BDNF and GDNF expression in discrete populations of nociceptors

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Running title: BDNF and GDNF in peptidergic nociceptors

Abstract

The brain derived neurotrophic factor (BDNF) and the glial cell line-derived neurotrophic factor (GDNF) are growth factors that promote the survival and differentiation of sensory neurons and intervene in the control of nociceptive neurotransmission. Both are synthesized by dorsal root ganglion (DRG) neurons and are anterogradely transported to the central terminals of the spinal cord dorsal horn.
To better investigate the specific expression pattern of BDNF and GDNF in nociceptors, we studied their localization in relationship to other established nociceptive markers in the mouse DRGs.
Our results can be summarized as follows:
1) BDNF and GDNF are expressed in distinct populations of small-to medium-sized DRG neurons, with BDNF three times more frequently expressed than GDNF (186.4±1.7 BDNF-immunoreactive (IR) cells/DRG vs 57.7±0.3 GDNF-IR cells/DRG; n= 3 mice); 2) A subset of BDNF-expressing neurons and a subset of GDNF-expressing neurons are of the peptidergic type; 3) BDNF-IR neurons are a subpopulation of calcitonin gene-related peptide (CGRP)-IR neurons (41.3±0.4%), also positive for substance P (SP) (42.3±0.1%), but not for somatostatin (SST); 4) GDNF-IR neurons are a subpopulation of CGRP-IR neurons (95.8±0.1%), also positive for SST (67.9±2.1%), but not SP; 5) Neither BDNF nor GDNF colocalized with the non-peptidergic marker IB4.
Our results show the existence of two subpopulations of peptidergic nociceptors characterized by the presence of CGRP, one expressing BDNF (plus SP), the other expressing GDNF (plus SST), suggesting a different role for these two neurotrophic factors in the discrimination of specific painful stimuli modalities.

key words: BDNF, GDNF, DRGs, nociceptors, peptides

Introduction

Primary sensory neurons of the dorsal root ganglia (DRG) are a heterogeneous population of neurons which detect and transduce information from a variety of specialized receptors, such as nociceptors, mechanoreceptors and proprioceptors (Basbaum et al., 2009). Differences among DRG sensory neurons are the cell body size, physiological properties, neurochemical phenotypes, and central/peripheral projections (Lawson 1992; Snider and McMahon 1998).

Nociceptors are small-to-medium size neurons and, based on their neurochemical content, are classically divided into peptidergic and non-peptidergic nociceptors, respectively, which express neuropeptides such as the calcitonin gene-related peptide (CGRP) and substance P (SP; McCarthy and Lawson, 1990), or typically bind the isolectin B4 (IB4; Silverman and Kruger, 1990).

Nociceptors depend on neurotrophic factors for their survival and differentiation (Klein, 1994; Buj-Bello et al., 1995; Snider and Silos-Santiago, 1996; Golden et al., 2010). However, increasing evidence indicates that neurotrophic factors are also able to directly and timely challenge neuronal excitability. In particular, the brain-derived neurotrophic factor (BDNF) and the glial-derived neurotrophic factor (GDNF) are widely implicated in the control of nociceptive neurotransmission under normal and pathological conditions (Bennett, 2001; Boucher and McMahon, 2001; Merighi et al., 2008; Merighi, 2015). Both are synthesized in small-to-medium size DRG neurons, coexist with several neuropeptides, and are anterogradely transported to the spinal cord dorsal horn (Zhou and Rush, 1996; Michael et al., 1997; Holstege et al., 1998; Ohta et al., 2001; Rind and von Bartheld, 2002; Salio et al., 2007; 2014).

The precise functional role of BDNF and GDNF on the transmission of nociceptive information still remains controversial as both antinociceptive and pronociceptive effects have been reported depending on the type of stimulus and the pathological pain model adopted. However, numerous studies in the last decade have importantly contributed to uncovering specific mechanisms which seem to follow a general scheme: while BDNF enhances the release of excitatory neurotransmitters (glutamate, SP, CGRP) and contributes to inflammatory and neuropathic pain (Coull et al., 2005; Lin et al., 2011; Bao et al., 2014; Chen et al., 2014; Zhang et al., 2014), GDNF reduces the central release of glutamate from activated nociceptors (Salio et al., 2014) and exhibits antinociceptive effects in different neuropathic pain models (Boucher et al., 2000; Sakai et al., 2008; Kimura et al., 2015).

A better knowledge of the cellular localization of BDNF and GDNF in nociceptive pathways is an important requirement for a better interpretation of their specific function. In the present study, we analyzed the expression of BDNF and GDNF in DRG nociceptors and investigated the pattern of their co-localization with established markers of nociceptor subpopulations.

Our results demonstrate the existence of two distinct subpopulations of peptidergic CGRP-positive nociceptors, one co-expressing BDNF and SP, the other GDNF and somatostatin (SST). The discrete expression of these neurotrophic factors into defined subpopulations of nociceptors suggest that they may be differently implicated in the discrimination of specific painful stimuli modalities.

2. Materials and methods

2.1 Animals

Immunohistochemical studies were carried out on male 3-week old CD1 mice (Light microscopy (LM): n=3; Electron microscopy (EM): n=3 for Araldite embedding; n=3 for Lowicryl embedding). Experiments were performed according to EU and national regulations on animal welfare and have been authorized by the Italian Ministry of Health (600.8/82.20/AG1826).

2.2 Tissue preparation for light and electron microscopy

Under deep pentobarbital anesthesia (60 mg⁄100 g), mice were perfused with Ringer solution followed by cold fixative solution. The latter consisted of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for LM, 1% paraformaldehyde+2% glutaradehyde in 0.2 M PB for EM Araldite embedding protocol and 4% paraformaldehyde + 0.1% glutaraldehyde + 0.2% picric acid in 0.2 M sodium PB for EM Lowicryl embedding protocol.

Lumbar DRGs and spinal cord segments were carefully dissected out, embedded in paraffin wax and cut at 6 μm with a microtome (RM 2125RT, Leica Microsystems, Wetzlar, Germany). Some spinal cord segments were cut with a vibratome (VT1000, Leica Microsystems) at 70 μm for LM, at 100 μm for EM, according to standard procedures (Cesa et al., 2003; Salio et al., 2005).

