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Spinal Substance P and CGRP staining in vincristine, cisplatin, streptozocin or constriction injury-induced neuropathies

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Abstract:

Substance P and CGRP-expressing neurons were quantified in dorsal horn of rats suffering from painful neuropathy after toxic-, metabolic- or chirurgically-induced injury. The CGRP immunoreactivities were increased in both models and the highest values were in diabetic and vincristine-induced rats. Immunostaining showed increase in SP immunoreactivity in diabetic rats. This study shows injury type-specific SP and CGRP liberation according to the etiology and delay after induction.

Keywords: *cisplatin, vincristine, sciatic nerve, diabetic rat, spinal cord*

Introduction:

Neurological toxicity is possibly characterized by a sensitive peripheral neuropathy possibly compounded by motor and/or sensitive lesions of the nervous system. Nociceptive circuitry can be chronically modulated in response to continuous stimuli. The mechanisms are various, not well understood and linked to the causes, metabolic, traumatic or toxic ones. For example either neuronal degeneration or lesions of microtubules causing axonopathy and demyelinization are respectively linked either to cisplatin or vincristine [1]. Moreover the mechanisms may additively or synergistically interact to induce a total state of neuronal dysfunction. This interlocking of mechanisms and their weakly differentiated expression may explain the relative homogeneity of neurological signs. This suggests that the deterioration of various common essential neuronal mechanisms may underlie the observed painful clinical signs after nerve injury.

One the different mechanism of neuropathic pain is damage or disruption of components of primary sensory neurons and redistribution of neurochemicals markers. These lesions are sited in peripheral nerves, dorsal root ganglion (DRG) or nerve roots. Nerve signals arising from the local injury and/or from supraspinal sites contribute to long-term changes and induction of chronic painful states. The amplification and persistence of pain are caused by a cascade of many events, including possible modifications of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), neurotrophins and kinases involved in phosphorylation processes.

We therefore set out to look for substance P and CGRP in spinal dorsal horn in four different chronic neuropathic states inducted either by a toxic, a traumatic or a metabolic nerve injury. Both models induced sensory deficits. We used drug-induced models where long-term and stable over time behavioral deficits are warranted: vincristine- and cisplatin-induced neuropathy [2,3], streptozocin-induced type 1 diabetes [4] and a surgical model, the chronic constriction injury of the sciatic nerve (CCI) [5]. Both animals showed mechanical hyperalgesia and cold allodynia. Furthermore CCI rats also showed mechanical allodynia. Cisplatin and vincristine-induced rats as diabetic rats presented hot hyperalgesia. However, in contrast with previously published results on SP and CGRP in neuropathic animals [6,7] no behavioural pain test have been conducted as it is known that noxious and non noxious stimuli are described to increase spinal SP or CGRP release in normal animals [8,9], complicating the analysis of the role of each peripheral nerve damage.

Materials and Methods:

Animals

In all studies, Sprague-Dawley adult male rats (180-200 g) (Charles River Laboratories, L'Arbresle, France) were used throughout. They were housed at constant temperature (22°C ± 2°C) and relative humidity (55%) under a fixed 12 h light/dark cycle. Food and water were

available *ad libitum*. During the experiments all efforts were made to minimize suffering of the animals, in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Creation of neuropathy

***** Cisplatin-induced neuropathy [2] Cisplatin treatment consisted of intraperitoneal injections of cisplatin (cisplatinum (II) diamine dichloride, Sigma Aldrich, France) diluted in 0.9% sodium chloride (B. Braun, Germany) for treated animals or vehicle for control animals at J1, J5, J8, J12 and J15. Cisplatin cumulated dose for treated animals was 15 mg/kg. To prevent cisplatin-induced nephrotoxicity, all the animals received a subcutaneous injection of saline after each injection of cisplatin (treated) or saline (control).

* Vincristine-induced neuropathy [3] The treatment consisted in injecting vincristine (150 µg/kg) every 2 days for 9 days, i.e. five injections of 150 µg/kg with a cumulated dose at the end of the experiments of 750 µg/kg. Vincristine sulfate (Oncovin® 1 mg/ml, Lilly, France) was diluted in 0.9% sodium chloride (B. Braun, Germany) to the correct concentration and then injected in the tail vein of the animals.

