Targeting the glial-derived neurotrophic factor and related molecules for controlling normal and pathologic pain

Targeting the glial-derived neurotrophic factor (GDNF) and related molecules for controlling normal and pathologic pain

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Introduction: GDNF and its family of ligands (GFLs) have several functions in the nervous system. As a survival factor for dopaminergic neurons, GDNF was used in clinical trials for Parkinson's disease. However, GFLs their receptors are potential targets for new pain-controlling drugs. Such a potential should not be underestimated because molecules with analgesic activities in rodents mostly failed to be effective in translational studies.

Areas covered: The circuitry, molecular and cellular mechanisms by which GFLs control nociception, their intervention in inflammatory and neuropathic pain, the problems related to effective GDNF delivery to the brain, the possibility to target the GFL receptor complex rather than its ligands, also by the use of non-peptidyl agonists, are discussed.

Expert opinion: In nociceptive pathways, an ideal drug should either: *i.* Target the release of endogenous GFLs from large dense-cored vesicles (LGVs) by acting *e.g.* onto the phosphatidylinositol-3-phosphate [PtdIns$_{(3)}$P] pool, which is sensitive to Ca^{2+} modulation; *ii.* Target the GFL receptor complex. Besides to XIB403, a thiol molecule that enhances GFRα family receptor signaling, existing drugs such as retinoic acid and amitriptyline should be considered for effective targeting of GDNF, at least in neuropathic pain. The approach of pain modeling in experimental animals is discussed.

Keywords: Artemin, BG00010, Capsaicin, Central sensitization, Chronic pain, GDNF, GFLs, Gliafin, Humans, Inflammation, Mouse, Neurturin, Persephin, Rat, Somatostatin, Substantia gelatinosa, XIB4035

1. Introduction

Chronic pain is a major clinical and economic issue, and several cross-sectional studies indicate that it affects up to 25-30% of the total adult population worldwide. Under this umbrella term are comprised several different types of long-lasting pain, including inflammatory pain (*e.g.* in arthritis), cancer pain, and neuropathic pain that follows to injury (*e.g.* an ischemic insult or a trauma) of the peripheral and/or central nervous system. The term is somewhat ambiguous, as its

current use implies the absence of a stimulus-to-pain correlation, *i.e.* a persistence of pain that outlasts its causative event. This always occurs in neuropathic pain and, sometimes but not always, also in inflammatory pain. Whereas the protective role of nociceptive pain appears obvious, inflammatory and neuropathic pain persisting after their causative event(s) is(are) ceased are a maladaptive response of the nociceptive system, and must be regarded as fully pathologic conditions. Hyperalgesia and allodynia typically characterize all types of chronic pain, whereas burning, paresthesia and dysesthesia are additionally present in neuropathic pain.

It is now widely accepted that an altered plasticity of the nociceptive system is responsible for the onset of chronic pain, resulting from peripheral and central sensitization of the nociceptors, the primary sensory neurons involved in the processing of pain-related stimuli [1]. In their simplest configuration, somatic and visceral pain pathways are made of a polyneuronal chain, having a nociceptor at its beginning, converging nociceptive information to the thalamus, and, from there, to the cerebral cortex (Figure 1). An important feature of the nociceptive system is that its responses to noxious stimuli (*i.e.* the actual or potential tissue damaging events) can be modulated by local circuit interneurons that, in normal conditions, calibrate the system response to the stimulus intensity and duration in time. One important site for this modulatory action is the substantia gelatinosa (lamina II) of the spinal cord and the spinal nucleus of the trigeminal nerve (SNuT). The nociceptors thresholds are lower than the stimulus intensity needed to damage tissues (*i.e.* they exert what is called a warning function), and, once activated, they release a cocktail of fast-acting (glutamate) and slow acting neurotransmitters (peptides, trophic factors) from their central terminals in the spinal cord and SNuT.

In nociceptive pain, only the nociceptors are activated, their response is short-lasting; pain has a high threshold, and, thus, plays its physiological protective function. In inflammatory pain, also non-nociceptive primary sensory neurons come into play, the system displays a long-lasting response (central sensitization), pain has a low threshold, and plays an important function in the repair process. Although acute inflammation produces a transient central sensitization that is

beneficial for tissue healing, chronic inflammatory pain is associated with a long-lasting and, sometimes, permanent central sensitization that persists after inflammation has ceased. Similarly, in neuropathic pain the nociceptive system becomes permanently sensitized, also as a consequence of neuronal damage, ectopic neuronal firing and microglia activation.

There are at present no satisfactory treatments for chronic pain as the medications of choice in clinical settings, such as non-steroidal anti-inflammatory drugs and opioids, are often inadequate and linked to undesirable side-effects. In addition, molecules with analgesic activities in rodents, up to now, unfortunately failed to be effective in translational studies, despite the urgent need for effective treatments in pathologic pain patients.

The glial-derived neurotrophic factor (GDNF) [2] is the first discovered member of a group of closely related neurotrophic factors that are collectively referred to as the GDNF family ligands (GFLs). Although the interest for the use GFLs in therapy was initially focused on neurodegeneration [3], artemin (ARTN), one of the GFLs, was long ago the first growth factor to be demonstrated to successfully reverse and normalize neuropathic pain in rats [4].

Here, I will discuss current experimental evidence and rationale for the development and use of GDNF, ARTN and their receptor complex ligands as new pain-controlling drugs.

