75^{NTR-/-}$ mice)	Rabbit polyclonal	1:800	Moses Chao (Skirball Inst.; NYU)	(Chalazonitis et al., 2008; Lee et al., 1992)
TH/rat (Native antigen from rat pheochromocytoma A)	Sheep polyclonal	1:225	Pel Freez # P60101 (AB_461070)	(Chalazonitis et al., 2008)
Antibodies used in sections or whole mounts of bowel				
5-HT (formaldehyde coupled to Keyhole Limpet hemocyanin; recognizes antigen formaldehyde-coupled to tissue protein)	Rabbit polyclonal	1:500–1000	Immunostar #20080 (AB_572263)	(Chen et al., 2001)
ANNA-1/human (autoantibodies to Hu proteins, primarily HuC/D)	Human IgG	1:32,000	Vanda Lennon (Mayo University) (AB_2314657)	(Lennon, 1994) (King et al., 1999)
HuC/D/human	Mouse monoclonal	1:60–75	Molecular Probes #A-21272 (AB_221448)	(Chalazonitis, D'Autreaux, et al., 2011)
CDNF/mouse (full length protein omitting signal sequence; no labeling in *Cdnf*$^{-/-}$ tissue)	Rabbit polyclonal	1:750	Päivi Lindholm-Pulkkila (University of Helsinki)	(Lindholm et al., 2007)
Smooth Muscle γ Actin (B4)/Chicken gizzard Actin	Mouse monoclonal	1:100	Santa Cruz Biotech SC-53142	(H. Li et al., 2019) (Sawtell & Lessard, 1989)
CGRP/rat (Conjugated to Keyhole Limpet hemocyanin); no labeling in *Cgrp*$^{-/-}$mice	Rabbit polyclonal	1:2000	Sigma #C8198 (AB_259091)	(Chalazonitis et al., 2008)
Kit/mouse Mouse myeloma cell line	Goat polyclonal	1:20	R&D Systems #AF1356 (AB_354750)	(Sanders, Ördög, Koh, Torishashi, & Ward, 1999)
kit Gln25-Thr519 (Ala207Glu)				
Dopamine (recognizes antigen aldehyde-coupled to protein)	Rat polyclonal	1:300	Protos Biotech Corp #NT-104DA rat	(Geffard et al., 1984)
DAT/rat (Peptide LTNSTLINPPQTPVEAGE; near N-terminal)	Rabbit polyclonal	1:400	Millipore #AB1591P (AB_90808)	(Chalazonitis et al., 2008)
TH/mouse (2 peptides from mouse (P24529) sequence)	Chicken Polyclonal IgY	1:1000	Abcam #76442 (AB_1524535)	(Coletto et al., 2019)
GABA (aldehyde coupled to Keyhole Limpet hemocyanin) No labeling in *Gad65*$^{-/-}$ *Gad67*$^{-/-}$ mice, which lack GABA	Rabbit Polyclonal	1:1000	Sigma-Aldrich #2052 AB_477652)	(Chalazonitis et al., 2008)