Neuropathic pain in neuropathy:
A combined clinical, neurophysiological and morphological study

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Chapter 1: Introduction and aims of present studies

Neuropathic pain (NP) is a major symptom which may be intractable in common neurological disorders such as neuropathy, spinal cord injury, multiple sclerosis and stroke. Pain is a complex sensation strongly modulated by cognitive influences, and understanding the underlying pathophysiological mechanisms in patients remains a challenge for pain specialists. The aim of my Phd-research was to show in according with present evidence-based studies the correlation between clinical manifestations of neuropathic pain and the underlying alteration of the different groups of fibers (Aβ, Aδ or C).

In the **second** chapter I revised the previous guidelines about neuropathic pain assessment. History and clinical examination are a requirement to confirm the presence of a NP, and also an important step in reaching an aetiological diagnosis for NP. History and bedside examination are still

fundamental to a correct diagnosis, while screening tools and questionnaires are useful in indicating probable NP. I argued in particular a recent technique, skin biopsy; I approached it at the beginning of my Phd during my stage at the I.R.C.S.S. C. Besta in Milan; then, I imported this procedure in our laboratory (Department of Pathological Anatomy, Sapienza University). We are now able to process skin biopsies and immunoassayed them with polyclonal anti-protein-gene-product 9.5 antibodies (specific for nerve fibers) using immunohistochemistry or immunofluorescence, which allowed demonstrating the extensive innervations of the epidermidis.

In the following chapters I approached some common conditions of neuropathic pain.

The **third** chapter is dedicated to the post-herpetic neuralgia, an exceptionally drug-resistant neuropathic pain. To investigate the pathophysiological mechanisms underlying postherpetic neuralgia we clinically investigated sensory disturbances, pains and itching, with

an 11-point numerical rating scale in 41 patients with ophthalmic postherpetic neuralgia. In all the patients we recorded the blink reflex, mediated by non-nociceptive myelinated Aβ-fibers, and trigeminal laser evoked potentials (LEPs) related to nociceptive myelinated Aδ- and unmyelinated C-fiber activation. We also sought possible correlations between clinical sensory disturbances and neurophysiological data. Neurophysiological testing yielded significantly abnormal responses on the affected side compared with the normal side. The blink reflex delay correlated with the intensity of paroxysmal pain, whereas the Aδ- and C-LEP amplitude reduction correlated with the intensity of constant pain . Allodynia correlated with none of the neurophysiological data. Our study shows that postherpetic neuralgia impairs all sensory fiber groups. The neurophysiological-clinical correlations suggest that constant pain arises from a marked loss of nociceptive afferents, whereas paroxysmal pain is related to Aβ-fiber demyelination. These findings might be useful for a better understanding of pain mechanisms in postherpetic neuralgia.

In the **fourth** chapter I treated the differential involvement of Aδ and Aβ fibers in neuropathic pain related to carpal tunnel syndrome (CTS). We studied 70 patients with a diagnosis of CTS (117 CTS hands). We used the DN4 questionnaire to select patients with neuropathic pain, and the Neuropathic Pain Symptom Inventory (NPSI) to assess the intensity of the various qualities of neuropathic pain. All patients underwent a standard nerve conduction study (NCS) to assess the function of non-nociceptive Aβ-fibres, and the cutaneous silent period (CSP) after stimulation of the IIIrd and Vth digits, to assess the function of nociceptive Aδ-fibres. In 40 patients (75 CTS hands) we also recorded LEPs in response to stimuli delivered to the median nerve territory and mediated by nociceptive Aδ-fibres. We sought possible correlations between neurophysiological data and the various qualities of neuropathic pain as assessed by the NPSI. We found that the median nerve sensory conduction velocity correlated with paroxysmal pain and abnormal sensations, whereas LEP amplitude correlated with spontaneous constant pain.

Our findings suggest that whereas paroxysmal pain and abnormal sensations reflect demyelination of non-nociceptive Aβ-fibres, spontaneous constant pain arises from damage to nociceptive Aδ-fibres.

In the **fifth** chapter I treated the mechanisms of pain in multiple sclerosis. In this clinical and neurophysiological study we sought information on the clinical characteristics and underlying mechanisms of neuropathic pain related to the disease. A total of 302 consecutive patients with multiple sclerosis were screened for neuropathic pain by clinical examination and the DN4 tool. In patients selected for having ongoing extremity pain or Lhermitte's phenomenon, we recorded somatosensory evoked potentials, mediated by Aβ non-nociceptive fibres, and LEP, mediated by Aδ nociceptive fibres. Of the 302 patients, 92 had pain (30%), and 42 (14%) neuropathic pain. Patients with neuropathic pain had more severe multiple sclerosis, as assessed by the expanded disability severity score, than those without pain. Whereas in patients with ongoing neuropathic pain laser evoked potentials were

more frequently abnormal than somatosensory evoked potentials we found the opposite in patients with Lhermitte's phenomenon. Our data underline the clinical importance of pain in multiple sclerosis and indicate that a more severe disease is associated with a higher risk of developing neuropathic pain. The prevalence of pain we found, lower than that reported in previous studies, may reflect the lower disease severity in our patients. Neurophysiological data show that whereas ongoing extremity pain is associated with spinothalamic pathway damage, Lhermitte's phenomenon is related to damage of non-nociceptive pathways. These findings may be useful in designing a new therapeutic approach to neuropathic pain related to multiple sclerosis.

The **sixth** chapter is dedicated to the mechanisms of pain in distal symmetric neuropathy. I and my colleagues performed a clinical, neurophysiological and histomorphological study on patients with neuropathic pain in distal symmetric neuropathy. In patients with distal symmetric polyneuropathy we assessed non-

nociceptive Aβ- and nociceptive Aδ- and C-afferents to investigate their role in the development of neuropathic pain. We screened 2240 consecutive patients with sensory disturbances and collected 269 patients with distal symmetric polyneuropathy (57% with pain and 43% without). All patients underwent the Neuropathic Pain Symptom Inventory to rate ongoing, paroxysmal and provoked pains, a standard NCS to assess Aβ-fibre function, LEPs to assess Aδ-fibre function, and skin biopsy to assess the unmyelinated innervations of the epidermidis. Patients with pain had the same age, but a longer delay since symptom onset than those without . Loss of intraepidermal innervation did not correlate with the presence of neuropathic pain. Whereas the LEP amplitude was significantly lower in patients with pain than in those without , NCS and intraepidermal fibre nerves data did not differ between groups. LEPs were more severely affected in patients with ongoing pain than in those with provoked pain. Our findings indicate that the impairment of Aβ-fibres has no role in the development of ongoing or provoked pain. In patients with ongoing pain

the severe LEP suppression and the correlation between pain intensity and LEP attenuation may indicate that this type of pain reflects damage to nociceptive axons. The partially preserved LEPs in patients with provoked pain suggest that thistype of pain is related to the abnormal activity arising from partially spared and sensitised nociceptive terminals. Because clinical and neurophysiological abnormalities followed similar patterns regardless of aetiology, pain should be classified and treated on mechanism-based grounds.

In the **seventh** chapter I treated the mechanisms of allodynia in distal symmetric polyneuropathy allodynia. Patients with painful neuropathy frequently complain of allodynia, i.e. pain in response to a normally non-painful stimulus. Many authors consider allodynia to be generated by sensitization of the second-order nociceptive neurons to Aβ-fibre input (central sensitization). With the hypothesis that patients suffering from this type of pain probably have a relative sparing of Aβ-fibres in comparison with patients with ongoing pain only, we sought aimed at seeking

information on mechanisms underlying allodynia. In 200 patients with distal symmetric polyneuropathy (114 with pain, 86 without) we assessed non-nociceptive Aβ- and nociceptive Aδ-afferents to investigate their role in the development of allodynia. After a detailed clinical examination and pain questionnaires patients underwent a standard nerve conduction study (NCS) to assess Aβ-fibre function, and LEPs to assess Aδ-fibre function. Forthy-four out of 114 patients with painful neuropathy suffered from allodynia. While NCS data did not differ between patients with and without allodynia, LEP amplitude was higher in patients with allodynia than in those without. Our data argue against a role of Aβ-fibres and central sensitization as the main mechanism for the development of allodynia in distal symmetric polyneuropathy. The partially preserved LEPs in patients with allodynia suggests that this type of pain might be related to the abnormal reduction of mechanical threshold of nociceptive terminals (peripheral sensitization).

In the **eighth** chapter I treated neuropathic pain in patient with crioglobulinemia. The study aimed at gaining information on peripheral neuropathy and neuropathic pain in patients with cryoglobulinaemia. We collected 48 consecutive patients with cryoglobulinaemia. All patients underwent a standard NCS to assess Aβ-fibre function, LEPs to assess Aδ-fibre function, and skin biopsy to assess C-fibre terminals. We used DN4 questionnaire to diagnose neuropathic pain, and the Neuropathic Pain Symptom Inventory to rate the intensity of the different qualities of neuropathic pain. Thirty patients had a peripheral neuropathy. Twenty-three had neuropathic pain as assessed by the DN4 questionnaire. NPSI questionnaire showed that the most frequent type of pain was the burning pain. Patients with peripheral neuropathy had an older age than those without . The duration of the disease correlated with the density of epidermal innervation as assessed by skin biopsy. The severity of the ongoing burning pain correlated with the amplitude of LEPs, but not with the density of epidermal innervation . Our findings showed that an older age is associated with the

development of peripheral neuropathy, and a longer duration of disease with a more severe peripheral nerve damage, as assessed by skin biopsy. The correlation between the intensity of ongoing pain and LEP attenuation indicate that neuropathic pain reflects damage to nociceptive axons.

In the **ninth** chapter I discussed the research on a peptide, the kiss-peptine, whose antagonist could be a new analgesic drug. More studies should be perform in the next future about it . Kisspeptin is a neuropeptide known for its role in the hypothalamic regulation of the reproductive axis. Following the recent description of kisspeptin and its 7-TM receptor, GPR54, in the dorsal root ganglia and dorsal horns of the spinal cord, we examined the role of kisspeptin in the regulation of pain sensitivity in mice. Immunofluorescent staining in the mouse skin showed the presence of GPR54 receptors in PGP9.5-positive sensory fibers. Intraplantar injection of kisspeptin (1 or 3 nmol/5 µl) induced a small nocifensive response in naive mice, and lowered thermal pain threshold in the hot plate test. Both

intraplantar and intrathecal (0.5 or 1 nmol/3 µl) injection of kisspeptin caused hyperalgesia in the first and second phases of the formalin test, whereas the GPR54 antagonist, p234 (0.1 or 1 nmol), caused a robust analgesia. Intraplantar injection of kisspeptin combined with formalin enhanced TRPV1 phosphorylation at Ser800 at the injection site, and increased ERK1/2 phosphorylation in the ipsilateral dorsal horn as compared to naive mice and mice treated with formalin alone. These data demonstrate for the first time that kisspeptin regulates pain sensitivity in rodents and suggest that peripheral GPR54 receptors could be targeted by novel drugs in the treatment of inflammatory pain.

In the **tenth** last chapter I gathered all the conclusion of the single studies. Here I tried to associate each quality of pain to an underling pathophysiological alteration, since the aim of y studies was to show the correlation between clinical manifestations of neuropathic pain and the underlying alteration of the different groups of fibers (A-β, A-δ or C).

Chapter 2: Neuropathic pain and methods to examine the somatosensory system

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1. Introduction

Injury to the nervous system causes loss of sensation in the territory innervated by the damaged nervous structure (nerve root, nerve fascicle, peripheral nerve, spinal segment, cortical structure, etc.). In a limited number of patients, such damage is followed by long-lasting, occasionally persistent, pain termed neuropathic pain (NP) in the damaged innervations territory. NP conditions consist of a series of different diseases and conditions ranging from nerve injury due to cancer over neuropathies following diabetes to diseases and lesions of the central nervous system (CNS). In addition to al long list of different aetiologies causing neropathic, these pains also differ in anatomical location and can localized anywhere from the peripheral nociceptor to the highest centres in the

brain. According to the International NP is defined as "Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" [1]. According to this, NP is now proposed to be defined the consequences of injury to the nervous system include a series of neurobiological events resulting in sensitization of those parts of the nervous system that have been deprived of their normal patterned afferent input. While primarily described for diseases and lesions affecting the peripheral nervous system, NP may also be a feature of a certain central disorders. Although probably more complex in nature, central share some of the same phenomena seen in peripheral NP, i.e. sensory loss in part of the territory with pain, and the paradox presence of lost sensibility and hypersensitivity to one or several sensory submodalities [2]. It is clear that NP is not a single disease but represents a syndrome, i.e. a constellation of specific symptoms and signs with multiple potential underlying aetiologies. Hence, an accurate neurological history and neurological examination, including sensory testing, is most important to reach a diagnosis and to postulate the presence of a NP

syndrome. The elucidation of underlying disease aetiology and the dissection of pain will in practice often occur simultaneously. The following is a brief description of steps in assessing a NP syndrome. The history will indicate whether the character and distribution of the pain is in accord with neuropathic criteria, and whether a relevant lesion or disease in the nervous system is probably responsible for the pain. The clinical examination will determine the presence of negative (loss of function) and positive (hyperalgesia and/or allodynia) sensory signs, for one or more sensory modalities affecting the somatosensory system, and their relevance to the underlying disease or lesion. Further diagnostic tests can be conducted to either document the presence of a specific underlying neurological or confirm a sensory lesion within the pain distribution.

1.2 Clinical symptoms and pathophysiological mechanisms

The symptoms and signs in NP can be divided into negative and positive phenomena. The negative symptoms and signs reflect the damage to the CNS resulting in partial

or complete sensory loss and numbness in the distribution of the nervous structure that has been damaged. The positive phenomena such as allodynia, hyperalgesia, and hyperpathia are all manifestations of hyperexcitability in the nervous system. Clinically, central NP is characterized by the presence of spontaneous ongoing pain and various types of evoked pains often occurring in different combinations. The examination of a patient complaining of pain aims at clarifying the underlying disease and understanding whether the pain is nociceptive, neuropathic, psychogenic, or a combination of the three types. Before suspecting neuropathic pain, the physician must exclude nociceptive pain . The diagnostic procedure is based on a meticulous medical history and systematic clinical examination. Laboratory tests and radiological examinations may be indicated to confirm or exclude the suspected disease. The patient's history should be searched to identify a possible association of the onset of pain with current diseases, trauma and surgery. Patients in whom NP is suspected must first undergo sensory examination. Tactile sense is best assessed with a piece of

cotton wool, pinprick sense with a wooden cocktail-stick, thermal sense with warm and cold objects (e.g. metal thermorollers), and vibration sense with a 128-Hz tuning fork. The sensory examination should search for negative (i.e., sensory loss) and positive (i.e., hyperalgesia and allodynia) sensory findings. The distributions of these sensory abnormalities should be neuroanatomically logical, namely, compatible with a definite lesion site. Despite being the basis of the assessment, clinical examination is not always sensitive enough to detect an underlying disease. Hence clinical, neurophysiological and radiological evaluations are complementary.

Ongoing pain. These pains are spontaneous and may be continuous or paroxysmal. The character of these pains differs, but it can be shooting, shock-like, aching, cramping, crushing, smarting, etc. Episodic, paroxysmal types of pain are second-lasting shooting, electric, shock-like, or stabbing in their character.

Evoked pains. The stimulus-evoked pains are classified according to the type of stimulus that provokes them, such as mechanical, thermal, or chemical stimuli.

In some patients, all these symptoms may be present; in others only one type of hypersensitivity is present. So a series of stimuli need to be applied to document or exclude abnormality. Evoked pains are usually brief, lasting only for the duration of the stimulation, but sometimes they can persist even after cessation of the stimulation causing after-sensations, which can last for minutes, hours, and even days. In such cases, the distinction between evoked and spontaneous types of pain can be difficult. Patients suffering from NP often complain of sensory deficits and different types of pain combined in various ways, such as electrical shock like sensations, or provoked by various stimuli, e.g. gentle brushing or cold water. The complex sensory profile of NP reflects the various pathophysiological mechanisms underlying. Although in some etiologic categories of NP specific types of pain may predominate, none of them are etiologic specific. Hence patients suffering from an identical disease may present

with heterogeneous sensory signs and symptoms. The diagnostic workup should therefore aim to detect specific sensory profiles through clinical examination, questionnaires dedicated to NP, and laboratory tools. Defining precise sensory profiles is crucial to successful NP management because they probably arise through different underlying mechanisms and thus probably respond differently to treatment [3,4]. Clinical examination and pain questionnaires dedicated NP can reliably distinguish precise sensory profiles in patients with NP whatever the cause. Current research findings indicate that whereas provoked pains probably arise through multiple mechanisms., the mechanisms responsible for spontaneous pains show no etiologic specific differences. Conversely, recent neurophysiological studies suggest that spontaneous paroxysmal pain reflects demyelination of non nociceptive, large myelinated fibers [5,6]. That specific sensory profiles can be distinguished across different neuropathic pain conditions, might be the starting point for a mechanism based classification of NP.

1 The functional and morphological assessment of the somatosensory system

1.1 Clinical examination and screening tools

History and clinical examination are a requirement to confirm the presence of a NP syndrome, and also an important step in reaching an aetiological diagnosis for NP. Several tools essentially based on pain descriptors have been proposed for the purpose of distinguishing NP from non-NP or characterizing multiple neuropathic phenotypes. The Douleur Neuropathique en 4 questions (DN4) contains seven items related to symptoms and three related to clinical examination [7]. A total score =4 out of 10 suggests NP. The DN4 showed 83% sensitivity and 90% specificity when compared to clinical diagnosis in the development study. The seven sensory descriptors can be used as a self-report questionnaire with similar results. The tool was developed and validated in French and translated into 15 languages. The DN4 has been used in epidemiological studies in general population and diabetics. The Neuropathic Pain Symptom Inventory (NPSI), the pain quality assessment tool devoted to NP

assessment, was originally validated in French and has been submitted to linguistic validation in 50 other languages. One study found that several NP dimensions of the NPSI were particularly sensitive to treatment effect. The structure of the NPSI makes it factorial suitable to capture different aspects of NP with presumably distinct mechanisms. The main advantage of screening tools is to identify potential patients with NP, particularly by non-specialists. However, these tools fail to identify 10–20% of patients with clinician diagnosed NP, showing that they cannot replace careful clinical judgment. Pain quality assessment measures are useful to discriminate amongst various pain mechanisms associated with distinct dimensions of NP experience . The NPSI is recommended to evaluate treatment effects on neuropathic symptoms or their combination , but should also be used in future trials to try to predict treatment outcome and better define responder profiles.