* Diabetic neuropathy [4] The rats (200–250 g) were rendered diabetic with an intraperitoneal (i.p.) injection of streptozocine (75 mg/kg) (Zanosar®, Upjohn, France) dissolved in distilled water. Diabetes was confirmed 3 weeks later by measurement of tail vein blood glucose levels with Glucotide™ and a reflectance colorimeter (Glucometer® 4, Bayer Diagnostics, France). Only rats with a final blood glucose level higher than 14 mM were included in the study.

* Chronic Constriction Injury [5] Briefly, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the left common sciatic nerve was exposed at the level of the middle of the thigh and four ligatures were loosely tied around the nerve using 4–0 braided silk thread (Ethicon Inc., Belgium) with a 1 mm space. The length of nerve thus affected was 4–5 mm. The surgical incision site was sutured and disinfected with povidone iodine. A sham operation was performed in the same manner except for the sciatic nerve ligation. Rats were tested two weeks after.

Immunohistochemistry

All procedures and lesions were approved by the local ethical committee and complied with the policy of the Society of Neuroscience on the use of animals in neuroscience research.

Within 24 h after the last vincristine or cisplatin injection, four weeks after the induction of diabetes and two weeks after the operation, rats were fixed by perfusion. Time-course curve of development of nociceptive signs are published elsewhere [2-5].

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and infused intracardially by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4). After infusion, L4-5 lumbar spinal cord was removed, post-fixed in the same fixative for 1 h at 4 °C and then transferred to 50° ethanol. The L4-5 spinal cord was identified by the lumbar enlargement and nerve roots. Sections of the lumbar spinal cord were paraffin-embedded, and the next day they were serially sectioned at 5 µm thickness in a transverse plane with a microtome. Tissue sections were then mounted on slides. Sections were deparaffined (xylene 3 × 5 min, 100° ethanol 2 × 10 min, 95° ethanol 2 × 10 min and distilled water 2 × 5 min). Deparaffined sections were microwaved in 10 mM citrate buffer (pH 6.1) to unmask antigenic epitopes for 10 min at 98°C and allowed to cool for 30 min at room temperature. Sections were washed for 5 min in distilled water and then in Tris-buffered Saline with 0.1% Tween-20 and 1% NaF (TBS Tween NaF). We hooped the sections with DAKO PEN.

Dako Cytomation standardized staining protocol was used. Sections were washed for 10 min in peroxidase-blocking solution (DakoCytomation) and washed for 10 min in TBS Tween NaF. The sections were incubated in primary polyclonal goat CGRP antibody (*SC-8856*, 1 : 200, Santa Cruz Biotechnology, Tebu-bio, France) and primary polyclonal goat Substance P antibody (*SC-9758*, 1 : 500, Santa Cruz Biotechnology, Tebu-bio, France) at 4°C overnight. Sections were then washed in TBS Tween for 15 min and incubated for 30 min at room temperature in biotinylated swine anti rabbit/mouse/goat antibody (Universal LSAB® + Kits DakoCytomation). After a further wash in PBS, tissue sections were incubated with streptavidin (Universal LSAB® + Kits DakoCytomation) for 30 min. After a final wash in TBS Tween, tissue sections were covered with the chromogen diaminobenzidine (Universal LSAB® + Kits DakoCytomation) for 5 min and then rinsed in distilled water for 2 × 5 min.

Sections were placed in montage aqueous milieu (Kits DakoCytomation) and examined under a Nikon labophot-2 microscope (Nikon, Tokyo, Japan). Sections were examined at magnification × 40 and × 100. Photographs of the dorsal horn were made with a camera. Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA) was used to optimize resolution, brightness and contrast of the images.

The density of fibbers immunoreactive for SP or CGRP in the superficial dorsal horn laminae was blindly determined with an image analysis system with coding of slides. Sections were viewed on a Nikon labophot-2 microscope at magnification × 100 for CGRP- and SP-immunostaining. We used LUCIA software (Cytogen GmbH, Sinn-Fleisbach, Germany). Immunoreactive structures (fibbers and varicosities) were separated from the background by an interactive method of thresholding. All objects in the field were automatically counted. The surface occupied by immunostained fibbers was expressed as the ratio of the number of black pixels to the total number of pixels in the selected area.

Statistical analysis

All the experiments were based on the same experimental scheme. For the study of CGRP and SP expressions, six animals were used, with a control for each treated rat in each treatment modality. The statistical analyses were used as mean +/- S.D. and performed with a Fisher F test and a Student *t* test. The results were considered significant for a probability level lower than 5% ($p<0.05$).

