Brain-derived neurotrophic factor amplifies neurotransmitter responses and promotes synaptic communication in the enteric nervous system

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ABSTRACT

Background: Besides its role in neuronal growth and differentiation, brain-derived neurotrophic factor (BDNF) has been implicated in the control of peristalsis where it serves to enhance gastrointestinal motility.

Aim: To unravel the cellular mechanisms governing BDNF's effect on motility.

Methods: Studies were performed in primary myenteric neuron cultures and whole-mount preparations derived from guinea pig ileum. Expression of BDNF and its tropomyosin-related kinase B (TrkB) receptor was assessed by immunohistochemistry. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in myenteric neurons were monitored using Fluo-4, and neurotransmitter release kinetics at enteric synapses were evaluated with FM1-43 imaging.

Results: Immunohistochemistry revealed the presence of BDNF and TrkB in mucosa, submucosal plexus and myenteric ganglia. Primary cultures also expressed BDNF and TrkB and were used to study the physiological effects of BDNF. None of the neurons studied displayed a $[Ca^{2+}]_i$ change when challenged with BDNF. However, BDNF exposure caused an enhancement of Ca^{2+} transients induced by serotonin and substance P, which was reversed by the Trk receptor blocker K-252a (0.1 μM). BDNF exposure also resulted in an amplification of spontaneous network activity which was reflected in an increased number of synaptic vesicle clusters. Furthermore, BDNF treatment facilitated FM1-43-labelled vesicle destaining in enteric terminals during field stimulation.

Conclusions: The findings demonstrate that BDNF is able to enhance rather than directly activate enteric nervous system signalling. Therefore, the promotion of motility by BDNF seems to result from its potent modulating role on enteric neuronal activity and synaptic communication.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin (NT) family that regulates growth, survival and differentiation of central and peripheral neurons.[1–3] The majority of cellular responses result from NT binding to tyrosine kinase receptors of the tropomyosin-related kinase (Trk) family, but NTs also act as ligands for the structurally unrelated pan-neurotrophin receptor (p75), a member of the tumour necrosis factor receptor superfamily.[4 5] BDNF binds with high affinity to the TrkB receptor and, although its long-term effects depend on gene regulation, studies in the central nervous system (CNS) have shown that BDNF also exerts a wide range of more rapid actions, including morphogenetic and chemotropic effects,[6 7] modulation of synaptic transmission[8 9] and acute neuronal excitation.[10 11] The latter is noteworthy as BDNF was shown to depolarise neurons directly by rapid gating of the $Na_v1.9$ Na^+ channel, thereby assigning BDNF the properties of a classical excitatory neurotransmitter.[12 13]

There is growing evidence that BDNF also plays a role in gut function. BDNF and the TrkB and p75 receptors are expressed in the enteric nervous system (ENS) and gut mucosa of a variety of species, including man.[14–20] Their expression is altered in some pathological conditions of the gut including Hirschprung disease and infantile hypertrophic pyloric stenosis.[21 22] In humans, a 2-week treatment with recombinant BDNF dose-dependently accelerated colonic transit and increased stool frequency.[23] In rats, acutely administered BDNF increased colonic motor activity.[24] Recently, involvement of endogenous BDNF in the peristaltic reflex of the rat colon was reported, where BDNF enhanced the release of 5-hydroxytryptamine (5-HT) and calcitonin gene-related peptide (CGRP) in response to mucosal stroking.[25] Although these findings suggest direct effects of BDNF on neuronal activity and excitability in the ENS, the interaction between BDNF and enteric neurons has not been studied.

In the present report, we examined the presence of BDNF and its high affinity receptor TrkB in the guinea pig ileum, and investigated its acute and modulating actions on Ca^{2+} signalling and its effect on synaptic organisation and vesicle recycling in cultured myenteric neurons in order to understand the mechanisms underlying BDNF's effect on motility.

MATERIALS AND METHODS

Primary myenteric neuron cultures

Cultured myenteric neurons were prepared from adult guinea pig ileum according to previously described methods.[26] Guinea pigs of either sex (250–700 g) were euthanised by a sharp blow to the head and exsanguination from the carotid arteries, a method approved by the Animal Ethics Committee of the University KULeuven. Longitudinal muscle–myenteric plexus (LMMP) preparations were dissected and digested in a protease and collagenase (Sigma, Bornem, Belgium) solution. After 30 min incubation (37°C), the suspension was placed on ice and spun at 500 *g*. Ganglia were picked and plated onto glass coverslips at the bottom of 12-well culture plates (Gibco, Merelbeke, Belgium). Culture medium (M199) enriched with 10% fetal bovine serum,

100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 30 mM glucose and 50 ng/ml of 7-s neuronal growth factor (NGF; Alomone Labs, Jerusalem, Israel) was changed every 2 days. Cultures were kept in a humified 5% CO_2 incubator (37°C). After a few days, neurons started growing in network-like structures reminiscent of the ganglionated plexus. Experiments were performed between day 7 and day 15. To evaluate BDNF's modulating effects, neurons were grown for 2, 24 or 48 h in growth medium with 50 ng/ml human recombinant BDNF (Invitrogen, Carlsbad, CA), a concentration based on literature data.[12 27] Following BDNF exposure, cells were immediately used for $[Ca^{2+}]_i$ or FM1-43 imaging.

Immunohistochemistry

For cryosections, segments of guinea pig ileum were removed and fixed in 4% paraformaldehyde (4 h, 4°C). Segments were washed, placed in phosphate-buffered saline (PBS) containing 30% sucrose as a cryoprotectant and stored overnight (4°C). Next day, ileum segments were embedded in Neg-50 (Richard-Allan Scientific, Kalamazoo, MI) and frozen in liquid nitrogen. Frozen 30 μm sections were cut, collected onto polysine-coated slides and left to dry (1 h at room temperature). For LMMP, pieces of guinea pig ileum were removed, opened and pinned flat on a sylgard dish. Mucosal layers were removed and LMMPs were fixed in 4% paraformaldehyde (1 h at room temperature). After washing, circular muscle was also removed. The cultured myenteric neurons were fixed in 4% paraformaldehyde (40 min at room temperature) and washed. All preparations were processed for permeabilisation and blocking of aspecific binding sites (2 h, PBS solution + 4% donkey serum and 0.5% Triton X-100). Next, preparations were transferred to the primary antibody solution (24 h, 4°C) and, after washing, exposed to secondary antibodies (table 1). The BDNF and TrkB antibody specificity was verified by preadsorption to the appropriate antigens according to the provider's instructions (Supplementary fig 1).

