Role of NGF in Neuronal Plasticity in the Lateral Reticular Nucleus in Chronic Inflammatory Pain

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Abstract: In pathological states, repetitive inputs from the ascending pathways involved in the genesis and integration of nociception, leads to molecular, anatomical and electrophysiological adaptive changes of these pathways, contributing to the development of pain chronicity. In the past, neurotrophic factors have been implicated in neuronal plasticity in adult central nervous system. We have previously described plastic changes associated with the up-regulation of NGF's high affinity receptor, TrkA, in the spinoreticular pathway in a chronic inflammatory pain model of arthritis induced by complete Freund's adjuvant. The present study investigated the role of central NGF in the maintenance of inflammatory pain. Analysis of TrkA and NGF expression revealed that they are expressed in the medial thalamus and several reticular nuclei of the brain stem such as the lateral reticular nucleus (LRt) and not in pathways classically described to be involved in the sensory-discriminative aspect of pain such as the lateral thalamus. In addition NGF was over-expressed in the LRt, lateral thalamus and cortex of polyarthritic rats. Using micro-injection of an adenoviral vector synthesizing NGF (or green fluorescent protein) in the LRt of normal animals, we showed that increased NGF levels in the LRt leads to the development of mechanical hypersensitivity and increased nocifensive behavior following an inflammatory stimulus. These results suggest that, NGF acts centrally as a possible molecular inducer of synaptic plasticity in the LRt in conditions of chronic inflammatory pain.

INTRODUCTION

Nerve Growth Factor (NGF) is a neurotrophic factor involved in the survival and differentiation of neuronal populations such as the cholinergic neurons of the basal forebrain, sympathetic neurons and small sensory nociceptive neurons in the dorsal root ganglia (DRG) during development [1]. In adulthood, along with Brain-Derived Neurotrophic Factor (BDNF), NGF, is implicated directly and/or indirectly in the neuronal plasticity of some areas of the brain such as the dentate gyrus and visual cortex which occurs in physiological and pathophysiological conditions [2-5]. The activity of NGF results, for example, from its retrograde transport to the cell body of the neurons in the DRG after binding to its high affinity receptor (TrkA) in peripheral target tissues, where it is synthesized and released [6]. This particular form of transport leads to the activation of intracellular signal transduction pathways which promote survival and/or plasticity of the projecting neuronal population.

Several lines of evidence indicate that central neuronal plasticity is a key element in the development and maintenance of chronic pain in animals and humans [7, 8]. Indeed, numerous forms of activation and neuronal remodeling take place at a supraspinal level in chronic pain conditions [9, 10]. Major advances in the understanding of the central processing of pain have been achieved in the last few decades using brain imaging techniques [11]. From these studies, it has become evident that there is not one common structure or master switch for the generation of pain but a complex pattern and parallel network activation which can probably be triggered by different afferent inputs via distinct anatomical pathways [12]. Among these pathways, two large families have been proposed: the 'medial' and the 'lateral' systems depending if they relay in the medial or lateral thalamus. The lateral system is essentially formed by spinothalamic and thalamo-cortical neurons (S1, S2) and is involved in the sensory discriminative aspect of pain. The spinal neurons of the 'medial system' project to : i) the lateral reticular nucleus (LRt), subnucleus reticularis dorsalis (SRD) or the parabrachial internal lateral subnucleus (PBil) [13]. Subsequently, these different nuclei send nociceptive information to various parts of the medial thalamus and are involved in the affective-motivational and cognitive-evaluative aspects of pain. Cortical areas involved in the 'medial system' are the insular cortex, the anterior cingulate cortex and pre-frontal cortex [14, 15].

We have previously shown that the phase of established chronic inflammatory pain, in the model of adjuvant induced

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arthritis (AIA), is associated with an increase in the expression of TrkA only in neurons of the spinoreticular pathway [16]. This result suggests a role for NGF in central neuronal plasticity in this model and, also questions the origin of NGF and TrkA in this nociceptive pathway. Therefore, we have attempted to identify the site of NGF synthesis in the brainstem and its potential effect on central pain processing. The spinoreticular neurons are located in deep layers of the spinal cord and project to several medial brainstem nuclei, such as medullary subnucleus reticularis and the lateral reticular nucleus (LRt), that have been found to be involved in the sensory-discriminative and/or the affective-motivational components of pain. However, virtually nothing is known regarding the role of NGF in these particular nuclei and how it influences pain transmission. Here, we identified the LRt as a major source of NGF synthesis in AIA. Furthermore, enhanced synthesis of NGF using an adenoviral vector in this nucleus increased noxious mechanical sensitivity and exacerbated nocifensive responses to a noxious inflammatory stimulus in naïve animals, suggesting that NGF is a crucial mediator of the neuronal plasticity of the spinoreticular pathway.

MATERIALS AND METHODS

Animal Model

Experiments were performed on 66 male Sprague-Dawley rats (Charles River, France), weighing 250-300g at the beginning of the experiment. Animals were anaesthetized with 4% halothane in nitrous oxide/oxygen mixture (2/1 v/v). AIA was induced by injection to each hindpaw of 50µl of complete Freund's adjuvant (CFA), containing 6 mg of *Mycobacterium butyricum* (Difco Laboratory, USA) suspended in 1ml of an emulsion of liquid paraffin- 0.9% NaCl-Tween 80 (6/4/1 by respective volumes), as previously described by Calvino *et al*., [17]. Control animals received 50µl of saline.

This model of chronic inflammatory pain has been largely described by several groups [18-20]. It leads to a strong peripheral inflammation and development of an auto-immune disease linked to the destruction of the cartilages of both hindpaws, forepaws and hips [20, 21]. Because of the auto-immune aspect of this disorder, injection of CFA in only one hindpaw induces a significant inflammation in other limbs, therefore the other side of the animal cannot be used as control consequently the CFA is classically injected on both sides in order to increase the reproducibility of the severity of the disease.

All experiments were carried out in accordance with the European Communities Council Directive of November 24 1986 (86/609/EEC) and the UK Home Office regulations. Guidelines on ethical standards for investigations of experimental pain in animals were followed (IASP, 1980). Accordingly, the number of arthritic animals was kept to a minimum. Rats were housed 3 to a large cage, to minimize the possibility of painful interactions [22]. They were kept at a constant temperature of 22°C, with a 12h alternating light-dark cycle. Food and water were available ad libitum. Food was made available on the floor of the cages to minimize potentially painful movements. Only the experiments using the viral vectors in naïve animals were performed in the UK. The experiments on arthritic animals were performed in France.