Results :

Animals were carefully handled to avoid suffering. None of the rats were tested to detect behavioural signs of hyperalgesia or allodynia. Nevertheless both animals displayed the evident behavioural or biological signs regardless of the type of neuropathy as changes in posture or hyperglycaemia. No death occurred in any of the groups.

Figures 1 and 2 present the density of fibers immunoreactive for substance P and CGRP expressed as the ratio of the number of black pixels to the total number of pixels in a selected area. The distribution of SP^+ and $CGRP^+$ nociceptive axons in normal spinal cord was restricted to lamina I and II in the spinal cord gray matter (Fig 1-E and Fig 2-E).

Cisplatin-induced neuropathy: CGRP staining was significantly increased in the treated group than in the control (26.5 ± 18.5% *vs.* 8.0 ± 2.0%, $p < 0.05$). No difference was observed for SP staining with the control group (24.3 ±5.9% *vs.* 19.3 ±0.6%).

Vincristine-induced neuropathy: only CGRP staining was significantly increased in treated than in control rats (46.0 ± 27.5% *vs.* 8.3 ± 4.4%, $p < 0.01$). No difference was observed for SP staining: 12.1 ± 3.6% *vs.* 19.3 ± 0.6%.

Diabetic neuropathy: the SP and CGRP staining were significantly enhanced than in the controls (29.2 ± 4.0% *vs.* 14.2 ±