Live cell imaging

$[Ca^{2+}]_i$–Fluo-4 imaging

Cultured myenteric neurons were loaded for 45 min in HEPES-buffered solution containing 10 μM Fluo-4 AM (Molecular Probes). After loading, cultured neurons were rinsed (HEPES solution) and transferred to a coverglass chamber mounted on an inverted confocal laser scanning microscope (Nikon TE 300-Noran Oz, Nikon Corp., Melville, NY). Fluo-4 (excitation Ar 488 nm line) signals were recorded in the 525/50 nm range. Neurons were identified 5 min prior to drug administration by a brief (5 s) K^+ depolarisation.[26] To analyse the recordings, regions of interest were drawn, average signals normalised and peak amplitude analysed, all using Igor Pro (Wavemetrics, Lakes Oswego, OR). A peak was considered if signals increased above baseline >5 times the resting noise level. A local perfusion system (1 ml/min) allowed us acutely to administer (anta-)agonist-containing solutions (50, 250 and 500 ng/ml BDNF; 0.1 μM ω-conotoxin MVIIA, Alomone Labs; 0.1 μM K-252a, Calbiochem, Darmstadt, Germany; and 10 μM 5-HT, 1 μM substance P (SP) and 100 μM hexamethonium, all from Sigma) for a set period of time. All concentrations were based on previous studies.[26 28] Amplitudes of agonist-induced Ca^{2+} rises were expressed as a percentage of the response to high K^+.

Table 1 Specifications of antibodies, with their host, dilution and provider

Antigen	Dilution	Source
BDNF, rabbit	1:600	Santa Cruz Biotechnologies, Santa Cruz, CA
TrkB, rabbit	1:600	Santa Cruz Biotechnologies, Santa Cruz, CA
Synaptophysin, rabbit	1:1000	Dr R. Jahn, Göttingen, Germany
PGP9.5, mouse	1:300	UltraClone Ltd, Cambridge, UK
ChAT, mouse	1:250	Chemicon International, Temecula, CA
nNOS, mouse	1:200	BD Transduction Laboratories, San Diego, CA
5-HT, mouse	1:20	DakoCytomation A/S, Glostrup, Denmark
Mouse-IgG Alexa 594, donkey	1:1000	Molecular Probes, Leiden, The Netherlands
Rabbit-IgG Alexa 488, donkey	1:1000	Molecular Probes, Leiden, The Netherlands
Rabbit-IgG AMCA, donkey	1:250	Jackson Immuno, West Grove, PA

AMCA, aminomethylcoumarin acetate; BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; 5-HT, 5-hydroxytryptamine; nNOS, neuronal nitric oxide synthase; PGP9.5, protein gene product 9.5; TrkB,tropomyosin-related kinase B.

FM1-43 imaging

Generally, FM1-43 (Molecular Probes) imaging was performed as previously described.[29] Coverslips containing cultured myenteric neurons were transferred to a recording chamber with two parallel (~10 mm) Pt/Ir wires for electrical field stimulation (EFS, 75 mA, 1 ms, WPI A385 stimulator, Hertfordshire, UK). All image sequences were recorded with TILLvisION software on an Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany) equipped with a PolyV monochromator (TILL Photonics, Gräfelfing, Germany) and PCO Sensicam-QE CCD camera (Kelheim, Germany). Recordings were made under constant perfusion with HEPES solution. Here, active synapses were labelled in the presence of 10 μM FM1-43 by three rounds of 300 action potentials (APs) at 20 Hz. The total exposure to FM1-43 was 135 s. After washing, destaining experiments were performed and complete destaining was obtained by exposure to high K^+; signals were background subtracted and normalised to their starting values using Igor Pro.

Statistics

Unless stated otherwise, all data are given as means (SEM). Differences between means were compared with independent Student t test or analysis of variance (ANOVA) with a Bonferroni post hoc test. The proportions of neurons responding in different conditions were compared with χ^2 tests. Differences were considered to be significant if $p<0.05$. N and n values represent the number of culture dishes and number of cells, respectively, unless specified otherwise. Statistical analysis was performed with Microsoft Excel (Microsoft, Redmond, WA) and GraphPad (GraphPad Software, San Diego, CA).

RESULTS

BDNF and its TrkB receptor are expressed in the guinea pig ileum

BDNF-like immunoreactivity was found in the basolateral region of subsets of mucosal epithelial cells and fibre-like structures in the centre of the mucosal villi (fig 1). The frozen sections further revealed the presence of BDNF in the myenteric and submucosal plexus of the guinea pig ileum. In LMMP preparations, punctuate BDNF-like immunoreactivity could be found in all myenteric ganglia and interganglionic connectives, as well as in the tertiary plexus. Staining for the TrkB receptor showed immunoreactivity in both myenteric and submucosal plexuses and on fibres situated in the centre of mucosal villi (fig 1). Subsets of enteroendocrine cells (EECs) present in the mucosal crypts also displayed TrkB-like immunoreactivity.

These EECs, however, could not be labelled with a 5-HT antibody, indicating that these cells differ from enterochromaffin cells. A punctate staining pattern similar to that for BDNF was detected in the tertiary plexus, myenteric ganglia and interganglionic connectives in LMMP preparations. In selective ganglia, the BDNF antibody clearly labelled individual neuronal cell bodies as well as neurites and varicosities. Intensely labelled neurons were present in 20.9% (3.4%) of myenteric ganglia (3.3 (0.2) neurons per ganglion, 200 ganglia from three animals). Double-labelling studies showed that a subset of BDNF-labelled neurons expressed nitric oxide synthase (NOS) while others expressed choline acetyltransferase (ChAT).

Presence of BDNF and TrkB in the myenteric neuron cultures

Neurons, identified by protein gene product 9.5 (PGP9.5) staining, displayed significant expression of BDNF in the cytosol, with some showing a clearly enhanced BDNF-like immunoreactivity (fig 2). Also immunolabelling for TrkB showed considerable staining in all identified neurons, labelling both neuronal cytosol and processes (fig 2). Again differences in staining intensity could be found. This indicates that an endogenous BDNF source as well as expression of TrkB was present in culture, which justifies the use of these cultures as a model system to study the physiological effects of BDNF on Ca^{2+} signalling and synaptic transmission.

Ca^{2+} signalling in cultured myenteric neurons is not acutely influenced by BDNF

To test whether BDNF's enhancing effect on gastrointestinal motility might result from rapid neuronal activation by BDNF as described in hippocampal neurons,[13] we studied the ability of BDNF to induce acute $[Ca^{2+}]_i$ changes in cultured myenteric neurons (fig 3). Neurons were first identified by high K^+ depolarisation, which induced a fluorescence rise of 2.99 (0.17). Five minutes later, cells were challenged with BDNF at different concentrations (50 ng/ml, n = 90, N = 10; 250 ng/ml, n = 79, N = 6; and 500 ng/ml, n = 72, N = 4) for 20 s and Fluo-4 emissions recorded for 100 s. None of the neurons responded with a Ca^{2+} rise to either of the BDNF concentrations, indicating that BDNF had no acute effect on $[Ca^{2+}]_i$ of cultured myenteric neurons.