Clinical and Behavioral Studies

Clinical Observations

Several parameters were measured on the day of sacrifice in order to assess the state of the disease: 1) the weight gain as measured by the difference between weights at injection and perfusion days, 2) the diameters of the ankles and the wrists, and 3) a mobility score with a 5-level scale: 4, the rat walks and runs normally; 3, the rat runs with difficulty, but walks normally; 2, the rat walks with difficulty; 1, the rat crawls; 0, the rat lies stationary [23].

Pain-Related Testing

The "foot-bend" procedure was used to evaluate the hyperalgesic state of the rat [24]. Each rat was held comfortably and the left hindpaw gently extended five times at 5 seconds intervals, on each occasion a rating of 1 if the animal emitted a squeak or 0 if not was given. This procedure was repeated on the right hindpaw and thus each animal obtain a score of 0 to 5 for each paw.

Thermal Hyperalgesia

Thermal response latencies were tested using the method described by Hargreaves *et al*., [25]. In brief, each animal was placed in a clear acrylic cubicle resting on a glass surface in a temperature controlled room (~22°C) and allowed to acclimatize for 15 minutes before testing. The withdrawal latencies were averaged over three consecutive tests, at least 5 minutes apart, in response to the thermal challenge from a calibrated (output of 190mW/cm^2) radiant light source. A cut-off of 20 seconds was imposed to prevent any significant tissue damage.

Mechanical Allodynia

Mechanical withdrawal thresholds were tested using a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). In brief, each animal was placed in a clear acrylic cubicle resting on a metal grid in a temperature controlled room (~22°C) and allowed to acclimatize for 15minutes before testing. Stimulation was applied via an actuator filament (0.5mm diameter) which under computer control applied a linear ramp of 2.5g/sec to the plantar surface of the paw. Paw withdrawal stops the stimulation and records the threshold. The withdrawal threshold is calculated as the average of three consecutive tests with at least 5 minutes between each test. A cut-off 50g was imposed to prevent tissue damage.

For these two tests, the left and right hind paws were tested alternately and measurements were taken on two days prior to surgery and then 3, 10, 14, 21, 28 and 31 days after virus administration.

Mechanical Hyperalgesia

Development of mechanical hyperalgesia was assessed using a noxious mechanical stimulus as previously described by Randall and Selitto [26]. Nociceptive thresholds, expressed in grams (g), were measured with an Ugo Basile analgesimeter (Varese, Italy) by applying an increasing pressure to the right hind paw of unrestrained rats until a struggle and/or squeak (vocalisation threshold) was elicited (a cut off

level of 750 g was applied). Animals were tested 10, 14, 21, 28 and 31 days after virus administration.

Formalin Test

The formalin test was performed 6 weeks after virus administration. Following acclimatization for 30 min in the testing chamber, rats received 50 µl of 5% formalin injected subcutaneously (s.c.) into the dorsal surface of the right hind paw. They were then replaced in the plexiglas box. Biting and licking of the injected paw was monitored by measuring the total duration of the response in seconds during 45 minutes following formalin administration

Adenovirus Vector Expressing NGF or GFP

Replication-defective recombinant adenoviruses were provided by Dr Smith (University of Kentucky) and were constructed as previously described [27, 28]. Briefly, plaque-purified adenoviruses were examined for replication-competence via PCR. Then, these viruses were amplified and purified by double cesium chloride gradient ultracentrifugation. The physical number of viral particles was determined by optical absorbency. The number of infectious particles was estimated by crystal violet staining using the agarose overlay method.

Injection of adenovirus vector expressing NGF or GFP were performed on 16 animals (n=8 per group). Animals were anaesthetized by intraperitoneal injection of a mixture of medetomidine (0.25mg/kg) and ketamine (60mg/kg) and placed in a stereotaxic frame (World Precision Instruments, Sarasota, Florida). Several incisions were performed in muscles at the base of the neck in order to be able to visualise the Obex. Two stereotaxic injections of viral vectors ($1.55x10^6$ VP/0.1 µl) were performed, on the right and the left side of the Obex, at the following coordinates: anteroposterior: the level of the Obex, lateral : 2mm, depth from the top of the Obex: 2mm. These coordinates were chosen according to the stereotaxic atlas from Georges Paxinos and Charles Watson and previous injections in the LRt [16]. Injections were performed using a Hamilton syringe (0.1 µl). Following the slow injection of viral vectors (10 min, followed by 10 min before the redrawal of the needle), the muscles and the skin were sutured and the animal allowed to return to his cage.

At the end of the formalin test, three randomly chosen animals from each group were perfused for histological analysis. The five remaining animals were deeply anaesthetized with pentobarbitone (50mg/kg, i.p.), killed by decapitation and the LRt was dissected for NGF titration.

Primary Antibody Used

The rabbit anti-TrkA antibody was a gift from Dr L. Reichardt. This antibody was raised against the ectodomain of TrkA and has been widely characterized [29] and used in the past, by several teams. In western-blot, this antibody recognizes only TrkA in pro-form (110 kDa) and its mature form (140 kDa) [29, 30]. It has been widely used in immunocytochemistry [31] and immunohistochemistry as well, [16, 32, 33].

Western Immunoblotting Using the Anti-NGF Antibody

Samples of submandibular gland were collected from two adult C57Bl6 mice and homogenized on ice in NP-40 based lysis buffer ((20 mM Tris (pH 8), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA and Complete protease inhibitor cocktail (Roche)). The protein concentrations of lysates were determined using a BCA Protein Assay Kit (Pierce, UK). Proteins (30µg/sample) were separated using 15% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then incubated with primary antibody, rabbit anti-mouse NGF 1:2000 Chemicon ab 927 (1:3000 in TBST, Upstate, USA), overnight at 4°C. After several T-BST washes, membranes were incubated with donkey anti-rabbit HRP-linked secondary antibody (1:5000, Amersham, UK) for 1hr at room temperature, and revealed using ECL-plusTM reagent (5 minutes) for detection by autoradiography.

Immunohistochemistry for TrkA and NGF was performed on 12 animals: 6 injected with saline and 6 injected with CFA. Animals were perfused at 21 days post-injection (PI) *i.e.* during the acute phase of AIA. Animals were deeply anaesthetized with pentobarbitone (50mg/kg, i.p.), and were perfused transcardially with 100ml of 0.1M Tris buffer pH 11, containing 0.9% sodium chloride (NaCl), followed by 500ml of 4% paraformaldehyde containing 15% saturated solution of picric acid in 0.1M phosphate buffer (PB), pH 7.4. Tissues were removed, postfixed in the same fixative for 12h and cryoprotected in 30% sucrose in PB. Coronal sections of 30µm were cut on a cryostat and collected in 0.1 M PB containing 0.9% NaCl (PBS), to be processed for immunohistochemistry. Immunohistochemistry for NGF required the incubation of sections in 0.01M citric acid pH6 for 10 minutes and several washes in PBS prior to incubation with antibodies. Tissue sections were incubated for 2 days at +4°C with the primary antibodies (rabbit anti-TrkA ectodomain, RTA, gift from Dr L. Reichardt, 1:6000; rabbit anti-mouse NGF 1:2000 Chemicon) in PBS containing 0.3% Triton X-100 (PBST). After 3 washes in PBST, the sections were incubated for 1h with the secondary antibody (biotinylated goat anti-rabbit 1:300, Vector). After 3 washes in PBST, the sections were incubated for 1h in 1:400 "Elite" streptavidin-biotin-peroxidase complex (Vector). Sections were washed 3 times in PBS, peroxidase activity was revealed by 3, 3' –diaminobenzidine (Vector) and sections were washed and mounted on gelatin-coated slides, dehydrated and coverslipped in Permount.