2.3 Double immunofluorescence staining

Deparaffinized lumbar DRG and spinal cord sections and spinal cord free-floating sections were pre-incubated in 0.02 M PBS containing 5% normal goat serum (PBS-5% NGS) or

PBS containing 6% bovine serum albumin (PBS-6% BSA) for 1 h, and then incubated overnight in appropriate mixtures of two primary antibodies. Sections were then incubated for 1 h in a mixture of two appropriate fluorochrome-conjugated secondary anti-species antibodies: IgG Alexa Fluor 488 or 594 (1:500, Life Technologies, Paisley, UK). Sections were finally washed in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

When it was necessary to use two primary antibodies raised in the same species (GDNF+CGRP and GDNF+SST), a paraformaldehyde vapor blocking method was used (Wang and Larsson, 1985; Salio et al., 2005). Sections were deparaffinized, hydrated preincubated in PBS-5% NGS for 1 h, and then overnight with rabbit anti-GDNF primary antibody. After washings in PBS, sections were incubated for 1 h with anti-rabbit Alexa Fluor 488 secondary antibody (1:500, Life Technologies). Sections were then washed in PBS, dehydrated, cleared in xylene and air-dried. Dried sections were placed for 2 h in a jar (1 L) containing 3 g of paraformaldehyde powder, at 80°C. Sections were then rinsed in PBS, preincubated in PBS-5% NGS for 1 h and incubated overnight with rabbit anti-CGRP or anti-SST primary antibody. After washings in PBS, sections were incubated for 1 h with an anti-rabbit Alexa Fluor 594 secondary antibody (1:500). Finally, sections were washed in PBS and mounted in Vectashield (Vector Laboratories).

2.4 EM postembedding immunostaining. Araldite and Lowicryl ultrathin sections were cut with an ultramicrotome (EM UC6; Leica Microsystems) and immunostained on grids following conventional postembedding protocols. In brief, sections were treated for 1 min with a saturated aqueous solution of sodium metaperiodate (Sigma), rinsed in 1% Triton X-100 in Tris buffered saline (TBS) 0.5 M, and then incubated for 1 h in 10% normal serum. Grids were then incubated overnight on drops of primary antibodies at optimal dilutions. After rinsing in TBS, they were incubated in a mixture of the appropriate gold conjugates (1:15), transferred into drops of 2.5% glutaraldehyde in cacodylate buffer 0.05 M, and finally washed in distilled water. Sections were counterstained with uranyl acetate and lead citrate before observation with a TEM (CM10; Philips, Eindhoven, The Netherlands).

2.5 Antibodies and controls.

The following primary antibodies were used: chicken anti-human BDNF (1:500; Promega, Madison, WI, USA; Salio et al., 2007), rabbit anti-GDNF (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Salio et al., 2014), rabbit anti-SST isoform 28 (1:2000; Merighi et

al., 1989), rabbit anti-CGRP (1:1000; Merighi et al., 1991), rat anti-SP (1:500; BD Biosciences, San Jose, CA, USA).

Lectin Griffonia simplicifolia (IB4), biotin conjugated (1:250; Sigma, St. Louis, MO, USA) was also used in immunohistochemical procedures.

Immunohistochemical controls consisting in omission of primary antibodies were routinely performed (Salio et al., 2005, 2014).

2.6 Quantification

Quantification of each given double immunostaining was performed by analyzing 3 DRGs/mouse. DRGs were serially sectioned *in toto* and one out of five sections in the series was subsequently processed for each double immunostaining combination. Such an approach allowed analysis of 4 sections separated by about 30 µm, thus reducing the risk of double counting the same neuron and allowing for exploration of the entire DRG. Individual sections were double immunolabeled for one of the following combinations: BDNF+GDNF, BDNF+CGRP, BDNF+SP, BDNF+SST, BDNF+IB4, GDNF+CGRP, GDNF+SP, GDNF+SST, GDNF+IB4.

Sections were photographed as single-stack images (capture images) at 20x with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems), and pairs of digital images of the same microscopic field were obtained with appropriate filter combinations to visualize the fluorescence labels. Single- and/or double labeled DRG neurons were visually counted from dual-color fluorescence images (Adobe Photoshop CS2 9, Adobe Systems, San Jose, CA, USA). Only neurons unambiguously distinct from the background were considered immunopositive. Results were expressed as mean (± SEM) number of cells per DRG or as mean percentages (± SEM) of double-labeled neurons versus the total number of single-labeled cells for each of the different combinations of primary antibodies, with n indicating the number of mice. Levene's test for equality of variances was performed to test homogeneity of variance across samples. Variances were considered different for $P<0.05$.

3. Results

3.1 Expression of BDNF and GDNF in nociceptors

BDNF- and GDNF-immunoreactivity (IR) was distributed in small-to medium-sized DRG neurons (Fig. 1A, B). No double-labeled neurons were observed (Fig. 1C), indicating that BDNF and GDNF were expressed in distinct populations of DRGs. The number of IR-cells

per DRG was counted and BDNF resulted three time more expressed than GDNF (186.4±1.7 BDNF-IR cells/DRG vs 57.7±0.3 GDNF-IR cells/DRG, n=3 mice). The variances of the numbers of both BDNF and GDNF neurons across different DRGs obtained from different mice were not statistically different at the Leven's test (P = 0.94 and P = 0.52, respectively) indicating homogeneous sampling.

Both BDNF-IR and GDNF-IR neurons were found to co-express CGRP (Fig. 1D, E). In particular, while less than half of the BDNF-IR cells also co-expressed CGRP (41.3±0.4%, n=3; see pie chart), almost all GDNF-IR cells were CGRP-IR (95.8±0.1%, n=3; see pie charts in Fig. 1).

As BDNF and GDNF were expressed by distinct populations of neurons, we then asked whether these nociceptors could be further characterized on the basis of their neuropeptide content. Thus, we double-immunostained DRGs with SP and SST. Interestingly, while about half of BDNF-IR cells were positive for SP (42.3±0.1%, n=3; pie chart; Fig. 1F), but none for SST (Fig. 1H), most of GDNF-IR neurons were positive for SST (67.9±2.1%, n=3; pie chart; Fig. 1G) but not for SP (Fig. 1I). This analysis revealed that BDNF- and GDNF-IR neurons were expressed in phenotypically distinct subpopulations of peptidergic neurons expressing SP and SST, respectively. Finally, neither BDNF-IR nor GDNF-IR neurons were IB4-positive (Fig. 1J-K).

3.2 Expression of BDNF and GDNF in primary afferent fibers (PAFs) projecting to the superficial dorsal horn

Peptidergic sensory neurons project to lamina I and lamina II outer (IIo) of the spinal dorsal horn, while non peptidergic neurons project deeper in lamina II inner (IIi) (Ribeiro da Silva, 2004). As BDNF and GDNF were expressed in distinct subpopulations of peptidergic nociceptors, we wondered whether they also projected separately in different laminae or sub-laminae of the dorsal horn.