BDNF enhances agonist-induced Ca^{2+} signalling

To test the hypothesis that, instead of activating neurons directly, BDNF could alter agonist-induced signalling in myenteric neurons, we exposed cultured neurons to BDNF (50 ng/ml) 2, 24 or 48 h prior to the experiment. Again Fluo-4-loaded neurons were first identified by high K^+ depolarisation (rise: 2.84 (0.33), n = 574, N = 51) and 5 min later challenged with the neurotransmitters 5-HT (10 μM; 10 s) or SP (1 μM; 10 s). These responses are mainly postsynaptic since ω-conotoxin MVIIA (0.1 μM), a blocker of the primarily presynaptically located N-type Ca^{2+} channel, had no effect on the agonist-induced Ca^{2+} peak amplitudes or the proportion of responding neurons. BDNF incubation did not alter the baseline fluorescence compared with neurons that were exposed to medium only (in arbitrary units: 23.9 (0.5), 24.4 (0.6), 23.9 (0.8) and 24.7 (0.7) for control, 2, 24 and 48 h, respectively, $p>0.05$, n = 574, N = 51). However, BDNF exposure caused a time-dependent enhancement of the responses induced by 5-HT and SP. First, the proportion of neurons responding to 5-HT increased (respectively: 92.2%, $p = 0.002$; 91.7%, $p = 0.007$; and 91.0%, $p = 0.007$ vs control: 74.2%) (fig 4E) and the $[Ca^{2+}]_i$ peak rose gradually with BDNF exposure (57.6% (0.8%), 63.3% (1.1%), 68.3% (1.0%) and 72.9% (0.9%) for control, 2, 24 and 48 h, respectively, $p<0.0001$, n = 256, N = 26) (fig 4A,C). Similarly, 24 and 48 h of BDNF incubation caused increased Ca^{2+} transients to SP (68.5% (0.9%) and 67.0% (0.7%), respectively) compared with control (61.6% (0.6%), $p<0.0001$, n = 215, N = 23) (fig 4B,D). Although the percentage of SP-responding neurons did not change, the proportion that displayed a second Ca^{2+} transient within 80 s after stimulus onset increased significantly after BDNF incubation (53.7%, $p = 0.02$ and 55.1%, $p = 0.01$ for 24 and 48 h, respectively vs control: 30.2%). The presence of K-252a (48 h), a blocker of Trk tyrosine kinase receptors, abolished the enhancing effect of BDNF (48 h) on 5-HT- (59.0% (0.9%), $p<0.0001$) and SP- (58.3% (0.6%), $p<0.0001$) induced Ca^{2+} transients (fig 4F) and also restored the number of 5-HT-responsive neurons close to the control level (77.2%, $p = 0.02$) (fig 4E). Treatment with K-252a alone did not affect the Ca^{2+} transient amplitudes or the number of responsive neurons.

BDNF promotes synaptic vesicle cluster density and spontaneous network activity in cultured myenteric neurons

To investigate if BDNF affects synaptic transmission in the ENS, we first examined the influence of BDNF on the number of synaptic contacts in the cultured myenteric neurons as identified with a synaptophysin antibody.[29] Cultured neurons were immunostained for synaptophysin and the neuronal marker PGP9.5. Changes in the density of synaptic contacts were determined by quantifying the number of synaptophysin-immunoreactive puncta per neuronal fibre length in control (N = 5; total fibre length: 6418 μm) and BDNF-treated cultured myenteric neurons (N = 5; total fibre length: 5051 μm). We found that 48 h of BDNF incubation increased the density of synaptic vesicle clusters (per 100 μm: 15.72 (0.45) vs 20.19 (0.66) in control and BDNF, respectively, $p<0.0001$) (fig 5).

To elucidate if the increased number of synaptic vesicle clusters was reflected in increased network activity, we performed Fluo-4 recordings (37°C) at normal (5 mM) or slightly elevated K^+ concentrations (6.5 mM). Neurons, identified by high K^+ depolarisation, displayed spontaneously occurring activity (fig 6A) that was increased by exposure to BDNF. First, the proportion of neurons that showed spontaneous responses increased (BDNF for 48 h: 61.0% vs control: 47.7%, $p = 0.04$) (fig 6B). Furthermore, BDNF-treated cultures showed a higher frequency (number of peaks/100 s) of activity in both 5 mM K^+ (3.04 (0.29) vs control: 2.21 (0.28), $p = 0.03$, n = 406, N = 30) and 6.5 mM K^+ (3.59 (0.26) vs control: 2.82 (0.21), $p = 0.01$, n = 345, N = 25) (fig 6C). K-252a abolished the increase in both the proportion of spontaneous active neurons (38.1%, n = 173, N = 9) and Ca^{2+} spike frequency (2.61 (0.26)). K-252a incubation, in the absence of BDNF, had no effect on Ca^{2+} spike frequency but was able to reduce the proportion of active neurons below control levels in the 6.5 mM K^+ condition. The use of the nicotinic receptor blocker hexamethonium (100 μM) reduced the proportion of spontaneously active neurons to about 20%, lowered the frequency (~1.5/100 s) and abolished the stimulating effect of the BDNF treatment irrespective of the experimental K^+ condition (Supplementary fig 2).