NGF *In Situ* Hybridization

In situ hybridization was performed at 21 days PI in 4 saline and 4 CFA-treated rats. Under deep pentobarbitone anesthesia (50mg/kg, i.p.), animals were perfused transcardially with 100ml of PBS, followed by 500ml of 4% paraformaldehyde pH 7.4. Following cryoprotection in 20% sucrose, the brains were sectioned at a thickness of 30µm in the coronal plane using a microtome (Leica, France). Tissue sections were collected into cold (-20°C) cryoprotective solution (sucrose 30%, ethylene glycol 30%, in 0.1M PB pH 7.4) and stored at -20°C until the day of hybridization. Every eighth coronal sections from each animal were processed simultaneously for free floating *in situ* hybridization of NGF mRNA. A 771 base 35S-labelled cRNA was transcripted from an ECoRI-linearized PBS-NGF plasmid [34] using T3 RNA polymerase in the presence of 35S UTP. The sense

transcript was generated from the same template using T7 RNA polymerase.

All sections were incubated with proteinase K (1µg/ml in 0.1M Tris buffer, pH 8.0, with 50mM EDTA) for 30 min at 37°C followed by 0.25% acetic anhydride in 0.1M triethanolamine for 10 min. Tissues were washed in 2X saline sodium citrate buffer (SSC) for 30 min. A pre-hybridization was performed for 1h at 60°C in pre-hybridization buffer (50% formamide, 10% dextran sulfate, 0.7% Ficol, 0.7% polyvinyl pyrrolidone, 7mg/ml bovine serum albumin, 0.15mg/ml tRNA, 0.33 mg/ml denaturized salmon sperm DNA, 40 µM DTT) and then the sections were hybridized for two days at 60°C in the same buffer, supplemented with radiolabelled ^{35}S cRNA probe (10^7 cpm/100 µl). Sections were allowed to cool down and given four 15 min washes in 4X SSC. Sections were incubated 30 min in RNAse A (20 µg/ml) at 45°C for 30 min. Sections were then successively washed 10 min in 4X SSC, 2X SSC, and 0.1 X SSC, one hour in 0.1 X SSC at 60°C and finally 10 min in 0.1 X SSC at room temperature. Sections were mounted on Superfrost plus slides and air dried. Sections were immersed in the emulsion LM-1 (Amersham) and kept at 4°C for one month. They were developed using D-19 developer (Kodak), counter colored with methyl blue and mounted in Depex.

NGF ELISA Titration

In a preliminary study, NGF ELISA titration was performed in rats injected with saline (n=12) or CFA (n=12) and killed at 5 days or 21 days PI (control n=6, CFA n=6 at each time-point). Under anaesthesia with 4% halothane in nitrous oxide/oxygen mixture (2/1 v/v), rats were guillotined. In a different experiment performed at 21 days PI, dissection of several cerebral structures (see Table **2**, n=8 per group), the right and the left LRt of each animal (see Fig. **4**, n=5 per group) were dissected on ice and rapidly frozen on dry ice.

All steps of the procedure were performed according to manufacturer's instructions (NGF Emax, Promega, Madison, USA). In brief, tissues were crushed in 400µl lysis buffer (Tris 20mM, NaCl 137mM, Nonidet P-40 1%, glycerol 10%, PMSF 1mM, aprotinin 10µg/ml, leupeptin 1µg/ml, sodium vanadate 0.5µM). Lysates were centrifuged for 30 min at 10,000g at +4°C. Primary antibody, polyclonal anti-NGF (1:1000), was coated overnight at +4°C on maxis or NUNC plates. After washes with TBST (Tris 20mM, NaCl 150mM, Tween 20 0.05%), non-specific sites were blocked with the buffer provided for one hour at room temperature. Samples and standard curve samples were coated for 6h at room temperature, washed 5 times and secondary antibody (anti-rat-HRP (1:2000)) was incubated for 2.5h at room temperature. After 5 washes in TBST, peroxidase activity was revealed by a chromogenic substrate (TMB) for 15 min. Optical density was measured at 450nm (Dynatek, France). Total protein content was titrated on the same lysates (BCA, Pierce, Rockford, USA) and concentrations were expressed in pg NGF per mg of total protein (BCA, Pierce).

Statistical Analysis

Changes in the NGF ELISA titration in the LRt of animals that received the GFP or NGF synthesizing viral vector were performed using Man-Whitney U-test. The analysis of changes in the time-course of sensory behaviour (thresholds of thermal, mechanical hyperalgesia or, nocifensive behaviour induced by intraplantar injection of formalin) was performed using a Kruskal-Wallis test followed by a Tukey post-hoc test. These statistical tests were performed using the software 'Sigma Stat'.

RESULTS

AIA is an inflammatory disorder induced by peripheral injection of Freund's adjuvant. It is characterized by several phases : i) pre-clinical from day 1 to the second week post-injection, ii) chronic inflammatory pain during weeks 3 and 4 post-injection, iii) post acute during weeks 5-8 post-injection and iv) recovery phase after the 8th week post-injection (see supplementary (Fig. **1)**, showing the time course of the behavioural changes of this model, personal experiments) [18-20]. In order to assess whether all AIA animals in this study were suffering from chronic inflammatory pain, clinical and behavioral observations were performed. At day 21, arthritic rats displayed a strong reduction of weight (-31g ± 11 in arthritic rats compared to +120g ± 4 in saline-injected animals), difficulties in walking (mobility score: 1.7 ± 0.2 in arthritic rats compared to 4.0 ± 0 in control animals), and increased ankle circumferences (4.6 ± 0.2 cm in arthritic rats compared to 2.9 ± 0.03 cm in control animals). The "foot-bend procedure" showed hyperalgesia in arthritic animals (flexion/extension scores: 3.4 ± 0.6 / 1.5 ± 0.6 (left/right, respectively) in arthritic compared to 0 ± 0 / 0 ± 0 in control animals).