Quantitative sensory testing is a psychophysiological measure of perception in response to external stimuli of controlled intensity. Detection and pain thresholds are

determined by applying stimuli to the skin in an ascending and descending order of magnitude. Mechanical sensitivity for tactile stimuli is measured using von Frey hairs or Semmes-Weinstein monofilaments, pinprick sensation with weighted needles and vibration sensitivity with a tuning fork or an electronic vibrameter; thermal perception and thermal pain are measured using a probe that operates on the Peltier principle . Most QST studies are still dedicated to the assessment of sensory small fibre function only, assuming that large fibre function was probably documented by standard clinical neurophysiology. This bias precludes any analysis on the relative importance of small vs. large sensory fibre function deficits in NP syndromes.QST is used for diagnosis and follow-up of small fibre neuropathy [8,9], and its usefulness is agreed in the early diagnosis of diabetic neuropathy. Quantitative sensory testing is particularly appropriate to quantify positive sensory phenomena, like mechanical and thermal allodynia and hyperalgesia, which may help characterize painful neuropathic syndromes, and predict or monitor treatment effects. In particular, pharmacological and non-

pharmacological treatment trials using QST found effects on dynamic mechanical allodynia, pinprick hyperalgesia and sensory loss, whereas treatment efficacy was predicted by thermal detection thresholds, vibration detection thresholds, heat hyperalgesia and dynamic mechanical allodynia [10,11]. Quantitative sensory testing can be used in the clinic along with bedside testing to document the sensory profile. Because abnormalities have often been reported in non-NPs as well, QST cannot be considered sufficient to separate differential diagnoses . QST is helpful to quantify the effects of treatments on allodynia and hyperalgesia and may reveal a differential efficacy of treatments on different pain components. Neurological examination in suspected NP should include assessment o motor, sensory, and autonomic phenomena in order to identify all signs of neurological dysfunction. Tactile sense is best assessed with a piece of cotton wool, pinprick sense with wooden cocktail stick, thermal sense with warm and cold objects (e.g., metal thermorollers) and vibration sense with a 128-Hz tuning fork (Table 1). To evaluate mechanical allodynia- hyperalgesia, we recommend the

use of simple tools such as a brush and at least one high-intensity weighted pinprick or von Frey filament (e.g. 128 mN). The evaluation of pain in response to thermal stimuli is best performed using the computerized thermotest, but we do not recommend the systematic measure of thermal stimuli except for pathophysiological research or treatment trials. A simple and sensitive tool to quantify pain induced by thermal stimuli in clinical practice is still lacking.

Fibres	Sensation	Testing		
		Clinical	QST	Laboratory[c]
Aβ	Touch	Piece of cotton wool	Von Frey filaments	NCS, SEPs
	Vibration	Tuning fork (128 Hz)	Vibrameter[a]	NCS, SEPs
Aδ	Pinprick, sharp pain	Wooden cocktail stick	Weighted needles	LEPs, IENF
	Cold	Thermoroller	Thermode[b]	None
C	Warmth	Thermoroller	Thermode[b]	LEPs, IENF
	Burning	None	Thermode[b]	LEPs, IENF

[a]Or other device providing graded vibratory stimuli.
[b]Or other device providing graded thermal stimuli.
[c]See Glossary.
Modified from [2].
doi:10.1371/journal.pmed.1000045.t001

Table 1. Summary of choice methods of assessing nerve. function per sensation

Neurophysiological tests: elettroneurography, somatosensory-evoked potentials and laser-evoked potentials

Nerve conduction studies (NCS) allows to investigate Aβ-fibres. Patient with suspected distal symmetric neuropathy usually underwent motor and sensory NCS using surface recording electrodes with standard placement. Amplitudes nerve action potentials of ulnar , median and sural sensory and conduction velocities are examined. Compound motor action potential amplitude and conduction velocity of peroneal, tibial, ulnar and median nerves are usually also examined. When appropriate , F-wave examination f the same nerves is carried out by delivering 20 random stimuli . Somatosensosory evoked potential (SEP) is obtained through the direct activation of a peripheral nerve fibre or its receptor, the relayed peripherally and centrally up to the primary somatosensory cortex. Most frequently, to elicit SEPs electrical stimuli are applied transcutaneously over a sensory or mixed sensory/motor nerve (e.g. *nervus*

medianus for upper limbs and *nervus tibialis* posterior for lower limbs). Electrical stimuli activate fast-conducting myelinated large nerve fibers (Aβ) with low electrical activation thresholds. The scalp activity related to the electrical stimulation of peripheral nerve fibers can be separated into short-, middle-, and late-latency brain responses. Pathological SEPs indicate the presence or damage along large size non-nociceptive afferents or the sensor nervous system. This technique is useful to determine the origin o a nerve lesion or to complete the exploration on the nervous system, but not to assess nociceptive pathway's function [12]. Current neurophysiological assessment of the trigeminal system comprises recordings of trigeminal reflex responses. The blink reflex assesses the ophthalmic division and consists of an early response, ipsilateral to the side of the stimulation (R1) and a late, bilateral, response (R2); the reflex responses are recorded after mechanical or electrical stimulation of the supraorbital region. The masseter inhibitory reflex studies the second an third trigeminal division; the early, SP1, and late , SP2, components are

elicited after mechanical or electrical stimulation of the maxillary or mandibular division. Trigeminal reflexes assess the function of large myelinated A-β afferents from all the trigeminal territories, aswell as their trigeminal central circuits in the midbrain, pons and medulla.

Large -size, non-nociceptive afferents (i.e., those that do not carry pain) have a lower electrical threshold than small-size, nociceptive afferents. Unless special techniques are used, i.e., experimental blocks or stimulation of special organs (cornea, tooth pulp, glans), electrical stimuli unavoidably also excite large afferents, thus hindering nociceptive signals. Hence standard neurophysiological responses to electrical stimuli, such as NCS and SEPs, can identify, locate, and quantify damage along the peripheral or central sensory pathways, but they do not assess nociceptive pathway function. For many years researchers have tried numerous techniques for selectively activating pain afferents. The currently preferred approach uses laser stimulators to deliver radiant-heat pulses that selectively excite the free nerve endings (Aδ and C) in the superficial skin layers. Consensus from over 200 studies now confirms

that late laser-evoked potentials (A-δ LEPs) are nociceptive responses. Late LEPs are the easiest and most reliable neurophysiological tools for assessing nociceptive pathway function and are diagnostically useful in peripheral and central neuropathic pain [13]. In clinical practice, their main limitation is that they are currently available in too few centres . Ultralate LEPs (related to C-fibre activation) are technically more difficult to record, and few studies have assessed their usefulness in patients with neuropathic pain. They are usually recorded after laser stimuli (biggers diameter and longer duration than Aδ stimuli setting) applied in trigeminal regions, where the density of full C fiber is higher and the distancy from the central nervous system is lower than any other region in the body . The radiant-heat pulse stimuli delivered by laser stimulators are absorpted by free nerve endings for Aδ- and C-fibers located within superficial skin layer. Aδ- and C-fibers possess different thermal activation thresholds. Thus , in function of the quantity of thermal energy that is delivered to the skin, it is possible to record brain responses that are either linked to Aδ nociceptor activation (late LEPs) or to

C-fiber nociceptors activation (ultralate LEPs). In scalp recordings, the early lateralized potential culminating at 150–180 ms over the temporal regions and inverting phase at the midline (N1/P1) would be dominated by opercular (and perhaps SI) activity, while the large vertex negative-positive response appears as the resultant of late insular and anterior cingulate activity [15]. Pain conditions that may benefit from an assessment with LEPs are neuropathies, radiculopathy, syringomyelia, multiple sclerosis and cerebral infarctions including Wallenberg's syndrome. Apart from allowing to discern Aβ from Aδ and or C fiber lesions, LEP can explore skin territories that are outside conventional sensory nerve territories (examined with NCS or SEPs) such as the face (trigeminal neuralgia) or the thoracic region (post-herpetic neuralgia); each dermatome that is not too hairy is accessible with the laser stimulus.

1.2 Skin biopsy and intraepidermal fibers quantification

Punch skin biopsy can quantify unmyelinated nerve fibres by measuring the density of intra-epidermal nerve fibres

(IENF). IENF loss has been shown in various neuropathies characterized by small-fibre axonal loss. Punch skin biopsy is easy to do, minimally invasive, and optimal for follow-up. Despite these advantages, it is useless in central pain and demyelinating neuropathy, and is currently available only in few research centres [15].

Skin biopsy is most commonly performed using a 3-mm disposable punch under sterile technique, after topical anesthesia with lidocaine. No suture is required (Fig. 1). A shallow biopsy (3-4 mm) is adequate to study epidermal nerve fibers, whereas a deeper biopsy (6-8 mm) is required to include sweat glands, hair follicles, and artero-venous anastomosis.

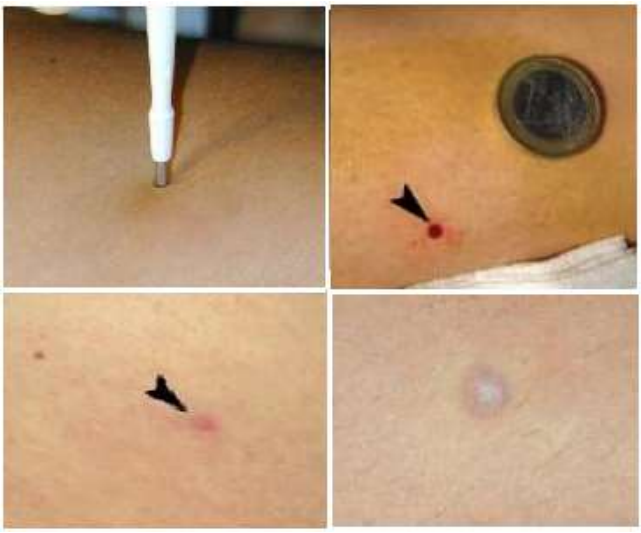


Fig. 1 Skin biopsy is most commonly performed using a 3-mm disposable punch under sterile technique, after topical anesthesia with lidocaine. No suture is required.

To optimize the sampling of such structures and myelinated fibers in hairy skin, particular attention should be paid to include a hair in the specimen [16] . The current technique was developed at the Karolinska Institute [17], and later standardizewd at the University of Minnesota [18] and at the Johns Hopkins University [19]. A less invasive sampling method is the removal of the epidermis alone by applying a suction capsule to the skin. With this

method, there is no bleeding, and local anesthesia is not needed. However, the method does not provide information on dermal and sweat gland nerve fibers. Moreover, thus far it has not been systematically used to investigate patients with small fiber neuropathy. This technique was developed at the University of Minnesota [20]. In most studies, hairy skin biopsies were obtained from the distal part of the leg (10 cm above the lateral malleolus), in some from the calf and the paraspinal region, and in many of them also from the upper lateral aspect of the thigh (20 cm below the anterior iliac spine) or other proximal locations where chosen to detect the length-dependent loss of nerve fibers, which is typical of axonal polyneuropathy. These sites may also be sampled in the case of a non-length-dependent ganglionopathy. When skin biopsy is taken from other body sites for evaluation of a unilateral process, a control biopsy from similar non-affected region should be taken.

After skin biopsy is performed, the specimen is immediately fixed in cold fixative for approximately 24 h at 4°C, then kept in a cryoprotective solution for one night,

and serially cut with freezing microtome or a crystat. The first and last few sections should not be used for nerve examination because of possible artefacts. Most studies for bright-field microscopy used 2% paraformaldehyde-lysine periodate (2% PLP), whereas most studies for indirect immunofluorescence with or without confocal microscopy used Zamboni's (2% paraformaldehde, picric acid) fixative. Either bright-fild immunohistochemistry ot immunofluorescence with or without confocal microscopy has been used, but the technique does not affect the reliability of skin biopsy in assessing intraepidermal nerve fiber (IENF) loss in neuropathy. However, no studies has been designed yet to compare the two techniques. Quantification of IENF density using bright-field immunohistochemistry was mostly based on the assessment of the number of fibers per linear measurement. Significant correlation with a stereologic technique supported the reliability of linear IENF density [21]. IENF are counted either under the light microscope at high magnification (i.e 40X objective) or using software for image analysis. The length of the epidermal surface is

measured using software for biological measures. The density is calculated in at least three sections as the number of IENF per length of the section (IENF/mm)(Fig.2). Other studies reported the IENF density per skin surface area [22]. Quantification of IENF density using confocal immunofluorescence technique is usually performed on images based on the stack of consecutive 2 μm optical sections (usually 16 sections) for a standard linear length of epidermis. The thickness of skin sections varies from 32 to 60 μm. Four epidermal areas are selected for confocal images acquisition, two images on each of two different sections excluding areas containing hair follicles and sweat ducts. For quantitative analysis, IENF are counted at high magnification (i.e 40X objective) (Figure 3) for light microscope or (20X) for epifluorescence microscope (Figure 4 and 5) or using a software for image analysis (e.g. Neurolucida, Microbrightfield) on digitized confocal images. In both bright-field and immunofluorescence methods, single IENF crossing the dermal-epidermal junction are counted, whereas secondary branching is excluded from quantification. No study provided

information or the rules for cunting IENF fragments, which have been comprehensively reviewed by Kennedy et al [23]. Intra- and interobserver variability, and interlaboratory agreement on IENF counts has been assessed [24].

The skin blister is an alternative technique to assess the epidermal innervations density. IENF density in blister roofs from foot and calf correlated with IENF density in skin biopsied from adjacent areas in 25 healthy subjects showing no systematic differences between skin biopsies and blisters (P=0.29) or between pairs of blisters from the same location (P=0.15) [22]. No side effects have been reported in published studies, but no study focused on safety was performed. The density of IENF at the distal leg ranged from 13.8 ± 6.7/mm (mean ± SD) to 9.8 ± 3.6/mm (mean ± SD).

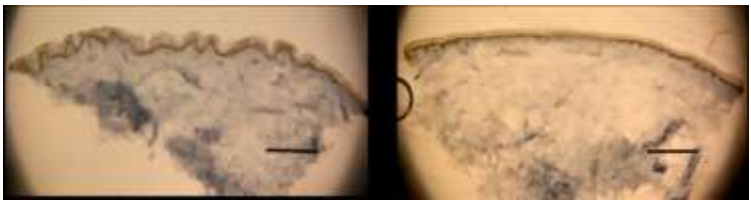


Fig. 2 Light microscope immunostainting (x5) skin biopsy 50 µm vertical sections from the proximal (on the left) and distal (on the right) areas of the leg, The derma- epidermal junction is evident: dark brown line, undulating in the thigh, more linear in the ankle. Only nerve fibers crossing the junction are counted for the IENF density. Bar =60µm.

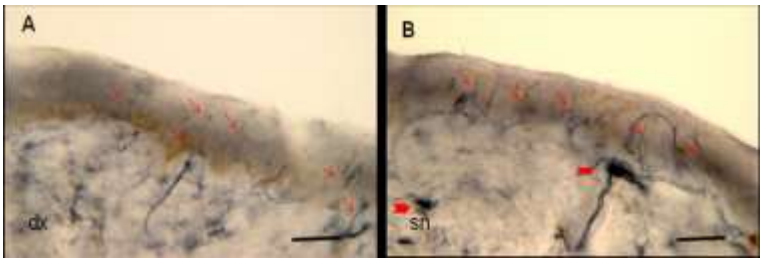
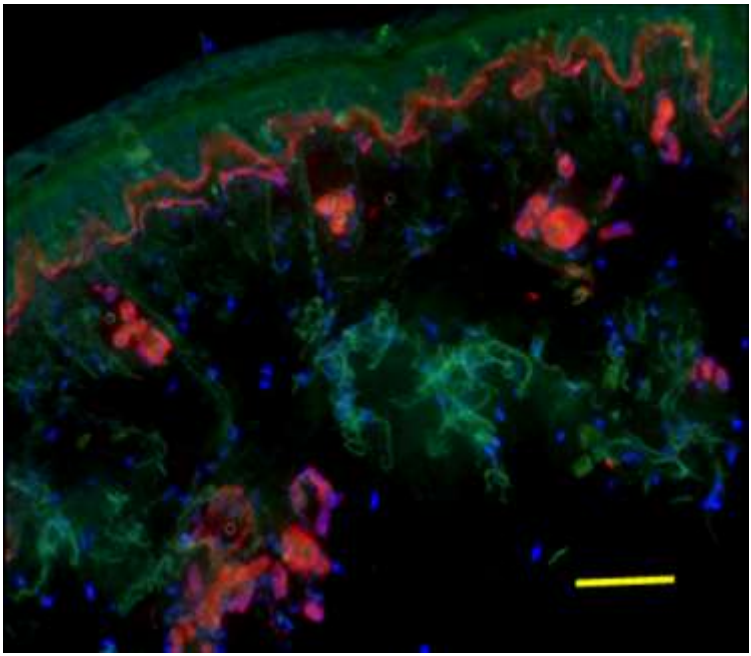
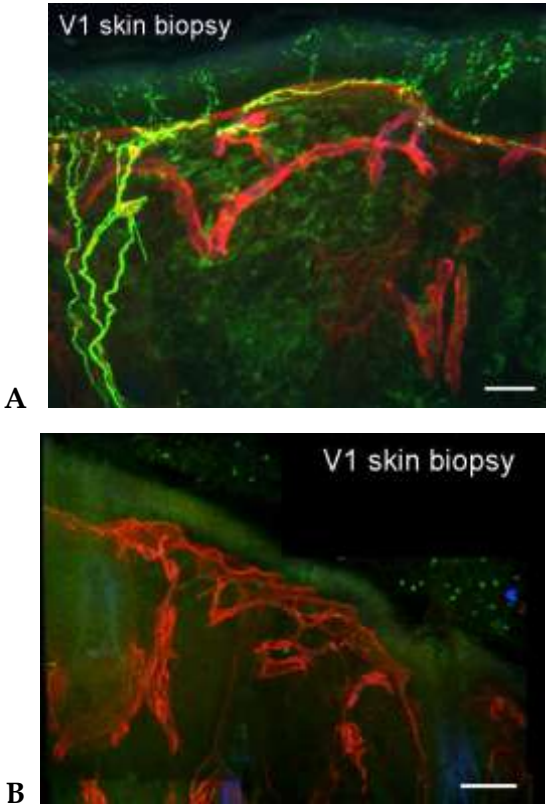


Fig. 3 Light microscope immunostainting (X40) skin biopsy tissue from the right (**A**) and left (**B**) side of the second trigeminal division in a patient with emifacial atrophy. Epidermal nerve fiber density measurements illustrated in 50 µm vertical sections, immunostained with the panaxonal marker anti-protein gene product 9.5 to demonstrate normal, fine, vertically arrayed unmyelinated nerve fibers within epidermis (arrows). And dermal nerve bundles (arrowheads) Bar =60µm.



Fg. 4 Epifluorescence microscope (x20) (using a software for image analysis- Neurolucida, Microbrightfield- on digitized confocal images) skin biopsy tissue from tigh of a voluntary. In the 20 µm vertical sections, immunostained with the panaxonal marker anti-protein gene product 9.5 to demonstrate normal, fine, green vertically unmyelinated nerve fibers within derma and epidermis.Vessels and basement membrane are in red (immunostained with the markers anti-collagen IV), nuclei are in blu. Yellow bar =80µm.



Fg. 5 Epifluorescence microscope (x40) (using a software for image analysis- Neurolucida, Microbrightfield- on digitized confocal images) skin biopsy tissue from the first trigeminal division in a normal subject (A) and in a patient with postherpetic neuroalgia: it is evident the severe loss of nerve fibers (green coloured after immunostained with PG 9.5) in figure B. In the 20 µm vertical sections, immunostained also with anti-collagene IV to demonstrate the epidermal basic membrane (red coloured) where the nerve fibres get fre of myelin; this marker also coloured basic membrane of lood vessels in the derma. White bar =60µm.

The largest normative study [25] included 188 healthy subjectsfrom three different laboratories (Maastricht, Ferrara, Milan) and stratified the study population per age and gender, providing normative values *per* decade.