2. GFLs and their receptors

All the four GFLs, *i.e.* GDNF, neurturin (NRTN), ARTN and persephin (PSPN) display potent neurotrophic activity and promote the survival of several different populations of central and peripheral neurons [5]. GFLs signal through a unique receptor complex that consists of a common receptor and a ligand-specific co-receptor [6]. The common receptor is the transmembrane receptor tyrosine kinase (RTK) rearranged during transfection (RET) that has a typical intracellular kinase domain and activates multiple cellular signaling pathways [7]. The ligand-specific co-receptor is a cell surface-bound GDNF family receptor α (GFRα) that binds (but not exclusively) one of the GFLs [3, 5]. Thus, GDNF preferentially binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3, and

PSPN to GFRα4. However, GDNF can also bind to GFRα2 and GFRα3, but with lower affinity, and still activates RET, although it remains to be established whether this signaling is physiologically relevant *in vivo*. All GFRα receptors are anchored to the cell plasma membrane through a glycosyl phosphatidylinositol (GPI) link [8]. GFLs are also able to signal in a RET-independent way and, among others, utilize the neural cell adhesion molecule (NCAM) as an alternative signaling receptor [9].

3. GFLs and the control of nociception

GFLs, like other neurotrophic factors, display pleiotropic functions that may be different during development, adulthood and in response to disease or injury. In addition, the nociceptive system is capable of remarkable plasticity. Thus, the definition of the role of GFLs in the normal processing of pain has been and still remains a complicated issue. In the following, I will mainly discuss the available data in the spinal nociceptive system, as it has been the most widely investigated part of the entire somatosensory system in relation to the intervention of GFLs in pain processing.

3.1 Localization studies

Of the GFL receptor complex ligands, only GDNF was detected in nociceptors although the three GFRα isoforms and RET have all been localized to dorsal root ganglia (DRGs).

Initial studies suggested that GDNF was expressed in small- to medium-sized DRG neurons and in fibers of laminae I–II of the spinal dorsal horn (SDH), likely after having been anterogradely transported along the central projections of these neurons [10]. Recently it was demonstrated that GDNF is normally expressed by a specific subpopulation of peptidergic calcitonin gene-related peptide (CGRP)/somatostatin 28 (SST_{28})-immunoreactive (IR) murine nociceptors and by their central terminals at type Ib glomeruli in SDH lamina II [11]. These neurons and their terminals were also decorated by the isolectin B4 (IB4) produced by the plant *Griffonia simplicifolia* [12], a marker of the cell membrane of the DRG neurons supported by GDNF during development [13]. Very likely, a similar pattern occurs in rat considering the mirroring distribution of brain-derived

neurotrophic factor (BDNF)/tyrosine kinase receptor B (TrkB) and GDNF in the two species [14, 15].

The GFL receptor complex components have been detected in rat [16-22] and mouse [11] DRGs and spinal cord. In rat, about half of unmyelinated IB4+ nociceptive C-fibers express GFRα1, and nearly 80% of the IB4+ nociceptors express either GFRα1 or GFRα2. Light microscopy studies showed that GFRα1 is mainly expressed in lamina II_o and GFRα2 in lamina II_i of the lumbar [21] and sacral spinal cord [17]. Notably, GFRα2-KO mice showed a normal acute pain response in the formalin test but a markedly attenuated persistent phase, indicating a deficit in the inflammatory pain response [23]. GFRα3 appeared to be mainly (if not exclusively) present in nociceptive peptidergic (CGRP+) DRG neurons and their peripheral unmyelinated C-fibers [24, 25]. These neurons were also reported to express RET and the transient potential vanilloid receptor type 1 (TRPV1) [26]. In mouse, the GFRα1/RET receptor complex was localized in non-peptidergic nociceptors and their terminals in SDH and, ultrastructurally, at type Ia glomeruli (Figure 2) [11]. Similarly to rodents, adult human DRG neurons express RET and the three GFRα isoforms. GFRα1 and RET were detected in both large and small sized cells. In the adult spinal cord, mRNAs were found encoding for GFRα1 and RET, but, notably, the GFRα2 mRNA was only detected in embryos [27]. RET was also localized to the human trigeminal ganglion [28].

Interspecies comparisons are difficult because the majority of rat work was focused on the survival/neuroprotective effect of the GDNF and to ascertain the plastic response of the sensory system to different types of injury, rather than considering a putative intervention as a pain modulator. The concurrent localization of GFRα1 and GFRα2 in DRGs is an important issue, since the latter also binds GDNF *in vitro* [5], thus leaving the possibility open for GDNF to act also on GFRα2-expressing neurons. However, it seems highly questionable that GDNF interacts *in vivo* with ligands other than GFRα1 [29], and it should be recalled that specific types of low-

threshold mechanoreceptors but not the nociceptors were identified by a unique co-expression pattern of RET and GFRα2 [30].

3.2 Functional studies

The concurrent presence of GDNF and its receptor complex after *in situ* analysis in adult rodents and humans is supportive for a modulatory role of GDNF in the somatosensory system. Therefore, while the intervention of GDNF in the maturation and maintenance of subsets of primary sensory neurons is well established [12, 13], today we begin to understand that it also modulates their functional properties once maturity is reached. After the initial suggestion that GDNF undergoes anterograde transport along primary afferent fibers projecting to periphery and spinal cord [10, 31], evidence was provided that GDNF can be actively secreted [32]. Initial cues on the modulatory role of GDNF in somatosensory system derived from the demonstration of its capability to enhance SOM expression [33] and release from DRGs *in vivo* [33-35], and capsaicin-stimulated release of CGRP from cultured DRG neurons [36]. The hypothesis that GDNF was as a modulator of the nociceptive information transfer between first- and second-order neurons in SDH was thus put forward [35], but still it remained controversial [25, 37] as both pro- and anti-nociceptive effects were reported, depending on the peripheral territory innervated (skin, muscle), type of pain (inflammatory, neuropathic), and stimulus (mechanical, thermal, noxious) – *see* 4. GFLs and pathological pain.