BDNF facilitates FM1-43 destaining in cultured myenteric neuron terminals

In order to evaluate the effect of BDNF on neurotransmitter vesicle cycling in enteric synapses, we used FM1-43 imaging.[29]

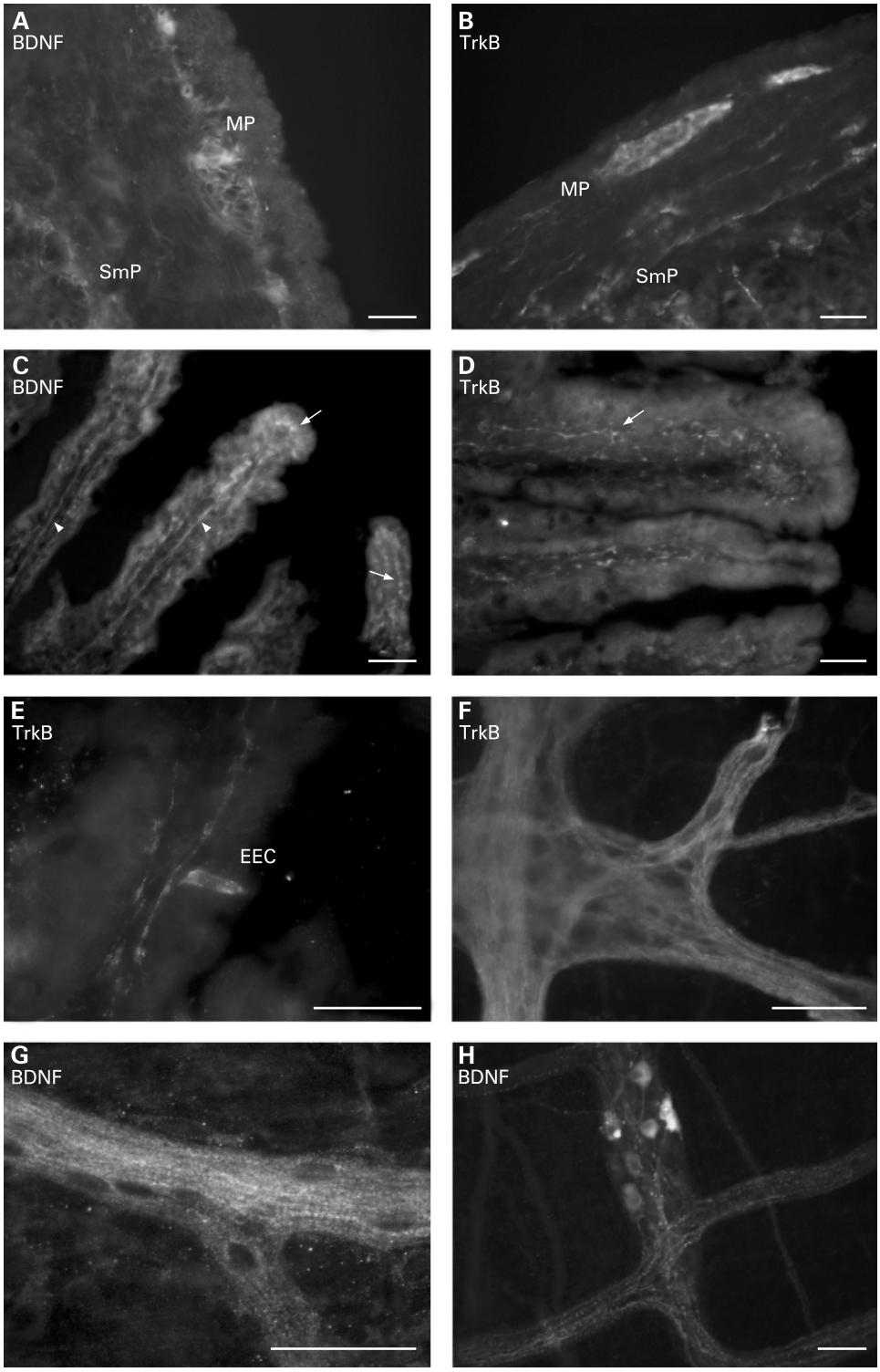

Figure 1 Brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B (TrkB) immunoreactivity in the guinea pig ileum. Guinea pig ileum frozen sections (A–E) and whole-mount longitudinal muscle–myenteric plexus preparations (F–H) were immunostained for BDNF and TrkB. (A, B) BDNF and TrkB immunoreactivity is present in the myenteric plexus (MP) and submucosal plexus (SmP). (C) BDNF immunoreactivity is found in mucosal epithelial cells (arrow) and in fasciculated bundles centred in the mucosal villi (arrowheads). (D) TrkB-immunoreactive fibres (arrows) are prominent in the mucosa. (E) Subsets of enteroendocrine cells present in the mucosal crypts display TrkB immunoreactivity. (F, G) TrkB and BDNF immunoreactivity is present in myenteric ganglia and interganglionic connectives, and (H) subsets of myenteric neurons show strong BDNF immunoreactivity in their cell bodies. Scale bars = 50 μm.

We first aimed to determine the effect of BDNF treatment on the number of recycling synaptic vesicles. The total fluorescence that was taken up during FM1-43 loading is a good estimate of the number of vesicles exocytosed or endocytosed during stimulation (three rounds of 300 APs, 20 Hz). We found no differences in the total fluorescence between control and BDNF-treated cultures (in arbitrary units: 12.59 (0.38) vs 12.26 (0.39) for control and BDNF, respectively, $p>0.05$) (fig 7C). Next, in order to evaluate the modulation of neurotransmitter release kinetics at ENS synapses, we analysed the fluorescence destaining profiles during specific electrical stimuli in control and BDNF-treated neurons. BDNF treatment for 48 h ($N = 7$, 234 terminals) enhanced the evoked vesicle turnover compared with the control ($N = 6$, 233 terminals), after both 40 APs (13% (1%) destained fraction vs control: 9% (1%)) and 400 APs (29% (2%) destained fraction vs control: 24% (2%)) stimuli (fig 7D).

DISCUSSION

The role of NTs in development,[30–34] survival[35–37] and pathological conditions[38–40] of the ENS is well documented, but little is