The authors reported that IENF density at the distal leg is lower in men than in women, that weight and height do not have any significant impact, and that values decline with age (Table 2). Norrnative reference values are available for bright-field imunohistochemistry but not yet for confocal immnofluorescence or blister technique. The most common side effect was a mild infection because of improver wound management recovering with topical antibiotic therapy. The only other complication reported was excessive bleeding which did not need suture.

Age (years)	Females (n = 97)		Males (n = 91)	
	0.05 Quantile values per age span	Median values per age span	0.05 Quantile values per age span	Median values per age span
20–29	6.7	11.2	5.4	9.0
30–39	6.1	10.7	4.7	8.4
40–49	5.2	9.9	4.0	7.8
50–59	4.1	8.7	3.2	7.1
60–69	3.3	7.9	2.4	6.3
≥70	2.7	7.2	2.0	5.9

Table 2. Intraepidermal nerve fiber (IENF) density normative values for clinical use (reproduced from Bakkers et al., Neurology)

2.4 Functional neuroimaging

Functional neuroimaging Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) measure with different methods cerebral blood flow (rCBF) or metabolic activity in defined brain regions. Activation studies investigate local synaptic changes specifically associated with a given task or a particular stimulus by comparing statistically activated and control conditions. Functional neuroimaging has disclosed a network of brain regions jointly activated by noxious stimuli (labeled -pain matrix). Activation of the lateral thalamus, SI-SII and posterior insula are thought to be related to the sensory-discriminative aspects of pain processing, whilst mid-anterior cingulate, posterior parietal and prefrontal cortices participate in the affective and attentional concomitants of pain sensation [26]. In unilateral spontaneous neuropathic pain, moderate but converging evidence from independent groups indicates decreased resting rCBF in contralateral thalamus, and reversal of this abnormality by analgesic procedures (but only case reports or small series with <20 patients [27]). Should this be confirmed in larger series,

thalamic hypoperfusion might be used in the future as a marker of NP and restoration of thalamic blood flow for treatment monitoring. In patients with provoked neuropathic pain, allodynia and hyperalgesia have been associated with amplification of the thalamic, insular, SI, SII and prefrontal–orbitofrontal responses, but not anterior–perigenual cingulate [28]. Neuropathic allodynia has been shown to enhance insular activity ipsilateral to pain [29] suggesting that a shift in hemispheric balance might contribute to the allodynic experience. Again, the total number of reported patients (n = 80) is still too small to support any diagnostic application; however, neuropathic allodynia has shown a different activation pattern than nonneuropathic allodynia which may open diagnostic perspectives.

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Chapter 3: Pathophysiology of pain in postherpetic neuralgia: A clinical and neurophysiological study

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Chapter based on: Pathophysiology of pain in postherpetic neuralgia: A clinical and neurophysiological study . A. Truini, F. Galeotti, M. Häänpää, R. Zucchi, A. Albanesi, A. Biasiotta,A. Gatti, G. Cruccu. Pain 2008.140: 405–410

1. Introduction

Postherpetic neuralgia (PHN) is an exceptionally drug-resistant neuropathic pain that persists after a herpes zoster rash has healed [31]. In PHN, the Abnormal sensory function may manifest as hypoesthesia, involving all sensory modalities, and pain. Most patients with PHN describe three types of pain: a constant deep, aching or burning pain, a paroxysmal, lancinating pain, and allodynia (i.e. pain provoked by normally non painful stimulus). Two-thirds of the patients report mechanical allodynia, and some patients have cold allodynia. Some patients also complain of itching that may be even more annoying than the pain itself [22,23]. PHN related pain

results from changes in peripheral and central nervous system somatosensory processing [9]. Although PHN most commonly involves the thoracic dermatomes, in 23% of patients it affects the ophthalmic division of the trigeminal nerve [32]. Current neurophysiological assessment of the trigeminal system comprises recordings of trigeminal reflex responses [6] and laser evoked potentials [11]. The blink reflex assesses the ophthalmic division and consists of an early response, ipsilateral to the side of the stimulation (R1) and a late, bilateral, response (R2), both mediated by large myelinated, A-β fibers [3]. Because the blink reflex is mediated by non-nociceptive A-β fibers, it provides no information on trigeminal nociceptive pathway function [3]. The best tool for assessing trigeminal nociceptive pathway function is laser stimulation [27]. Laser generated radiant heat pulses selectively excite free nerve endings in the superficial skin layers, activate myelinated A-δ and unmyelinated C-fibers [28], and evoke scalp potentials generated by the opercular-insular cortex and cingulate gyrus [12]. Although skin biopsy studies have shown a severe loss of epidermal-free nerve endings

in the affected dermatomes, such studies used exclusively a pan-neuronal marker (PGP 9.5), which does not allow differentiating the nerve endings of myelinated (Aδ) from those of unmyelinated (C) neurons [21]. Previous neurophysiological studies investigated the A-β fiber-mediated blink reflex [20] and A-δ LEPs [29] in patients with PHN. No studies have systematically assessed neurophysiological responses related to non-nociceptive and nociceptive fibers in patients with PHN, or tried to correlate neurophysiological abnormalities reflecting specific fiber damage with PHN pain; this information might be useful for a better understanding of pain mechanisms. To seek information on trigeminal nerve function and pain mechanism in ophthalmic PHN, we assessed myelinated and unmyelinated fiber function by recording the blink reflex to measure A-beta fiber function, and LEPs to measure A-delta and C-fiber function, in patients with ophthalmic PHN. We then determined the diagnostic accuracy of the neurophysiological testing and the correlation of neurophysiological data, clinical sensory deficits, and pain.

2. Methods

2.1. Patients

We did a prospective, cohort study, recruiting consecutive patients with ophthalmic PHN from January 2006 to March 2008. The reference standard for the diagnosis of trigeminal PHN was the IHS diagnostic criteria: pain in the distribution of the ophthalmic division of the trigeminal nerve, herpetic eruption in the same territory, pain that precedes herpetic eruption by less than 7 days, and pain that persists after 3 months [16]. Exclusion criteria were neurological or dermatological disease other than PHN, cognitive impairment, diabetes, and herpes zoster-related corneal damage. Forty-one patients with ophthalmic PHN aged 50–88 years (mean 72.7; 19 F, 22 M) fulfilled inclusion criteria. Patients had a disease duration of 3–30 months (median: 5 months). All patients were receiving drugs for neuropathic pain. All patients gave their informed consent to undergo the procedure and the research was approved by the local Ethical Committee.

2.2. Clinical examination

All patients underwent a general and neurological examination. Sensory disturbances were carefully assessed. Patients were examined for negative (tactile, pinprick, and thermal hypoesthesia) and positive symptoms (constant pain, paroxysmal pain, itching, mechanical and cold allodynia, and pinprick hyperalgesia). Patients were instructed to rate positive and negative sensory disturbances on an 11-point numerical rating scale ranging from 0 (no disturbance) to 10 (worst possible disturbance). The presence and the severity of negative symptoms were assessed by comparing the affected side with the mirror image of the normal side. Although we used a preformatted questionnaire all the questions and the clinical tests were always performed randomly. To avoid missing data we checked at the end of the whole examination if all items were fulfilled.

2.3. Neurophysiological examination

All patients underwent blink reflex recordings. Evaluation methods adhered to those indicated by the International

Federation of Clinical Neurophysiology (IFCN) [7]. The blink reflex was evoked by electrical stimulation (0.1 ms, 25–45 mA) of the supraorbital nerve through surface electrodes. EMG signals were recorded from the orbicularis oculi through surface electrodes. We measured the latency of R1 of each side. To study LEPs we used a previously reported technique [5]. In brief, we used a neodymium:yttrium-aluminium- perovskite laser (Nd:YAP) (wavelength 1.34 mm, pulse duration 2–20 ms, maximum energy 7 J) with fiber-optic guidance. Laser pulses of relatively high intensity (119–178 mJ/mm2), short duration (5 ms), and small diameter (5 mm), elicited pinprick sensations related to Aδ fiber input. Laser pulses of lower intensity (38–76 mJ/mm2), relatively long duration (10 ms) and large diameter (10 mm), elicited purely warmth sensations related to C-fiber input. Laser pulses were directed to the supraorbital skin. The laser beam was shifted slightly after each stimulus. The interstimulus interval was varied pseudorandomly (10–15 s). Subjects lay on a couch and wore protective goggles. They were instructed to keep their eyes open and gaze

slightly downwards. To determine the laser perceptive threshold we delivered a series of stimuli at increasing and decreasing intensity, and defined the perceptive threshold as the lowest intensity at which the subjects perceived at least 50% of the stimuli. The main LEP complex, N2–P2, was recorded through disk electrodes from the vertex (Cz) referenced to the nose. From 10 to 20 trials devoid of artifacts were collected and averaged off line. We measured peak latency and amplitude (peak-to-peak) of the main N2–P2 vertex complex. We do not report data About the earlier, negative (N1) component, because in our laboratory, in patients, it is less reproducible than the N2–P2 vertex complex, and thus it is not routinely recorded for clinical purposes. For both blink reflex and LEPs to distinguish Abnormal from normal data we used the unaffected side as a control. The blink reflex responses were considered Abnormal when the R1 latency exceeded 1.2 ms the latency of the normal side or was Absent [7]. LEPs were considered Abnormal when Absent [29]. All neurophysiological recordings were performed by technical staff and stored on disk or printed. Two authors,

blind to the side of disease, measured the responses and assessed abnormalities.

3. Statistics

We used the Kolmogorov–Smirnov test to assess the normal distribution. Paired t-test was used to analyze the between-side differences of normally distributed data such as the latency of the R1 blink reflex, A-δ LEP, and C-LEP, and the laser perceptive thresholds. The Wilcoxon matched-pair test was used for amplitude of Ad and C-LEPs, which did not show a normal distribution. The diagnostic accuracy of neurophysiological testing was evaluated with Fisher's exact test, with the calculation of sensitivity and specificity and their 95% confidence intervals (CI). We studied the correlations between the side-to-side difference of neurophysiological responses and sensory disturbances (tactile, pinprick, and thermal hypoesthesia, itching, constant and paroxysmal pain, and mechanical allodynia) with the nonparametric Spearman's R correlation coefficient. $P < 0.01$ was considered significant. All results are reported as means ± SD.

4. Results

4.1. Clinical findings

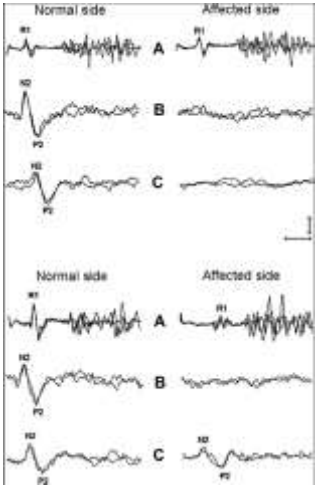
Most patients reported sensory deficits involving all sensory modalities simultaneously. Of the 41 patients studied, 29 reported tactile hypoesthesia (mean rating 4.4 ± 1.6), 30 pinprick hypoesthesia (mean rating 4.6 ± 1.7), and 24 thermal hypoesthesia (mean rating 4.8 ± 1.7). Among sensory symptoms, 24 patients reported paresthesias (mean rating 5.6 ± 1.5), and 26 itching (mean rating 5.8 ± 2.4). Whereas most patients (29 of 41 patients) complained of constant pain, 18 patients had mechanical dynamic allodynia, 16 patients paroxysmal pain, 9 patients hyperalgesia and 8 patients cold allodynia (mean ratings, for constant pain 5.4 ± 1.8, mechanical dynamic allodynia 5.5 ± 1.6, paroxysmal pain 6.2 ± 1.9, hyperalgesia 5.2 ± 1.2, and cold allodynia 4.1 ± 1.2). 4.2. Neurophysiological findings Of the 41 patients who underwent blink reflex testing, R1 blink reflex was Absent in 17 and delayed in 16 patients. In the 24 patients with normal or delayed responses, the latency of the R1 blink reflex was far longer after stimulation of the affected side than after stimulation

of the normal side ($P < 0.001$, paired t-test) (Fig. 1). The laser perceptive threshold related to A-δ fibers was significantly higher after stimulation of the affected side than after stimulation of the normal side ($P < 0.001$). The mean A-δ LEP latency after stimulation of the affected side was not significantly delayed ($P > 0.20$). The A-δ LEP amplitude was lower after stimulation of the affected side than after stimulation of the normal side ($P < 0.001$, Wilcoxon test) (Fig. 1). Of the 41 patients tested, 22 patients had Absent responses on the affected side, and four of them also on the normal side. The laser perceptive threshold related to C-fibers was significantly higher after stimulation of the affected side than after stimulation of the normal side ($P < 0.001$, paired t-test). The mean C-LEP latency after stimulation of the affected and normal side was similar ($P > 0.2$). The C-LEP amplitude was lower after stimulation of the affected side than after stimulation of the normal side ($P < 0.001$, Wilcoxon test). In 27 of the 41 patients, laser stimulation of the affected side failed to evoke reproducible brain potentials (Fig. 1). In 12 of these patients, C-LEPs were not reproducible or markedly

dampened also after stimulation of the normal side. Abnormal neurophysiological responses were strongly associated with affected side ($P < 0.0001$; Fisher's exact test). All but three patients had at least one Abnormal response on the affected side; the sensitivity was 93% (CI: 80–98). Twelve patients had abnormal responses on the controlateral side, thus yielding a specificity of 71% (CI: 55–84). Positive and negative predictive values were 0.76 and 0.91. 4.3. Correlations The side difference in amplitude of both A-delta and C-LEPs correlated with the intensity of constant pain ($P < 0.01$, Spearman's R correlation coefficient) (Fig. 2A and B). The side difference in R1 latency correlated with the intensity of paroxysmal pain ($P < 0.001$) (Fig. 2C). The side difference in C-fiber perceptive threshold correlated with the magnitude of thermal hypoesthesia ($P < 0.001$).

The correlation between the side difference in C-fiber perceptive threshold and intensity of itching only approached the statistical significance ($P = 0.02$). We also analyzed the following correlations, which did not reach statistical significance: the side difference in R1 latency

with magnitude of tactile hypoesthesia (P > 0.03), the side difference in perceptive threshold and amplitude of A-δ LEPs with magnitude of pinprick hypoesthesia (P > 0.03), the side difference in amplitude of C-LEPs with magnitude of thermal hypoesthesia and intensity of itching (P > 0.05); the intensity of mechanical allodynia with any of the three neurophysiological responses (P > 0.05); magnitude of sensory deficits and intensity of pain (P > 0.1).



ig. 1. Neurophysiological assessment.. Jpper panel: this representative patient ha onstant pain as the predominant type of ain as assessed by the numerical rating cale. On the affected side R1 is minimally elayed whereas LEPs are absent. Lower anel: this representative patient had aroxysmal pain. On the affected side R1 is ignificantly delayed, A-delta LEP is bsent, C-LEP has a slight amplitude eduction. (A) Blink reflex, (B) A-delta LEP C) C-LEP. Horizontal calibration: 10 ms fo A), 200 ms for (B and C). Vertical alibration: 200 μV for (A), 20 μV for (B and).

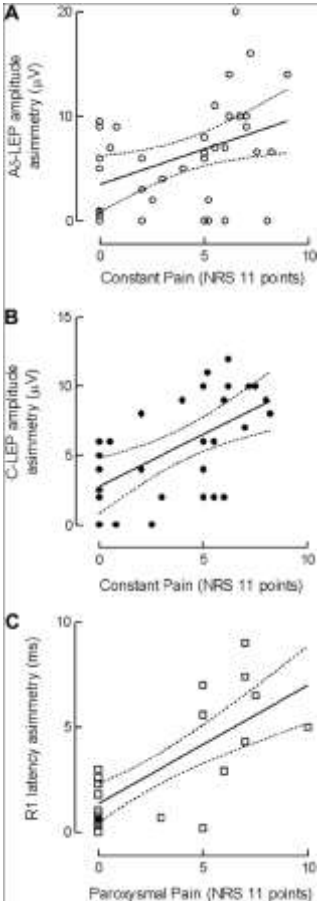


Fig. 2. Correlations between neurophysiological abnormalities and pain. (A and B) The intensity of constant pain, as assessed by the numerical rating scale (NRS), correlated with the side-to-side difference in A-delt LEP ($P < 0.01$, $R = 0.4166$) and C-LEP amplitude (P 0.01, $R = 0.5762$). (C) The intensity of paroxysmal pain had a correlation with the side-to-side difference in R1 latency ($P < 0.001$, $R = 0.6404$). Dashed lines indicate 95% confidence intervals fror he mean

5. Discussion

Our neurophysiological and clinical study assessed function of the three sets of cutaneous afferents (Aβ, Aδ, and C) in PHN. We found strong correlations between the neurophysiological Abnormalities reflecting specific fiber damage and the various types of pain.

5.1. Trigeminal nerve function in PHN

Most patients had severe tactile, pinprick, and warmth hypoesthesia. Consistently, we found that all the three neurophysiological responses (the R1 blink reflex, A-δ LEP, and C-LEP) were strongly abnormal. Although the N1-LEP (which we did not record) might have been more sensitive to disclose small latency delays, because both A-delta and C-LEPs were Absent or reduced in amplitude rather than being delayed, we believe that the small-fiber dysfunction originates from varicella-zoster-induced degeneration of the dorsal root ganglion cells. A relatively small increase in the latency of the R1 blink reflex may sometimes originate from an Aβ cell loss because of the reduced spatial-

temporal summation at central synapses. Our patients, however, had strong delays (some had an R1 latency of 15–20 ms), which are typical of demyelination [3,18]. Twelve patients had abnormal C-LEPs, and four abnormal A-δ LEPs, even after the stimulation of the non affected side. Eight of these patients were aged around 80 years. A previous study from our group found an age-related decrease in A-δ LEP amplitude [30]. Our findings in this study now suggest an age related decrease also for C-LEPs. LEP abnormalities after stimulation of the normal side possibly depend on mild neuronal loss or dysfunction in the peripheral nerves or in the brain with advancing age [13]. Another important point in interpreting our findings is that all our patients were receiving drugs acting on the nervous system that can dampen LEPs [4]. Alternatively, the bilaterally Abnormal finding may reflect bilateral dysfunction analogous to the bilateral Abnormality in EMG recordings [14], quantitative somatosensory testing [15] and neuropathological studies [33] in some patients with unilateral herpes zoster. The occurrence of these bilateral Abnormalities restricts the specificity of

neurophysiological testing to 71%; however, the sensitivity was high (93%), and thus neurophysiological testing would be useful in diagnosing zoster sine herpete.