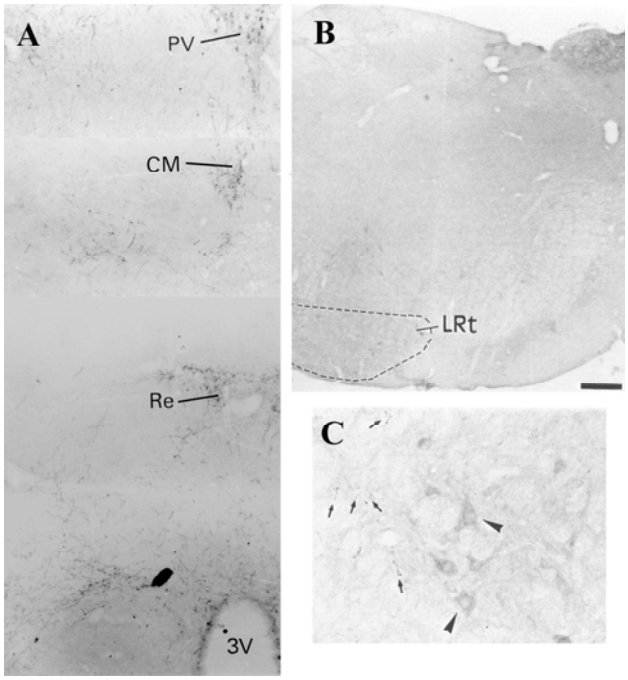

Fig. (1). TrkA-immunoreactive labelling in the medial thalamus of a naïve rat (**A**) and in the lateral reticular nucleus of the brain stem (LRt) in one representative arthritic rat, at low (**B**) and high power photomicrograph (**C**). Both TrkA-IR fibers (small arrows) and neurons (arrowheads) are seen in the LRt (Lateral reticular nucleus). Scale bar: **A**: 250µm, **B**: 100µm, **C**: 30µm. PV: Paraventricular thalamic nucleus, CM: Central medial thalamic nucleus, Re: Reuniens thalamic nucleus, 3V: Third ventricule.

TrkA-Like Immunoreactivity

Nuclei of the medial thalamus such as the mediodorsal, the reuniens nucleus and the laterodorsal nucleus displayed similar numbers of TrkA-immunoreactive (IR) fibers in both control and arthritic rats (Table **1**, Fig. **1A**). Both fibers and neuronal TrkA-IR profiles were also present in the paraventricular nucleus and the abundance of IR fibers was more pronounced in this nucleus than in any other thalamic nuclei examined (Fig. **1A**). Furthermore, the ventrobasal complex of the thalamus showed no IR neurons and rare IR fibres (1-2 fibers per 30µm section). In the brainstem, multipolar neuronal profiles and varicose fibers were TrkA-IR in the lateral reticular nucleus (LRt, Figs. **1B,C**) and the density of IR fibers was more pronounced in this nucleus than any other brainstem nuclei examined. Few TrkA-IR fibers were observed in the nucleus gracilis, the nucleus of the solitary tract or in the medullary subnucleus reticularis dorsalis nucleus (SRD, 1-2 fibers per 30µm section). TrkA-IR neuronal profiles were also present in the gracilis nucleus (Table **1**). In contrast, no TrkA-IR profiles could be seen in the cuneate nucleus. Importantly, comparison of labeling in control and arthritic rats showed no difference in the distribution of TrkA-IR in any sites described above.

NGF-Like Immunoreactivity

Two forms of mouse NGF were identified: a 'pro-form' referred as 7S (designating its sedimentation coefficient) is a hight molecular weight (130kDa) made of two copies of the subunits α, β and γ. The antibody we used was produced using the 2.5S mouse NGF protein (13 kDa). In order to better characterize the antibody against NGF, we used in this study an other western-blot analysis in tissues of mouse submandibular gland, where the 2.5S mouse NGF protein is abundantly expressed. Using western-blot analysis, we observed that, in the mouse submandibular gland, this antibody recognizes two bands: the mature 13kDa form of NGF and an unknown band at 16 kDa (Fig. **2A**).

Using this antibody, we did not observe any NGF immunoreactivity in the major sites of NGF synthesis previously described (where pro-NGF is synthesized), such as hippocampal and cortical neurons in control and arthritic animals. In contrast, we observed a fine and punctate NGF-IR staining labeling in the cell bodies and dendrites of neurons projecting to these target structures and in varicose fibers in control rats (Figs. **2B,C**). These IR neurons are those previously described, *i.e.* neurons of the septum and the basal forebrain (for example, see Figs. **2B,C**). As a consequence, the NGF-immunoreactivity observed in this study may be the mature form of NGF which binds to TrkA and is retrogradely transported in vesicles within projecting cells. The antibody used does not recognize in immunohistochemistry the pro-form of NGF (pro-NGF), but rather its cleaved form (mature NGF). The immunoreactive varicosities are probably the NGF located in vesicles, being transported in the axons of the projecting cells. Finally, no IR for NGF could be seen in thalamic, cuneate or gracilis nuclei, the medullary, the lateral reticular and the solitary nuclei, in either control or arthritic animals.

The spinal cord showed no NGF labeling in control animals (Fig. **2D**). In contrast, fine punctate labeling was observed in neurons of laminae V-VI of the dorsal horn, exclusively at lumbar levels L3, L4 and L5 in arthritic rats (1-4 neurons per 30µm section, Figs. **2E-F**). Such NGF-IR neurons were not seen in other laminae or at other lumbar levels (data not shown). Our hypothesis is that in arthritic rats, an increased amount of NGF is synthesized in projecting structure of deep dorsal horn neurons and that NGF is retrogradely transported from this structure to the lumbar lamina V (Fig. **2F**).