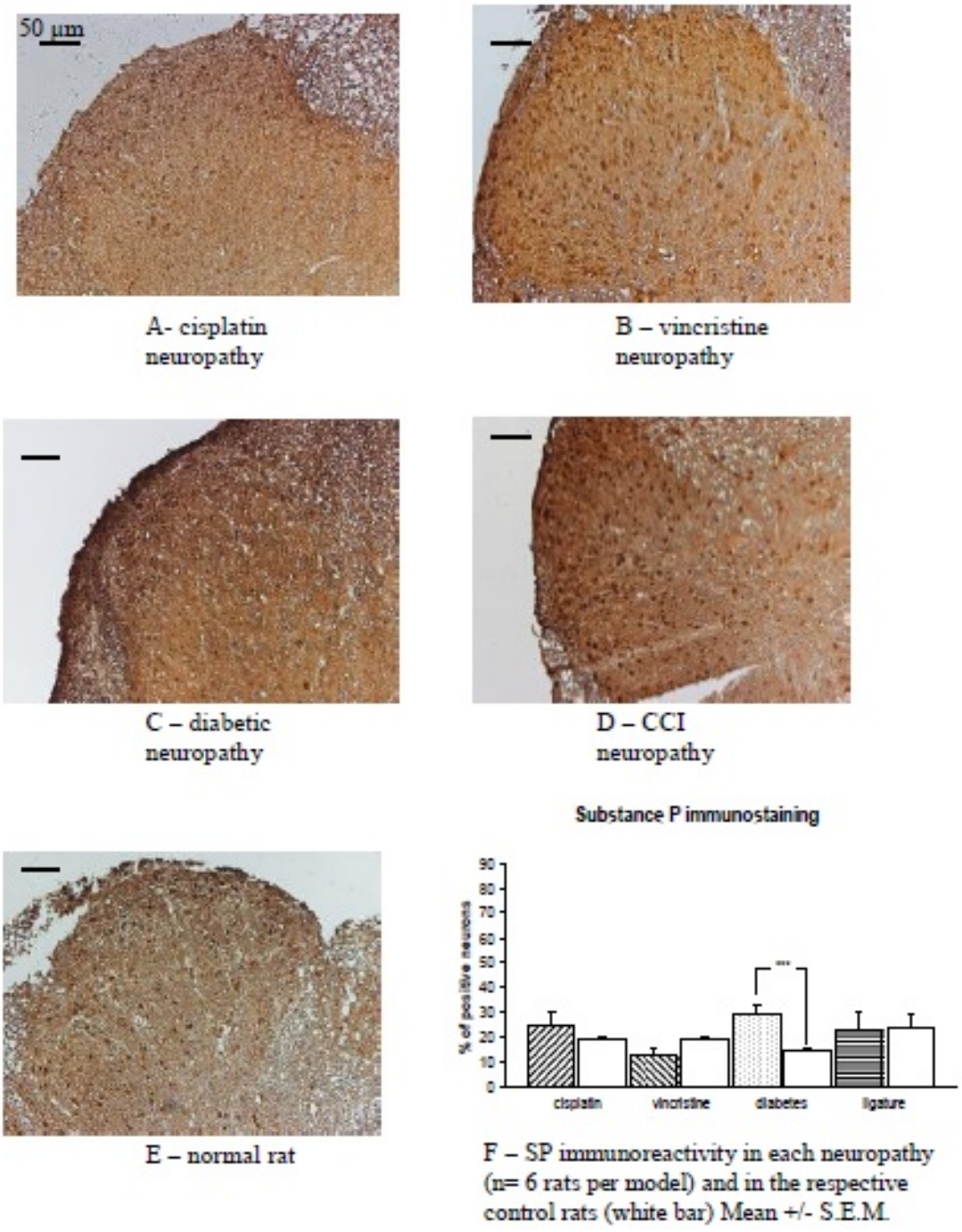

Figure 1: Substance P immunostaining in dorsal horn after various toxic inductions
Photomicrographs of substance P immunostaining in the superficial dorsal horn laminae were made 24 h after cisplatin (A), vincristine (B), diabetes (C), CCI-induction (D) and NaCl 0.9% (E), scale bar = 50 µm. Scale bar = 50 µm. (F) Comparison of substance P immunoreactivity after quantitative measurements (mean ±S.E.M) of the converted black and white digital image density of fibres immunoreactive for substance P in the spinal dorsal horn of various neuropathic rats (hatched bars) and corresponding control rats (empty bars).
The density was expressed as the ratio of the number of black pixels to the total number of pixels in a selected area. Symbols denote statistical differences in the density versus respective control rats (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student *t*-test).