Recently, this issue has been clarified in a mouse slice preparation [11]. In this context, capsaicin was capable to release GDNF from peptidergic CGRP/SST_{28}-IR nociceptors, and GDNF was shown to exert a tonic inhibitory control on the glutamate excitatory drive of SDH neurons after ERK1/2 phosphorylation assay, real-time Ca^{2+} imaging and patch-clamp experiments. The reduction of the capsaicin-evoked $[Ca^{2+}]_i$ rise and of the frequency of miniature excitatory postsynaptic currents (mEPSCs) in SDH neurons was specifically abolished after ablation of GFRα1 by enzymatically cutting its membrane GPI anchor [8] . In the same work, the selective localization of GDNF in

large dense-cored vesicles (LGVs) was also demonstrated. These observations led to conclude that GDNF released from peptidergic CGRP/ SST_{28}-IR nociceptors acutely depressed neuronal transmission in SDH signaling to non-peptidergic IB4+ nociceptors at glomeruli that either display GDNF/SST_{28} (type Ib)-IR or GFRα1/IB4 labeling (type Ia). These data are of potential pharmacological interest as they highlight a novel modality of cross talk between nociceptors that may be relevant for discrimination of pain modalities (Figure 2) and strengthened the idea that GDNF could enter the scene as a potential therapeutic target in the control of pain.

4. GFLs and pathological pain

4.1. Animal studies

Animal studies aiming to elucidate the role of GFLs in pathological pain have been carried out in rats and mice and were, for the most, focused onto GDNF. They substantially converged to demonstrate that GDNF has different effects in inflammatory and neuropathic pain, and that hyperalgesic effects (Table 1) are seen in the peripheral nervous system, whereas analgesic effects (Table 2) occur in central neurons. Only limited data are available for ARTN and even less for NRTN. Several discrepancies can be found when comparing these studies. At least some of these discrepancies can be related to the use of animals not only of different species, but also strains and sexes, as substantial differences under these respects were observed *e.g.* in the anti-depressive response to ketamine an NMDA receptor antagonist widely employed in the control of pain [38].

4.1.1. ARTN

Anti-ARTN antibodies have been reported to partly revert the inflammatory pain that follows a local injection of complete Freund's adjuvant (CFA) in rat [39]. Mice overexpressing ARTN in keratinocytes displayed an increased sensitivity to several types of pain-inducing stimuli, and the intraplantar injection of ARTN in wild-type controls resulted in transient thermal hyperalgesia [40]. Anti-ARTN antibodies in rats and mice were instead inefficacious in blocking neuropathic pain that

followed spinal nerve ligation (SNL) [39], in keeping with the observation that subcutaneous ARTN blocked the mechanical and thermal hyperalgesia in the same experimental paradigm in rats [4].

In brief, these observations converge to indicate that ARTN is pronociceptive at periphery in inflammatory pain and antinociceptive in spinal cord under neuropathic conditions.

4.1.2. GDNF

4.1.2.1. Inflammatory pain

When inflammatory pain was elicited after challenge of cutaneous, muscular or articular afferents a tissue-specific response to GDNF was observed (Table 1).

Cutaneous afferents have been challenged with direct intraplantar/intradermal injections of GDNF or CFA. Intraplantar injection of GDNF induced thermal [41] or mechanical hyperalgesia, the underlying mechanism being primarily associated with IB4+ and GFRα1 expressing nociceptors [42-44]. However, CFA-induced mechanical and thermal hyperalgesia were reversed or blocked by GDNF in another study [45]. Besides to behavioral effects, CFA upregulated the GDNF protein/mRNA content of DRGs. Notably, after surgical plantar incision, mechanical and thermal hyperalgesia were associated with a downregulation of the GDNF mRNA in skin [46].

The inflammatory response to locally administered GDNF in the first set of studies was substantially inferred after behavioral responses, and it cannot be excluded that the proalgesic effects of GDNF were indirect and linked *e.g.* to an overproduction of interleukin-18 (IL18) at the injection site. Notably, IL18 is a known mediator of pain [47] and can be upregulated by GDNF in skin [48] . Thus, the physiological relevance of this type of experimental paradigm can be questioned. Interpretation is further complicated by the possibility that deeper tissues and not the skin are involved in GDNF-induced edema, therefore being responsible of the inflammatory behavior [49] . On the other hand the studies implying an anti-nociceptive effect of GDNF in cutaneous inflammatory pain are in line with the functional observations in a slice model of inflammation [11].

Mechanical hyperalgesia was observed in several studies where GDNF was injected intramuscularly [50-52], its cellular/molecular substrates being associated with IB4+ and GFRα1-IR nociceptors [50]. Another study has shown that anti-GDNF antibodies blocked the mechanical hyperalgesia induced by muscle lengthening contraction, the underlying mechanism involving a cyclooxygenase-2 (COX2)-dependent upregulation of the GDNF mRNA [53].

Anti-GNDF antibodies also reduced the delayed thermal hyperalgesia that followed CFA intraarticular injection [54]. Finally, a proalgesic role for GDNF was also inferred in a very recent study where inflammatory pain was induced after experimental bone carcinogenesis [55].

In brief, it therefore appears that deep-tissues' inflammatory pain is positively regulated by GDNF, whereas the picture for cutaneous pain is much less well defined.

4.1.2.2. Neuropathic pain

GDNF was clearly antinociceptive in neuropathic pain (Table 2). All studies employing the classic experimental paradigms to induce this type of pain converged to demonstrate that GDNF was capable to block heat/mechanical hyperalgesia and allodynia. In these experiments the growth factor was mainly administered intrathecally [56-60] or intraspinally [61, 62], but also directly into the DRGs [62, 63], in contact with the lesioned nerve [56], or intramuscularly [64]. It is worth nothing that allodynia and thermal hyperalgesia were also blocked after direct injection of GDNF into the locus coeruleus [65], *i.e.* targeting the descending pain inhibitory system. Relevant to the present discussion, not only the intact molecule was employed [56-60, 65], but GDNF was also delivered with different types of viral vectors [61-64] or using a slowly releasing chitosan matrix [56]. Finally, an antinociceptive action for GDNF was proposed in a study on induced herpetic neuralgia [66].