5.2. Pathophysiology of pain in PHN

Whereas our patients' neurophysiological abnormalities correlated with the severity of pain, their clinical sensory deficits did not, presumably because neurophysiological investigations are more accurate and objective in assessing nerve fiber damage than patients' subjective reports of sensory deficits. An interesting finding concerns the clinical-neurophysiological correlations for the spontaneous pain, that patients with PHN typically describe as a constant, aching, burning pain. In our patients we found that the intensity of constant pain correlated with LEP abnormalities related to A-delta and C-fibers, thus suggesting that the constant pain is related to thermal-pain pathway damage. This finding is in line with several clinical studies reporting that in patients with PHN constant pain is associated with heat pain deficits [10]. It also agrees with skin biopsy studies in patients with

constant pain reporting a severe loss of epidermal-free nerve endings [23,24]. In patients with PHN the loss of small ganglion neurons may provoke long-term changes in the central nervous system, including hyperactivity of the second-order neurons of the nociceptive pathway [10]; such hyperactivity of dorsal horn cells has been reported in animal studies following complete primary afferent loss of a spinal segment [19]. We cannot, however, completely rule out that only very few surviving and sensitized C nociceptors may induce constant pain. Besides constant pain, patients with PHN usually complain of paroxysmal pain, described as electrical shock like, shooting or stabbing pains. The correlation of paroxysmal pain with blink reflex abnormalities in our patients indicates that paroxysmal pain is associated with A-beta-fiber dysfunction. Our results agree with the previous animal studies reporting an increased spontaneous ectopic discharge recorded in sensory myelinated axons after nerve injuries [2]. These results suggest that paroxysmal pain may be related to high-frequency discharges of impulses abnormally generated in demyelinated A-β

fibers. Whether the high-frequency bursts in demyelinated A-β fibers are sufficient to provoke pain per se or rather after ephaptic transmission to the neighboring C-fibers [1], or through a WDR neuron involvement [8] is still an open matter. We found no significant correlation of neurophysiological abnormalities with mechanical allodynia (too few patients had cold allodynia). It has been suggested that the incidence of allodynia may correlate inversely with the severity of small-fiber or spinothalamic deafferentation [25], i.e., that patients with allodynia tend to have higher LEPs than those without provoked pain [11]. In this report, the intensity of constant pain correlated with LEP attenuation, while that of provoked pain did not, which may in part support these assumptions. Probably this type of pain arises through multiple mechanisms and their relative contributions to pathophysiology of mechanical allodynia differ among subjects and may vary over the course of PHN [10].

Because the correlation between the severity of itching and the C-LEP perceptive threshold and amplitude asymmetry only approached the statistical significance, we cannot

draw reliable lines of reasoning on the pathophysiological mechanism of itching in PHN. Whereas our C-LEPs reflect general damage to unmyelinated fibers, many studies showed that itching is specifically mediated by C-pruriceptors [17,26]. Our study in a broad spectrum of patients with trigeminal PHN provides evidence that PHN impairs non-nociceptive as well as nociceptive trigeminal fibers.

The correlation between specific fiber damage and the various clinical types of pain indicates that PHN pain arises through several distinct pathophysiological mechanisms.

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Chapter 4: Differential involvement of Aδ and Aβ fibers in neuropathic pain related to carpal tunnel syndrome.

Chapter based on: Differential involvement of Ad and Ab fibres in neuropathic pain related to carpal tunnel syndrome. A. Truini, L. Padua, A. Biasiotta, P. Caliandro, C. Pazzaglia , F. Galeotti, M. Inghilleri, G. Cruccu. PAIN 145 (2009) 105–109

1. Introduction

Carpal tunnel syndrome (CTS), an entrapment neuropathy of the median nerve at the wrist [5], frequently manifests with neuropathic pain [12]. The commonly used test for diagnosing CTS is nerve conduction study (NCS) because it identifies and quantifies damage to the median nerve [17]. NCS nevertheless has the disadvantage of assessing non-nociceptive large myelinated fibres (Aβ-fibres) alone and provides no information on nociceptive fibre function [11]. Clinical assessment of patients with neuropathic pain relies on psychometric measures such as pain questionnaires. The clinician administered DN4 questionnaire is a 10-item screening tool that indicates

neuropathic pain when the score is ≥4 [8]. The self-administered neuropathic pain symptom inventory (NPSI) measures the intensity of the various qualities of neuropathic pain [7]. Current neurophysiological assessment of neuropathic pain relies on recording laser-evoked potentials (LEPs) [14]. Laser generated radiant heat pulses selectively activate Aδ and C mechanothermal nociceptors, and evoke scalp potentials related to small myelinated (Aδ) fibres [31]. LEPs are the most reliable and agreed methods of investigating nociceptive fibre function in patients with pain [11]. A simpler neurophysiological tool for acquiring information on nociceptive fibre function is the cutaneous silent period (CSP), an inhibitory response evoked in hand muscles by painful digital nerve stimulation [30]. Although the CSP is used to investigate nociceptive Aδ-fibre function in the upper limb, its nociceptive origin remains controversial [16]. Even though CTS is a common peripheral nerve disorder, no studies have investigated the role of nociceptive and non-nociceptive fibres in CTS-related neuropathic pain. We designed this prospective clinical and neurophysiological

study to gain more information on the frequency, quality and underlying mechanisms of CTS-related neuropathic pain. To investigate the different pathways involved in the development of pain we studied non-nociceptive Aβ-fibre function by standard nerve conduction study and nociceptive Aδ-fibre function by LEPs and CSP. We then determined the possible correlations between neurophysiological data and the various qualities of pain, as assessed by the NPSI.

2. Methods

2.1. Clinical examination

We conducted a prospective study from December 2006 to June 2007 recruiting consecutive patients with CTS from two university neurological outpatient clinics. Seventy patients, aged 25–81 years (mean 54 years; 8 males, 62 females), corresponding to 117 hands with CTS were enrolled in the Department of Neurological Sciences, Sapienza University, and in the Department of Neurosciences, Catholic University, both in Rome. One staff member examined the patients clinically and

administered the questionnaires and another did neurophysiological testing. The physician who assessed the results of neurophysiological testing was blinded to clinical findings and questionnaire results. All patients gave their informed consent to undergo the procedure. The research was approved by the two local Ethical Committees. The CTS diagnosis was based on established criteria and recommendations of the American Academy of Neurology (AAN) and the American Association of Electrodiagnostic Medicine (AAEM) [1,17]. The patient's history was recorded and a complete neurophysiological examination was performed to exclude the presence of other diseases that could cause or contribute to CTS, such as diabetes, polyneuropathy, hypothyroidism or acromegaly. We included only patients with idiopathic CTS. The Boston Carpal Tunnel Questionnaire was used to obtain a patient-oriented validated measurement [24]. In brief, it evaluates two CTS domains: ''symptoms" assessed on an 11-step scale; and ''functional status" assessed on an 8-step scale. The DN4 questionnaire for neuropathic pain was administered to all patients, for each hand separately.

Patients who scored ≥4 on the DN4 completed the NPSI questionnaire. The NPSI subscores were calculated for the five clinical symptoms: constant burning (superficial), constant pressing (deep), paroxysmal, provoked pain (allodynia, hyperalgesia) and abnormal sensations (paresthesias and dysesthesia). Although many patients had some kind of neuropathic pain complaint, only 4 were taking drugs (pregabalin and amitriptyline or duloxetine).

2.2. Neurophysiological examination

For nerve conduction testing we used a protocol inspired by the AAN and AAEM recommendations [1,17]. In brief, the testing comprised median nerve sensory conduction velocity in the Ist and IIIrd digit-wrist segments and ulnar nerve sensory conduction velocity in the Vth digit. Subjects who had normal median nerve sensory conduction velocities underwent the comparative test radial -median nerve sensory conduction velocity [27]. Motor nerve conduction was studied by stimulating the median and ulnar nerves at the wrist and the elbow and recording from thenar muscles and abductor digiti minimi. From the nerve

conduction study results CTS hands were classified into six severity groups: extreme, absent motor and sensory responses; severe, absent sensory response and abnormal distal motor latency; moderate, abnormal digit- wrist sensory nerve conduction velocity and abnormal distal motor latency; mild, abnormal digit-wrist sensory nerve conduction velocity and normal distal motor latency; and minimal, abnormal comparative test only. For the CSP study the IIIrd and Vth digits were stimulated with electrical shocks (80 mA, 0.5 ms) delivered through ring electrodes. Electromyographic (EMG) signals were recorded from the abductor digiti minimi muscle through surface electrodes. During EMG recordings, subjects were instructed to maintain an approximately maximum voluntary isometric contraction of the target muscle with the aid of EMG acoustic and visual feedback. Signals were samplified (bandwidth 50 Hz–5 kHz), full-wave rectified, averaged (6 trials) and stored. Onset and offset latency measurements were taken at the initial and final intersections of the averaged signal and a baseline indicating 80% of the background EMG level [16].

In accordance with the recommendations of the International Federation of Clinical Neurophysiology (IFCN) [21], instead of measuring CSP suppression, we measured its duration, a CSP variable that depends less on background muscle contraction levels. To study LEPs we used a neodymium:yttrium–aluminium– perovskite (Nd:YAP) laser (wavelength 1.34 mm, pulse duration 2–20 ms, maximum energy 7 J). The median nerve territory on the palm of the hand was stimulated by laser pulses at relatively high intensity (119–178 mJ/mm2), short duration (5 ms), and small diameter (5 mm) eliciting pinprick sensations. The laser beam was shifted slightly after each stimulus. The interstimulus interval was varied pseudorandomly (10–15 s). Subjects lay on a couch and wore protective goggles. They were instructed to keep their eyes open and gaze slightly downwards. To determine the laser perceptive threshold we delivered a series of stimuli at increasing and decreasing intensity, and defined the perceptive threshold as the lowest intensity at which the subjects perceived at least 50% of the stimuli. The main A-delta LEP complex, N2–P2, was recorded

through disc electrodes from the vertex (Cz) referenced to the nose. From 10 to 20 trials devoid of artefacts were collected and averaged offline. We measured peak latency and amplitude (peak-topeak) of the main N2–P2 vertex complex. We analyzed the correlation between the main neurophysiological data (IIIrd digit-sensory action potential and IIIrd digit-sensory nerve conduction velocity, IIIrd digit CSP duration, and LEP amplitude) and the five NPSI subscores (burning, pressing, paroxysmal, provoked pain and Abnormal sensations).

3. Statistical analysis

Non-parametric Mann–Whitney test was used to analyze the differences in clinical, demographic and neurophysiological data between painful and non-painful CTS hands. Chi-square test was used to assess the frequency of the various qualities of neuropathic pain, and frequency differences in CTS score severity between the painful and non-painful CTS hands. Correlations between neurophysiological data (IIIrd digit-sensory nerve action potential and conduction velocity, IIIrd digit CSP duration,

and LEP amplitude) and the NPSI score related to the five clinical symptoms (burning, pressing, paroxysmal, and provoked pain and abnormal sensations) were calculated with the non-parametric Spearman's R correlation coefficient and P values adjusted with Bonferroni correction for multiple correlations. P values of <0.01 were considered significant. We reported in the text both P values (i.e. before and after Bonferroni correction) only for the variables that were significant after the correction. All data are reported as means ± SD.

4. Results

4.1. Clinical results

Of the 117 hands from the 70 patients with CTS examined, the DN4 questionnaire identified 76 hands with neuropathic pain, and 41 without. No differences were found for age and Boston Carpal Tunnel Questionnaire scores between pain and non-pain groups (P > 0.2, Mann–Whitney test) (Table 1). NPSI analysis showed that 51 CTS hands had burning pain (mean rating 3.5 ± 3.2), 42 pressing pain (mean rating 2.5 ± 2.6), 56 paroxysmal pain (mean

rating 3.1 ± 2.5), 39 provoked pains (mean rating 2.0 ± 3.6), and 62 Abnormal sensations (5 ± 3.3). The various kinds of pain differed significantly in frequency (P = 0.0002, chi-square test), provoked pains being less frequent than paroxysmal pain and abnormal sensations.

Painful hands (n = 76)	
Non-painful hands (n = 41)	
Age (years)	54.3 ± 13.4 54.7 ± 15.8
IIrd digit SNAP amplitude (μV)	13.1 ± 9.3 15.3 ± 10.0
IIrd digit SNCV (m/s)	38.9 ± 7.3 40.5 ± 8.0
IIrd digit CSP duration (ms)	51.2 ± 17.8 49.0 ± 14.3
Laser perceptive threshold (mJ/mm2)*	75.7 ± 24.9 55.7 ± 16
N-LEP latency (ms)	190.5 ± 16.7 190.5 ± 14.6
LEP amplitude (μV)**	10.6 ± 8.4 15.9 ± 5.9

CTS: carpal tunnel syndrome; Min: minimal; Mild; Mod moderate; Sev: severe;

Ext: extreme; SNAP: sensory nerve action potential; SNCV sensory nerve conduction

velocity; CSP: cutaneous silent period; LEP: laser-evoke potentials.

P = 0.004.

* P = 0.002.

Table 1. Summary of clinical and neurophysiological data

4.2. Neurophysiological results

Of the 117 hands from the 70 patients with CTS tested, the median nerve sensory action potential was Absent in 15 CTS hands, the CSP in three (all patients who had extreme CTS, two with pain and one without). LEPs were absent in 15 CTS hands (14 with pain and one without). No difference was found in the frequency of the different CTS severity grades between neuropathic and non-neuropathic groups ($P = 0.13$, chi-square test). Neither NCS data nor CSP duration differed between patients with and without pain ($P > 0.2$, Mann–Whitney test). Nor did the LEP latency differ between the two groups of patients ($P > 0.2$) but the perceptive threshold was higher and the LEP amplitude lower in CTS hands with pain than in those without ($P = 0.004$ and $P = 0.002$) (Fig. 1).

4.3. Pain correlations

The NPSI subscores related to burning and pressing pain correlated only with LEP amplitude (burning pain: $R = -0.5036$, $P = 0.0004$, after Bonferroni correction $P = 0.008$; pressing pain: $R = -0.5311$, $P < 0.0001$, after Bonferroni

correction P = 0.002; other neurophysiological variables P > 0.1, Spearman's R correlation coefficient). Conversely, the NPSI subscores related to paroxysmal paroxysmal pain and to Abnormal sensations correlated only with median nerve sensory conduction velocity (paroxysmal pain: R = -0.5022, P < 0.0001; after Bonferroni correction P = 0.002; abnormal sensations: R = -0.4292, P = 0.0003; after Bonferroni correction P = 0.006; other neurophysiological variables P > 0.1) (Fig. 2). We found no significant correlation between the NPSI subscores related to provoked pain and any of the neurophysiological variables tested (P > 0.5).

5. Discussion

Our prospective clinical and neurophysiological study provides new information on the frequency, quality, and pathophysiological mechanisms underlying neuropathic pain in a large cohort of patients with CTS. A previously unreported finding is that median nerve conduction abnormalities, reflecting non-nociceptive Aβ-fibre damage, correlated with paroxysmal pain and abnormal sensations.

Conversely LEP abnormalities, reflecting nociceptive Aδ-fibre damage, correlated with burning and pressing pain.

5.1. Clinical findings

The 65% frequency of neuropathic pain, as assessed by the DN4 questionnaire in our 117 patients with CTS is in line with the 50% reported in a previous clinical study in 1123 patients with CTS assessed with a dichotomous categorical score (yes or no) [27]. A distinctive feature of our study is that we analyzed the various clinical qualities of neuropathic pain in CTS. This analysis, according to the NPSI, showed that provoked pains, including hyperalgesia and allodynia, are less frequent and less severe than paroxysmal pain, and abnormal sensations in patients with CTS. Given that patients with and without CTS-related pain had similar disease severity and similar Boston Carpal Tunnel Questionnaire scores we agree with others that pain related to CTS depends on factors other than disease severity [27].

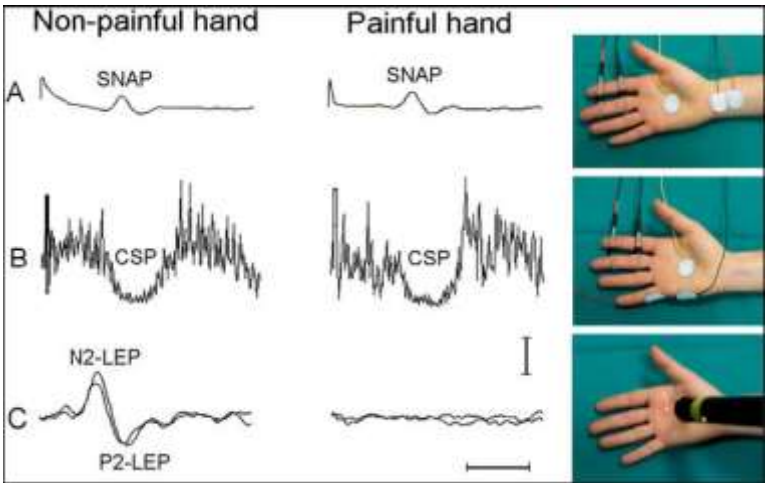

Fig. 1. Neurophysiological assessment in a representative CTS hand with (right) and without pain (left). (A) sensory nerve action potential (SNAP) after IIIrd digit stimulation. Calibration: 2 ms/10 μV. (B) cutaneous silent period (CSP) after IIIrd digit stimulation. Calibration: 50 ms/100 μV. (C) laser-evoked potentials (LEPs) after median nerve territory stimulation. Calibration: 200 ms/10 μV. While SNAP latency and amplitude and CSP duration were similar in the two CTS hands, in the CTS hand with pain (predominantly burning pain) LEPs were absent.

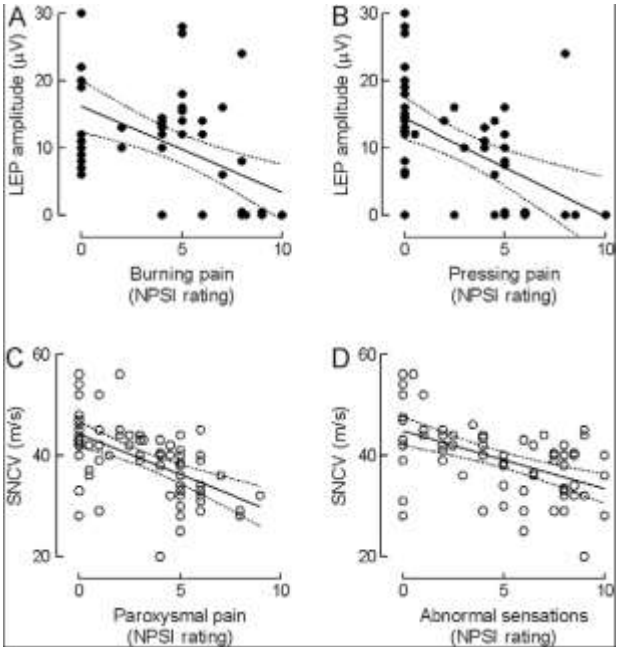

Fig. 2. Correlations between neurophysiological abnormalities and pain. The intensity of spontaneous burning pain (A) and spontaneous pressing pain (B) correlated with the A-delta LEP amplitude (R = -0.5036, P = 0.008; R = -0.5311, P = 0.002). The intensity of paroxysmal pain (C) and abnormal sensations (D) correlated with the median nerve sensory conduction velocity (R = -0.5022, P = 0.002; R = -0.4292, P = 0.006). Dashed lines indicate 95% confidence intervals from the mean.

5.2. Neurophysiological findings

The CSP was absent only in 3 of the 117 hands we tested, all from patients who had extreme CTS and absent LEPs. Several studies reported that in severe entrapment neuropathies the CSP, a response mostly mediated by A-delta fibres, is usually spared and is abolished only by complete nerve transection [30], probably because compression mainly damages large myelinated fibres, and tends to spare small fibres [25]. Our data confirm that the CSP is a useful tool for documenting residual nerve continuity in severe entrapment neuropathies [30]. Although LEPs are usually recorded after applying stimuli to hairy skin, a recent study, using the same type of laser stimulator, showed that laser stimuli applied to glabrous and hairy skin yield similar psychophysical and electrophysiological responses [15]. Whereas LEP amplitude differed in CTS hands with and without pain, CSP did not. These findings support the view that LEPs are highly sensitive to nociceptive pathway impairment and the most reliable diagnostic tool for neuropathic pain [11].