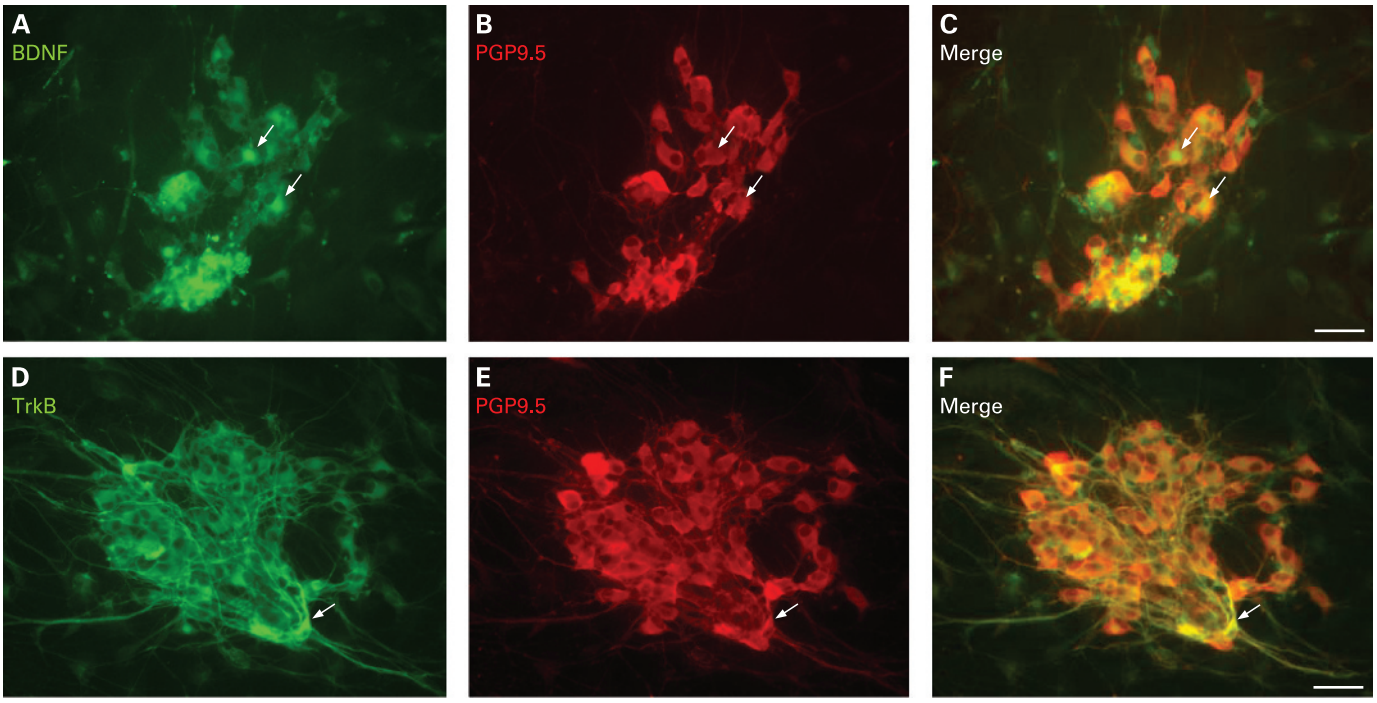

Figure 2 Cultured myenteric neurons express brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B (TrkB). Cultured myenteric neurons were immunostained for BDNF (A–C), TrkB (D–F) and the neuronal marker protein gene product 9.5 (PGP9.5) (A–F). Both BDNF and TrkB immunoreactivity co-localised with PGP9.5 staining (overlap in yellow), indicating that expression was present in myenteric neurons in cultures. Expression profiles varied, with some neurons showing intense BDNF or TrkB immunoreactivity (arrows). Scale bars = 50 μm.

known about the physiological role these NTs play in adult gut. The latter is of increasing interest, since the prokinetic effects of exogenous BDNF on gastrointestinal motility have been shown in animals and in man.[23 24] The first indication of gastrointestinal effects originated from clinical applications in neurodegenerative disorders such as amyotrophic lateral sclerosis and diabetic neuropathy in which diarrhoea was the principal side effect.[41 42] In a paper that addressed the underlying mechanisms, it was shown that mucosal stroking released BDNF in the sensory part of the reflex and that this endogenous BDNF played a role in mediating peristalsis.[25] Yet, the target for BDNF to increase gastrointestinal motility has not been identified at the cellular level. In the present study we show that BDNF is able to enhance agonist-induced signalling and synaptic transmission, which suggests that these modulating rather than acute excitatory effects underlie the motility changes caused by BDNF.

BDNF and TrkB have previously been shown to be present in the gastrointestinal tract of several species, including humans,

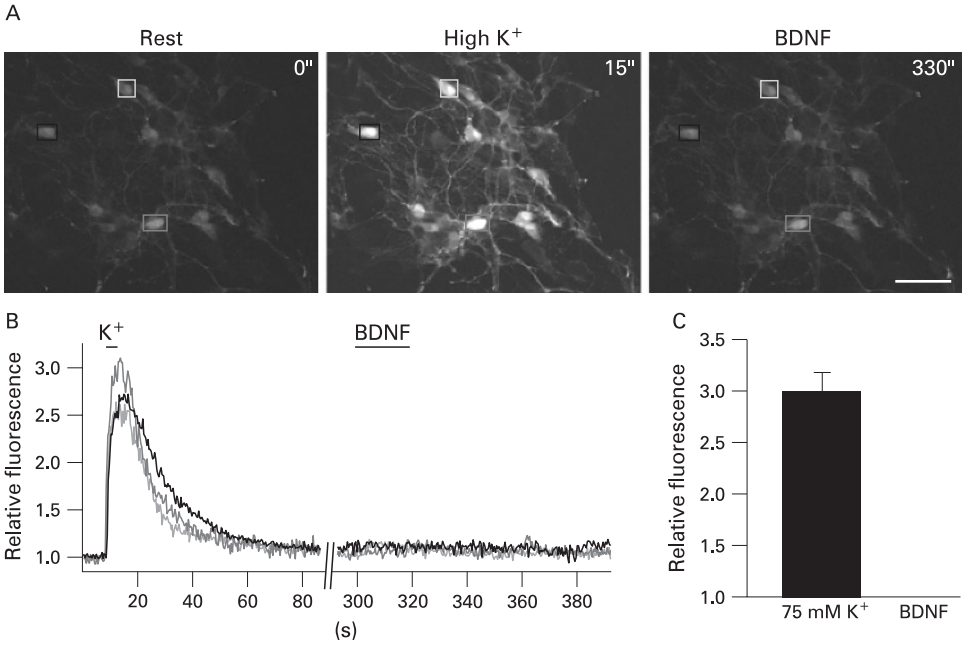

Figure 3 Brain-derived neurotrophic factor (BDNF) has no acute effects on Ca^{2+} signalling in cultured myenteric neurons. (A) Representative time-lapse fluorescence images of cultured myenteric neurons loaded with Fluo-4 at three time points. Scale bar = 100 μm. (B) Typical relative Fluo-4 fluorescence plotted against time of three neurons, as indicated with greyscale-matched regions of interest (in A). Note that neurons, identified with high K^+ depolarisation, show no increase in $[Ca^{2+}]_i$ after stimulation with BDNF. (C) The normalised average amplitudes of the high K^+- and BDNF-induced Ca^{2+} transients in cultured myenteric neurons.