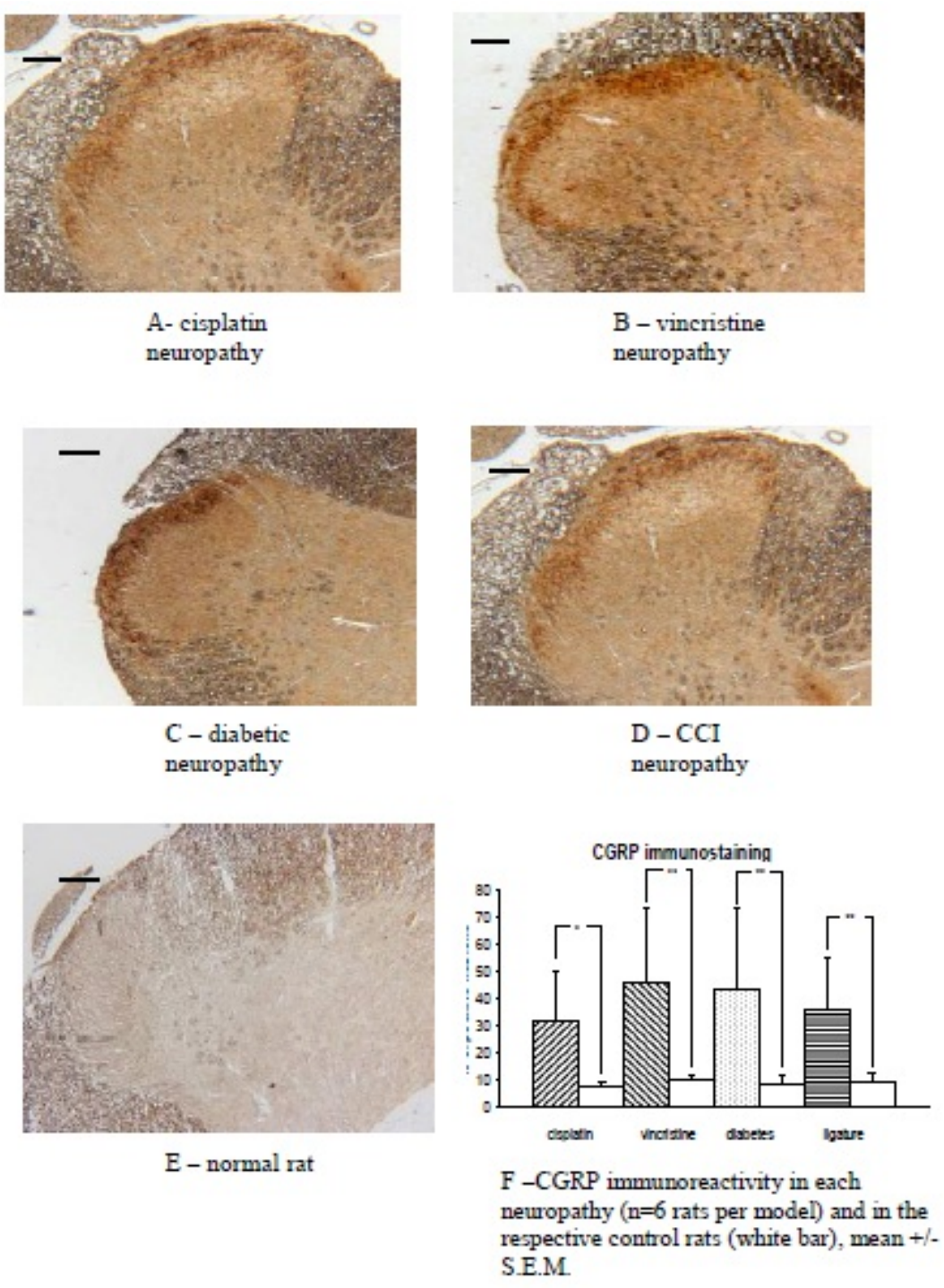

Figure 2: CGRP immunostaining in dorsal horn after various toxic inductions

Photomicrographs of CGRP immunostaining in the superficial dorsal horn laminae were made 24 h after cisplatin (A), vincristine (B), diabetes (C), CCI-induction (D) and NaCl 0.9% (E). Scale bar = 50 µm. (F) Comparison of CGRP immunoreactivity after quantitative measurements (mean ±S.E.M) of the converted black and white digital image density of fibres immunoreactive for CGRP in the spinal dorsal horn of various neuropathic rats (hatched bars) and corresponding control rats (empty bars).

The density was expressed as the ratio of the number of black pixels to the total number of pixels in a selected area. Symbols denote statistical differences in the density versus respective control rats (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student *t*-test).

1.3%, $p < 0.001$ and 46.4 ± 29.2% *vs.*7.8 ± 4.9%, $p < 0.01$, respectively).

CCI-induced neuropathy: After sciatic nerve ligation, a higher significant CGRP staining was observed versus the control group (29.7 ± 21.4% *vs.* 8.2 ± 3.4%, $p < 0.01$) in opposite to that observed with substance P (22.3 ± 8.0% *vs.* 23.7 ± 5.4%). The density was similar for the contralateral side in all nerve injured animals (data not shown).

Discussion:

This report shows that the different types of nerve injury may result in differences in SP and CGRP staining. When we compare to the respective vehicle groups, we observed that the differences in immunostaining are linked to the delay after the last injection of the injury: 9 days and 3 weeks for anticancer drugs, 2 weeks after surgical injury (Chronic Constriction Injury) and 4 weeks after induction of a metabolic dysfunction (diabetes).

The experimental conditions chosen in our study corresponded to that described in previously published studies and the delays of assay correspond to the time to appearance of classical significant behavioural changes [2-5]. As diabetes, chronically administered anticancer drugs represented gradual and repeated insults to the peripheral nerves whereas nerve ligation produced a great nerve stress nevertheless not reproducible in human. Such high vincristine and cisplatin doses may moreover exaggerate the conditions of treated patients with malignancy. However, contrary to what is generally described about SP and CGRP in neuropathic animal models, we don't perform behavioural tests in this study as both the noxious and non-noxious stimuli used in behavioural tests were known to be involved in spinal SP or CGRP release. As variations of the SP and CGRP levels may also be considered as responses to stimuli of the tests, it would be difficult even impossible to correlate the variations of SP and CGRP immunostaining to the nervous damages [8,9].

Substance P is a quite specific indicator of C-fibbers neurons and CGRP is expressed by all types of primary afferents in the rat. Substance P and CGRP are released on appropriate stimulation and play an important role in pain signal transmission by potentiating nociceptive signalling and revealing pain-related behaviour. Among various aetiologies of nerve injury, it is known that a peripheral inflammation modify SP and CGRP contents in both DRG neurons and primary sensory afferents [6]. Dysfunction is generally not confined to degeneration at the site of aggression, but also extends to the primary sensory neurons within the dorsal horn as a result of the axon reaction [7]. Damages to small myelinated Aδ- and unmyelinated C-fibbers are implicated in hyperalgesia and allodynia. These damages to the central nervous system linked with an anatomical reorganization of spinal terminations of surviving axons and with an ectopic activity may contribute to persistent input in the spinal cord, playing an important role in central sensitization and leading to the persistence of pain.