Conversely, rather than being a pure nociceptive response the CSP may also have an Aβ-fibre-mediated component [29]. Accordingly, it is not suppressed by opiates given at a dose that induces pain relief and suppresses the nociceptive RIII reflex in the biceps femoris muscle [16], and does not correlate with pain in patients with peripheral neuropathy [32].

5.3. Pain correlations

We found that superficial burning and deep pressing pain correlated inversely with LEP amplitude, thus suggesting that these types of pain are due to nociceptive pathway damage. Our data are in line with previous studies that found a close link between pain and nociceptive fibre damage in painful polyneuropathy as assessed by psychophysiological [22], neurophysiological [10], or neuropathological (skin and nerve biopsies) [20,34] investigations in patients with different types of peripheral neuropathy. Therefore CTS and polyneuropathy share similar pathophysiological mechanisms underlying ongoing pain. Animal studies demonstrated that

peripheral nerve injury causes spontaneous hyperexcitability of nociceptive afferents (peripheral sensitization) [13,19,35]. Microneurographic studies in humans reported that in patients with peripheral neuropathies burning pain is associated with spontaneous, anomalous discharges in afferent fibres [6,26]. We therefore hypothesize that in patients with CTS spontaneous constant pain arises from abnormal, spontaneous hyperactivity originating in damaged axons of nociceptive fibres. However, we cannot exclude the possibility that nociceptive pathway damage may provoke long-term changes in the central nervous system, including hyperactivity of the second order neurons of the nociceptive pathway (central sensitization) [13,23,28] that may act as a concurrent mechanism. The NPSI items corresponding to paroxysmal pain, and abnormal sensations correlated with sensory nerve conduction velocity, thus indicating that these sensory disturbances are associated with Aβ-fibre damage. This finding confirms the notion that paresthesias and non-painful sensory disturbances are caused by abnormal non-nociceptive A-

beta-fibre activity [18]. The mechanisms responsible for paroxysmal pain are far more controversial. In particular CTS is a condition characterised by chronic focal compression that induces demyelination, which mainly affects Aβ-fibres [25]. Our finding of a correlation between Aβ-fibre damage and paroxysmal pain is therefore coherent with the pathophysiological mechanisms of CTS. However, in an earlier study we found that also in patients with postherpetic neuralgia paroxysmal pain was associated with delayed Aβ-fibre-mediated responses [33]. These data raise the possibility that regardless of the disease paroxysmal pain may be invariably related to Aβ-fibre damage. Consistently with previous animal studies describing spontaneous ectopic discharges recorded in Aβ-fibre axons after nerve injuries [2,3,9], we suggest that paroxysmal pain is related to high frequency bursts generated in demyelinated Aβ-fibres. Whether these high-frequency bursts in demyelinated Aβ-fibres are sufficient to provoke pain per se or do so only after ephaptic transmission to the neighbouring C fibres, or through the

involvement of wide dynamic range neurons is an open matter [33].

NPSI item related to provoked pains correlated with none of the neurophysiological data. Many studies proposed alternative mechanisms for allodynia/hyperalgesia. Provoked pains may arise through multiple mechanisms even in the same disease (and may vary over the course of disease), thus their relative contributions may differ among subjects [13].

Our study shows that a specific type of neuropathic pain is differentially associated with nociceptive and non-nociceptive fibre damage and thus arises through different pain mechanisms. If these findings hold true in other neuropathic pain conditions, showing that the various types of neuropathic pain are invariably caused by similar mechanisms regardless of the disease, they could be useful in designing new treatment strategies targeted to the type of pain [4].

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Chapter 5: Mechanisms of pain in multiple sclerosis: a combined clinical and neurophysiological study

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Chapter based on: Mechanisms of pain in multiple sclerosis: a combined clinical and neurophysiological study. A Truini, F Galeotti, S La Cesa, S Di Rezze, A Biasiotta, G Di Stefano, E Tinelli, E Millefiorini, A Gatti, G Cruccu. Pain. In press

Introduction

Neuropathic pain is common in patients with multiple sclerosis (MS). According to previous published studies its prevalence ranges from 26% to 58% [17,18]. Although MS patients may suffer from various types of neuropathic pain, the most frequent are the ongoing extremity pain and the Lhermitte's phenomenon [18]. Ongoing extremity pain—often called "dysesthetic extremity pain" in the MS literature [17]—is a chronic form of pain in MS patients [18,19], described as a continuous burning pain that is typically bilateral, affecting the legs and feet, and that is usually worse at night. Lhermitte's phenomenon is defined as a transient short-lasting sensation related to neck

movement and felt in the back of the neck, lower back or in other parts of the body [1,18]. Clinical assessment of patients with neuropathic pain relies on psychometric measures such as pain questionnaires. The clinician-administered DN4 questionnaire is a 10-item screening tool that indicates neuropathic pain when the score is ≥4 [5]. Electrically-elicited somatosensory evoked potentials (SEPs) are commonly used in patients with sensory disturbances due to multiple sclerosis [23]. SEPs are mediated by non-nociceptive Aβ-fibres, the afferent input is relayed through the dorsal columns of the spinal cord and the medial lemniscus in the brainstem, and provide no information on nociceptive pathways, [7,26]. The most reliable and agreed neurophysiological method for investigating nociceptive fibre function in patients with neuropathic pain is laser evoked potential (LEP) recording [7,11]. Laser-generated radiant heat pulses selectively activate Aδ and C mechano-thermal nociceptors [26], and evoke scalp potentials related to small myelinated (Aδ) fibres. The afferent volley is conducted along small-

myelinated (Aδ) primary sensory neurons, and relayed to ascending nociceptive spinal pathways and brain [7,9,26].

Although patients with multiple sclerosis frequently experience neuropathic pain, effective treatment awaits research clarifying the underlying mechanisms. In this clinical and neurophysiological study we sought information on the clinical characteristics and the underlying mechanisms of the two commonest types of neuropathic pain related to multiple sclerosis: ongoing neuropathic pain and Lhermitte's phenomenon. To do so we collected MS patients, and identified by clinical examination and DN4 questionnaire patients with the different types of neuropathic pain. Then to assess nociceptive and non-nociceptive pathway function in these two types of neuropathic pain we investigated SEPs and LEPs.

Methods

We conducted a period prevalence study collecting consecutive patients with a definite diagnosis of multiple sclerosis from the outpatient clinic at the Department of

Neurology and Psychiatry at Sapienza University, Rome. Patients with a clinical isolated syndrome (patients presenting with acute or subacute episode of neurological disturbance due to a single white-matter lesion [16]) were also included. Two neurologists (one from the multiple sclerosis outpatient service and the other from the neuropathic pain unit) examined the patients clinically and administered the questionnaire, and two neurophysiologists performed the evoked potential recordings. All patients gave their informed consent to undergo the procedure. The research was approved by the Institutional Review Board.

Clinical examination

All patients underwent a detailed neurological examination using bedside tools. Touch was investigated with a piece of cotton wool and von Frey hairs, vibration with a tuning fork (128 Hz), pinprick sensation with a wooden cocktail stick. In all patients laser stimuli were used for a quantitative evaluation of warm and pinprick sensations. The diagnosis of multiple sclerosis was based

on Polman criteria [20]. In all patients the expanded disability severity score (EDSS) was collected to rate the severity of multiple sclerosis. Patients were asked to report pain experienced within one month of assessment. Being too difficult to ascertain its causal or casual association with MS, headache alone was not considered. All patients with pain other than headache completed the DN4 questionnaire for neuropathic pain. In every patient a definite diagnosis of neuropathic pain was supported by the patient history, the clinical examination (including the DN4) showing the positive and negative sensory signs with a logical neuroanatomical distribution and laboratory tests (MRI and neurophysiological testing) [27]. We then divided patients into three groups: without pain, with nociceptive pain, and neuropathic pain. Patients were instructed to rate pain intensity on an 11-point numerical rating scale ranging from 0 (no disturbance) to 10 (worst possible disturbance).

Neurophysiological testing

We studied somatosensory evoked potentials after median and tibial nerve stimulation using surface recording electrodes with standard placement. Methods used adhered to those recommended by experts of the International Federation of Clinical Neurophysiology [7]. In brief, electrical stimuli were applied to the median and tibial nerve with saline-soaked pads at a frequency of 4 Hz (stimulus duration 0.1 ms; intensity: sensory plus motor threshold; bandpass: 10–1000 Hz). Early cortical somatosensory evoked potentials (N20 or P40) were recorded from Pc and Cz versus Fz, and two series of 1000 artefact-free trials were averaged online for each nerve tested. To study laser evoked potentials we used a neodymium:yttrium–aluminium–perovskite (Nd:YAP) laser (wavelength 1.34 mm, pulse duration 2–20 ms, maximum energy 7 J). Foot and hand were stimulated by laser pulses at relatively high intensity (127–203 mJ/mm2), short duration (5 ms), and small diameter (5 mm) eliciting pinprick sensations. The laser beam was shifted slightly after each stimulus. The interstimulus interval was varied

pseudorandomly (10–15 s). Subjects lay on a couch and wore protective goggles. They were instructed to keep their eyes open and gaze slightly downwards. To determine the laser perceptive threshold we delivered a series of stimuli at increasing and decreasing intensity, and defined the perceptive threshold as the lowest intensity at which the subjects perceived at least 50% of the stimuli. The early, lateralized component, N1, and the main complex, N2–P2, were recorded through disc electrodes from the temporal areas (Tc) referenced to frontal area (Fz) and vertex (Cz) referenced to the nose. From 10 to 20 trials devoid of artefacts were collected and averaged offline. We measured peak latency and amplitude (peak-to-peak) of the temporal N1 component and the N2–P2 vertex complex. In all patients median and tibial nerve somatosensory evoked potentials and hand and foot laser evoked potentials were recorded bilaterally and considered abnormal when stimulation applied to at least one limb yielded abnormal results. Neurophysiological data were compared with normative ranges in our laboratory [7].

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess group differences for age, duration of disease in patients with neuropathic pain, nociceptive pain, and without pain. We used the non-parametric Kruskal-Wallis analysis to test group differences for pain intensity and EDSS. Mann-Whitney test was used to compare the severity of neuropathic and nociceptive pain. Because age, duration of disease and EDSS might influence the development of pain in concert, a logistic regression analysis was used to assess which of these factors predict the development of pain. In the logistic regression analysis the three variables were divided in two groups according to the median value. Then data for patients without pain were compared with those for patients with nociceptive and neuropathic pain. We used the chi-square test to assess the differences in frequency of pain in the various clinical courses of multiple sclerosis, and the Fisher exact test for the neurophysiological abnormalities across ongoing extremity pain and Lhermitte's phenomenon. P values of <0.05 were

considered to indicate statistical significance. All results are reported as means ± SD.

Results

We consecutively collected data for 302 patients (211 F, 91 M, mean age 39.4 ± 10.9 years, mean EDSS: 2.0 ± 2.3; mean duration of disease from the diagnosis (years): 8.0 ± 7.2). In 239 patients multiple sclerosis had a relapsing-remitting course, in 10 primary progressive, in 43 secondary progressive, and 10 patients had a clinical isolated syndrome. Of the 302 patients studied, 92 patients had pain (30%). According to clinical examination and DN4 questionnaire 42 patients (13.9%) experienced neuropathic pain. The other patients suffered from back pain, muscle spasm, and other musculoskeletal pains. Of the 42 patients with neuropathic pain 8 had trigeminal neuralgia (2.6%), 15 ongoing extremity pain (5.0%), and 19 Lhermitte's phenomenon (6.3%). The intensity of neuropathic pain did not differ from that of nociceptive pain (6 ± 1.9 vs 5.6 ± 1.4, P = 0.8, Mann-Whitney test). However Kruskall-Wallis analysis showed that trigeminal neuralgia was the most

severe type of pain, and the Lhermitte's phenomenon the less severe (P <). Patients with trigeminal neuralgia had a paroxysmal, electrical shock-like sensation in the face, usually triggered by light mechanical touch. All these patients had a mild tactile hypoesthesia. Three patients also had an increased pinprick threshold to laser stimuli. Patients with ongoing extremity pain had ongoing, predominantly burning, pain mainly affecting the legs. All patients had thermal-pain sensory deficits as assessed by the laser perceptive threshold examination, and milder sensory deficits affecting touch and vibration sensations. Patients with Lhermitte's phenomenon felt an electrical sensation, spontaneous or related to neck flexion, radiating down the spine and the limbs. Most patients with Lhermitte's phenomenon had only mild deficit of touch and vibration sensations, affecting the distal part of the body. Notwithstanding the abnormalities of the MRI and neurophysiological testing four patients had no clinically evident sensory deficits, as assessed by bedside tools. Chi-square test showed that neuropathic pain was less frequent

in patients with a clinical isolated syndrome and relapsing-remitting form than the other clinical forms ($P < 0.05$).

ANOVA showed that patients with pain (nociceptive and neuropathic) were older and with a longer duration of disease. Kruskall-Wallis analysis showed that patients with pain (nociceptive and neuropathic) had a higher EDSS and a longer duration of disease ($P < 0.0001$). Logistic regression analysis identified EDSS as the only factor significantly associated with neuropathic pain (OR 2.3; CI 95%:1.1-4.9; $P = 0.03$).

Ten patients with ongoing extremity pain and 18 with Lhermitte's phenomenon underwent neurophysiological testing. SEPs were abnormal in 3 out of 10 patients with ongoing extremity pain (30%) and in 13 out of 18 patients with Lhermitte's phenomenon (72%)(Figure 1,2). Conversely, LEPs were abnormal in 9 patients with ongoing extremity pain (90%) and in 3 patients with Lhermitte's phenomenon (16.6%) (Figure 1,2). Whereas the abnormality frequency of SEPs was significantly higher in patients with Lhermitte's phenomenon ($P = 0.002$, Fisher exact test), the abnormality frequency of LEPs was

significantly higher in patients with ongoing extremity pain (P = 0.02) (Figure 3). In all patients with ongoing extremity pain and most of those with Lhermitte's phenomenon magnetic resonance imaging (MRI) showed a spinal lesion. In most cases MRI scans could not precisely distinguish whether the lesion involved the anterolateral or the dorsal columns. The eight patients with trigeminal neuralgia did not undergo the evoked potential testing because of the low number and the peculiarities of the trigeminal system.

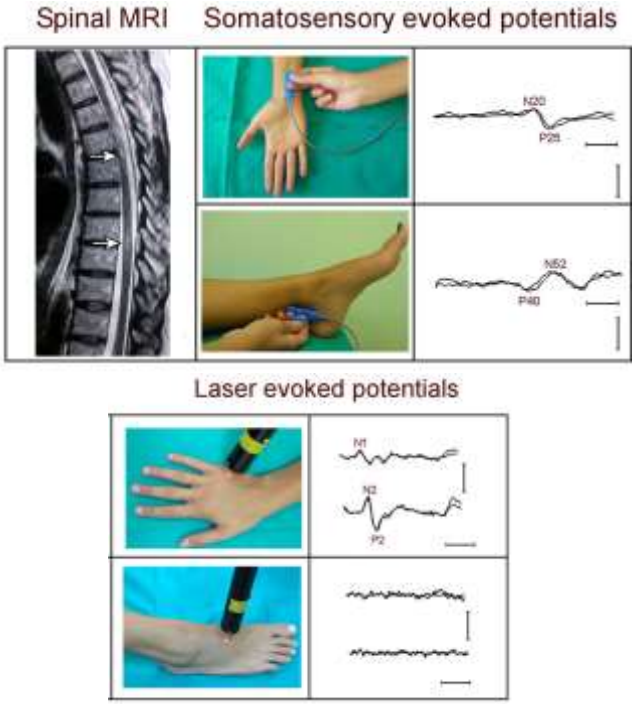
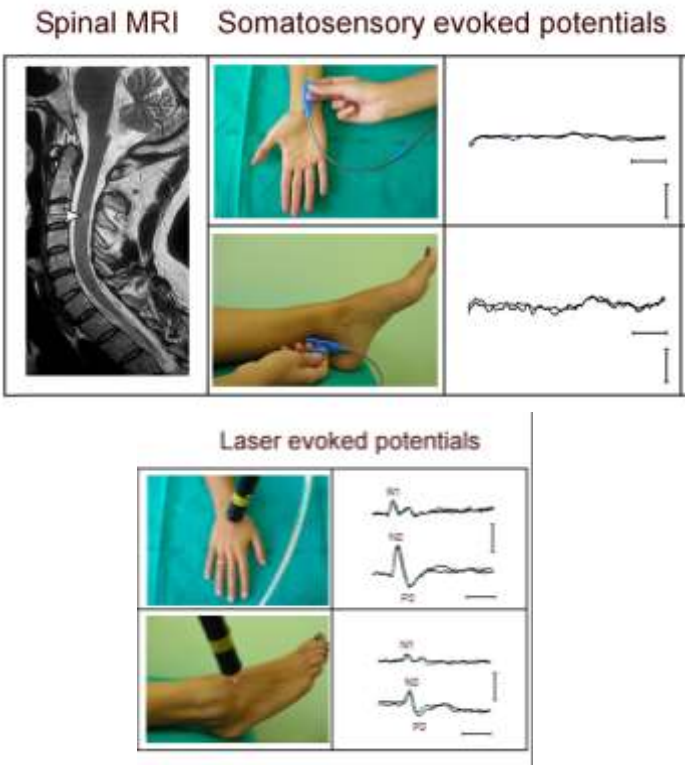


Figure 1. Spinal MRI and neurophysiological assessment in ongoing extremity pain. MRI scans shows demyelinating lesions affecting thoracic spinal cord in a patient with ongoing extremity pain. In this patient while somatosensory evoked potentials (SEPs) were spared, laser evoked potentials (LEPs) after foot stimulation were absent. LEPs: 20μV/200ms. Median nerve SEPs: 4μV/5ms; Tibial nerve SEPs: 4μV/10ms



- **Figure 2. Spinal MRI and neurophysiological assessment in Lhermitte's phenomenon.** MRI scans shows a demyelinating lesions affecting cervical spinal cord in a patient with Lhermitte's phenomenon. In this patient while LEPs were spared, SEPs after median and tibial nerve stimulation were absent. LEPs: 20μV/200ms. Median nerve SEPs: 4μV/5ms; Tibial nerve SEPs: 4μV/10ms.

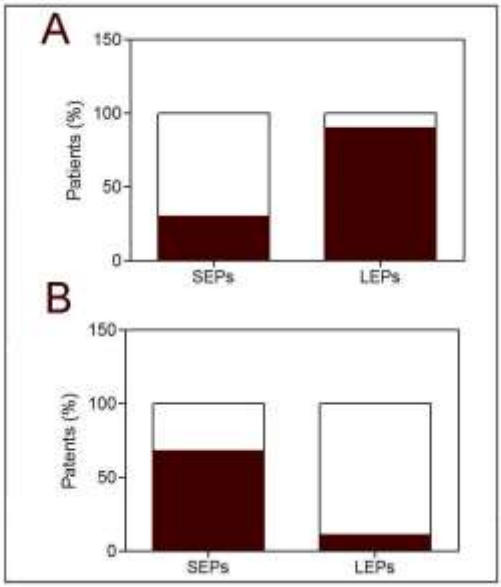


Figure 3. Prevalence of the neurophysiological Abnormalities in patients with in ongoing extremity pain and Lhermitte's phenomenon. A: Graph showing that in patients with ongoing extremity pain the abnormality frequency of LEPs was significantly higher than that of the SEPs (P = 0.02, Fisher exact test). B: Graph showing that in patients with Lhermitte's phenomenon the abnormality frequency of the SEPs was significantly higher than that of the LEPs (P = 0.002, Fisher exact test).