BDNF-IR fibers were distributed in laminae I-IIo and formed a dense network of neuronal varicose processes. GDNF-IR fibers were instead observed in lamina IIo, below the dorsal band occupied by BDNF-IR fibers (Fig. 2A). Both BDNF- and GDNF-IR fibers were distributed dorsally to the IB4-labeled band within lamina IIi (Fig. 2B, C).

This laminar organization was also clearly distinguishable in double immunostainings. As observed in DRGs, both BDNF and GDNF were highly co-expressed with CGRP in nociceptive fibers of laminae I-II (Fig. 2D-E). Moreover, BDNF+SP-IR was observed in the

same peptidergic fibers (Fig. 2F) located dorsally to the fibers expressing SST (Fig. 2G). In contrast, GDNF+SST-IR fibers (Fig. 2H) were distributed ventrally to SP-IR fibers (Fig. 2I).
Electron microscope experiments confirmed the presence of two distinct subpopulations of peptidergic nociceptors organized in peptidergic synaptic glomeruli of the type Ib (GIb). Indeed, co-localization between BDNF+SST-IR (Fig. 3A-B) or GDNF+SP-IR (Fig. 3C-D) was never observed in over 50 immunolabeled profiles. The central terminal of these glomeruli was characterized by groups of small agranular synaptic vesicles and scattered dense core vesicles (DCVs; Ribeiro da Silva, 2004). Both neurotrophic factors and peptides were selectively localized to DCVs (see inserts in Fig. 3).

4. Discussion

The classification of DRG neurons and, in particular, of nociceptors has undergone considerable refinement during the last decades (Le Pichon and Chesler, 2014).The seminal distinction between peptidergic and non-peptidergic neurons has been progressively split into a number of ramifications leading to the identification of several phenotypically distinct neuronal subpopulations.
In the present study, we have updated nociceptor classification in terms of their “neurotrophic content”. In particular, we have focused our interest on two specific neurotrophic factors, BDNF and GDNF, which are synthesized in primary sensory neurons, anterogradely transported to the dorsal horn and synaptically released onto dorsal horn neurons (Michael et al 1997; Kerr et al 1999; Otah et al, 2001; Salio et al., 2007, 2014). All these features strongly suggest that these molecules may act as neuromodulators at the interface between first and second order sensory neurons, as a growing body of evidence highlights the importance of these growth factors as key regulators of nociceptive processing mechanisms at the spinal cord level (Merighi et al., 2008; Merighi, 2015).
Our data demonstrate that both BDNF and GDNF are expressed in small-to-medium sized peptidergic primary sensory neurons expressing CGRP, but not in IB4-positive non-peptidergic neurons. The presence of these trophic factors in peptidergic DRG neurons is consistent with previous data (Michael et al., 1997; Luo et al., 2001; Salio et al., 2007; Salio et al., 2014). However, the level of expression and the cell distribution of these two trophic factors exhibit several outstanding differences, which can be summarized as follows: 1) BDNF and GDNF are expressed in distinct cell populations of DRG neurons; 2) the BDNF population is larger than the GDNF one; 3) the level of co-localization between the neurotrophic factors and CGRP is quantitatively different. Indeed, while virtually all

GDNF-expressing neurons also express CGRP, BDNF was only found in 41% of CGRP-positive neurons. The proportion of non-CGRP BDNF expressing neurons appears here higher than previously reported (Michael et al., 1997), but the discrepancy can simply reflect species difference (mouse *vs* rat). All the same, it indicates that a significant number of BDNF neurons do not belong either to classical peptidergic (CGRP-expressing) or to non-peptidergic (IB4-positive) nociceptors, therefore suggesting that BDNF can also be expressed by non-nociceptive sensory neurons. Conversely, GDNF was chiefly expressed in peptidergic nociceptors, supporting a role as a pain modulator.

With the purpose of fully characterizing the populations of peptidergic neurons identified by the neurotrophic factors, we investigated their respective relationships to SP and SST, identified markers of distinct and mutually exclusive CGRP subpopulations (Ribeiro-Da-Silva, 2004). Our quantitative analysis demonstrated that BDNF- and GDNF-expressing neurons specifically co-express SP and SST, respectively. Interestingly, the proportion of BDNF neurons expressing SP (about 40%) closely overlaps that of neurons co-expressing BDNF+CGRP. As SP neurons represent a subpopulation of CGRP neurons, virtually all BDNF+SP neurons should also contain CGRP (BDNF/SP/CGRP neurons). Similarly, as most of GDNF neurons express SST and SST co-localizes with CGRP, then the majority of GDNF+CGRP neurons should also contain SST (GDNF/SST/CGRP neurons). In summary, CGRP neurons constitute a large heterogeneous population of nociceptors; when classified according to their neurotrophic content, about 30% of CGRP neurons contain BDNF+SP and about 20% contain GDNF+SST (Fig.4).

We observed that the central projections of these distinct neuronal populations reach different laminae in the superficial dorsal horn of the spinal cord. Interestingly, GDNF/SST occupies an intermediate band at the interface between the more superficial projection site of BDNF/SP fibers and the deeper termination of IB4-positive fibers in lamina IIi. That fibers expressing different neurotrophic factors and peptides have distinct projection sites in the superficial dorsal horn clearly indicates that they should play a different functional role in conveying nociceptive information to supraspinal centers under normal and/or pathological conditions. The idea that growth factors such as BDNF and GDNF may act as modulators of substantia gelatinosa circuits during the course of normal pain processing, has been supported by a series of anatomical and functional slice studies in rat or mouse in our laboratory (Bardoni et al. 2007; Merighi et al. 2008; Salio et al., 2014). In parallel, several studies have demonstrated the intervention of BDNF or GDNF in the onset of neuropathic or inflammatory pain (Boucher et al., 2000; Coull et al., 2005; Sakai et al.,