Table 1. Relative abundance of TrkA-immunoreactive fibers, neuronal profiles and neurons showing NGF mRNA in various brain areas analysed. Results indicated as - absence, +/- very low abun-dance, + low abundance, ++ relatively abundant and +++ very abundant. These results are given for control animals and show that TrkA and NGF are expressed in Some cortical areas, in some nuclei of the medial thalamus, of the brainstem but are absent in the lateral thalamus. No difference was observed in arthritic rats

	TrkA-IR		NGF mRNA
	Fibers	**Neurons**	
Medial thalamus			
PV	+++	++	++
MD	+	-	+
LD	++	-	++
CL	-	-	-
CM	-	-	-
Re	+	-	+
Rh	-	-	-
Lateral thalamus			
VL	+/-	-	-
VP	+/-	-	-
VM	+/-	-	-
Rt	-	-	-
Gracilis	+	-	+/-
Cuneate	-	-	++
LRt	++	+	+++
MRN	-	-	-
SRD	+	-	+
Solitaris	+	+	+
Somesthesic Cortex	+	+	+
Cingular cortex	+	+	+

NGF *In Situ* Hybridization

As previously described [35, 36], NGF mRNA was abundant in the hippocampus. In the thalamus, NGF mRNA

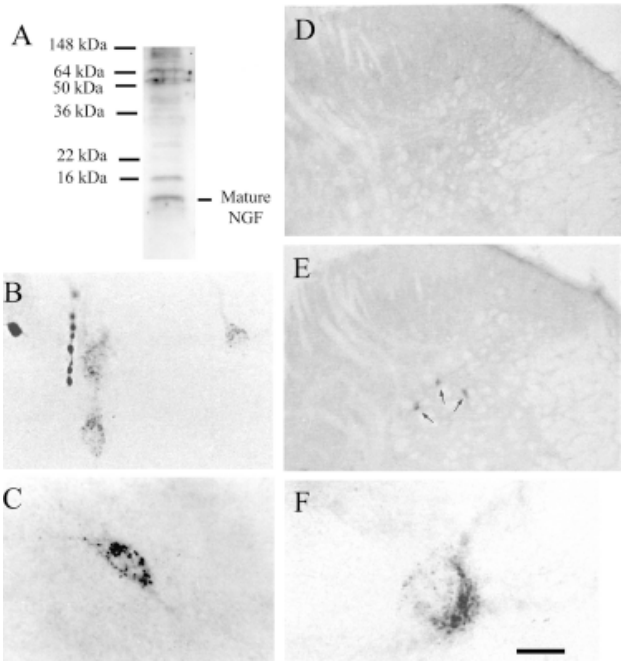

Fig. (2). NGF western-blotting on mice submandibular gland (A) and immunohistochemistry on rat brain and spinal cord (**B**-**F**). **A**: Western-blot analysis of NGF showing that the antibody used in this study recognizes the mature form of NGF at 13 kDa in the mouse submandibular gland. **B, C**: NGF-immunoreactivity in the basal forebrain of one representative control animal. **C** is a higher power magnification of **B**. NGF-IR consists in a dense labelling of varicosities and varicose fibers in the basal forebrain and fine punctuate labelling in neuronal cell bodies (**C**), suggesting that the staining is located in the cell bodies and processes of NGF-sensitive neurons. **D**-**F**: NGF-immunoreactivity in the spinal cord of a naive (**D**) and an arthritic rat (**E, F**). No labelling could be detected in the spinal dorsal horn of control animals (**D**). In contrast, in arthritic rats during the acute phase of AIA (3 weeks PI), a fine punctuate labelling was observed in neurons located in laminae V-VI of the dorsal horn, at lumbar levels L3-L5 (**B**, arrows). **F** is a high power photomicrograph of **E**. Scale bar: **B**: 10μm, **C**: 15μm, **D, E**: 100μm, **F**: 25μm.

was located in neurons of the dorsolateral, the anteroventral and reuniens nuclei (Table **1**). In the brainstem, few positive neurons were seen in the nucleus of the solitary tract and in the subnucleus reticularis dorsalis (2-3 neurons per section). In contrast, a large number of neurons present in the LRt were positive for NGF mRNA (Fig. **3B**). Importantly, most labeled neurons were located in the parvocellular part of this nucleus (LRtPC, Fig. **3A**). No NGF mRNA was seen in the medullary reticular, gracilis or cuneate nuclei. Finally, no difference could be detected between the distribution and the number of NGF mRNA positive cells between control and arthritic rats.

No NGF mRNA could be observed in the spinal dorsal horn of control or arthritic rats confirming, as previously discussed (see result section: 'NGF-like IR'), that the observed staining of NGF in arthritic rats is due to retrogradely transported NGF and is not due to local synthesis of NGF in the spinal dorsal horn.

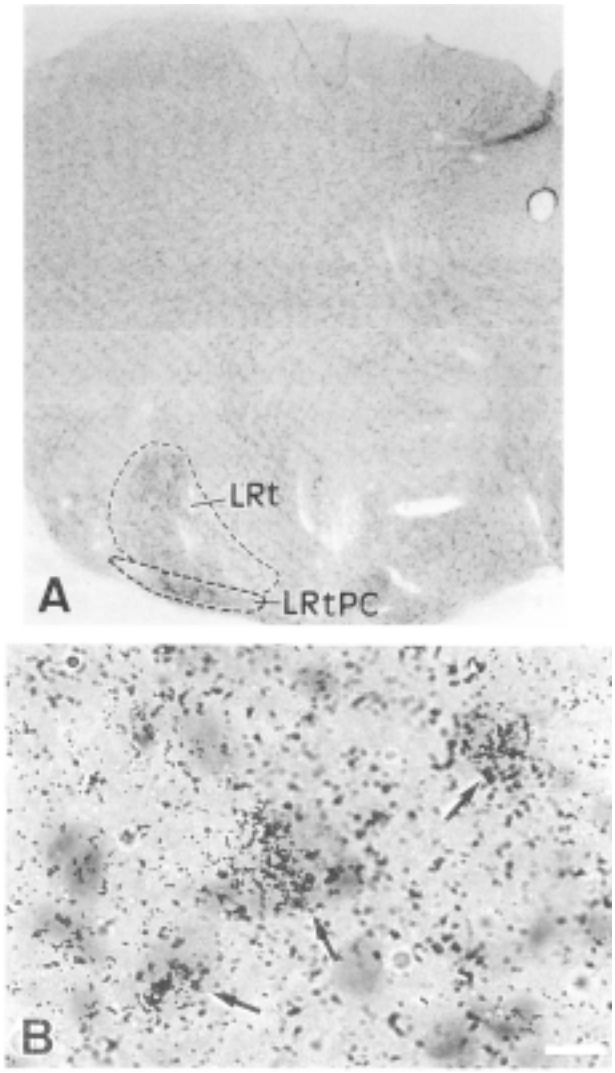

Fig. (3). In situ hybridization of NGF mRNA in the lateral reticular nucleus (LRt) of the brain stem, at the level of the obex in a control animal. **A**: Low power photomicrograph showing localization of the LRt and a higher signal of hybridization in the parvocellular part (LRtPC). **B**: High power photomicrograph showing a high density of silver grains covering cell bodies of LRt neurons (arrows). Scale bar: **A**: 250μm **B**: 10μm.

NGF ELISA Titration

In a preliminary study, using ELISA, we measured changes in NGF concentration only in the LRt of arthritic rats and control animals, at two different time-points during the development of the disease (5 days: pre-clinical phase and 21 days: period of established chronic pain). During the pre-clinical phase of the disease (5 days PI), there was no change of NGF concentration, 100% ± 29 and 96% ± 15 in control and arthritic rats, respectively. However, during the acute phase of the disease (21 days PI), NGF concentrations in the LRt of arthritic rats was twice those of control rats (215% ± 55 in arthritic rats compared to 100% ± 12 in control animals).