4.1.3. NRTN

NRTN was used in parallel to ARTN and GDNF in a mouse study where inflammatory pain was induced by CFA, or the GFLs were locally administered by intraplantar injections [41]. As a

decrease of expression was observed after CFA, it can be tentatively hypothesized that NRTN was antinociceptive in this type of pain.

4.2. Human studies

Although limited in numbers, human studies appear to confirm pre-clinical animal data. Initial observations on the presence of growth factors in patients suffering from interstitial cystitis or bladder cancer demonstrated, among others, an increase of urinary GDNF [67]. More recently [68], urinary GDNF was shown to decrease in response to treatment of refractory bladder pain with the analgesic onabotulinum toxin A (OnabotA), although its basal levels in patients were not significantly different from those in normal subjects. The decrease in urinary GDNF after OnabotA indicated a local process of release, thus suggesting that GDNF could have a role in visceral pain in general, and in urinary bladder pain in particular. Observations in bladder pathologies thus appear in line with the notion that GDNF intervenes as a pronociceptive factor in peripheral inflammatory pain of deep tissues' afferents. In the search of useful biomarkers for pain conditions, GDNF and related molecules were also investigated in cerebro-spinal fluid (CSF) and blood. In patients suffering from chronic migraine or fibromyalgia, the CSF content of GDNF and SOM were reduced [69]. In a subsequent study in long-term pain patients, however, GDNF resulted to be increased in CSF but decreased in blood, implying that changes of the GDNF levels in CNS are not traceable peripherally under chronic pain conditions [70]. To explain their results, authors have hypothesized that a major component of blood GDNF comes from sources other than CNS, as GDNF does not cross the blood brain barrier (BBB). Finally, in another recent preliminary report the CSF levels of GDNF were seen to be higher in patients suffering from neuropathic pain when compared with those of patients suffering from arthritic back pain [71]. These observations are more difficult to be grouped in a coherent frame encompassing a clear relationship of GDNF and pain. In other studies such a relationship was even lacking. Patients suffering from painful and non-painful necrotic vasculitic neuropathies had increased levels of GDNF and GFRα1 mRNAs in sural nerve biopsies,

but values of both mRNAs were not significantly different in relation to the presence or absence of pain [72]. Similarly, in a study on the course of UV-induced skin inflammatory hyperalgesia, RT-PCR analysis revealed an up-regulation of the ARTN but not GDNF, GFRα1, or GFRα3 mRNAs [73].

Under this context, ARTN (BG00010) was very recently employed in the first-in-human, double-blind, placebo-controlled, randomized, dose-escalation study on subjects with unilateral sciatica: after enrolment of forty-eight subjects, BG00010 was generally well tolerated, but, at present, not yet associated with any clear, dose-dependent trends in Likert pain scores [74].

5. Delivery of GFLs to the brain

Much of what we know about delivery of GFLs to the brain comes from pre-clinical and clinical studies on Parkinson's disease (PD) or other neurodegenerative conditions, see *e.g.* [3].

However, it is worth noting here that the goal of GFL-based therapies in neurodegenerative diseases is to exploit the growth factor activity of the molecules, whereas in the case of effective pain-controlling drugs is the modulatory activity to be targeted.

5.1. Protein based-therapy

Bioavailability is, in general terms, surely one of the most important issues in protein-based therapies [75]. GDNF is a large basic protein of 134 amino acids of 15 kDa and cannot be transported across the BBB. Consequently, the direct delivery of GDNF to the brain was the primary focus of translational research. Delivery strategies have included the direct administration of the molecule, its encapsulation in microspheres composed of biodegradable polymers to achieve a controlled release over a prolonged period of time, DNA nanoparticle gene transfer, and convection-enhanced delivery [76-79]. Interactions of GDNF with components of the extracellular matrix ,its activation of receptors other than RET/GFRα1, induction of anti-GDNF antibodies by recombinant GDNF are all factors hampering the use of the full molecule in therapy. Interestingly, it was demonstrated that insect cell-derived GDNF:tetanus toxin C retains its bi-functional activity

in mammalian CNS *in vivo* and improved the delivery of GDNF to spinal cord following intramuscular- or intrathecal administration [80].

5.2 GFL mimetics and small molecules targeting the GFLs' receptors

The term neurotrophic mimetics is used to indicate small molecule agonists of the neurotrophic factors. Being of small size, these molecules should have the advantage of crossing the BBB and should not induce anti-antibodies production. Whereas several mimetics exist for nerve growth factor (NGF) or BDNF, gliafin is the only GDNF mimetic available today. Gliafin (153-ETMYDKILKNLSRSR-167; UniProtKB entry no. Q07731) is a small peptide that displays GDNF-like activity and was recently demonstrated to induce efficient differentiation and functional integration of transplanted stem cell derivatives [81]. It has not been tested yet as an alternative to GDNF in local delivery experimental paradigms of inflammatory or neuropathic pain, but it deserves further attention as a possible GDNF therapeutic substitute.

In parallel, progress in knowledge of the biochemistry of GFL receptors and the use of high-throughput screening will hopefully led to discover GFRα specific agonists/antagonists and or to develop additional small molecules mimicking the activity of the GFLs [3]. Today, XIB4035, a 456.5 Da quinol binding to GFRα1, is the only available "ligand" of GFRα1 reported to induce RET autophosphorylation in Neuro-2A cells [82]. The molecule displaces GDNF from GFRα1 with an IC_{50} of 10 mM. Very recently it was demonstrated not to be a true GFRα1 agonist, but rather to enhance the GFRα family receptor signaling [83]. In this study XIB4035 was administered topically and found effective to treat small fiber neuropathy in diabetic female rats.