2008; Lin et al., 2011; Chen et al., 2014; Kimura et al., 2015). Several lines of evidence indicate that BDNF mainly displays pronociceptive effects contributing to development of hyperalgesia and central sensitization (Kerr et al., 1999), as well as neuropathic pain symptoms (Coull et al 2005). Theoretically, the co-expression of BDNF with SP, an excitatory neuropeptide (Hunt and Mantyh, 2001), supports this notion. However, we have previously demonstrated that activation of the preferred SP receptor NK1 as well as of the BDNF receptor TrkB, may enhance the release of glycine and GABA in lamina II (Vergnano et al., 2004; Ferrini et al., 2007; Bardoni et al., 2007). Therefore the emerging picture is surely more complex and led us to hypothesize that at least two parallel channels of processing for nociceptive information exist in the gelatinosa, whereby high molecular weight modulators can activate one or the other in relation to functional status. Interestingly, as TrkB receptors are expressed (among others) by the same fibers containing BDNF/SP/CGRP (Salio et al., 2005), the release of the neurotrophic factor likely activates and maintains an autocrine loop which enhances the release of SP/CGRP and glutamate onto dorsal horn neurons. This loop, however, somehow should activate the parallel inhibitory path mediated by BDNF (see Merighi et al., 2008 for further discussion). Differently from BDNF, GDNF mainly acts on dorsal horn neurons as a negative modulator of nociceptive information at least in slice preparations (Salio et al., 2014). Although pronociceptive effects have been described at the peripheral level (Malin et al., 2006), the antinociceptive effects of GDNF on central neurons have been well established at least in neuropathic pain (Boucher et al.,2000; Unezaki et al., 2012; Meng et al., 2015). However, it remains puzzling that, with a remarkable parallelism to BDNF, pronociceptive effects have been reported in experimental models of inflammatory pain (see for review Merighi, 2015). Clearly, our knowledge of the gelatinosa circuitry is insufficient to fully grasp the complex network of interactions between these (and other) modulators as it emerged in the last decades. In this perspective, the co-expression of GDNF with the inhibitory peptide SST suggests that primary sensory neurons containing GDNF/SST/CGRP may exert specific control on the transmission of nociceptive information to dorsal horn neurons. Supporting this suggestion, we found that the GDNF receptor complex GFRalfa1/Ret and the SST receptor SST2R were both expressed by IB4-labeled non-peptidegic fibers and that the activation of these receptors reduced the release of glutamate onto lamina II neurons (Bencivinni et al., 2011; Salio et al., 2014): considering that GDNF/SST/CGRP fibers terminate immediately dorsally to IB4-labeled fibers, they are ideally positioned to exert a tight control on non-peptidergic fibers activity.

4.1 Conclusions

Detecting the phenotypic diversity of nociceptors represents a necessary prerequisite for comprehensive understanding of their function in pain transmission. Here we have demonstrated that the neurorotrophic factors BDNF and GDNF identify two discrete subpopulations of peptidergic nociceptors which project independently to the spinal dorsal horn. The presence of different subsets of peptidergic nociceptors suggests the existence of a sophisticated system to encode specific nociceptive stimuli and discriminate pain modalities.

Acknowledgements

This work was supported by a grant from Compagnia San Paolo (Fondi di Ateneo 2012).

The authors have no conflicts of interest with regard to the studies described in this manuscript.

References

Bao, Y., Hou, W., Liu, R., Gao, Y., Kong, X., Yang, L., Shi, Z., Li, W., Zheng, H., Jiang, S., Li, C., Qin, Y., Hua, B., 2014. PAR2-mediated upregulation of BDNF contributes to central sensitization in bone cancer pain. Mol Pain. 10, 28-38.

Bardoni, R., Ghirri, A., Salio, C., Prandini, M., Merighi, A., 2007. BDNF-mediated modulation of GABA and glycine release in dorsal horn lamina II from postnatal rats. Dev Neurobiol. 67, 960-975.

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and molecular mechanisms of pain. Cell 139, 267–284.

Bencivinni, I., Ferrini, F., Salio, C., Beltramo, M., Merighi, A., 2011. The somatostatin analogue octreotide inhibits capsaicin-mediated activation of nociceptive primary afferent fibres in spinal cord lamina II (substantia gelatinosa). Eur J Pain. 15, 591-599.

Bennett, D.L., 2001. Neurotrophic factors: important regulators of nociceptive function. Neuroscientist 7, 13–7.

Boucher, T.J., McMahon, S.B., 2001. Neurotrophic factors and neuropathic pain. Curr Opin Pharmacol. 1, 66-72.

Boucher, T.J., Okuse, K., Bennett, D.L., Munson, J.B., Wood, J.N., McMahon, S.B., 2000. Science 290, 124-7.

Buchman, V.L., Horton, A., Rosenthal, A., Davies, A.M., 1995. GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron 15, 821-8.

Cesa, R., Morando, L., Strata, P., 2003. Glutamate receptor delta2 subunit in activity-dependent heterologous synaptic competition. J. Neurosci. 23, 2363–2370.

Chen JT, Guo D, Campanelli D, Frattini F, Mayer F, Zhou L, Kuner R, Heppenstall PA, Knipper M, Hu J., 2014. Presynaptic GABAergic inhibition regulated by BDNF contributes to neuropathic pain induction. Nat Commun. 5, 5331.

Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W., De Koninck, Y., 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438, 1017-21.

Ferrini, F., Salio, C., Vergnano, A.M., Merighi, A., 2007. Vanilloid receptor-1(TRPV1)-dependent activation of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse. Pain 129, 195-209.

Golden, J.P., Hoshi, M., Nassar, M.A., Enomoto, H., Wood, J.N., Milbrandt, J., Gereau, R.W., Johnson, E.M. Jr., Jain, S., 2010. RET signaling is required for survival and normal function of non peptidergic nociceptors. J. Neurosci. 30, 3983-3994.

Holstege, J.C., Jongen, J.L., Kennis, J.H., Rooyen-Boot, A.A., Vecht, C.J., 1998. Immunocytochemical localization of GDNF in primary afferents of the lumbar dorsal horn. Neuroreport 9, 2893-2897.

Hunt, S.P., Mantyh, P.W., 2001.The molecular dynamics of pain control. Nat Rev Neurosci. 2, 83-91.

Kerr, B.J., Bradbury, E.J., Bennett, D.L., Trivedi, P.M., Dassan, P., French, J., Shelton, D.B., McMahon, S.B., Thompson, S.W., 1999. Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. J Neurosci. 19, 5138-5148.

Kimura, M., Sakai, A., Sakamoto, A., Suzuki, H., 2015. Glial cell line-derived neurotrophic factor-mediated enhancement of noradrenergic descending inhibition in the locus coeruleus exerts prolonged analgesia in neuropathic pain. Br. J. Pharmacol. 172, 2469-78.

Klein, R., 1994. Role of neurotrophins in mouse neuronal development. FASEB J. 8, 738–744.

Lawson, S.N., 1992. Morphological and biochemical cell types of sensory neurons. In: Scott, S.A. (Ed.). Sensory neurons. 1st ed. New York, Oxford University Press. Pp. 27–59.

Le Pichon, C.E., Chesler, A.T., 2014. The functional and anatomical dissection of somatosensory subpopulations using mouse genetics. Front Neuroanat. 8, 21.

Lin, Y.T., Ro, L.S., Wang, H.L., Chen, J.C., 2011. Up-regulation of dorsal root ganglia BDNF and trkB receptor in inflammatory pain: an in vivo and in vitro study. J. Neuroinflammation 8, 126-147.