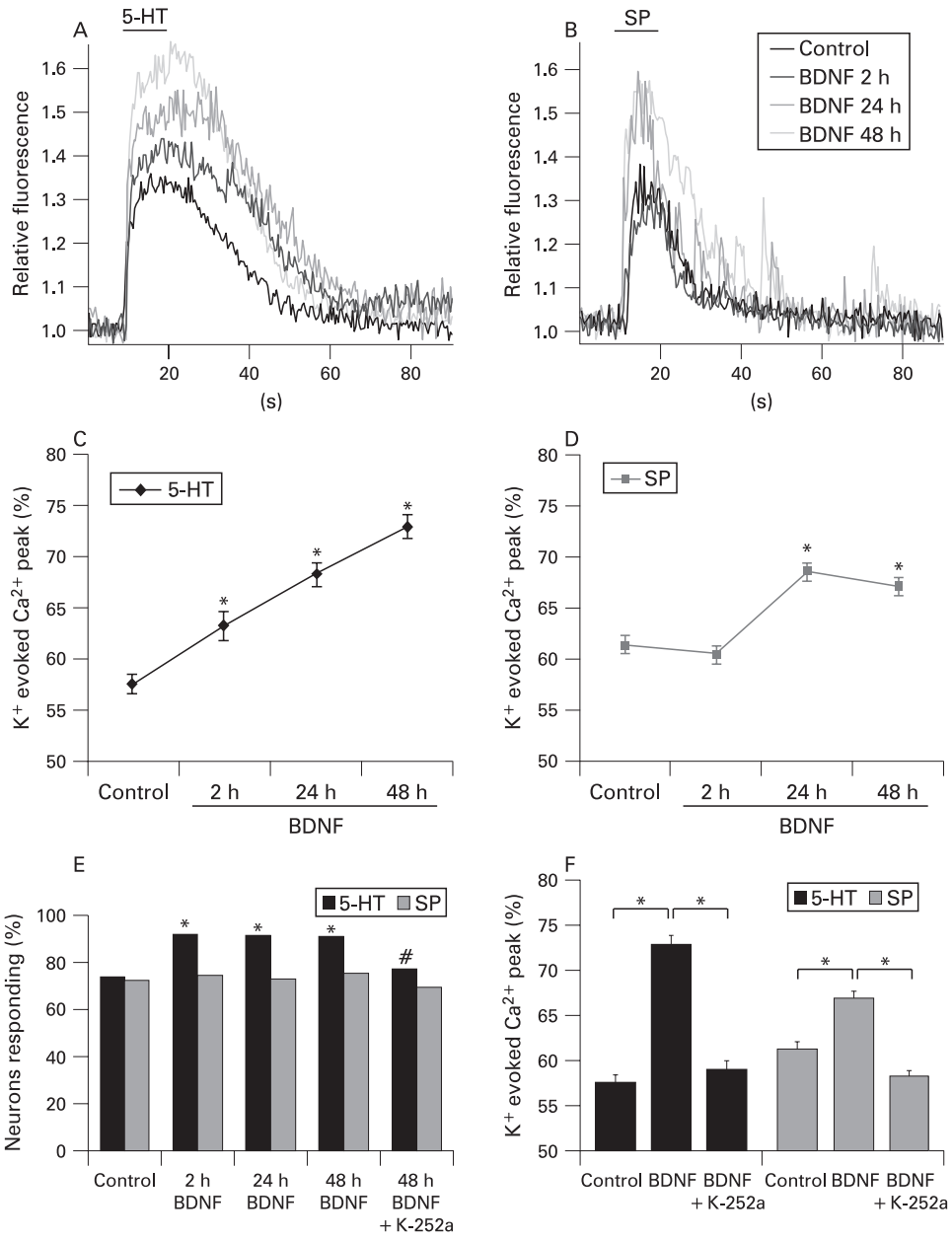

Figure 4 5-Hydroxytryptamine (5-HT)- and substance P- (SP) induced Ca^{2+} signalling is enhanced in brain-derived neurotrophic factor (BDNF)-treated cultured myenteric neurons. (A, B) Representative tracings of 5-HT- (A) and SP- (B) evoked Ca^{2+} transients of cultured myenteric neurons after incubation with bone-derived neurotrophic factor (BDNF). Relative Fluo-4 fluorescence is plotted against time. Traces are grey value coded for BDNF incubation time. (C, D) Normalised average amplitudes of the 5-HT- (C) and SP- (D) induced Ca^{2+} transients in cultured myenteric neurons after exposure to BDNF for 2, 24 and 48 h (*$p<0.05$ vs control, analysis of variance (ANOVA), Bonferroni post hoc). BDNF treatment caused a time-dependent enhancement of the Ca^{2+} transients to 5-HT and SP stimulation. (E) Proportion of neurons responding with a Ca^{2+} transient to 5-HT (black) or SP (grey) stimulation after incubation with BDNF for either 2, 24 or 48 h, or with BDNF+K-252a for 48 h (*$p<0.05$ vs control, #$p<0.05$ vs 48 h BDNF, χ^2). (F) Normalised average amplitudes of the 5-HT- and SP-induced Ca^{2+} transients after exposure to control, BDNF or BDNF+K-252a (*$p<0.05$, ANOVA, Bonferroni post hoc). Co-treatment with K-252a completely reversed the enhancement of the Ca^{2+} transients to 5-HT and SP stimulation.

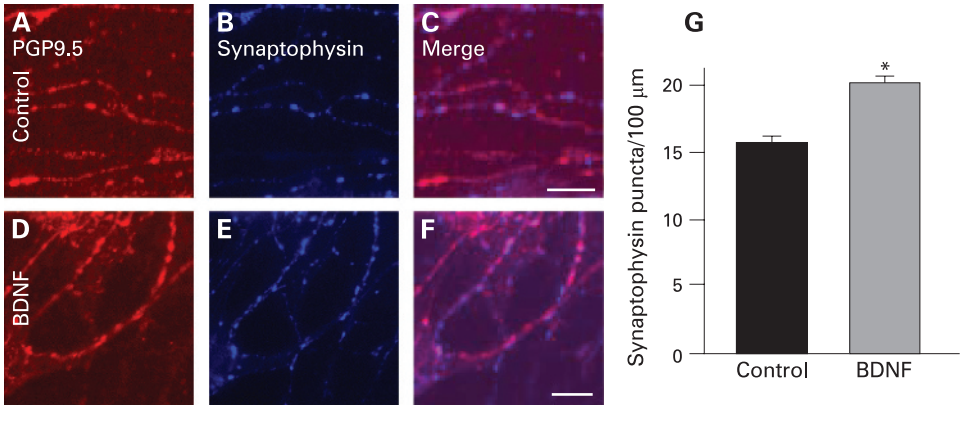

Figure 5 Brain-derived neurotrophic factor (BDNF) increases synaptic vesicle cluster density in cultured myenteric neuron fibres. (A–F) Representative images of co-labelling with anti-protein gene product 9.5 (PGP9.5) and anti-synaptophysin in control and BDNF-treated cultured myenteric neurons. Changes in the density of synaptic vesicle clusters were determined by quantifying the number of synaptophysin puncta per neuronal fibre length. (G) Average number of synaptic vesicle clusters per 100 µm neuronal fibre in control and BDNF-treated cultures (*$p<0.05$ vs control, Student t test). Scale bars = 10 µm.