In chicken embryonic sympathetic neurons *in vitro*, GFRα1 and GFRα2, as well as the common signal transducing receptor RET, were all down-regulated by retinoic acid [84]. Interestingly, more recent work on mouse DRG neurons showed that LE135, a retinoid acid receptor antagonist, produces pain through direct activation of TRP channels [85].

6. Conclusion

Although far from being ready for translational trials, animals studies, primarily in neuropathic pain, are strongly supportive of the possibility to develop effective new pain controlling strategies based on the intervention of GFLs in the modulation of nociceptive circuitry. As information has been added to a better comprehension of the neuronal circuitry involved in the growth factor modulation of glutamate release at the synapses between first- and second-order sensory neurons, and targeting GFRα receptors appear to be at hand, GFLs should deserve more attention in the immediate future as potential new drugs to target pathologic pain.

7. Expert opinion

Today there are no drugs that address ARTN, GDNF, or the GFRα isoforms in the clinical management of chronic pain. Here, I have presented the data *in vivo* that show the intervention of GFLs in the rodent somatosensory and visceral nociceptive pathways under normal conditions and after experimental inflammatory or neuropathic pain. In the last years, many promising analgesic molecules in rodent pain models had limited or no translation into successful proof-of-concept clinical trials, to the point that several companies dropped their search efforts in finding novel analgesic drugs [86]. In line with this unfortunate tendency, there is at present only one phase 1 study supporting the development of BG00010 for the treatment of neuropathic pain [74].

There is a general consensus that central sensitization at spinal and supraspinal levels is the key event in the development of pathological pain [1]. Two main lines of thought can then be followed to direct pain drug discovery strategies: the first (and most commonly followed) aims at producing compounds that can cross the BBB to directly interfere with central sensitization mechanisms, the second intends to hit the primary afferent neurons at periphery, in order to reduce or block central sensitization without the unwanted CNS side effects [87].

Under this perspective ARNT, GDNF and related small molecule derivatives appear to be amenable to the development of new therapeutic strategies by primarily hitting neuropathic pain. Based on the previously discussed evidence of animal studies, and considering that GDNF has been demonstrated

to undergo both retrograde and anterograde transport in neurons [10, 31] it seems reasonable that, broadly-speaking, a GDNF-related therapy may be effective at both peripheral and central levels. In addition, as minimally invasive topical or systemic routes of administration other than the direct delivery into the central or the peripheral neurons have proved to be efficacious in animal models [4, 64, 81-83], it seems desirable that these routes are explored in clinical settings too.

GDNF is not the only growth factor that intervenes in the modulation of the nociceptive input to the spinal cord, as BDNF is today generally acknowledged to act a pronociceptive factor [88]. Remarkably both factors are selectively stored in LGVs at central and, likely, peripheral terminals of nociceptors, BDNF being co-stored with the pronociceptive peptide substance P [14] and GDNF with the antinociceptive peptide SOM [11]. Noteworthy, SOM is upregulated by GDNF [13, 34, 35, 56, 89], and the levels of both are reduced in patients suffering from chronic pain syndromes [69]. An interesting option for developing new drugs is thus targeting the release of these pain-related messengers from LGVs, as their exocytosis is differentially regulated than that of the small synaptic vesicles storing the fast-acting transmitter glutamate that is responsible for acute pain. In nociceptive pathways, an ideal drug to target the release of endogenous GFLs from LGVs could act *e.g.* onto the phosphatidylinositol-3-phosphate [PtdIns$_{(3)}$P] pool, which is sensitive to Ca^{2+} modulation [90].

An additional strategy would be to better exploit the efficacy of existing drugs that are currently used in different clinical settings and, therefore, would require a less-intensive research effort to be targeted to chronic pain treatment. One of these drugs could be the tricyclic antidepressant amitriptyline, as it was shown to decrease the release of BDNF [91], and, at the same time, increase that of GDNF at least in glia [92]. If one considers the picture emerged from a more precise dissection of the nociceptive circuitries in the substantia gelatinosa of the spinal cord (Figure2), amitriptyline has the potential to target at the same time a pro- (BDNF) and an antinociceptive (GDNF) factor at this very important relay station. Notably, a very recent study has shown that

systemic (intraperitoneal) amitriptyline reversed the effects of allodynia and hyperalgesia induced by chronic constriction injury (CCI) in mice [93], and case reports in the clinical literature indicate that high doses of topical amitriptyline are beneficial in the treatment of neuropathic pain although systemic adverse effects should be taken into account [94] .

Retinoids, through their activation of retinoic acid receptors and retinoid X receptors, regulate diverse cellular processes, and pharmacological intervention in their actions was proved to be useful in the treatment of certain skin disorders and cancers. The aforementioned links between retinoic acid and chronic pain, albeit at present only established *in vitro*, will hopefully also deserve further attention in the future.

The discussion of the failure of translational research in pain is beyond the purpose of this contribution, and the crucial limitations in animal pain models, their behavioral testing, and the experimental paradigms used to elicit pain were well outlined recently [95]. Still it remains confusing to many researchers that what is often tested in the laboratory is nociception rather than pain, and under this perspective an overcoming of the conventional reflex-based behavioral tests is mandatory with, at least, the complementary use of *e.g.* performance-based tests, facial mimics analysis, *etc.*

Also the discussion on the opportunity to use spontaneous models of pain, such as pet animals that are widely diffused today and the subject on an increasing veterinary attention, is not a new one. Under this perspective, a further amelioration and refinement of the *ex-vivo* preparations will enable pain researchers to perform quicker and less expensive high-throughput screening experiments of drugs with potential antinociceptive effect. These antinociceptive drugs could be then tested for their potential analgesic effects in controlled veterinary trials on small animals suffering from inflammatory or neuropathic pain, and, finally, translated into human clinical trials.