Discussion

In this period prevalence study in patients with neuropathic pain related to multiple sclerosis identified by clinical examination and responses to the DN4 questionnaire, we found that a higher EDSS and a more severe clinical course (such as secondary and primary progressive) increase the risk of developing neuropathic pain. We also provide new neurophysiological evidence that ongoing extremity pain is associated with LEP abnormalities, thus suggesting that this type of pain arises from nociceptive pathway damage. Conversely, Lhermitte's phenomenon is associated with SEP abnormalities, thus presumably arises from damage to the non-nociceptive Aβ-fibre pathway.

Clinical findings

The overall prevalence of pain assessed within the past month was 30% and that of neuropathic pain was 14%. Both values are lower than those (53-79% and 58% respectively) reported in previous studies [2,4,18,24]. These discrepant results probably depend partly on our patients'

clinical characteristics given that our patients were younger, the EDSS was lower, the duration of disease was shorter and the clinical course less severe than those reported in the literature. Another possible explanation is that previous studies over-rated the prevalence of pain: according to a recent systematic review [18] studies on pain in MS frequently assessed the presence of pain only by a mail questionnaire, and some studies included any type of pain, nor did they use a validated questionnaire to diagnose neuropathic pain. The DN4 questionnaire that we used in our study, specifically designed to diagnose neuropathic pain, increases diagnostic specificity and thus reduces false-positive diagnoses, particularly for ongoing pain. DN4 is a validated screening tool to diagnose neuropathic pain with a high specificity and sensitivity (about 80%) [5]. However we also used clinical examination in addition to DN4 to identify patients with neuropathic pain because it might fail to identify patients with trigeminal neuralgia and Lhermitte's sign. Indeed these patients frequently complain only of paroxysmal, electrical shock like sensations, and no other clinically-

evident sensory disturbances or pains can be detected in the region affected by pain. We found that patients with higher EDSS and a more severe clinical MS course (primary and secondary progressive courses) are at increased risk for the development of neuropathic pain. This finding is in line with several previous clinical studies and suggests that the more numerous the lesions the higher the probability of pain developing [18,22].

Neurophysiological findings and pain mechanisms

We showed that a specific type of sensory disturbance was associated with a specific afferent pathway damage as assessed by neurophysiological testing. Previous neurophysiological studies have already investigated LEPs in patients with multiple sclerosis showing their high diagnostic sensitivity in patients with sensory disturbances [13,23]; however these studies have not analysed pain characteristics or investigated possible correlations between LEP abnormalities and pain. We found that ongoing extremity pain is associated with LEP abnormalities, thus suggesting that this type of pain is

related to nociceptive pathway damage. In all patients, MRI imaging showed cervical or thoracic spinal cord damage. Accordingly, we hypothesize that in our patients ongoing extremity pain arises from spinothalamic tract lesions. As the underlying mechanism we conjecture deafferentation of thalamic nuclei [9,12]. We found that Lhermitte's phenomenon is associated with SEP abnormalities, thus suggesting that this type of pain is related to non-nociceptive Aβ-fibre pathway damage. Because most patients had cervical spinal cord lesions as assessed by MRI imaging, and reported pain due to neck movement, Lhermitte's phenomenon probably arises from a demyelinating lesion in the dorsal columns of the cervical spinal cord. This hypothesis is a common belief among clinicians [14,21], and has also been supported by a previous MRI study [10], but this is the first study confirming it with a functional investigation such as evoked potentials. Our finding that Lhermitte's phenomenon, a paroxysmal, electrical-shock-like sensation, is associated with dorsal column damage, is consistent with previous neurophysiological studies in peripheral

neuropathic pain conditions (i.e. postherpetic neuralgia and carpal tunnel syndrome) showing that this type of pain is associated with Aβ-fibre demyelination [27,28]. These data suggest that, regardless of aetiology, paroxysmal pain is related to Aβ-fibre damage. Consistently with previous animal studies describing spontaneous ectopic discharges recorded in Aβ-fibre axons after nerve injuries [6,15,29], we conjecture that paroxysmal pain is related to high-frequency bursts generated in demyelinated Aβ-fibres. Whether these high-frequency bursts in demyelinated Aβ-fibres are sufficient to provoke pain per se or do so only after ephaptic transmission to neighbouring nociceptive fibres, or by involving wide dynamic range neurons is an open matter. Our findings in patients with multiple sclerosis lend further support to the view that neuropathic pain should be classified according to sensory profiles rather than aetiology [3,8]. This approach could minimize the pathophysiological heterogeneity within study groups and clinical trials, thus making it easier to identify a positive treatment response and opening the way to new therapeutic approaches.

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Chapter 6: Mechanisms of pain in distal symmetric polyneuropathy: A combined clinical, neurophysiological and morphological study.

Chapter partly based on: Mechanisms of pain in distal symmetric polyneuropathy: A combined clinical and neurophysiological study. A. Truini, A. Biasiotta, S. La Cesa , G. Di Stefano, F. Galeotti, M.T. Petrucci, M. Inghilleri, C. Cartoni, M. Pergolini, G. Cruccu. Pain (150)2010 :516-521.

1. Introduction

Distal symmetric polyneuropathy is a common neurological condition that has manifold causes, including systemic diseases, metabolic disorders, and exogenous toxic substances [17]. Sensory disturbances include hypaesthesia involving the various sensory modalities, and pain. Functional assessment of patients with neuropathic pain relies on psychometric measures, such as pain questionnaires and sensory testing, and dedicated neurophysiological tests. A validated and commonly used pain questionnaire is the self-administered Neuropathic

Pain Symptom Inventory (NPSI), a tool designed to measure the intensity of the various neuropathic pain qualities [5,26]. The reference standard test for diagnosing neuropathy is the nerve conduction study (NCS). NCS nevertheless has the disadvantage of assessing non-nociceptive, large myelinated fibres (Aβ fibres) alone and provides no information on nociceptive pathway function [7]. Current neurophysiological assessment of nociceptive pathways relies on recording laser-evoked potentials (LEPs) [7]. Laser-generated radiant heat pulses selectively activate Aδ and C mechano-thermal nociceptors, and evoke scalp potentials related to small myelinated (Aδ) fibres [29,30]. Although laser stimuli activate both Aδ and C mechano-thermal nociceptors, LEPs related to C-fibres cannot be reliably recorded after foot stimulation [7,30–32]. LEPs are the most reliable and agreed neurophysiological method for investigating nociceptive fibre function in patients with pain [7]. Although damage to small nerve fibres is widely assessed by quantifying intraepidermal nerve fibres from a skin biopsy a recent study showed that intraepidermal nerve fibre density did not correlate with

neuropathic pain [10]. The pathophysiological mechanisms underlying neuropathic pain remain debatable. Although most investigators postulate that neuropathic pain always arises from nociceptive pathway damage [20,22], others – invoking the undeniable inhibitory effect of Aβ-fibre input on central nociceptive transmission [8,24] – have suggested that Aβ-fibre loss may provoke pain by disinhibiting nociceptive pathways [9,14]. An earlier study reporting that a pre-existing neuropathy selectively involving Aβ-fibres is a risk factor for the development of chronic postherpetic pain [2] suggests that Aβ-fibre loss could be a contributing factor in this neuropathic pain condition. This pathophysiological mechanism receives further support from a recent clinical and neurophysiological study in patients with peripheral neuropathy showing that pain correlates with Aβ-fibre damage, as assessed with nerve conduction studies (NCS) [19]. More information is therefore, needed on pathophysiological mechanisms underlying neuropathic pain related to distal symmetric peripheral neuropathy. This information could be used to develop more effective therapeutic approaches for a pain

condition that is notoriously difficult to treat. We designed this clinical, neurophysiological and histomorphological study in patients with distal symmetric polyneuropathy to investigate the role of clinical features, such as age and duration of disease, and the role of primary afferent neurons (non-nociceptive Aβ and nociceptive Aδ afferent fibres) on the development of neuropathic pain. We assessed clinical variables including the various qualities of neuropathic pain with the NPSI and non-nociceptive Aβ-fibre function with standard nerve conduction study (NCS), nociceptive Aδ-fibre function with laser-evoked potentials (LEPs), and nociceptive C-fibre damage with skin biopsy and intraepidermal nerve fibres (IENF) quantification.

2. Methods

We screened 2240 patients with sensory disturbances consecutively referred to our institution from October 2006 to June 2011, and collected 269 patients with distal symmetric polyneuropathy (57% with pain and 43% without). The diagnosis of distal symmetric polyneuropathy was based on clinical, biological, and electrodiagnostic findings, adhering to the criteria proposed by England et al. [13]. Patients with symmetrical reduction or absence of ankle reflexes, decreased distal sensation, and abnormal NCS or LEPs were included in this study. Exclusion criteria were diagnosis of inflammatory or inherited neuropathies, sensory disturbances due to neurological diseases other than distal symmetric polyneuropathy, cognitive impairment. We chose to exclude patients with inflammatory or inherited neuropathies because these kinds of neuropathies seldom manifest with focal or multifocal sensory disturbances, and thus we decided to avoid a retrospective selection of this kind of patients. Aetiologies varied widely: chemotherapy-induced neuropathy (50 patients), diabetic neuropathy (89

patients), peripheral neuropathy of unknown origin (51 patients), monoclonal gammopathy-related neuropathy (79 patients). All patients underwent clinical examination, a nerve conduction study, LEP recordings and 69 of them skin biopsies with quantification of somatic IENFs. One staff member examined the patients clinically and administered the NPSI questionnaire and others did neurophysiological testing, with those recording NCS being blinded to LEP data and vice versa. The research was approved by the local Ethical Committee.

2.1. Clinical examination

All patients underwent a detailed neurological examination using bedside tools. Touch was investigated with a piece of cotton wool, vibration with a tuning fork (128 Hz), and pinprick sensation with a wooden cocktail stick. In all patients laser stimuli were used for a quantitative evaluation of warm and pinprick sensations. Gait impairment, and muscle strength were assessed with the Medical Research Council score. Patients were also

asked to report dysautonomic symptoms. Patients were grouped according to the clinically documented presence or absence of neuropathic pain rated ≥4 on the 0–10 numerical rating scale and persisting since at least one month [12]. All the patients with pain were taking pain medications: 40.5% was taking gabapentin or pregabalin, 19.5% duloxetine, 10.5% a combination therapy with pregabalin and duloxetine, 7.5% tramadol, 16.5% a combination of tramadol and pregabalin, 4.5% oxycodon, and 3% amytriptiline. Patients with pain completed the NPSI questionnaire. The NPSI subscores were calculated for the various types of pain: ongoing pain (burning and pressing pain), paroxysmal, provoked pain and abnormal sensations (paraesthesias and dysaesthesia).

2.2. Neurophysiological examination and skin biopsy

Patients underwent motor and sensory NCS using surface recording electrodes with standard placement. Methods used adhered to those recommended by experts of the International Federation of Clinical Neurophysiology [18].

NCS comprised sensory nerve action potentials (SNAPs) and conduction velocities recorded from sural, ulnar and superficial radial nerves. Other nerve function variables examined were compound motor action potential (CMAP) amplitude and peroneal, tibial and ulnar nerve conduction velocities. To study LEPs, we used a neodymium:yttrium-aluminium- perovskite (Nd:YAP) laser (wavelength 1.34 mm, pulse duration 2–20 ms, maximum energy 7 J). The dorsum of the right foot and the left hand was stimulated by laser pulses at relatively high intensity (150–200 mJ/mm2), short duration (5 ms), and small diameter (5 mm) eliciting pinprick sensations. The laser beam was shifted slightly after each stimulus. The interstimulus interval was varied pseudo-randomly (10–15 s). Subjects lay on a couch and wore protective goggles. They were instructed to keep their eyes open and gaze slightly downwards. To determine the laser perceptive threshold, we delivered a series of stimuli at increasing and decreasing intensities, and defined the perceptive threshold as he lowest intensity at which the subjects perceived at least 50% of laser stimuli. The early,

lateralized component, N1, and the main complex, N2–P2, were recorded through disc electrodes from the temporal areas (Tc) referenced to frontal area (Fz) and vertex (Cz) referenced to the nose. From 10 to 20 trials devoid of artefacts were collected and averaged offline. We measured peak latency and amplitude (peak-to-peak) of the temporal N1 component and the N2–P2 vertex complex. NCS and LEP data were compared with normative ranges established in our laboratory. Patients underwent skin biopsies from the proximal region of the thigh (20 cm below the anterior iliac spine) and the distal region of the leg (10 cm Above the lateral malleolus, with the sural nerve territory). Biopsies were taken after local anesthesia using a 3 m disposable punch under sterile technique. Three sections randomly chosen from each biopsy were immunoassayed with polyclonal anti-protein-gene- product 9.5 antibodies using the free-floating protocol for bright field immunohistochemistry [19,20]. The linear density of intraepidermal fibers was calculated following the rules reported by the guidelines of the European Federation of the Neurological Societies [21].

IENF data were compared with normative ranges established in our laboratory.

2.3. Statistical analysis

We used Mann–Whitney U-test to analyze the differences in neurophysiological and clinical data between patients with and without pain. Chi-square test was used to assess the frequency of the various qualities of neuropathic pain across the different aetiologies. We used the nonparametric Spearman's R correlation coefficient to correlate the intensity of ongoing and provoked pain, the most frequent qualities of pain, with foot-LEP amplitude, IENF density, and the intensity of Abnormal sensations with sural-SNAP, conduction velocity from sural nerve and foot-LEP amplitude. P values of <0.05 were considered significant. All results are reported as means ± SD.

3. Results

Of the 269 selected patients, all having distal, symmetric sensory disturbances mostly had a predominantly sensory neuropathy and 153 had pain . Whereas delay since

symptom onset was longer in patients with pain than in those without (3.8 ± 3.1 years vs 2.3 ± 1.7; P = 0.01, Mann–Whitney test), no difference was found between the two groups in age (65.2 years vs 63.6 years; P > 0.30) (Table 1). Although clinical assessment showed that most patients, regardless of pain, had sensory deficits involving all sensory modalities, pinprick and thermal thresholds assessed with laser stimuli were significantly higher in patients with pain than in those without (P < 0.001). Nine patients (seven with pain, two without) had an increased laser perceptive threshold but a clinically normal pinprick sensation.

No difference was found in the frequency of neuropathic pain and its different qualities between the various aetiologies (P > 0.5, χ2-test). NPSI analysis showed that nearly all patients had ongoing pain: 119 had burning pain (mean rating 6.9 ± 2.0). Of the 153 patients with pain 63 had also provoked pain (mean rating 5.8 ± 3.8) (Table 2). The various kinds of pain differed significantly in frequency (P < 0.0001, χ2-test), burning pain being more frequent and severe than the other types of pain. Patients

with provoked pain had lower pinprick and thermal laser thresholds than patients with ongoing pain ($P < 0.0001$, Mann–Whitney test).

Whereas LEP amplitude (both the N1 component and the N2–P2 complex) significantly differed between patients with and without pain ($P < 0.0001$), NCS and IENF density data did not ($P > 0.50$) (Fig. 1A, 1B and 2); nor did LEP latency and sensory conduction velocities differ in the two groups. All patients with distal symmetric neuropathy showed loss of intraepidermal nerve fibers (Table 1 and Fig. 1B).

Clinical data	
Age (years)	63.6 ± 11.1 65.2 ± 9.1
Delay since symptom onset (years)	2.3 ± 1.7 3.8 ± 3.1*
Warm threshold after hand stimulation (mJ/mm2)	37.3 1.7 48.6 ± 13.7**
Pinprick threshold after hand stimulation (mJ/mm2	6.5±27.1 90.7± 41.8**
Warm threshold after foot stimulation (mJ/mm2)	54.4 ± 10 71 ± 14.6**
Pinprick threshold after foot stimulation (mJ/mm2	3.6±30.8 104.8± 44.3**
NCS data	
Ulnar SNAP amplitude (µV)	4.4 ± 4.0 3. 9 ± 4.3
Ulnar SNCV (m/s)	48.1 ± 5.9 47.8 ± 4.6
Sural SNAP amplitude (µV)	3.6 ± 2.8 4.4 ± 5.3
Sural SNCV (m/s)	46.9 ± 3.5 47.3 ± 3.4
LEP data	
Latency of hand N1 (ms)	173.8 ± 14.9 179.9 ± 9.2
Amplitude of hand N1 (µV)	3.3 ± 2.9 1.5 ± 2**
Latency of hand N2 (ms)	222.2 2 ± 24.9 228.1 ± 21.3
Amplitude of hand N2–P2 (µV)	14.8 ± 13.3 6.5 ± 8.1**
Latency of foot N1 (ms)	206.1 ± 14.9 207.6 ± 24.8
Amplitude of foot N1 (µV)	2 ± 2.2 0.5 ± 1.2**
Latency of foot N2 (ms)	261.6 ± 26.4 276.8 ± 43.4

Amplitude of foot N2–P2 (μV)	12.4 ± 10.7	2.6 ± 5.4**

NCS, nerve conduction study; SNAP, sensory nerve actio potential; SNCV, sensory nerve conduction velocity; LE laser-evoked potentials.

ENF density (nerve fibres/mm)

Thigh 5.8± 3

Ankle 3± 2.3

P < 0.01.

* P < 0.0001.

Table 1. Clinical and neurophysiological data in patients with painful and non-painful neuropathy.

Comparisons between neurophysiological data in patients with the two more frequent and severe types of pain, namely ongoing and provoked pains, showed that the mean foot-LEP amplitude in patients with ongoing pain was lower than that in patients that have also provoked pain (7.4 vs 0.5 μV; $P < 0.0001$) (Fig.1 and 3). While the intensity of ongoing burning pain correlated inversely with the foot-LEP amplitude ($R = -0.4113$; $P = 0.0005$, spearman's R correlation coefficient), the intensity of provoked pain did not ($P > 0.2$) (Fig. 3). Neither sural-SNAP amplitude nor foot-LEP amplitude correlated with the intensity of abnormal sensations ($P > 0.2$); sural nerve conduction velocity approached statistical significance ($R = -0.2459$; $P = 0.09$). The frequency of NCS and LEP abnormalities was similar in the various aetiologies ($P > 0.5$, $\chi 2$-test).

4. Discussion

In this prospective clinical and neurophysiological study in a large cohort of patients with distal symmetric

polyneuropathy, we found that the development of pain depends not on age but on the duration of disease. We also provide neurophysiological evidence that non-nociceptive Aβ-fibre injury has no role in the development of neuropathic pain. Among the various pain qualities we studied, the most frequent, ongoing and provoked pains arise through different pathophysiological mechanisms. The correlation between ongoing burning pain with LEP suppression indicates that this type of pain is associated with axonal damage whereas the partially preserved LEPs in patients with provoked pain suggests that provoked pain is related to sensitized nociceptive nerve terminals. Our findings in patients with pain related to distal symmetric polyneuropathy may open the way to improved therapeutic strategies based no longer on aetiology but on the underlying pathophysiological mechanisms.