In a second experiment performed at 21 days PI, during the phase of established chronic pain, we confirmed these

results and found that in arthritic rats NGF content was also significantly increased in other areas of the brain involved in the transmission of painful stimuli, such as the medial thalamus, the somatosensory and cingulate cortex compared to control rats (Table **2**). However, no significant differences were seen in the ventro-lateral thalamus or the dorsal reticular nucleus between NGF levels of the two groups (Table **2**).

Consequences of NGF Over-Expression in the LRt Using an Adenoviral Vector

In order to evaluate whether an increase in the level of NGF in certain brain nuclei involved in pain (such as the LRt), modulates pain threshold or enhances evoked pain, we performed bilateral injections of an adenoviral vector synthesizing either NGF or Green Fluorescent Protein (GFP) into the LRt. This nucleus was chosen because it was the site of increased NGF expression in arthritic rats. Using histochemical analysis we observed cells transfected by the viral vector (GFP positive cells) in the LRt, and also along the tract of the needle (Fig. **4A**, n=3 per group). Due to the failure of our antibody to recognize the pro-form of NGF, sites of NGF synthesis could not be determined by immunohistochemistry. Therefore, tissues were freshly dissected (n=5 in each group), homogenized and NGF was titrated by ELISA. NGF titration showed a 5-fold increase of NGF content in the LRt of animals that received the NGF-expressing vector compared to the GFP-expressing vector (Fig. **4A**, 479 % ± 142 % in NGF-viral vector animals versus 100 % ± 54% in GFP-viral vector animals, $p<0.05$, Man-Whitney u-test).

Following the increase of NGF synthesis in the LRt, behavioral analysis showed that this treatment did not induce mechanical allodynia (Figs. **5A, B**) or thermal hyperalgesia (Figs. **5C, D**) in these animals compared with the GFP-treated animals. The Kruskal-Wallis test showed a lack of differences between the groups and time-points. However, the "NGF-treated" animals displayed an increased (but not statistically significant) mechanical hyperalgesia 28 days post injection of the viral vector (Fig. **5E**). In addition, treatment with NGF-synthesizing viral vector induced a more rapid onset and exacerbation of the second phase of nociceptive behavior in the formalin test (Fig. **5F**). In contrast, the first phase, which is known to be due to mechanisms of peripheral sensitization, was not altered (Fig. **5F**). The Kruskal-Wallis test showed differences between groups and the post hoc tests showed only a difference at the time point: 15 min post formalin injection between the GFP and NGF treated animals ($p<0.05$ Tukey post hoc test).

DISCUSSION

Our study first compared the densities of NGF and TrkA immunoreactivity as well as NGF mRNA expression in thalamic and brainstem structures, the main targets of ascending spinal nociceptive pathways, in a model of inflammatory pain compared with naïve animal. We observed that the lateral reticular nucleus (LRt), a brain stem nucleus involved in the integration of non sensory-discriminative information and the affective-motivational aspect of pain, is a major site of NGF synthesis. In addition, we have established that over-expression of NGF in the LRt using an adenoviral vector in naïve animals, enhances their sensitivity to noxious mechanical and especially inflammatory stimulus, suggesting that NGF could be one of the triggers for synaptic plasticity regulating pain perception in this nucleus probably leading to an enhancement of the activity of neurons in deep laminae of the spinal cord.

Retrograde Transport of Mature NGF from Brain Stem Nuclei to the Spinal Cord

NGF is synthetized from four alternatively sliced transcript (A, B, C and D), but the expression of transcripts C and D is very limited as they are only found in the cerebral cortex and the heart, where they represent less than 10% of all RNAs coding for NGF [37]. Therefore, transcripts A and B encounter for most of the NGF synthetized and give rise to two pre-pro-NGF proteins of 34 kDa and 27 kDa respectively. The signal pre-peptide of these pre-pro proteins are cleaved, forming pro-NGF proteins with a molecular weight of 32 kDa and 25 kDa [37]. The pro-forms of NGF (more or less glycosylated) are cleaved producing the mature form of NGF, which is biologically active and has a molecular weight of 13kDa (see supplementary data 2 for a synthetic cartoon of NGF synthesis). We used western-blot analysis to better characterize the antibody against NGF used

Table 2. NGF ELISA Titration in Several Brain Areas in Naïve and Arthritic Rats, During the Acute Phase of the Disease (3 Weeks Post Injection). Results are Expressed as pg of NGF/mg of Total Protein. N=6 Per Group. * p< 0.05 and ** p< 0.01, t-Test. Statistically Significant Increased NGF Content is Observed in the Medial Thalamus, Ventral Aspect of the Brainstem, and the Somatosensory and Cingular Cortex

	Control	AIA 3
Medial Thalamus	71.5 ± 1.0	**107.4 + 18.2 ***
Ventro-lateral Thalamus	45.3 + 7.2	78.3 +10.3
Reticular nucleus of the brainstem (dorsal part)	163.6 + 16.8	144.0 + 10.8
Reticular nucleus of the brainstem (ventral part)	80.1 + 2.6	**131.8 + 19.6 ****
Somatosensory cortex	76.2 + 4.4	**91.3 + 5.7 ***
Cingular cortex	167.6 + 6.1	**192.5 + 6.8 ***

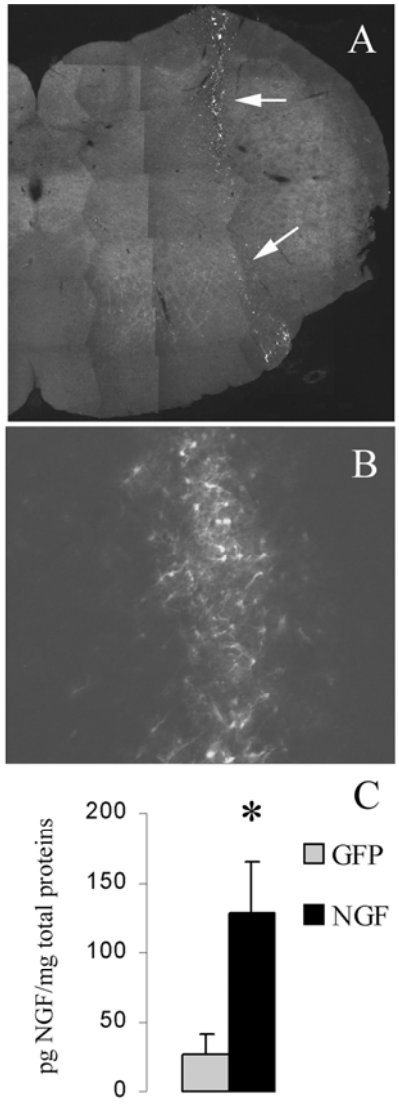

Fig. (4). Biochemical and anatomical consequences of the injection of adenoviral vector synthesizing GFP or NGF in the LRt. **A**: Fluorescent observation of the GFP showing the spread of cells transfected by the viral vector in the LRt and along the tract of the needle made during the injection. **B**: High power magnification of A. **C**: NGF ELISA titration in the LRt of animals that received the GFP or NGF synthesizing viral vector, 5 weeks after the injection of vector showing the up-regulation of NGF induced by the NGF viral vector. n=5 per group, * $p<0.05$, Man-Whitney U-test.

in this study. As expected, in the mouse submandibular gland, it recognized the mature 13kDa.