GFLs	Species	Type of experiment	Type of pain	Site/Mechanism of action
ARTN	Rat males (Sprague-Dawley) Mouse females (C57BL/6J)	Intraplantar injection of CFA	Mechanical hyperalgesia partly reversed by anti-ARTN antibodies	[39]
ARTN	ARTN-OE and wild-type mice	Intraplantar injection of ARTN	Increased sensitivity to heat, cold, capsaicin and mustard oil in ARTN-OE mice Transient thermal hyperalgesia in wild-type mice	[40]
ARTN	Mouse	Intraplantar injection of ARTN	Cold hyperalgesia	Activation of TRPM8-GFRα3 in sensory neurons [96]
ARTN, GDNF, NRTN	Mouse (C57BL/6J)	Intraplantar injection of CFA Intraplantar injection of GFLs	Thermal hyperalgesia	Decrease of GDNF and NRTN expression after CFA, potentiation of TRPV1 response in *isolated sensory neurons* [41]
GDNF	Rat males (Sprague-Dawley)	Intraplantar injection of CFA	Thermal hyperalgesia blocked by GDNF	Increased GDNF content and upregulation of TRPV1 in DRGs [45]
ARTN, GDNF	Rat males (Sprague-Dawley)	Plantar incision	Mechanical hyperalgesia Thermal hyperalgesia	ARTN mRNA upregulation in skin and muscle; GDNF mRNA downregulation in skin, upregulation in muscle [46]
GDNF	Rat females (Wistar)	Intradermal injection of GDNF	Mechanical hyperalgesia	IB4+ nociceptors [42]
GDNF	Rat males (Sprague-Dawley)	Intradermal injection of GDNF	Mechanical hyperalgesia	IB4+ nociceptors [43] Activation of PKCε [44] Hyperalgesia blocked by DORs [97]
GDNF	Rat males (Sprague-Dawley)	Intramuscular injection of GDNF Ergonomic muscular injury	Acute/chronic muscle pain	GFRα1 at IB4+ nociceptors [50]
GDNF	Rat males (Sprague-Dawley)	Muscle lengthening contraction	Mechanical hyperalgesia blocked by anti-GDNF antibodies	COX2-dependent upregulation of GDNF mRNA [53]
GDNF	Rat males (Sprague-Dawley)	Intramuscular injection of GDNF	Mechanical hyperalgesia of Aδ-fibres but not C-fibres	ASIC-dependent, TRPV1-independent [51]
GDNF	Mouse males (C57BL/6J)	Muscle lengthening contraction	Mechanical hyperalgesia	[52]

		Intramuscular injection of GDNF		
GDNF	Rat males (Sprague-Dawley)	Intraarticular injection of CFA	Delayed thermal hyperalgesia decreased by anti-GNDF antibodies	[54]
GDNF	Rats	Induction of bone cancer	Mechanical and thermal hyperalgesia reduced by intrathecal lentivirus-mediated GDNF RNAi	Downregulation of GDNF protein levels, Reduction of SP-IR Downregulation of the pERK⁄ERK [55]

TABLE 1: Experimental data on the intervention of GFLs in inflammatory pain
Abbreviations: ARTN = artemin; ASIC = acid-sensing ion channel; CFA = complete Freund's adjuvant; COX2 = Cyclooygenase 2; DORs = δ-opioid receptors; DRGs = dorsal root ganglia; ERK = extracellular-signal-regulated kinase; GFL = GDNF family of ligands; GFRα: GDNF family receptor α; GNDF = glial-derived neurotrophic factor; IB4 = isolectin B4; IR = immunoreactivity; NRTN = neurturin; OE = overexpressing; pERK = phosphorylated form of extracellular-signal-regulated kinase; PKCε = protein kinase Cε; RNAi = RNA interference; SP = substance P; TRPV1 = transient receptor potential channel vanilloid type 1

GFLs	Species	Type of experiment	Type of pain	Site/Mechanism of action of GLFs
ARTN	Rats males (Sprague-Dawley)	SNL	Mechanical and thermal hypersensitivity blocked by ARTN (*subcutaneous*)	Reversal of the increase of GFRα3 and of the loss of IB4+ nociceptors in DRGs and reduction of SNL-potentiated capsaicin CGRP release [4]
ARTN	Rat males (Sprague-Dawley) Mouse females (C57BL/6J)	PSL	Mechanical hyperalgesia not reversed by anti-ARTN antibodies	[39]
GDNF	Rats females (Sprague-Dawley)	Partial transection of sciatic nerve branches	Neuropathic pain and allodynia blocked by GDNF (continuous – *intrathecal* or *peripheral* within a chitosan matrix)	Increased expression/secretion of SOM from DRG neurons [56]
GDNF	Rats	PSL or SNL	Neuropathic pain blocked by GDNF (*intrathecal*)	Reversal of the injury-induced plasticity of several sodium channel subunits [57]
GDNF	Rat males (Sprague-Dawley)	CCI SNL	Mechanical and thermal hypersensitivity blocked by GDNF (*intrathecal*)	Reduction of GDNF content and percentages of RET-IR neurons in DRGs [58]
GDNF	Rats	CCI	Reduction of mechanical allodynia by GDNF	Effect of GDNF abolished by NCAM antisense oligodeoxynucleotides or C3d, an NCAM-mimetic peptide [98]
GDNF	Rat males (Sprague-Dawley)	SNL	Development of thermal and tactile hypersensitivity prevented by GDNF (*intrathecal*)	Attenuation of reduction of IB4+/ increase of ATF-3/galanin in DRGs [59]
GDNF	Rats (Wistar)	SNL	Neuropathic pain mechanical allodynia and thermal hyperalgesia attenuated by lentiviral-mediated GDNF transfer (*intraspinal*)	Reversal of IB4+ downregulation and ATF-3 upregulation in DRGs [61]
GDNF	Rat	SNL	Mechanical hyperalgesia and allodynia attenuated by lentivector transduced MSCs (*intraDRGs*)	MSC secretion of GDNF [63]
GDNF	Rats males (Sprague-Dawley)	CCI	Mechanical allodynia and thermal hyperalgesia attenuated by adenoviral-mediated GDNF transfer (*intramuscular*)	Protective action associated with anti-inflammation and prohibition of microglia activation [64]
GDNF	Rats males (Sprague-Dawley)	CCI	Allodynia and thermal hyperalgesia blocked by injection of GDNF in	Enhancement of descending noradrenergic inhibition [65]