Concerning vincristine and cisplatin-induced neuropathies, 9 days and 3 weeks respectively after beginning the toxic disturbance, only CGRP immunostaining was significantly increased but SP spinal release was almost unmodified. The CGRP reactivity in small- and large-diameter sensory nerves is important [10]. However some authors described that vincristine decreases SP reactivity because of impairment of the axonal transport and degeneration of transganglionic neurons [11]. However they do not presented the delay after injection.A local regeneration of A-fibbers during the time window between each vincristine injection may be also evoked. Cisplatin neuropathy is a neuronopathy and the DRG is the primary site of damage with a peptide concentration increase attributed to the damage to the axonal transport system [12]. Thus our observed CGRP increase in spinal cord may be explained by a regional

distribution of CGRP from regenerating DRG neurons terminating in superficial laminas of the dorsal horn.

Following CCI, an increase of CGRP and no modification of SP were observed as for toxic-induced neuropathies. Concerning CGRP, we described as Herzberg et al. [25], a spinal release of CGRP after CCI, attributed to the descending facilitation mechanism maintaining neuropathic pain [26]. In a model of inflammatory pain following complete Freund's adjuvant injection into the rat knee, a similar significant increase in the number of ipsilateral CGRP-IR small DRG neurones was observed [27]. A bilateral increase in CGRP-immunoreactive fibbers was also described in the dorsal horn of the lumbar spinal cord in arthritic rats [28].

About SP post-CCI changes, conflicting results are reported in literature. Kajander and Xu showed decreased SP levels at 60 days after CCI of the sciatic nerve [19]. Malmberg and Basbaum also observed decreased SP levels up to 14 days, but no change 70 days after the partial nerve ligation [20]. Other authors reported that the spinal nerve lesion resulted in a significant decrease in the SP release from high-threshold sensory neurons in the dorsal horn and *de novo* release of the peptide from low-threshold fibbers [6,21]. The distal peripheral axotomy is described to cause a substantial decrease in SP release in laminae I and II [21]. After a peripheral injury, the small-diameter C-sensory cells down-regulate the SP content, which is expressed *de novo* in Aβ fibers [22]. It has also been reported that the DRG SP content is time-dependent and that the elapsed time between the stimulating toxic treatment and the increase in cellular SP content is about 72 hours [23]. However, all these previously described low SP contents were observed after multiple behavioural tests whose nociceptive and not nociceptive stimuli are known to disturb the neuropeptide's release in nervous system. Our observed absence of modification in SP immunostaining in CCI is however consistent with that previously observed by Kajander and Xu who found significant decreases in CGRP and SP in the ipsilateral spinal cord 10 days after nerve transection [19]. In a delayed but inflammatory pain model such as polyarthritis, the pattern of SP staining shows a significant upregulation of the SP release in lamina I neurons [24]. In our work, the absence of SP modification may be related to the short delay after the nerve ligation, i.e. two weeks.

Type 1 diabetes neuropathy was tested four weeks after streptozocin injection. We observed an increased SP and CGRP immunostaining in the dorsal horn. It was reported that a persistent pain state as diabetes may upregulate spinal peptides such as SP and CGRP via a long-lasting neuroinflammation or neuroimmune activation of neuroglials cells in the spinal cord [16]. Effectively the toxic induction was distant, i.e. four weeks, of the SP and CGRP measures allowing the establishment of new convergent but not entirely functional synapses that not allow reestablishment of normal protective pain. There are conflicting results in the literature about effect of diabetes. A relative preservation of the numbers of neurones expressing SP and CGRP was previously observed [13]. Attenuated release of SP in diabetic rats along with hyperalgic responses and a progression to hypoalgesia, not explored there, have been described by Calcutt et al. [17]. A release of SP levels in diabetic rats has also been described with hyperalgesic responses A link with hyperglycemia could also be made [18]. Nevertheless an upregulation of SP in large myelinated Aβ fibbers was likely observed during allodynia, which is reduced by intrathecal injections of tachykinin receptor antagonists [8]. This could be also linked to the fact that our model of streptozocin-induced diabetic rats showed allodynia especially to heat [4]. This was not the case in all the literature

and suggests a contribution of SP in this pathological state.