Luo, X.G., Rush, R.A., Zhou, X.F., 2001. Ultrastructural localization of brain-derived neurotrophic factor in rat primary sensory neurons. Neurosci Res. 39, 377-84.

Malin, S.A., Molliver, D.C., Koerber, H.R., Cornuet, P., Frye, R., Albers, K.M., Davis, B.M., 2006. Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. J Neurosci. 26, 8588-8599.

McCarthy, P.W., Lawson, S.N., 1990. Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. Neuroscience 34, 623–632.

Meng, F.F., Xu, Y., Dan, Q.Q., Wei, L., Deng, Y.J., Liu, J., He, M., Liu, W., Xia, Q.J., Zhou, F.H., Wang, T.H., Wang, X.Y., 2015. Intrathecal injection of lentivirus-mediated glial cell line-derived neurotrophic factor RNA interference relieves bone cancer-induced pain in rats. Cancer Sci. 106, 430-437.

Merighi, A., Polak, J.M., Fumagalli, G., Theodosis, D.T., 1989. Ultrastructural localization of neuropeptides and GABA in rat dorsal horn: a comparison of different immunogold labeling techniques. J. Histochem. Cytochem. 37, 529 –540.

Merighi, A., Polak, J.M., Theodosis, D.T., 1991. Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. Neuroscience 40, 67– 80.

Merighi, A., Salio, C., Ghirri, A., Lossi, L., Ferrini, F., Betelli, C., Bardoni, R., 2008. BDNF as a pain modulator. Prog. Neurobiol. 85, 297-317.

Merighi, A., Bardoni, R., Salio, C., Lossi, L., Ferrini, F., Prandini, M., Zonta, M., Gustincich, S., Carmignoto, G., 2008. Presynaptic functional trkB receptors mediate the release of excitatory neurotransmitters from primary afferent terminals in lamina II (substantia gelatinosa) of postnatal rat spinal cord. Dev Neurobiol. 68, 457-475.

Merighi, A., 2015. Targeting the glial-derived neurotrophic factor and related molecules for controlling normal and pathologic pain. Expert Opinion Ther Targets. 1-16.

Michael, G.J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D.L., Yan, Q., Priestley, J.V., 1997. Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. J. Neurosci. 17, 8476-90.

Ohta, K., Inokuchi, T., Gen, E., Chang, J., 2001. Ultrastructural study of anterograde transport of glial cell line-derived neurotrophic factor from dorsal root ganglion neurons of rats towards the nerve terminal. Cells Tissues Organs 169, 410-421.

Ribeiro-da-Silva, A., 2004. Substantia gelatinosa of the spinal cord. In: Paxinos, G. (Ed.).The rat nervous system, San Diego: Elsevier Academic, pp. 129–148.

Rind, H.B., von Bartheld, C.S., 2002. Anterograde axonal transport of internalized GDNF in sensory and motor neurons. Neuroreport 13, 659-664.

Sakai, A., Asada, M., Seno, N., Suzuki, H., 2008. Involvement of neural cell adhesion molecule signaling in glial cell line-derived neurotrophic factor-induced analgesia in a rat model of neuropathic pain. Pain 137, 378-388.

Salio, C., Lossi, L., Ferrini, F., Merighi, A., 2005. Ultrastructural evidence for a pre- and postsynaptic localization of full-length trkB receptors in substantia gelatinosa (lamina II) of rat and mouse spinal cord. 22, 1951-66.

Salio, C., Averill, S., Priestley, J.V., Merighi, A., 2007. Costorage of BDNF and neuropeptides within individual dense-core vesicles in central and peripheral neurons. Dev. Neurobiol. 67, 326-338.

Salio, C., Ferrini, F., Muthuraju, S., Merighi, A., 2014. Pre-synaptic modulation of spinal nociceptive transmission by glial cell line-derived neurotrophic factor (GDNF). J. Neurosci. 34, 13819-13833.

Silverman, J.D., Kruger, L., 1990. Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. J. Neurocytol. 19, 789–801.

Snider, W.D., McMahon, S.B., 1998. Tackling pain at the source: new ideas about nociceptors. Neuron 20, 629–32.

Snider, W.D., Silos-Santiago, I., 1996. Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 351, 395–403.

Sasaki, A., Matsumura, S., Katano, T., Nakazawa, T., Nishio, N., Andoh, T., Yamamoto, T., Nakatsuka, T., Kuraishi, Y., Ito, S., 2012. Involvement of Tyr1472 phosphorylation of NMDA receptor NR2B subunit in postherpetic neuralgia in model mice. Mol Pain 8, 59.

Wang, B.L., Larsson, L.I., 1985. Simultaneous demonstration of multiple antigens by indirect immunofluorescence or immunogold staining. Novel light and electron microscopical double and triple staining method employing primary antibodies from the same species. Histochemistry 83, 47–56.

Vergnano, A.M., Salio, C., Merighi A., 2004. NK1 receptor activation leads to enhancement of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse. Pain 112, 37-47.

Zhang, H.H., Zhang, X.Q., Xue, Q.S., Yan-Luo, Huang, J.L., Zhang, S., Shao, H.J., Lu, H., Wang, W.Y., Yu, B.W., 2014. PLoS One 9, e85536.

Zhou, X.F., Rush, R.A., 1996. Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. Neuroscience 74, 945-953.

Legends

Figure 1. Distribution of neurotrophic factors in DRGs and colocalization with nociceptive markers. (A-C) BDNF (red, A) and GDNF (green, B) are expressed in small to medium size DRG neurons, but no co-expression was observed (C). (D) Double-immunostaining for BDNF (green) and CGRP (red) in small to medium DRG neurons. (E) Double-immunostaining for GDNF (green) and CGRP (red) in small to medium DRG cells. (F) Double-immunostaining for BDNF (green) and SP (red) in small to medium DRG neurons. (G) Double-immunostaining for GDNF (green) and SST (red) in small to medium DRG neurons. Note, the pie charts in figures D, E, F, G illustrate the extent of co-expression (yellow) of peptides (CGRP, SP, SST; green) in trophic factor-containing neurons and of trophic factors (BDNF and GDNF; red) in peptidergic neurons. (H) Double-immunostaining for BDNF (green) and SST (red; no co-expression). (I) Double-immunostaining for GDNF (green) and SP (red; no co-expression). (J) Double-immunostaining for BDNF (green) and IB4 (red; no co-expression). (K) Double-immunostaining for GDNF (green) and IB4 (red; no co-expression). Scale bars: 50 μm.