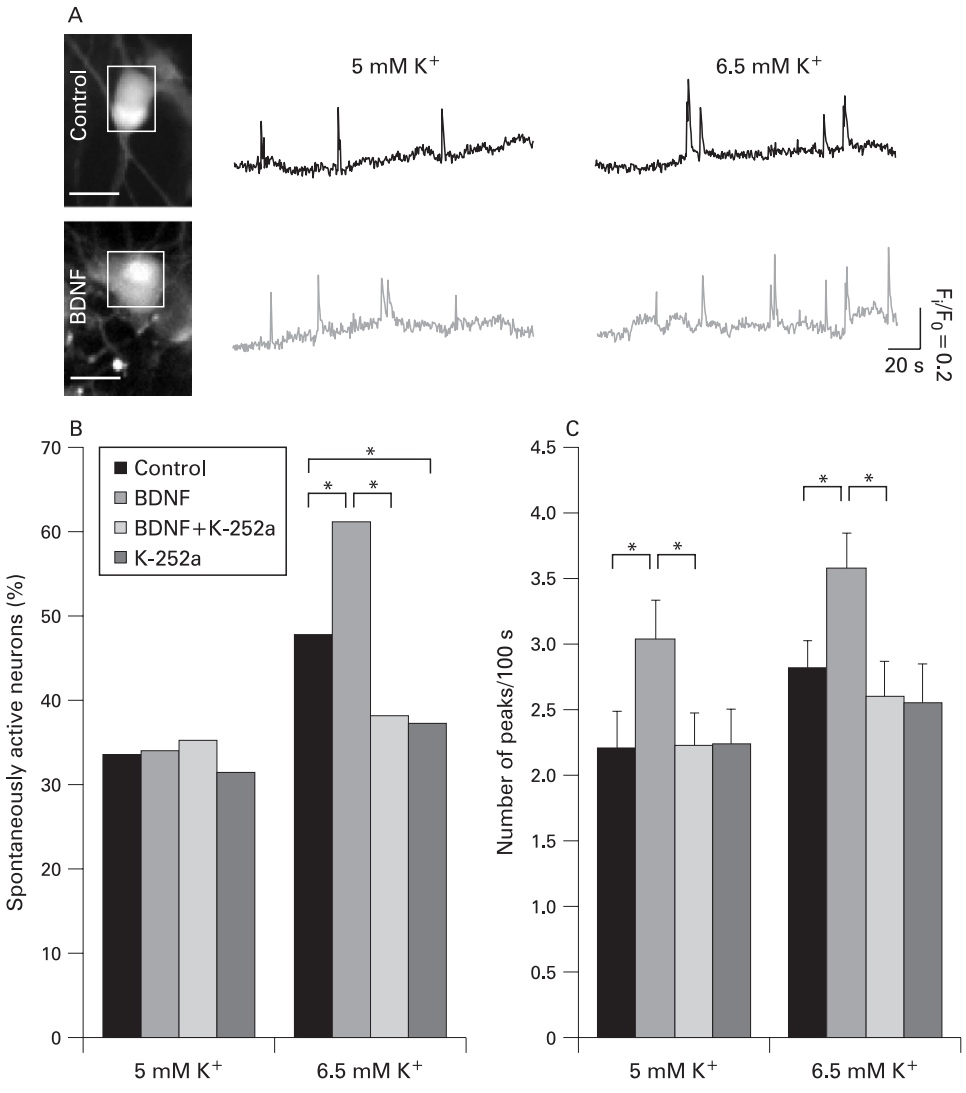

Figure 6 Brain-derived neurotrophic factor (BDNF) promotes spontaneous network activity via tropomyosin-related kinase B. (A) Representative tracings of Fluo-4 recordings in normalised and depolarised conditions (5 and 6.5 mM K^+, respectively) of control cells and cells exposed to BDNF, K-252a or both for 48 h. (B) Proportion of neurons taking part in the spontaneous network activity in control and treated cultures (*$p<0.05$, χ^2). (C) Average frequency of spontaneously occurring Ca^{2+} transients in control and BDNF-treated cultures (*$p<0.05$, analysis of variance, Bonferroni post hoc).

but a detailed localisation has not yet been evaluated.[14–20] Here, we demonstrate that BDNF- and TrkB-like immunoreactivity is present throughout the ENS of the guinea pig ileum. Apart from the expression in neurons, mucosal epithelial cells prove to be a potential source of BDNF. Together with the TrkB-like immunoreactivity on nerve fibres in the mucosal villi, this suggests a role for BDNF–TrkB signalling in the gut wall. It is conceivable that these BDNF-expressing epithelial cells are the major source of endogenous BDNF that is released during mucosal stroking.[25] The whole-mount LMMP preparations also revealed punctuate BDNF- and TrkB-like immunoreactivity in myenteric ganglia and interconnective strands similar to the staining patterns for synaptic proteins.[29] Expression of TrkB and BDNF at synaptic terminals has also been shown in the CNS,[43–45] which is in line with its presynaptic actions as shown by the FM1-43 imaging experiments. In addition to the punctuate staining, we could clearly find BDNF-like immunoreactive neurons that were either ChAT or NOS positive in selective ganglia within the myenteric plexus. TrkB- and BDNF-like immunoreactivity was also present in the cultures of enteric neurons. Although expression levels differ per neuron, it remains elusive why, compared with the whole-mount tissues, such a high number of neuronal cell bodies express BDNF-like immunoreactivity. In the CNS, BDNF can be upregulated in response to damage, ischaemia and stress. Such mechanisms may also occur in the ENS; however, preliminary studies mimicking some of these conditions failed to alter enteric neuronal BDNF expression.