From our present study, it can be concluded that CGRP immunoreactivity may correspond to a first phase observed after a neurotoxic stimulus. Only observed after repeated injections of the two anticancer toxic drugs and after a nerve constriction, without the possible perturbation by stimuli from behavioural tests, these results are compatible with a role for CGRP only in the initiation and the development of spinal sensitization observed after a nerve injury. In the early time window, the nociception pathways using CGRP are rapidly involved, SP being released only in a pain maintenance phase during the development of neuropathic pain as for diabetic rats [29]. SP may be the neurotransmitter most noticeably affected a long time after a toxic induction. Effectively, SP staining in the dorsal horn is higher than controls in both models and may prolong the spinal excitatory effects of CGRP [30]. It would be now important to further investigate whether the increased immunostaining corresponds with increased protein content in the same cords and whether there is also increased mRNA and protein synthesis in the matching DRG. An increase in CGRP content could improves the central facilitation mechanism leading to hyperexcitability of dorsal horn nociceptive circuitry. In adult animals, the expression of CGRP is also regulated by nerve growth factors, responsible for the increase in CGRP expression [31]. The spinal release of SP is directly involved in the descending facilitation mechanism maintaining neuropathic pain. Thus the increased SP immunostaining following chronic painful states may also result from the absence of the supraspinal descending inhibitory signals to the lumbar dorsal horn which, under physiological conditions, suppress the transmission of noxious message at the spinal level.

Conclusion:

From this limit amount of data, we suggest a great dependence on the type and location of the injury and on the time delay after the toxic aggression. These points would necessitate further explorations of both the regeneration of the sensory axons and the possibilities to guide regenerating axons, according to the different nerve injuries, delays after the injury and nociceptive behaviours. Pharmacological evaluation of new analgesic treatments might furthermore be linked to these new developments.

References:

[1] S. Quasthoff, H. Hartung, Chemotherapy-induced peripheral neuropathy, J. Neurol. 249 (2002) 9-17.

[2] N. Authier, J.P. Gillet, J. Fialip, A. Eschalier, F. Coudore, An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections, Exp. Neurol. 182 (2003) 12-20.

[3] N. Authier, J.P. Gillet, J. Fialip, A. Eschalier, F. Coudore, A new animal model of vincristine-induced nociceptive peripheral neuropathy, Neurotoxicol. 24 (2003) 797-805.

[4] C. Courteix, A. Eschalier, J. Lavarenne, Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain, Pain 53 (1993) 81-88.

[5] G.J. Bennett, Y.K. Xie, A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man, Pain 33 (1988) 87-107.

[6] V.S. Seybold, L.G. Abrahams, Characterization and regulation of neurokinin1 receptors in primary cultures of rat neonatal spinal neurons, Neurosci. 69 (1995) 1263-1273.

[7] S. Kobayashi, Y. Kokubo, K. Uchida, T. Yayama, K. Takeno, K. Negoro, H. Nakajima, H. Baba, H. Yoshizawa, Effect of lumbar nerve root compression on primary sensory neurons and their central branches: changes in the nociceptive neuropeptides substance P and somatostatin, Spine 30 (2005) 276-282.

[8] M.A. Coudore-Civiale, C. Courteix, J. Fialip, M. Boucher, A. Eschalier, Spinal effect of the cholecystokinin-B receptor antagonist CI-988 on hyperalgesia, allodynia and morphine-induced analgesia in diabetic and mononeuropathic rats, Pain 88 (2000) 15-22.

[9] X.Y. Hua, K.F. Salgado, G. Gu, B. Fitzsimmons, I. Kondo, T.T. Bartfai, T.L. Yaksh, Mechanisms of antinociception of spinal galanin: how does galanin inhibit spinal

sensitization? Neuropeptides 39 (2005) 211-216.

[10] S.K. Joshi, J.P. Mikusa, G. Hernandez, S. Baker, C.C. Shieh, T. Neelands, X.F. Zhang, W. Niforatos, K. Kage, P. Han, D. Krafte, C. Faltynek, J.P. Sullivan, M.F. Jarvis, P. Honore, Involvement of the TTX-resistant sodium channel Nav 1.8 in inflammatory and neuropathic, but not post-operative, pain states, Pain 123 (2006) 75-82.

[11] B. Csillik, Z. Janka, I. Boncz, J. Kalman, A. Mihaly, L. Vecsei, E. Knyihar, Molecular plasticity of primary nociceptive neurons: relations of the NGF-c-jun system to neurotomy and chronic pain, Ann. Anat. 185 (2003) 303-314.