4.1. Clinical findings

When we assessed the various clinical qualities of neuropathic pain in distal symmetric polyneuropathy

according to the NPSI, we found that burning pain was the most frequent (96% of patients) and severe (mean rating 6.9) type of pain, followed by provoked pains (hyperalgesia-allodynia). No other pain types were frequent enough to allow us to test any reliable correlation with neurophysiological measures. Our finding that age did not differ between patients with and without pain seems to contrast with previous studies showing that the incidence of neuropathic pain conditions peaks in the elderly [11]. These reported data probably merely reflect the higher frequency of peripheral nerve diseases in the elderly, rather than an age-related development of neuropathic pain (e.g. the prevalence of peripheral neuropathy rises from 2.4% in the general population to 8% in subjects older than 55 years) [23]. The lack of a significant association between pain and age suggests that the age-related changes in the somatosensory system, reported in many clinical studies [16], have no influence on the development of pain in distal symmetric peripheral neuropathy.

Patients (%)	Intensity 0–10 points (mean ± SD)
Burning pain	96 6.9 ± 2.0
Pressing pain	38 4.6 ± 1.0
Paroxysmal pain	26 6.0 ± 2.0
Provoked pain	44 5.8 ± 3.8
Abnormal sensations	59 4.5 ± 1.9

Table 2. NPSI data.

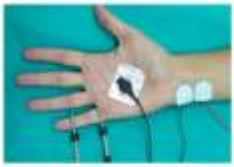
Stimulation and Recording	Non-painful Neuropathy	Painful Neuropathy
Ulnar NCS 	SNAP  2 ms 10μV	SNAP  2 ms 10μV
Hand LEPs 	N1 Tc-Fz N2 Cz-nose P2 200ms 10μV	N1 Tc-Fz N2 Cz-nose P2 200ms 10μV
Sural NCS 	2 ms 10μV	2 ms 10μV
Foot LEPs 	N1 Tc-Fz N2 Cz-nose P2 200ms 10μV	Tc-Fz N2 Cz-nose P2 200ms 10μV

Fig. 1A. Neurophysiological assessment in patients with painful and non-painful neuropathy. Neural signals recorded in patients with painful and non-painful neuropathy (black lines) are superimposed to neural signals recorded in a representative normal subject (blue lines). While nerve conduction study data were similar in the two patients, the patient with pain had Abnormal LEPs. NCS: nerve conduction study. SNAP: sensory nerve action potential. LEP: laser-evoked potentials.

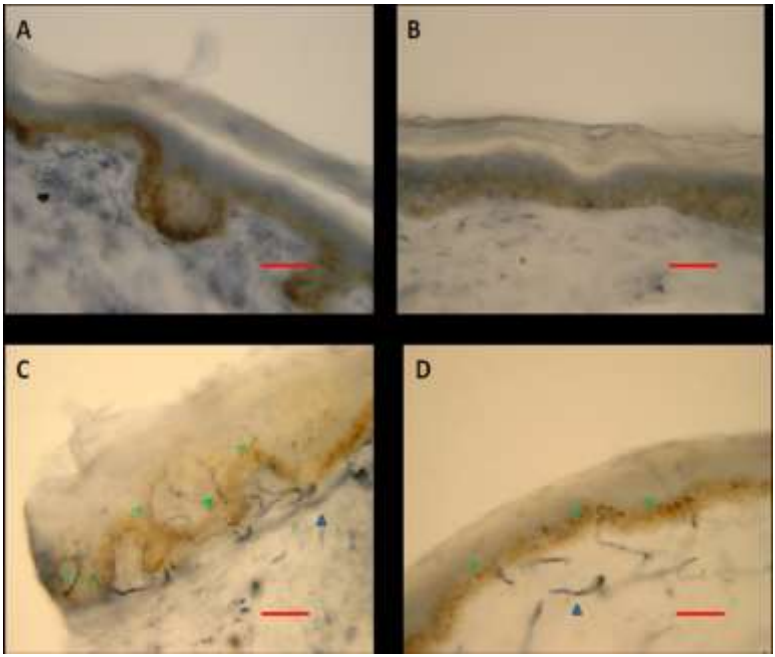


Fig 1B. **A** and **B** skin biopsies images (X40) (from thigh and ankle respectively)showing severe abnormalities in patient with distal symmetric neuropathy- complete loss of IENF and subepidermal plexus-, compared to control (**C,D**). Green arrows show single nerve fibers arising from subepidermal neural plexus bundles (blue arrowshead) and penetrate the basement membrane, losing their Schwann cell sheath; then, as naked axons, they cross th entire epidermis reaching the stratum corneum with an even distribution in hairy skin, and cluster at the apex of dermal papillae in glabrous skin (**C**). Red s**cale bar = 70 µm.**

A previously unreported finding was that in patients with pain delay since symptom onset is longer than in those without pain. Because our patients' pain was associated with nociceptive pathway damage, we suggest that in distal symmetric polyneuropathy the damage to nociceptive pathways usually follows non-nociceptive Aβ-fibre injury. Hence the longer the disease lasts the greater the likelihood of nociceptive fibre damage developing and provoking neuropathic pain.

4.2. Role of Aβ fibres

NCS studies disclosed no differences between patients with and without pain. Nor did NCS data correlate with pain intensity. These findings exclude the possibility that Aβ-fibre damage plays any noteworthy role in pain associated with distal symmetric peripheral neuropathy. The previously proposed theory that Aβ fibre loss disinhibits nociceptive pathways thus provoking pain [2,9,14] originates from the ''gate control theory" and is

indirectly supported by evidence that high-frequency low-intensity electrical stimulation applied to peripheral nerves or spinal cord attenuate pain [8].

Because the Aβ fibre damage in our patients was unrelated to the development of pain (at least of ongoing and provoked pains), we propose that although the input from Aβ fibres modulates pain transmission in the central nervous system, Aβ fibre loss does not per se provoke pain.

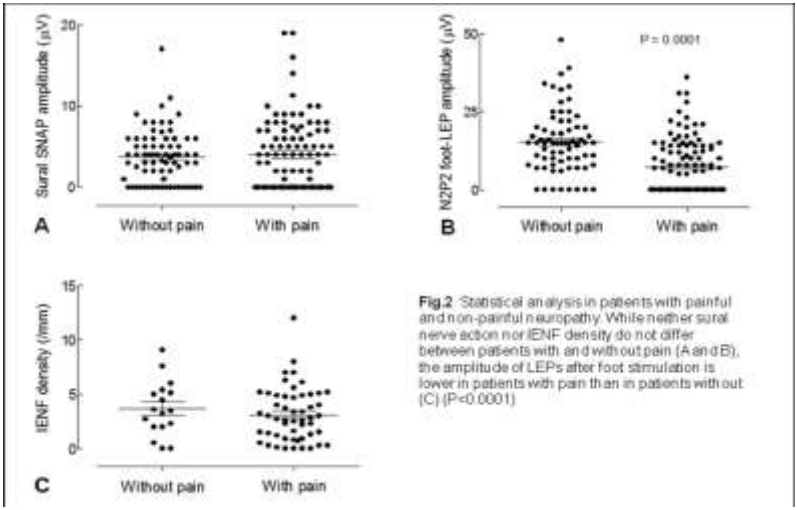


Fig.2 Statistical analysis in patients with painful and non-painful neuropathy. While neither sural nerve action nor IENF density do not differ between patients with and without pain (A and B), the amplitude of LEPs after foot stimulation is lower in patients with pain than in patients without (C) (P<0.0001)

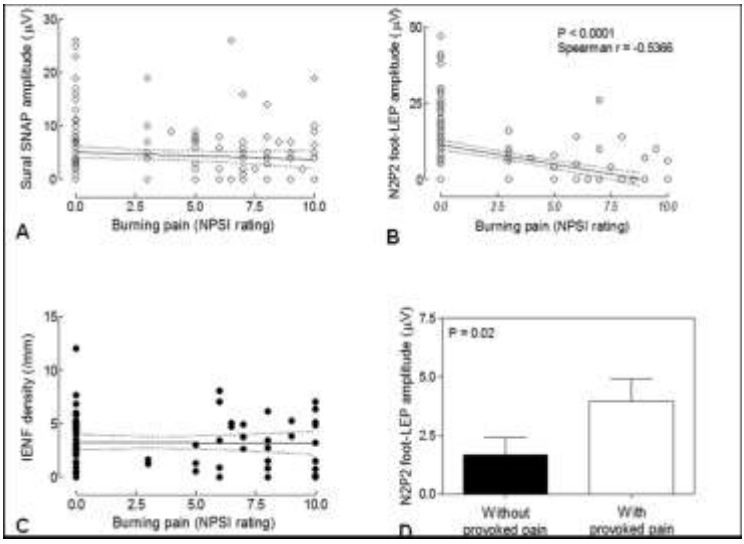


Fig. 3 Statistical analysis in patients with burning pain, the most frequent quality of ongoing pain and Sural nerve amplitude (A), LEP amplitude (B) and IENF density (C): only foot LEP amplitude significantly correlated with the severity of ongoing pain (R = -0.4113; P = 0.0005). Foot LEP amplitude was bigger in patients who complained also provoked pain (D).

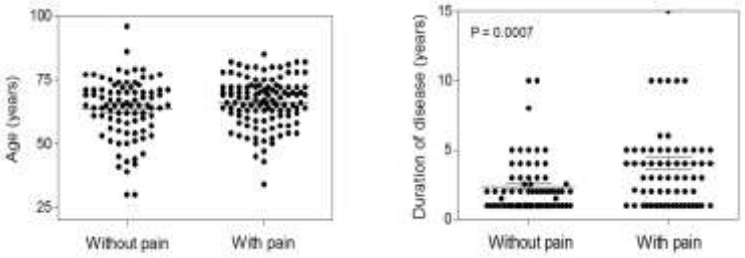


Fig. 3 Symptom onset was longer in pts with pain than in those without (3.8 ± 3.1 y vs 2.3 ± 1.7; p=0.01).

4.3. Ongoing pain

When we investigated the pathophysiological mechanisms underlying the various qualities of neuropathic pain by comparing LEP data in patients distinguished by the NPSI we found far smaller amplitudes in patients with ongoing pain than in those without pain and the correlation between LEP attenuation and pain intensity Intensity was highly significant. Whereas the intensity of burning pain, the most frequent quality of ongoing pain, correlated inversely with the foot-LEP amplitude, it did not with the sural sensory nerve action potential nor with IENF. This neurophysiological finding shows that ongoing pain in

distal symmetric polyneuropathy is related to the damage of nociceptive axons. In their study on central pain, Garcia-Larrea et al. [15] found that ongoing pain was associated with markedly reduced amplitude LEPs. Whereas ongoing pain in patients with central pain presumably depends on a classic deafferentation mechanism, this explanation cannot hold true for ongoing pain in distal symmetric peripheral neuropathy. In length-dependent neuropathy, the short distance between dorsal root ganglion cells and dorsal horn prevents an anatomical deafferentation of the second-order neurons. We conjecture that the development of ongoing pain involves other pathophysiological mechanisms. Microneurographic studies in humans reported that in patients with peripheral neuropathies burning pain is associated with spontaneous, anomalous discharges in afferent fibres [4,25]. Skin biopsy studies describe reduced intraepidermal nociceptive terminals in patients with ongoing pain related to peripheral neuropathy [27,34]. In agreement with skin biopsy studies, we found that patients with ongoing pain had severely reduced amplitude LEPs (laser stimuli activate the

intraepidermal nociceptive terminals, rather than the nerve axons). We therefore hypothesize that ongoing pain reflects the abnormal, spontaneous activity originating in damaged nociceptive fibre axons that have lost their intraepidermal terminals. Two previous studies by our group showed that in patients with postherpetic neuralgia and carpal tunnel syndrome, ongoing pain correlated with LEP abnormalities and paroxysmal pain correlated with Aβ-fibre damage [32,33]. Our present findings on ongoing pain in patients with distal symmetric polyneuropathy agree with these two previous studies; in this study we could not reliably assess a possible correlation between paroxysmal pain and A-beta fibre damage, as assessed with NCS, because only a few patients with distal symmetric polyneuropathy had paroxysmal pain.

4.4. Provoked pain

Although we found reduced amplitude LEPs also in patients with provoked pain, the LEP attenuation was significantly lower than that in patients with ongoing pain. This difference replicates in peripheral neuropathy the

findings by Garcia-Larrea et al. [15] in patients with central pain. The partially preserved LEPs in patients with provoked pain suggest that provoked pain reflects peripheral sensitisation. Over the past decades, ample evidence underlines a possible role for sensitised nociceptive terminals as primary determinants of pain in humans. Previous studies directly demonstrated abnormally reduced C nociceptor thresholds to mechanical stimuli in patients with provoked pain [6,25]. Whether central sensitisation also participates in the development of provoked pain remains open to question. In patients with peripheral neuropathy the spontaneous firing of nociceptive fibres, demonstrated by microneurographic recordings [4], may sensitise central nociceptive neurons to mechanically evoked input [28]. In patients with chronic neuropathic pain, differential nerve blocks showed that provoked pain was abolished concurrently with loss of tactile sensations at a time when unmyelinated fibres were still unaffected [21]. Our neurophysiological findings suggest, though do not prove, that provoked pain arises through still intact, and sensitised nociceptive nerve

terminals. According to our hypothesis in distal symmetric polyneuropathy,provoked pain is due to an abnormal lowered mechanical threshold of intraepidermal nerve terminals and conversely ongoing pain is related to the spontaneous activity in damaged nociceptive axons that have lost their intraepidermal terminals. All patients with pain were taking medications. Although these medications reduced pain intensity of about 50% in most of them, no drug completely abolished any of the different types of pain complained by patients. Thus we believe that our data are not significantly influenced by treatment.

4.5. Aetiology-independency

Pain onset was not influenced by the aetiology. Whereas delay since symptom onset was longer in patients with pain than in those without, no difference was found between the two groups in age (Fig. 3). This observation, together the neurophysiological evidence of damage of nociceptive pathway in patients with neuropathic pain, leads to conclude that in distal symmetric polyneuropathy the injury of nociceptive fibre follows the injury of non-

nociceptive fibres. So distal symmetric polyneuropathy could be complicated by neuropathic pain as much as its duration. Loss of IENF does not reflect the presence of neuropathic pain: unmyelinated fibre function is available even if IENF are decreased, and *viceversa* it could be damaged even if they are visible. Loss of intraepidermal fibres is a biomarker of neuropathy.

In this large cohort of patients with distal symmetric peripheral neuropathy with and without pain, we found no differences in pain frequencies, pain type, and LEP or NCS abnormalities according to aetiology. Conversely, regardless of the aetiology, our clinical and LEP studies identified two main ''pain phenotypes" [3]: patients with ongoing pain and LEP suppression and patients who also complain of provoked pains, with a less severe LEP attenuation. This difference strongly suggests that different types of pain arise through distinct mechanisms regardless of aetiology, thus calling for a change in the way we classify and treat patients with neuropathic pain in clinical practice. As recent European guidelines [1] recommend, we agree that instead of grouping patients by aetiology,

they should be grouped according to the various qualities of pain. This approach might minimize pathophysiological heterogeneity within the groups under study and increase the power to detect a positive treatment result.

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Chapter 7: **In patients with distal symmetric polyneuropathy allodynia is mediated by sensitization of peripheral nociceptors**

Introduction

In patients with peripheral neuropathy neuropathic pain manifests with spontaneous and provoked symptoms (Fig. 1). Spontaneous symptoms include ongoing pain and paroxysmal pain, while provoked pain frequently consists of mechanical dynamic allodynia, i.e. pain in response to a normally non-painful brushing. The reference standard test for diagnosing peripheral neuropathy is the nerve conduction study (NCS). NCS nevertheless has the disadvantage of assessing non-nociceptive, large-myelinated fibres (Aβ fibres) alone and provides no information on nociceptive pathway function. Current neurophysiological assessment of nociceptive pathways

relies on recording laser evoked potentials (LEPs). Laser-generated radiant heat pulses selectively activate Aδ and C mechano-thermal nociceptors, and evoke scalp potentials related to small myelinated (Aδ) fibres. LEPs are the most reliable and agreed neurophysiological method for investigating nociceptive fibre function in patients with pain [1,2].

Although allodynia is a frequent complaint in patients with peripheral neuropathy, its underlying mechanisms is still debated. Most authors consider allodynia to be generated at the central level [3]. In patients with peripheral neuropathy the spontaneous firing of damaged nociceptive afferents may evoke ongoing pain and as a secondary effect sensitize second-order nociceptive neurons to Aβ-fibre input, thus inducing allodynia [3,4]. However some observations suggested that provoked pains, including allodynia, may be due to an abnormal reduction of the mechanical threshold in sensitized nociceptors.

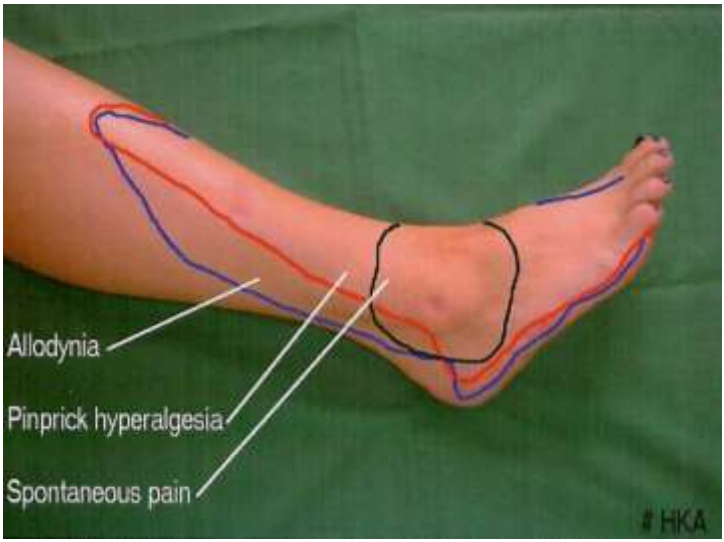

Fig.1 In patients with peripheral neuropathy neuropathic pain manifests with spontaneous and provoked symptoms. Spontaneous symptoms include ongoing pain and paroxysmal pain, while provoked pain frequently consists of mechanical dynamic allodynia, i.e. pain in response to a normally non-painful brushing.

More information on the pathophysiological mechanisms underlying allodynia related to distal symmetric polyneuropathy could be used to develop more effective therapeutic approaches for this type of pain. In this prospective clinical and neurophysiological study in

patients with distal symmetric polyneuropathy we investigated the role of non-nociceptive Aβ and nociceptive Aδ afferent fibres on the development of mechanical dynamic allodynia. To do so we investigated Aβ-fibre function with standard nerve conduction study (NCS) and nociceptive Aδ-fibre function with laser evoked potentials (LEPs).