			locus coeruleus	
GDNF	Rats males (Sprague-Dawley)	CCI and electroacupuncture analgesia	Heat hyperalgesia	Exacerbation of hyperalgesia [89] or block of analgesia by antisense GFRα1 oligodeoxynucleotides [99]
GDNF	Rat	PSL	Tactile allodynia reduced by gamma knife (GK) irradiation	Increase of GDNF and Iba-1 protein, a macrophage marker, in peripheral nerves [100]
GDNF	Mouse males C57BL/6J	CCI	Thermal hyperalgesia attenuated by GDNF (intrathecal)	GDNF counteracted the downregulation of E-cadherin/p120ctn in dorsal horn [60]
GDNF	Mouse males C57BL/6J	SNL	Mechanical hyperalgesia partly reversed by lentiviral vector-mediated GDNF overexpression (*intraspinal* or *intraDRGs*)	[62]
GDNF	Y1472F-KI and wild type C57BL/6J mice	Induced herpetic neuralgia	Spontaneous pain and allodynia	Decrease in GDNF-IR fibers in spinal cord, reduction of NMDA-induced current in spinal cord slices by GDNF [66]

TABLE 1: Experimental data on the intervention of GFLs in neuropathic pain
Abbreviations: ARTN = artemin; ATF-3 = activating transcription factor 3; CCI = Chronic constriction injury of sciatic nerve; CGRP = calcitonin gene-related peptide; DRGs = dorsal root ganglia; GFL = GDNF family of ligands; GNDF = glial-derived neurotrophic factor; IB4 = isolectin B4; IR = immunoreactive; NCAM= neural cell adhesion molecule; MSCs = mesenchymal stem cells; PSL = partial ligation of sciatic nerve; SOM = somatostatin; RET = receptor tyrosine kinase (RTK) rearranged during transfection; SNL = spinal nerve ligation.

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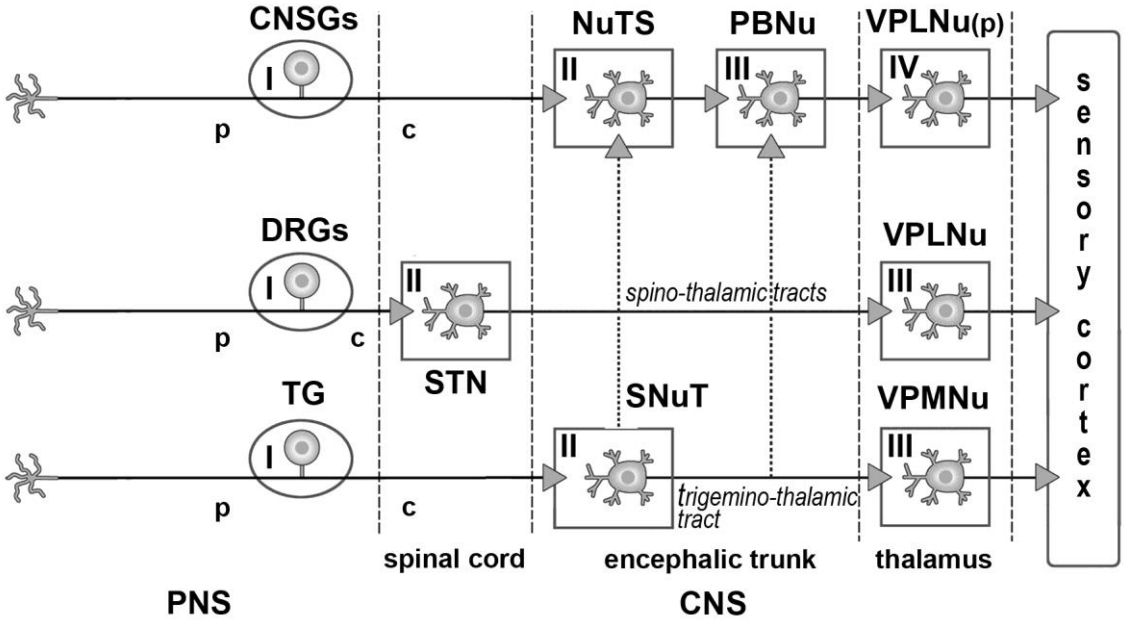

Figure 1

Simplified scheme of the main nociceptive pathways in mammals.

Nociceptive pathways consist of polyneuronal chains of neurons that originate from a pseudounipolar primary sensory neurons neuron (nociceptor) in the peripheral nervous system. Primary sensory neurons are localized in CNSGs, DRGs or TG. Nociceptive somatic inputs from all the parts of the body except the head are transferred to second-order spino-thalamic projection neurons in the dorsal horn of the spinal cord. These neurons give rise to the spino-thalamic tracts that reach the third-order neurons in VPLNu. Nociceptive somatic inputs from the head are relayed to the SNuT and then, along the trigemino-thalamic fibers, to the VPMNu. Nociceptive visceral afferents mainly derive from CNSGs associated to the vagus and glossopharyngeal nerves and are the relayed to the NuTS, the PBNu, and, finally the VPLNu(p). There is a small contingent of visceral afferents also in DRGs (less than 5%). From the thalamus, the nociceptive input is finally transferred to the sensory cortex where it gives rise to the sensation of pain. There are indirect connections (dashed lines with arrows) between the somatic and visceral pathways, which are the anatomical basis for the so called referred visceral pain, a condition in which visceral pain is perceived as originating from somatic structures. For simplicity the descending inhibitory pathways are not represented, as well as the connections with the limbic system that is responsible for the emotional and affective components of pain.