Using Ca^{2+} imaging we showed that unlike in hippocampal neurons,[13] BDNF could not induce acute Ca^{2+} changes in cultured myenteric neurons and therefore could not be attributed 'neurotransmitter' properties as in the CNS.[13] This indicated that the increase of peristalsis caused by BDNF,[23 25] apart from possible regional- and species-dependent differences, was due to a mechanism other than acute neuronal excitation. One possibility, namely that BDNF enhanced neurotransmitter-induced Ca^{2+} signalling, was investigated by challenging cultured myenteric neurons with 5-HT and SP after several hours of exposure to BDNF. This resulted in a time-dependent enhancement of the Ca^{2+} transients. Not only individual responses, but also the proportion of neurons responding to 5-HT increased, while the number of SP-responsive neurons remained constant. The acute and synchronous nature of 5-HT and SP responses suggests a major involvement of direct postsynaptic actions, although a contribution of secondary transmitter release cannot be fully excluded. The different effect of BDNF on the number of 5-HT and SP responders may result from diversity in control of receptor expression or, alternatively, BDNF may differentially target the way ligand-gated ion channels ($5\text{-}HT_3$) and G-protein-coupled receptors (tachykinin

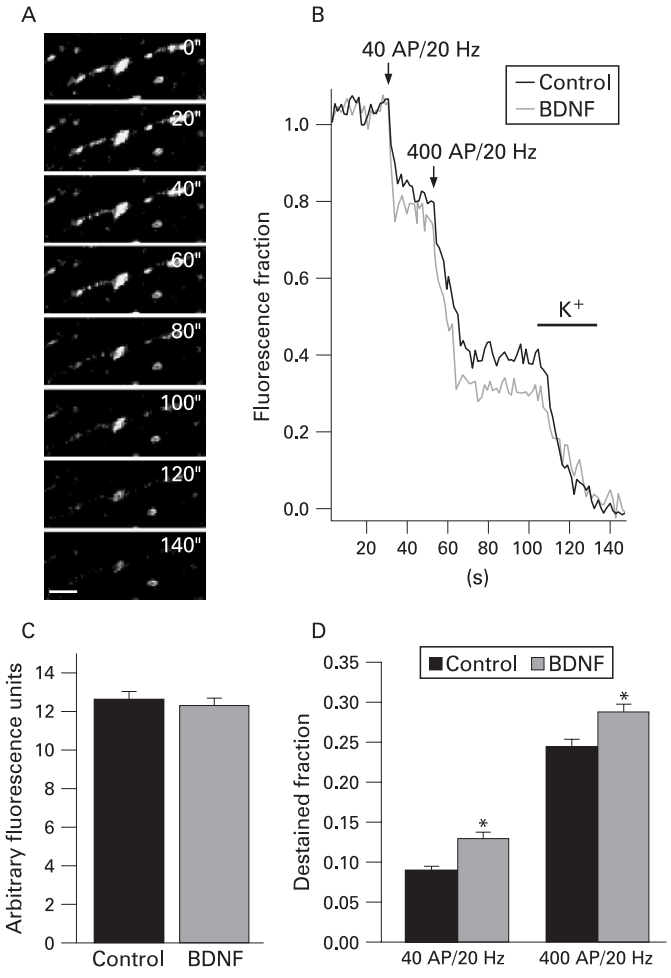

Figure 7 Evoked vesicle turnover is enhanced in brain-derived neurotrophic factor (BDNF)-treated cultured myenteric neurons. (A) Consecutive images of enteric release sites that were labelled with the vesicle recycling marker FM1-43. During a 140 s recording the neurons were stimulated with three different stimuli (40 action potentials (AP), 400 AP and high K^+). Scale bar = 5 μm. (B) Representative traces of FM1-43 destaining from control and BDNF-incubated neurons. Electrical stimuli are indicated with arrows, and complete destaining was obtained by exposing the neurons to high K^+. (C) Total fluorescence taken up by 900 APs in control and BDNF conditions; the arbitrary fluorescence units reflect the number of vesicles being recruited during neurotransmission. (D) Average destained fraction after 40 APs or subsequent 400 APs for control and BDNF-treated neurons. The electrical stimuli destained larger fractions from the neurons after 48 h of BDNF incubation (*$p<0.05$ vs control, Student t test).

receptors) couple to intracellular Ca^{2+} changes, employing Ca^{2+} influx and intracellular Ca^{2+} stores, respectively. At least for 5-HT, data from the CNS demonstrate a close modulatory relationship between BDNF and the serotonin system.[46–51] The amplification of the 5-HT and SP responses and the increase in spontaneous activity elicited by BDNF incubation were completely abolished by K-252a, a specific inhibitor of plasma membrane neurotrophin receptors,[52] which underscores the involvement of BDNF's high affinity Trk receptor.

BDNF plays a crucial role in the control of synaptic transmission and plasticity in the CNS. Mice with reduced levels of BDNF exhibit fewer neurotransmitter-containing vesicles that are ready to fuse, pronounced synaptic fatigue and reduced expression levels of synaptobrevin and synaptophysin.[53 54] Similarly, mice lacking the TrkB receptor have decreased density of synaptic vesicles in hippocampal synapses.[55] BDNF's role in synaptic transmission in the ENS has received only little attention, which is surprising as this may easily be the pathway by which BDNF influences gastrointestinal motility.[23 25] An increase in synaptic vesicle number and/or a higher efficacy of synaptic transmission is likely to enhance peristalsis, for example via facilitated 5-HT or CGRP release.[25] We studied the effects of BDNF on synaptic vesicle clusters and vesicle release dynamics in cultured myenteric neurons using immunocytochemistry and FM1-43 imaging. BDNF was found to increase the density of synaptophysin-identified synaptic vesicle clusters. Since the majority of vesicular release in the ENS is excitatory (ACh, ATP, 5-HT, etc.), one can assume that the net effect of an increased number of synaptic contacts is stimulatory in nature, as confirmed by the hexamethonium experiments, in which the BDNF-evoked enhancement of spontaneous activity was abolished. Using FM1-43, we show that after BDNF treatment, a larger fraction of vesicles can be released from enteric neuron terminals by short bursts of stimuli. BDNF thus promotes enteric synaptic efficacy as it also does in the CNS.[56] The facilitation of vesicle release can account for the increased network activity in the absence of a direct excitatory effect of BDNF, and may underlie the observed effects of BDNF on release of 5-HT and CGRP during initiation of the peristaltic reflex.[25] Synaptic facilitation is an important mechanism to stimulate peristaltic activity, and is attractive from a therapeutic point of view. This mode of action reinforces the signal but relies on natural stimuli to start the reflex and therefore does not necessarily cause spasm or perpetual uncontrolled stimulation of propulsion. However, one has to be cautious to think of BDNF as a pro-motility agent at this moment because the effects of BDNF on the gastrointestinal tract are only starting to be discovered.

In summary, we showed that BDNF and its receptor TrkB are expressed on epithelial cells and on neurons in the myenteric plexus. Although BDNF had no direct excitatory effects on enteric neurons, it enhances neuronal responsiveness to neurotransmitters such as 5-HT and SP, increases synaptic vesicle cluster density and facilitates the release of synaptic vesicles from the ENS. All these modulating effects suggest that BDNF plays an important stimulatory role in long-term regulation of presynaptic and postsynaptic activity, which is probably to increase gastrointestinal motility as described in animals and in man.[23 25]

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