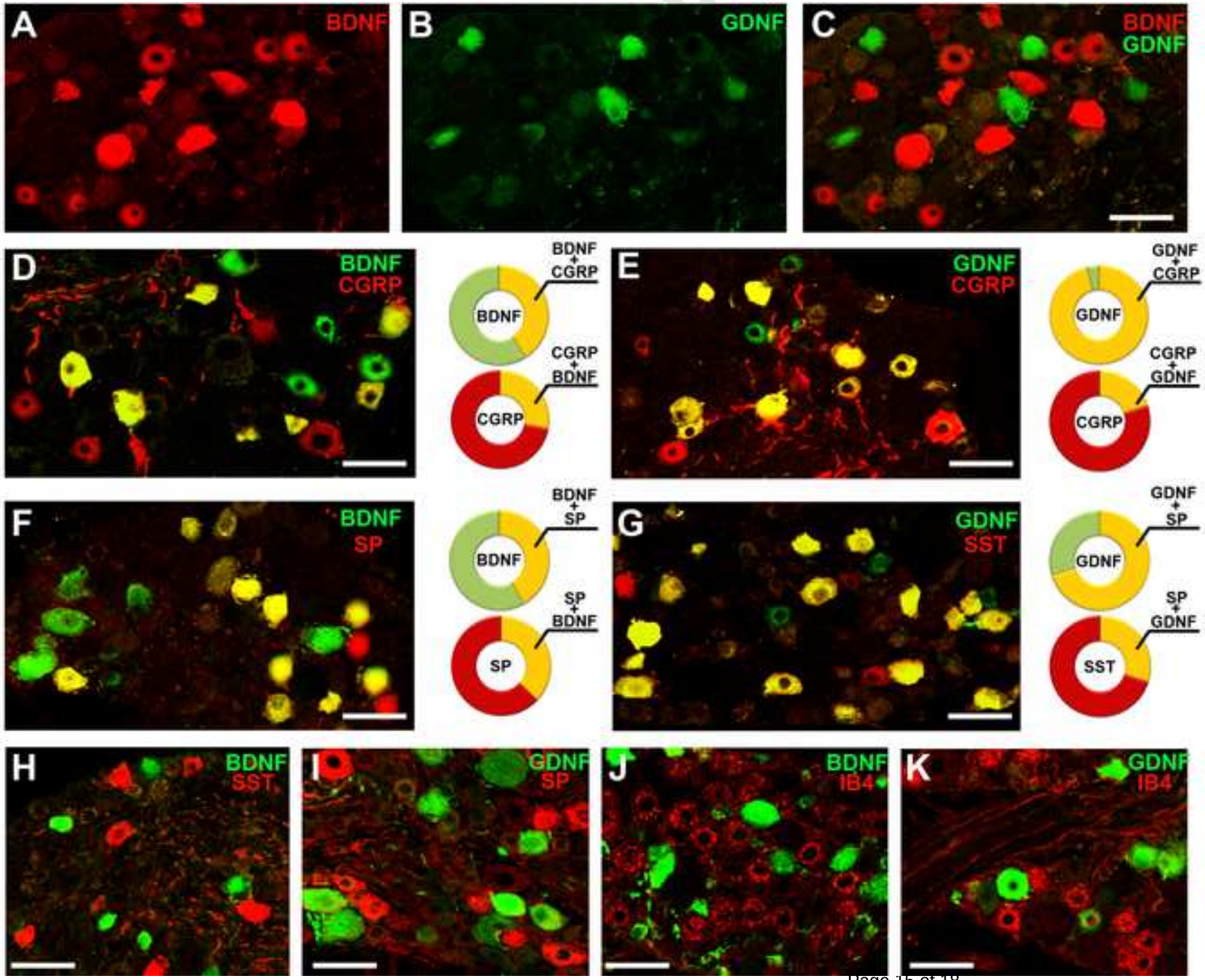
Figure 2. Distribution of neurotrophic factors and co-localization with nociceptive markers in spinal dorsal horn. (A) BDNF (red) and GDNF (green) are distributed in two different populations of nociceptive PAFs. (B) BDNF-IR fibers (green) are localized in laminae I-IIo while IB4-positive fibers (red) are distributed in lamina IIi. (C) GDNF-IR fibers (green) in lamina IIo do not overalp with IB4-positive fibers (red) in lamina IIi. (D) BDNF (green) and CGRP (red) are extensively co-expressed (yellow) in nociceptive peptidergic terminals of laminae I-IIo. (E) GDNF (green) and CGRP (red) are extensively co-expressed (yellow) in nociceptive peptidergic terminals of laminae IIo. (F) BDNF (green) and SP (SP) are co-expressed in a population of PAFs distributed in laminae I-IIo. (G) BDNF (red) and SST (green) are expressed in two different populations of peptidergic PAFs, with SST localized more ventrally in lamina IIo. (H) GDNF (red) and SST (green) were extensively co-expressed in lamina IIo. (I) GDNF (green) and SP (red) are expressed in two different populations of peptidergic PAFs, with SP localized more dorsally in lamina I-IIo. Scale bars: 200 μm.

Figure 3. Ultrastructural localization of neurotrophic factors and peptides in mouse spinal dorsal horn. (A) and (B) Double immunogold staining for BDNF (10-nm gold particles) and SST (20-nm gold particles; see inset). In (A) BDNF-immunoreactive peptidergic type I glomerular terminal (GIb) is surrounded by several unlabeled dendrites (d).BDNF is exclusively localized in DCVs scattered in the central terminal (see inset). In (B) a SST-immunoreactive GIb terminal is surrounded by unlabeled dendrites (d) and one vesicle-containing dendrite (v1). Note that terminals are either labeled for BDNF or SST. (C) and (D) Double immunogold staining for GDNF (10-nm gold particles) and SP (20-nm gold particles; see inset). In (C) GDNF-immunopositive GIb glomerulus is surrounded by several unlabeled dendrites (d). GDNF shows a selective localization in DCVs (inset). In (D) A SP-immunoreactive central terminal in a type Ib glomerular arrangement (GIb) is surrounded by three unlabeled dendrites (d) and a vesicle-containing dendrite (v1) and makes synapse with a unlabeled dendrite (arrowheads). Note that terminals are either labeled for GDNF or SP. Scale bars: 250 nm; insets A, C: 10 nm; B, D: 20 nm.

Figure 4. The Venn diagram depicts the relationship between different populations of sensory neurons in the mouse DRG, characterized according to their neurotrophic and peptidergic content. We observed two major populations of neurons identified by CGRP and BDNF (gray solid circles). These two populations largely overlap, and the overlapping neurons also express SP (BDNF/SP/CGRP neurons). The phenotype of BDNF neurons which do not express CGRP remains to be addressed. Within CGRP neurons instead, GDNF is expressed by a group of cells which do not contain BDNF (vertical lines). Most of these neurons express SST (horizontal lines; GDNF/SST/CGRP neurons).