In our study, NGF immunoreactive labeling was absent in previously described sites of NGF synthesis (*i.e.* hippocampus and cerebral cortex, [38]) where NGF is synthesized as a pro-NGF form. Nonetheless, we observed dense labeling of NGF in varicosities and fine punctate staining in the cell bodies of these NGF-dependent neurons. The most parsimonious explanation is that the antibody used in this study only recognized the transported form of NGF in vesicles following endocytosis at the site of synthesis.

In addition, in some cases (such as in the medial thalamus, cortex and LRt), NGF ELISA showed changes in protein content whereas NGF mRNA levels were unaffected. These apparent discrepancies can be explained as i) ^{35}S in situ hybridization may show variation of mRNA but is not the most sensitive method to demonstrate changes in mRNA content. ii) An increase in protein level is not always due to an increased mRNA level, but translational and post-translational mechanism may induce such increased protein concentration with stable mRNA levels.

In the spinal cord, we observed a punctuated NGF staining in neurons of laminae V-VI of the dorsal horn exclusively at lumbar levels L3, L4 and L5 in arthritic rats compared to control rats, where we previously described an increase of TrkA immunoreactivity [16]. We have also previously characterized these TrkA positive neurons as neurons of the spinoreticular pathway [16]. Finally, NGF ELISA titration demonstrated an increased NGF content during the chronic inflammatory phase of arthritis in the LRt where this pathway terminates. Therefore, we hypothesize that NGF synthesis is increased in the LRt, captured and retrogradely transported by spinoreticular neurons to their cell bodies in the deep layers of the spinal cord in this model of sustained inflammatory pain. As previously described in cortical neurons [39, 40], increased NGF content could enhance TrkA synthesis and might explain the increased TrkA-IR described in our previous article [16].

TrkA and NGF are Expressed in Nuclei Involved in Pain Modulation

Analysis of TrkA immunoreactivity confirmed that the ventrobasal (VB) complex of the thalamus and the dorsal column nuclei displayed few, if any, TrkA-IR [32]. We also found only rare TrkA-IR fibers in the VPL and the nucleus gracilis in contrast to Sobreviela *et al.*, [41]. Finally, no NGF mRNA could be observed in the VB complex and rare positive neurons were seen in the nucleus gracilis, as previously described [42]. Therefore, we conclude that NGF and its receptor are poorly expressed in structures participating in the lemniscal pathway which is mainly involved in sensory discrimination.

A proportion of TrkA positive spinothalamic neurons terminate in the medial thalamus, a structure that receives various exteroceptive and visceral inputs and is thought to hold a pivotal position between the spinal cord and the cerebral cortex [43]. In this study, we observed that the paraventricular thalamic (PV) nucleus and the reuniens nucleus have a strong density of TrkA-IR fibers and TrkA-IR neuronal profiles, associated with a large expression of NGF mRNA only in the PV nucleus. Another medial thalamic nucleus, the mediodorsal nucleus, also receives TrkA-IR afferents, but with a weaker density than the previous two. These three nuclei receive information from the spinal cord including nociceptive inputs [44]. Although the precise function ascribed to medial nuclei is still unclear, several lines of evidence suggest that they are involved in the non-discriminatory aspect of sensory information and nociception [45]. Differences in the expression of NGF and TrkA observed in lateral and medial thalamic nuclei suggest that NGF may be more likely implicated in the regulation of the activity of structures involved in the non-discriminatory aspect of exteroceptive and/or visceroceptive information rather than the epicritic sensory aspect.