[12] I. Barajon, M. Bersani, M. Quartu, M. Del Fiacco, G. Cavaletti, J.J. Holst, G. Tredici, Neuropeptides and morphological changes in cisplatin-induced dorsal root ganglion neuronopathy. Exp. Neurol. 138 (1996) 93-104.

[13] D.W. Zochodne, V.M.K. Verge, C. Cheng, H. Sun, J. Johnston, Does diabetes target ganglion neurones? Progressive sensory neurone involvement in long-term experimental diabetes, Brain 124 (2001) 2319-2334.

[14] D.R. Tomlinson, P. Fernyhough, L.T. Diemel, Neurotrophins and peripheral neuropathy. Philos. Trans. R. Soc. Lond. B Biol. Sci. 351 (1996) 455-462.

[15] G. Pittenger, A. Vinik, Nerve growth factor and diabetic neuropathy. Exp. Diab. Res. 4 (2003) 271-285.

[16] E. Hansson, Could chronic pain and spread of pain sensation be induced and maintained by glial activation ? Acta Physiol. 187 (2006) 321-327.

[17] N.A. Calcutt, C.O. Stiller, H. Gustafsson, A.B. Malmberg, Elevated substance-P-like immunoreactivity levels in spinal dialysates during the formalin test in normal and diabetic rats, Brain Res. 85 (2000) 20–27.

[18] N.A. Calcutt, J.D. Freshwater, A.P. Mizisin, Prevention of sensory disorders in diabetic Sprague-Dawley rats by aldose reductase inhibition or treatment with ciliary neurotrophic factor, Diabetologia 47 (2004) 718-724.

[19] K. Kajander, J. Xu, Quantitative evaluation of calcitonin gene-related peptide and substance P levels in rat spinal cord following peripheral nerve injury, Neurosci. Lett. 186, (1995) 184-188.

[20] A.B. Malmberg, A.I. Basbaum, Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates, Pain 76 (1998) 215-222.

[21] M. Malcangio, M.S. Ramer, T.J. Boucher, S.B. McMahon, Intrathecally injected neurotrophins and the release of substance P from the rat isolated spinal cord, Eur. J. Neurosci. 12 (2000) 139-144.

[22] K. Noguchi, Y. Kawai, T. Fukuoka, E. Senba, K. Miki, Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons, J. Neurosci. 15 (1995) 7633-7643.

[23] O.J. Igwe, c-Src kinase activation regulates preprotachykinin gene expression and substance P secretion in rat sensory ganglia. Eur. J. Neurosci. 18 (2003) 1719-1730.

[24] P. Honoré, P.M. Menning, S.D. Rogers, M.L. Nichols, P.W. Mantyh, Neurochemical plasticity in persistent inflammatory pain, Prog. Brain Res. 129 (2000) 357-363.

[25] U. Herzberg, E. Eliav, J.M. Dorsey, R.H. Gracely, I.J. Kopin, NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve, Neuroreport 8 (1997) 1613-1618.

[26] L.R. Gardell, T.W. Vanderah, S.E. Gardell, R. Wang, M.H. Ossipov, J. Lai, F. Porreca, Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation, J. Neurosci. 23 (2003) 8370-8379.

[27] P.C. Staton, A.W. Wilson, C. Bountra, I.P. Chessell, N.C. Day, Changes in dorsal root ganglion CGRP expression in a chronic inflammatory model of the rat knee joint: differential modulation by rofecoxib and paracetamol, Eur. J. Pain 11 (2007) 283-289.

[28] S. Kar, R.G. Rees, R. Quirion, Altered calcitonin gene-related peptide, substance P and enkephalin immunoreactivities and receptor binding sites in the dorsal spinal cord of the polyarthritic rat. Eur. J. Neurosci. 6 (1994) 345-354.

[29] J.H. Jang, T.S. Nam, K.S. Paik, J.W. Leem, Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. Neurosc. Lett. 360 (2004) 129-132.

[30] T.J.S. Shi, J. Li, A. Dahlstrom, E. Theodorsson, S. Ceccatelli, I. Decosterd, T. Pedrazzini, T. Hokfel, Deletion of the neuropeptide Y Y1 receptor affect pain sensitivity, neuropeptide transport and expression, and dorsal root ganglion neuron numbers, Neurosci. 140 (2006) 293-304.

[31] G. Ruiz, JE. Baños, The effect of endoneurial nerve growth factor on calcitonin gene-related peptide expression in primary sensory neurons, Brain Res. 1042 (2005) 44-52.