Below, the projections of the peptidergic primary afferent fibers (containing BDNF or GDNF) and non-peptidergic fibers (binding IB4). Three main areas of projections can be identified: an external site (lamina I and IIo) for BDNF/SP/CGRP-containing fibers, an intermediate one for GDNF/SST/CGRP-containing fibers (between lamina IIo and IIi), and a inner one for IB4-fibers (lamina IIi).

Figure


A
BDNF
B
GDNF
C
BDNF
GDNF
D
BDNF
CGRP
BDNF + CGRP
BDNF
CGRP + BDNF
CGRP
E
GDNF
CGRP
GDNF + CGRP
GDNF
CGRP + GDNF
CGRP
F
BDNF
SP
BDNF + SP
BDNF
SP + BDNF
SP
G
GDNF
SST
GDNF + SP
GDNF
SP + GDNF
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H
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SST
I
GDNF
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J
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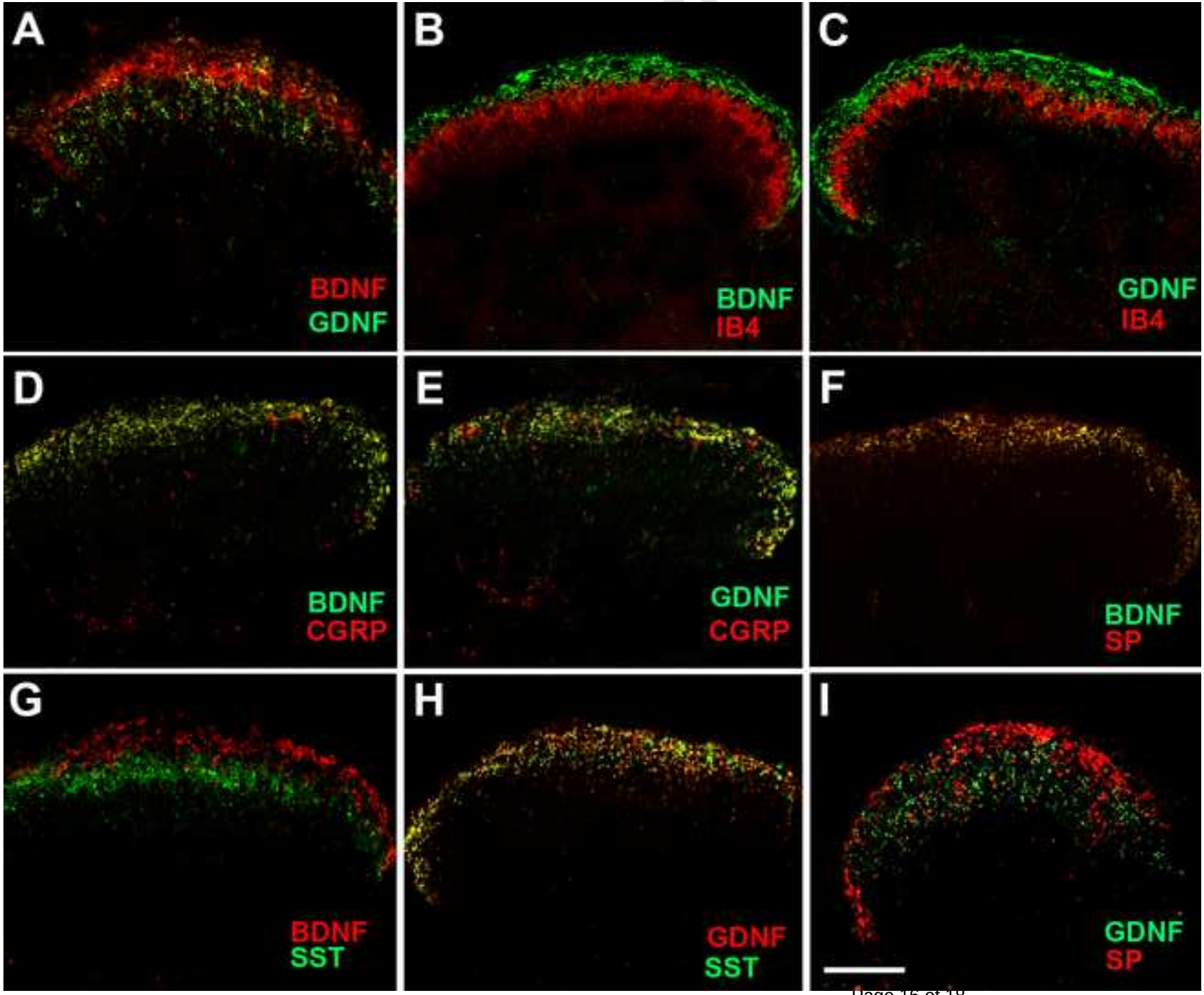
IB4
K
GDNF
IB4