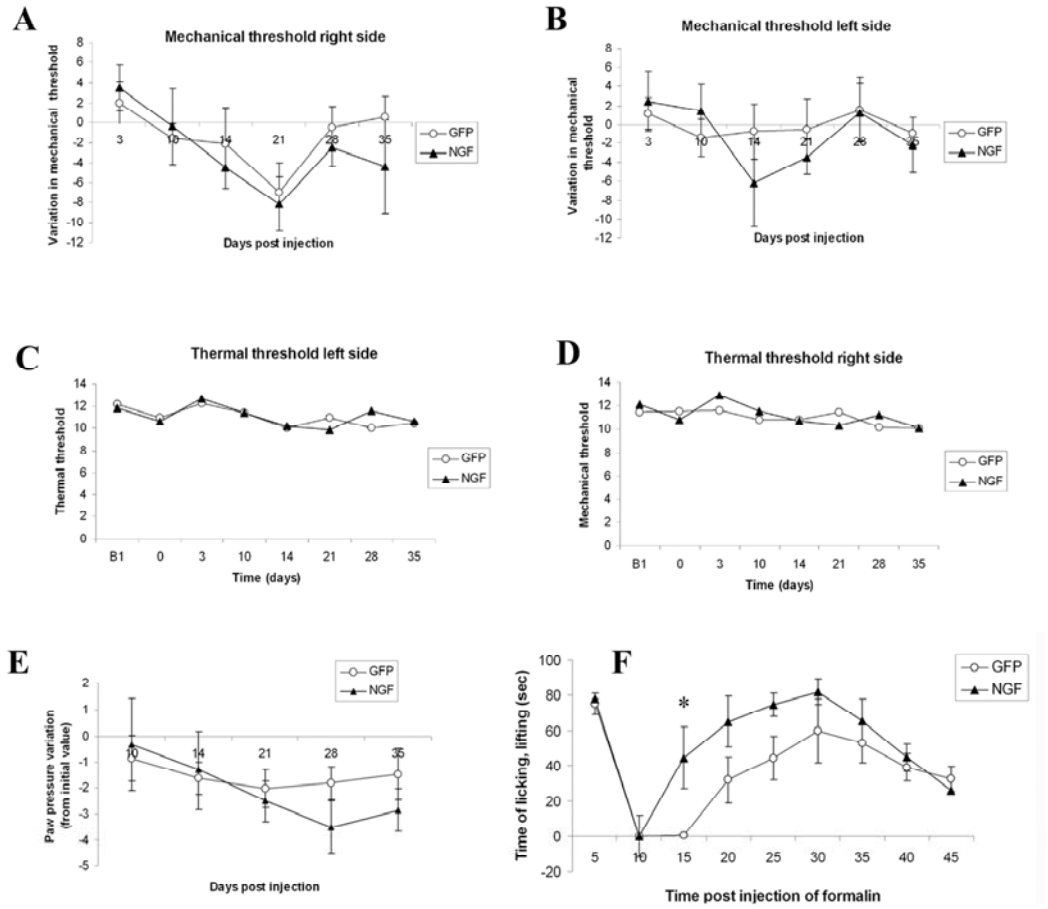

Fig. (5). Behavioral consequence of the up-regulation of NGF in the LRt following injection of adenoviral vectors expressing GFP or NGF. **A-E**: Time-Course variation of the mechanical allodynia (**A, B**), thermal hyperalgesia (**C, D**) and mechanical hyperalgesia (**E**) in days before (B: baseline), the day of the injection of viral vector (0) or following this injection. **F**: Time-course of the nocifensive behaviour (lifting, licking the injected paw) induced by intraplantar injection of formalin, in animals that received the stereotaxic injection of adenoviral vector 5 weeks before. n=8 per group. Results are expressed as mean ± sem and were compared using a Kruskal-Wallis test followed by a Tukey post-hoc test. * $p<0.05$ compared to animals that received the GFP-expressing viral vector at the same time-point. Note that the threshold of thermal hyperalgesia and mechanical allodynia are not affected by the NGF viral vector (**A-D**). In contrast, the animals injected with the NGF expressing viral vector display an enhanced sensitivity to the test of mechanical hyperalgesia (**E**) and to an inflammatory stimuli (formalin test, F).

Functional Significance of NGF Up-Regulation in the Spino-LRt Pathway

In order to determine whether the increased NGF content in one of the nuclei involved in the non-discriminatory aspect of pain, such as the LRt, was able to modulate pain-related behavior, we injected an adenoviral vector over-expressing NGF in the LRt of naïve animals. We observed that over-expression of NGF enhances the response to a noxious mechanical stimulus as shown by decreased vocalization thresholds and increased licking and biting behavior induced by intraplantar formalin administration, which are both supraspinally-mediated pain responses. In contrast, NGF over-expression did not alter sensitivity as measured by spinally-mediated pain response to certain stimuli (*i.e.* Von Frey and thermal test) suggesting that the LRt might participate in the modulation of nociceptive responses organized supraspinally as previously described [46-48]. Nonetheless, it is also plausible that NGF over-expression changes the characteristic of the spinal inhibitory pathway from the LRt on spinal nociceptive transmission (see below) and probably induces plasticity of the spinal Wide Dynamic Range neurons (WDR).

Neurons of the LRt receive inputs resulting from visceral and noxious stimuli from at least half of the body surface [48]. Such stimuli and subsequent increases in the activity of these neurons lead to changes in expression of genes such as c-fos [49]. The LRt sends descending inhibitory efferents to the spinal cord [50-52], trigeminal nucleus [53], as well as collateral branches to the periaqueductal grey matter (PAG) [54]. Conversely, the PAG and the nucleus raphe magnus (NRM) send inhibitory information to the LRt and a correlation of the neuronal activity between the LRt and the rostro ventral medulla (RVM) following mobilization of inflamed hind paw has been described [55] suggesting that both nuclei modulate the activity of the LRt [56]. Finally, as revealed by electrical stimulation of the LRt, this nucleus is involved in the regulation of supraspinally organized nociceptive responses elicited by painful peripheral stimuli

[46, 48]. Taken together, these data suggest that the LRt plays a complex role in the transmission and the control of pain supraspinally.

In this study, we observed that NGF over-expression in the LRt, significantly reduced the time of the interphase and increased the severity of the second phase of the formalin test. The interphase is the time between the first phase (due to peripheral sensitization, 0-5 min post-injection) and the second phase (known to be due to spinal mechanisms of enhanced synaptic strength, 15-40 min post-injection) of the pain response seen in this test. This interphase has previously been described as a consequence of an active inhibition of WDR by descending inhibitory pathways [57, 58]. Furthermore, the second phase is mediated in part by sensitization of WDR. Therefore, we postulate that increased NGF concentrations in arthritic rats, or virus-injected naïve animals, could reduce the descending inhibition coming from the LRt and enhance the activity of deep spinal WDR neurons, ultimately leading to an exacerbation of pain sensation. Neurons of the spinoreticular pathway are located in the deep layers of the spinal cord [59] and undergo molecular changes that alter their electrophysiological properties in arthritic animals. For example, we have previously observed an increase in TrkA-IR and retrograde transport of NGF in these neurons [16]. Other authors have also described up-regulation of c-fos [60], pro-dynorphine [61], enkephaline [62] and NK1 internalisation [63]. Most interestingly, these neurons have non-linear adaptive properties [64-66] and in arthritic rats, they display an unusual pattern of ectopic activity and expansion of their receptive fields [67, 68]. *In vitro*, these neurons can fire tonically during a depolarizing current but also continue to fire after the depolarizing current ends [69], suggesting that the membrane properties of these neurons are critical and lead to their sustained electrophysiological activity. We hypothesize that NGF, which has previously been described as a trigger for synaptic plasticity in the cortex and hippocampus [70-73], is retrogradely transported from the LRt and induces plastic changes in the membrane properties of the WDR neurons located in deep layers of the dorsal horn, participating in the long-lasting pain perception in arthritic rats. Such changes may only occur in models of persistent inflammatory pain which allow sufficient time for NGF synthesis.

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ABBREVIATIONS

3V	=	Third ventricle
BDNF	=	Brain-derived neurotrophic factor
CFA	=	Complete Freund`s adjuvant
CL	=	Centrolateral thalamic nucleus
CM	=	Central medial thalamic nucleus
IR	=	Immunoreactivity
LD	=	Laterodorsal thalamic nucleus
LRt	=	Lateral reticular nucleus
LRtPC	=	Lateral reticular nucleus, parvocellular part
NGF	=	Nerve growth factor
PBil	=	Parabrachial internal lateral subnucleus
PI	=	Post injection
PV	=	Paraventricular thalamic nucleus
Re	=	Reuniens thalamic nucleus
Rh	=	Rhomboid thalamic nucleus
Rt	=	Reticular thalamic nucleus
SRD	=	Subnucleus reticularis dorsalis
VL	=	Ventrolateral thalamic nucleus
VM	=	Ventromedial thalamic nucleus
VP	=	Ventroposterior thalamic nucleus

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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