Abbreviations: **CNS** = central nervous system; **CNSGs** = cranial nerve sensory ganglia; **DRGs** = dorsal root ganglia; **NuTS** = nucleus tractus solitarii; **PBNu** = parabrachial nucleus; **PNS** = peripheral nervous system; **STN** = spino-thalamic neuron; **SNuT** = spinal nucleus of the trigeminal nerve; **VPLNu** = ventro-postero lateral nucleus of the thalamus; **VPLNu(p)** = ventro-postero lateral nucleus of the thalamus – parvocellular part; **VPMNu** = ventro-postero medial nucleus of the thalamus. Roman numerals indicate the order of the neurons along their polysynaptic chain.

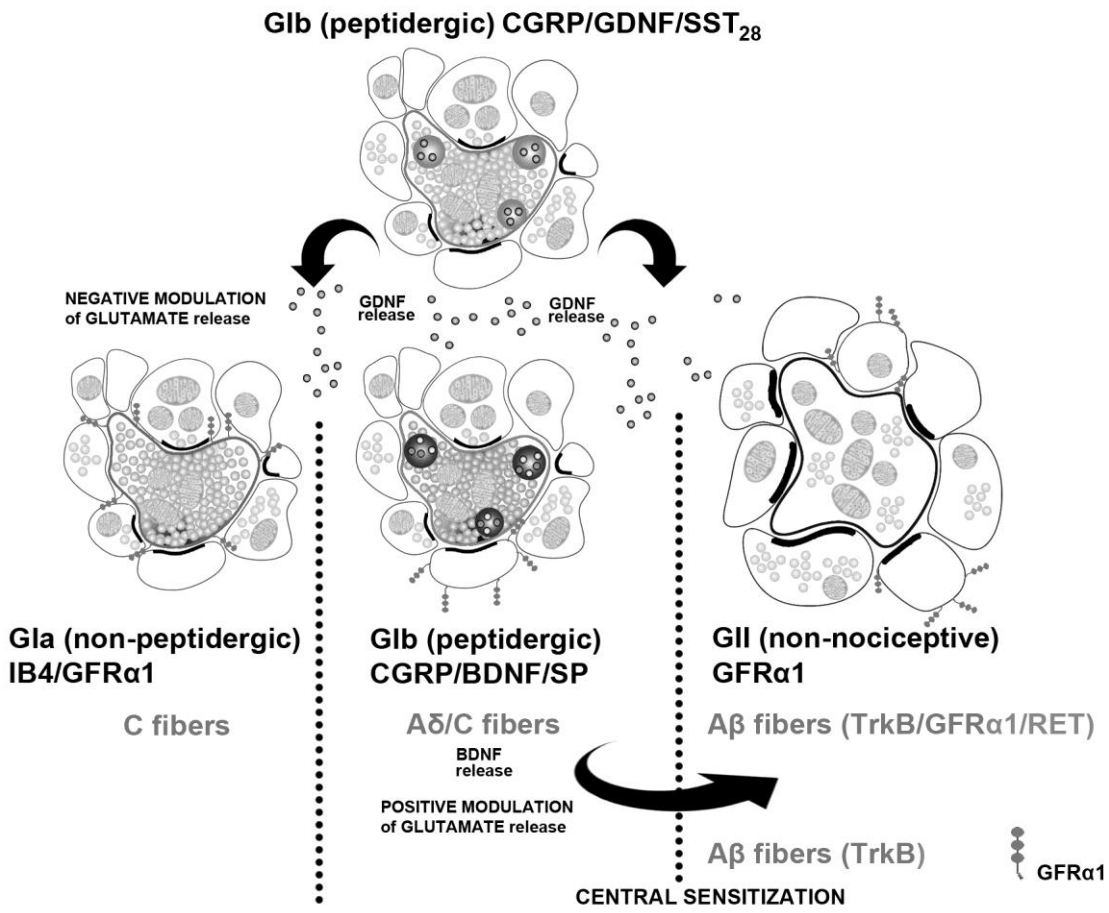

Figure 2

Sites of potential GDNF modulation of nociceptive input in substantia gelatinosa (lamina II) of the spinal dorsal horn.

GDNF is released from LGVs in peptidergic (CGRP+/SST_{28}+) type Ib glomeruli made by Aδ/C fibers in substantia gelatinosa. Once released, GDNF can negatively modulate glutamate release by acting on GFRα1-IR dendrites at three different types of glomeruli. Under conditions of nociceptive physiological pain GDNF counteracts the positive modulatory action of BDNF by acting on the subpopulation of Aδ/C fibers expressing BDNF which are engaged in peptidergic (CGRP+/SP+) type Ib glomeruli displaying GFRα1-IR at their peripheral dendrites. GDNF can also negatively modulate the glutamate release from non-peptidergic, IB4+ C fibers at type Ia glomeruli. These fibers have been shown to be the preferential target for the anti-nociceptive action of GDNF in animal models of neuropathic pain (see text). When central sensitization occurs, BDNF recruits non-nociceptive Aβ fibers at type II glomeruli. Since at least some of these fibers express the GFRα1/RET complex they can be an additional potential target for the antinociceptive effects of GDNF. For details see [11, 15].

Abbreviations: BDNF = brain-derived neurotrophic factor; CGRP = calcitonin genre-related peptide; SP = substance P; SST_{28} = 28 amino acid somatostatin; GFRα1 = GDNF family receptor α1; IB4 = isolectin B4; RET = receptor tyrosine kinase (RTK) rearranged during transfection; TrkB = tyrosine kinase receptor B