Figure


A
BDNF
GDNF
B
BDNF
IB4
C
GDNF
IB4
D
BDNF
CGRP
E
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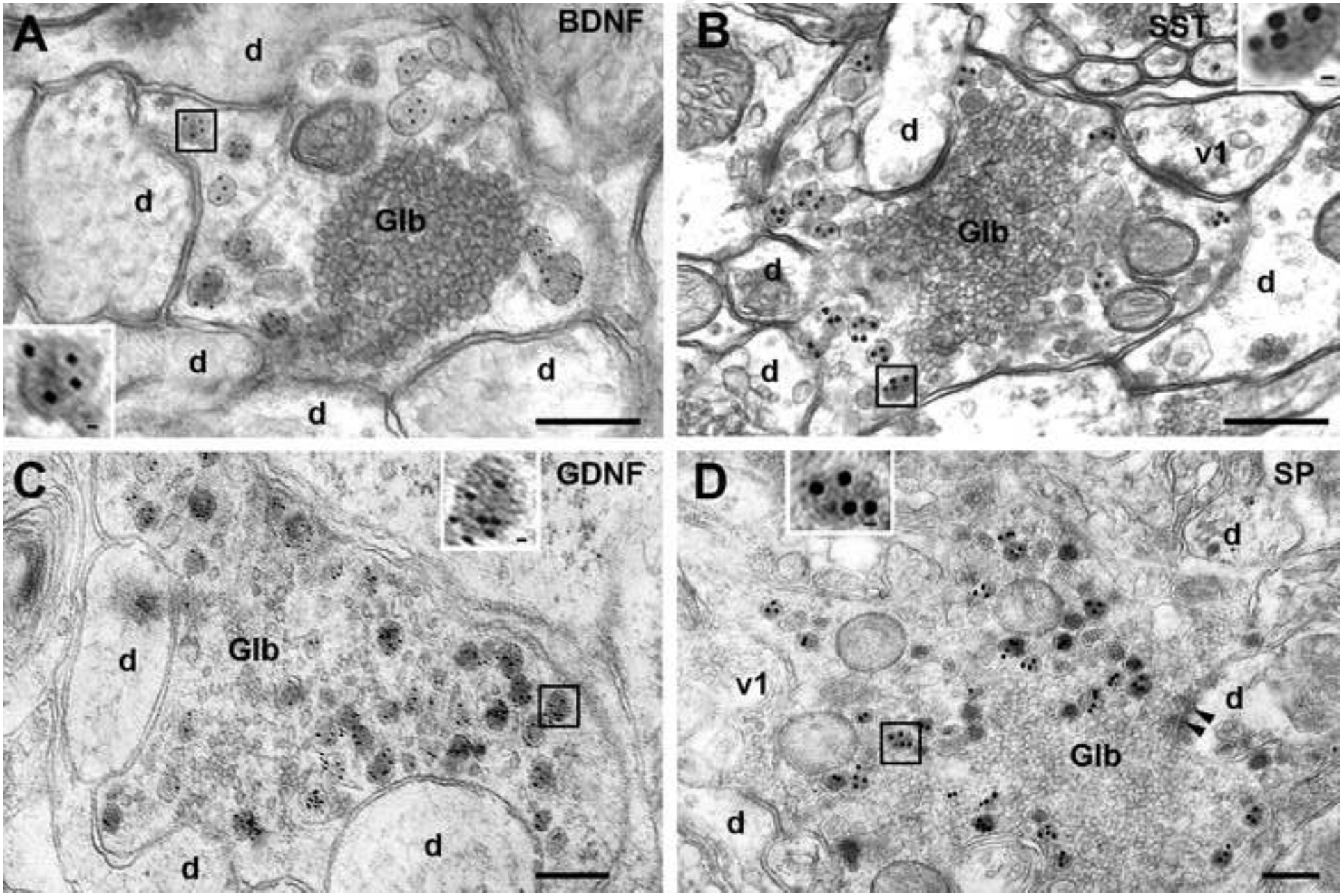
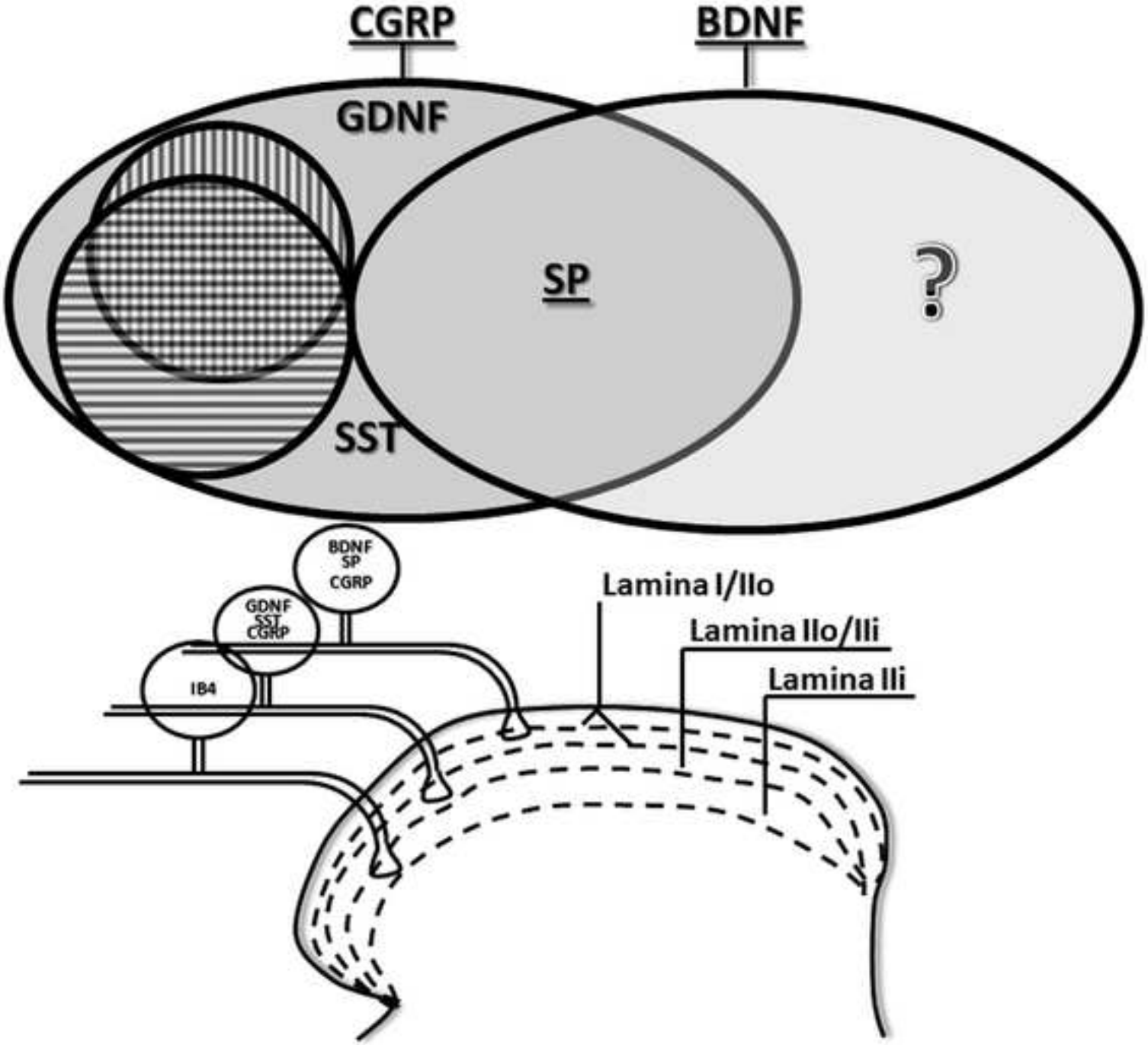
CGRP
F
BDNF
SP
G
BDNF
SST
H
GDNF
SST
I
GDNF
SP

Figure



Figure


CGRP
BDNF
GDNF
SP
?
SST
BDNF
SP
CGRP
GDNF
SST
CGRP
IB4
Lamina I/IIo
Lamina IIo/IIi
Lamina IIi