Methods

We prospectively collected 200 patients with distal symmetric polyneuropathy (114 with pain and 86 without). The diagnosis was based on clinical, biological, and electrodiagnostic findings, adhering to the criteria proposed by England et al. (i.e. patients with symmetrical reduction or absence of ankle reflexes, decreased distal sensation, and abnormal NCS or skin biopsy). We included patients with four different aetiologies: diabetes-related neuropathy (70), chemotherapy-induced neuropathy (53), cryoglobulinemia related neuropathy (30), neuropathy of unknown origin (47). Exclusion criteria were sensory

disturbances due to neurological diseases other than distal symmetric polyneuropathy and cognitive impairment. To limit the heterogeneity of aetiologies we also excluded patients with different kinds of neuropathy, when less than 10 patients was collected. Two staff members examined the patients clinically and others did neurophysiological testing, with those recording NCS being blinded to LEP data and vice-versa. The research was approved by the local Ethical Committee. All patients underwent clinical examination using bedside tools. Patients were grouped according to the clinically documented presence or absence of neuropathic pain, as assessed by the DN4 questionnaire. The DN4 questionnaire is clinician-administered screening tool that comprises various clinical items including allodynia and indicates neuropathic pain when the score is ≥4. Patients with neuropathic pain were further divided in two groups: with and without allodynia, as assessed by the dedicated items of the DN4 (all patients without allodynia suffered from ongoing pain). Patients also underwent motor and sensory NCS using surface recording electrodes with standard placement. Methods used adhered to those

recommended by experts of the International Federation of Clinical Neurophysiology [18]. NCS comprised sensory nerve action potentials (SNAP) and conduction velocities recorded from sural, ulnar and superficial radial nerves. Other nerve function variables examined were compound motor action potential (CMAP) amplitude and peroneal, tibial and ulnar nerve condition velocities. We studied LEPs using a neodymium:yttrium-aluminium-perovskite (Nd:YAP) laser. The dorsum of the right foot and the left hand were stimulated by laser pulses at relatively high intensity (150–200 mJ/mm2), short duration (5 ms), and small diameter (~5 mm) eliciting pinprick sensations. Subjects lay on a couch and wore protective goggles. To determine the laser perceptive threshold we delivered a series of stimuli at increasing and decreasing intensity, and defined the perceptive threshold as the lowest intensity at which the subjects perceived at least 50% of laser stimuli. The early, lateralized component, N1, and the main complex, N2-P2, were recorded through disc electrodes from the temporal areas (Tc) referenced to frontal area (Fz) and vertex (Cz) referenced to the nose. From 10 to 20 trials

devoid of artefacts were collected and averaged offline. We measured peak latency and amplitude (peak-to-peak) of the temporal N1 component and the N2-P2 vertex complex. NCS and LEP data were compared with normative ranges established in our laboratory.

Statistical analysis

Mann-Whitney U-test was used to analyse the differences in neurophysiological data between patients with and without pain according to the DN4, and (within the group of patients with pain) neurophysiological differences between those with and without allodynia. The differences of frequency of neuropathic pain and allodynia across the different aetiologies were analysed with the Chi-square test and those of neurophysiological data with the Kruskal-Wallis test. P values of < 0.05 were considered significant. All results are reported as mean ± SD.

Results

Of the 200 selected patients, all having distal, symmetric sensory disturbances most had a predominantly sensory neuropathy, 114 with pain and 86 without. Although clinical assessment showed that most patients, regardless of pain, had sensory deficits involving all sensory modalities, pinprick and thermal thresholds assessed with laser stimuli were significantly higher in patients with pain than in those without ($P < 0.001$, Mann-Whitney test). In the group of patients with pain DN4 identified 44 patients with allodynia, 70 without. Chi square test showed no differences in frequency of pain and allodynia across the different aetiologies ($P > 0.5$). Kruskal-Wallis test showed no differences in neurophysiological data across the different aetiologies ($P > 0.5$). Whereas LEP amplitude (both the N1 component and the N2-P2 complex) was significantly lower in patients with pain than in those without ($P < 0.0001$, Mann-Whitney test), NCS data did not differ ($P > 0.2$); nor did LEP latency and sensory

conduction velocities differ in the two groups (P > 0.2). Comparisons between neurophysiological data in patients with allodynia showed that while the mean foot-LEP amplitude was higher in patients with allodynia than in those with ongoing pain only, (P = 0.006), NCS data (including the sural SNAP amplitude) did not differ (P > 0.2).

Discussion

In this prospective clinical and neurophysiological study in a large cohort of patients with distal symmetric polyneuropathy we found that neuropathic pain is associated with a damage of nociceptive pathway as assessed by LEP recordings. Furthermore the partially preserved LEPs in patients with allodynia suggests that this type of pain is related to partially preserved and sensitised nociceptive nerve terminals. In this study we investigated a large cohort of patients with distal symmetric polyneuropathy due to different causes. When we investigated neurophysiological differences between

patients with and without allodynia we did not distinguish across the specific aetiology, because previous studies showed that neuropathic pain does not depend on the aetiology [5]. Also in this study we found no differences in pain frequencies, LEP or NCS abnormalities according to aetiology. Hence we believe that to seek information on pain mechanisms patients should not be grouped by aetiology. In our cohort of patients with distal symmetric polyneuropathy we also included patients with cryoglobulin-related neuropathy. Although in this condition nerve damage is due to the vasculitis, and thus multiple mononeuropathy should be expected, all our patients presented with distal symmetric sensory disturbances. This feature is probably due to the low temperature at which cryoglobulins precipitate. The extremity temperature is usually lower that the rest of the body, thus increasing the possibility of cryoglobulin precipitation causing vascular occlusion and nerve damage. All patients with pain were taking medications. Although these medications reduced pain intensity of about 50% in many of them, no drug completely abolished

any of the different types of pain complained by patients. Thus we believe that our data are not significantly influenced by treatment. We found that while the amplitude of LEPs was significantly smaller in patients with pain, NCS data did not differ. These findings support the current knowledge on neuropathic pain. Previous clinical, neurophysiological, and neuropathological investigations showed that in patients with peripheral neuropathy of various aetiologies neuropathic pain is invariably associated with nociceptive pathway damage and unrelated to Aβ-fibre damage [5]. We may hypothesize that in patients with distal symmetric polyneuropathy pain arises from damaged and dysfunctioning nociceptive fibres. Comparisons between neurophysiological data in patients with and without allodynia showed that NCS data did not differ. This finding argue against the possible role of Aβ-fibres. Whether allodynia were mediated by Aβ-fibres, a partial, though statistically significant, preservation of this type of afferent fibres should be detected in a large sample of patients. The lack of any differences in Aβ-fibre mediated

NCS between patients with and without allodynia suggest that this set of fibre is dissociated from allodynia. Although we found a reduced amplitude of LEPs also in patients with allodynia the LEP attenuation was significantly lower than that in patients without this type of pain. This finding indicates that patients with allodynia have partially spared nociceptive afferents, and suggests that allodynia might be mediated by peripheral sensitization of nociceptors, manifesting with an abnormal lowered mechanical threshold of intraepidermal nerve terminals. Over the past decades, ample evidence underline a possible role for sensitised nociceptive terminals as primary determinants of pain in humans. Previous studies directly demonstrated abnormally reduced C nociceptor thresholds to mechanical stimuli in patients with provoked pain [6]. In patients with postherpetic neuralgia many studies showed that allodynia correlates with the sparing of thermal sensation [7], thus suggesting the need of a relative sparing of thermal-pain afferent fibres. Support for the peripheral sensitization as the main mechanism for allodynia comes also from

placebo-controlled trials showing that topical application of lidocaine and capsaicin provides significant pain relief [8]. Admidettly our data cannot exclude the possibility that central sensitization participates in the development of allodynia, but strongly suggest that it is unnecessary. Our study showing that allodynia is associated with partially preserved nociceptive afferent fibres and unrelated to Aβ-fibres could be useful in designing new treatment strategies targeted to this type of pain.

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Chapter 8: Clinical, neurophysiological and skin biopsy study of peripheral neuropathy related to cryoglobulinaemia

Introduction

In addition to Meltzer's triad of purpura, weakness and arthralgias, the neurological system could be involved in a significant proportion of patients affected by cryoglobulinemia in different ways, as peripheral neuropathy, cranial nerve involvement and vasculitic central nervous system involvement [1] (Table 1 and 2). The neurological complications are predominantly in the peripheral nervous system and are mainly associated with mixed cryoglobulinaemia [1]. Peripheral neuropathy range from pure sensory axonopathy to mononeuritis multiplex [2]. The most frequently described form is a distal sensory or sensory-motor peripheral neuropathy [3]. Prevalence of

peripheral neuropathy varied widely. In a prospective study of 321 patients with chronic HCV infection, 50% of whom were cryoglobulin positive, clinically symptomatic sensory or motor peripheral neuropathy was found in 9% [4]. In a study of 26 HCV-mixed cryoglobulinaemia patients [5], neurologic examination revealed a neuropathy in 48% of subjects, while electrophysiologic variables were altered in 82%. The *tempo* of the vasculitic neuropathy may be subacute, chronic, or acute on chronic [6]. The exacerbation of neuropathy occurs simultaneously with the failure of other organs, as a result of the increased activity of the underlying vasculitis. In patients with distal polyneuropathy, nerve conduction studies are in keeping with a predominantly axonal process, mainly affecting the sensory nerves. Neuropathological data show axonal degeneration, differential fascicular loss of axons, signs of demyelinization and small-vessel vasculitis, with mononuclear cell infiltrates in the perivascular area (Fig. 1a) [7].

Clinical manifestations	Type I	Type II	Type III
Arthralgia ≫ arthritis	+	++	+++
Purpura	+, usually nonpalpable	+++, palpable	+++, palpable
Gangrene/acrocyanosis	+++	+	±
Hyperviscosity	+++	±	−
Hematologic	++	±	±
Renal	+	++	+
Neurologic	+	++	++
Liver	±	++	+++
Lung	−	+	+

Table 1. Salient Clinical features of the crioglobulinemia syndromes: cryoglobulinaemic vasculitis (CV) is a systemic vasculitis associated with serum positive cryoglobulins that is, immune complexes composed of rheumatoid factor (RF) monoclonal or polyclonal against polyclonal IgG (type II or type III cryoglobulins, respectively) or immunoglobulins without RF activity (type I), which reversibly precipitate or form a gel at a temperature below 37°C. Type I and II CV are usually linked to non- malignant B-cell lymphoproliferation, type III often triggered by chronic hepatitis C virus infection.

Clinical manifestations	Type I	Type II	Type III
Cryocrit	>5%	<5% (1–2)	<5% (1–2)
C3	Normal	Decreased	Decreased
C4	Normal	Decreased	Decreased
CH_{50}	Normal	Decreased	Decreased
RF	+	++	++
Autoantibodies (ANA, ENA, AMA, etc.)	–	++	++
Hepatitis B	–	+	+
Hepatitis C	–	+++	+++

RF, rheumatoid factor; ANA, antinuclear antibodies; ENA, antibodies to extractable nuclear antigens [such as Sm, RNP, Ro (SS-A), La (SS-B), Jo-1, Scl-70)]; AMA, antimitochondrial antibodies. Hematologic features include thrombosis and bleeding and spurious/artifact thrombocytosis. Adapted from Refs. 1,2,10–13.

Table 2. Laboratory features of the crioglobulinemia syndromes

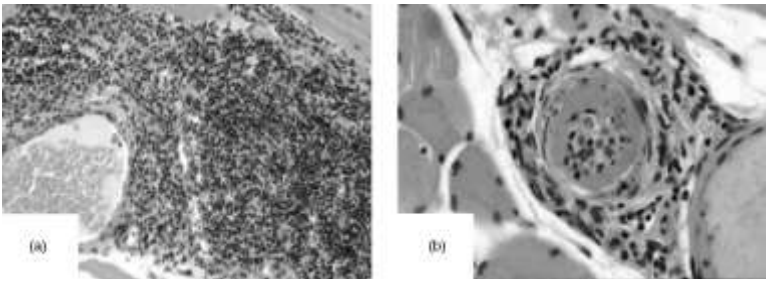

Fig. 1. Transversal section of superficial peroneal nerve biopsy from patients with hepatitis C virus-associated mixed cryoglobulinaemia peripheral neuropathy. (a) Perivascular inflammatory mononuclear cell infiltrates. Mononuclear cells did not invade the blood vessel wall (-25). (b) Necrotizing arteritis with perivascular and transluminal inflammatory cell infiltration and concurrent wall fibrinoid necrosis (-400). Images from Rev Neurol (Paris) 2002; 158:920–924.

Polyneuropathy usually presents with painful, asymmetric paresthesias that later become symmetric [8]. The pain is positively associated with the presence of vasculitis [8].

We designed this clinical, neurophysiological and histopathological study in patients with distal symmetric neuropathy related to cryoglobulinemia in attempt to make correlations between clinical features, such age and

duration of disease, and to investigate the role of primary afferent neurones (non-nociceptive Aβ and nociceptive Aδ and C afferent fibers) on the development of neuropathic pain. We assessed clinical variables including the various qualities of neuropathic pain with the NPSI, Aβ -fibers function with nerve conduction study (NCS), Aδ function with laser evoked potentials (LEPs) and C-fibers with skin biopsy and IENF density.

2.Methods

We prospectively collected 48 patients with cryoglobulinemia. The diagnosis was based on clinical, pathologic and serologic findings, adhering to the criteria proposed by Ferri C et al. [9]. All patients underwent clinical examination, nerve conduction study, LEP recordings and skin biopsy. One staff member examined the patients clinically and administered the NPSI questionnaire ; others did neurophysiological testing and skin biopsy, with all the members being blinded each other . We collected 30 patients with peripheral neuropathy: 23

with neuropathic pain and 7 without. The diagnosis of distal symmetric polyneuropathy was based on clinical, biological, and electrodiagnostic findings, adhering to the criteria proposed by England et al. [10]. Patients with symmetrical reduction or absence of ankle reflexes, decreased distal sensation, and abnormal NCS or LEPs were included in this study. Exclusion criteria were diagnosis of inflammatory or inherited neuropathies, sensory disturbances due to neurological diseases other than distal symmetric polyneuropathy cryoglobulinemia related, cognitive impairment. The research was approved by the local Ethical Committee.

2.1 Clinical examination

All patients underwent a detailed neurological examination using bedside tools. Tactile sensation was investigated with a piece of cotton wool, vibration with a tuning fork (128 Hz), and pinprick sensation with a wooden cocktail stick. In all patients laser stimuli were used for a quantitative evaluation of warm and pinprick

sensations. Gait impairment, and muscle strength were assessed with the Medical Research Council score. Patients were also asked to report dysautonomic symptoms. Patients were grouped according to the clinically documented presence or absence of neuropathic pain rated ≥4 on the 0–10 numerical rating scale and persisting since at least one month [11]. Patients with pain completed the NPSI questionnaire. The NPSI subscores were calculated for the various types of pain: ongoing pain (burning and pressing pain), paroxysmal, provoked pain and abnormal sensations (paraesthesias and dysaesthesia).

2.2. Neurophysiological examination and skin biopsy

Patients underwent motor and sensory NCS using surface recording electrodes with standard placement. Methods used adhered to those recommended by experts of the International Federation of Clinical Neurophysiology [12]. NCS comprised sensory nerve action potentials (SNAPs) and conduction velocities recorded from sural, ulnar and superficial radial nerves. Other nerve function variables

examined were compound motor action potential (CMAP) amplitude and peroneal, tibial and ulnar nerve conduction velocities. To study LEPs, we used a neodymium:yttrium-aluminium- perovskite (Nd:YAP) laser (wavelength 1.34 mm, pulse duration 2–20 ms, maximum energy 7 J). The dorsum of the right foot and the left hand was stimulated by laser pulses at relatively high intensity (150–200 mJ/mm2), short duration (5 ms), and small diameter (˜5 mm) eliciting pinprick sensations. The laser beam was shifted slightly after each stimulus. The interstimulus interval was varied pseudo-randomly (10–15 s). Subjects lay on a couch and wore protective goggles. They were instructed to keep their eyes open and gaze slightly downwards. To determine the laser perceptive threshold, we delivered a series of stimuli at increasing and decreasing intensities, and defined the perceptive threshold as the lowest intensity at which the subjects perceived at least 50% of laser stimuli. The early, lateralized component, N1, and the main complex, N2–P2, were recorded through disc electrodes from the temporal areas (Tc) referenced to frontal area (Fz) and vertex (Cz)

referenced to the nose. From 10 to 20 trials devoid of artefacts were collected and averaged offline. We measured peak latency and amplitude (peak-to-peak) of the temporal N1 component and the N2–P2 vertex complex. NCS and LEP data were compared with normative ranges established in our laboratory. Patients underwent skin biopsies from the proximal region of the thigh (20 cm below the anterior iliac spine) and the distal region of the leg (10 cm above the lateral malleolus, with the sural nerve territory). Biopsies were taken after local anesthesia using a 3 mm disposable punch under sterile technique. Three sections randomly chosen from each biopsy were immunoassayed with polyclonal anti-protein-gene- product 9.5 antibodies using the free-floating protocol for bright field immunohistochemistry [13,14]. The linear density of intraepidermal fibers was calculated following the rules reported by the guidelines of the European Federation of the Neurological Societies [15]. IENF data were compared with normative ranges established in our laboratory.

2.3. Statistical analysis

We used Mann–Whitney U-test to analyze the differences in neurophysiological and clinical data between patients with and without pain. Chi-square test was used to assess the frequency of the various qualities of neuropathic pain. We used the nonparametric Spearman's R correlation coefficient to correlate the intensity of ongoing-burning pain and allodynia, the most frequent qualities of pain, with foot-LEP amplitude, IENF density. P values of <0.05 were considered significant. All results are reported as means ± SD.

3. Results

Of the 30 selected patients, all having distal, symmetric sensory disturbances mostly had a predominantly sensory neuropathy and 23 had pain as assessed by the DN4 questionnaire. The NPSI showed that the most frequent types of pain were the ongoing burning pain and allodynia. NPSI analysis showed that nearly all patients

had ongoing pain: 14 had burning pain (mean rating 6.5 ± 2.0). Of the 14 patients with pain 10 had also allodynia (mean rating 5.8 ± 3.8). The various kinds of pain differed significantly in frequency ($P < 0.0001$, $\chi 2$-test), burning pain being more frequent and severe than the other types of pain. Patients with peripheral neuropathy had an older age than those without (P=0.03, Mann Whitney test) (Fig. 2).

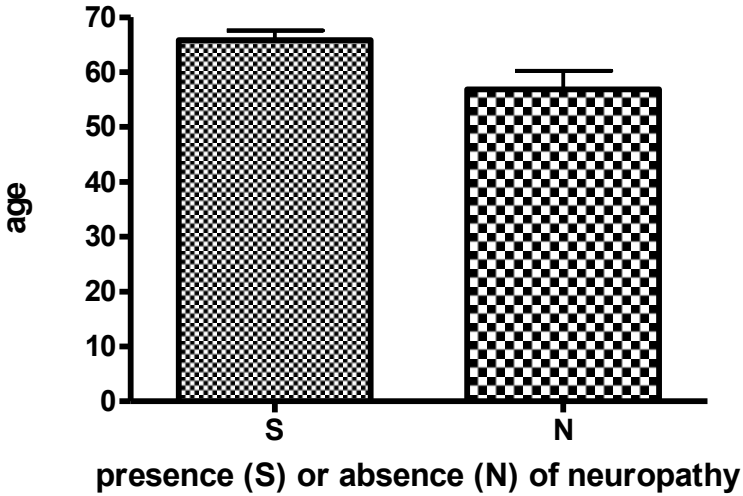

Figure 2. Patients with peripheral neuropathy had an older age than those without (P=0.03, Mann Whitney test)

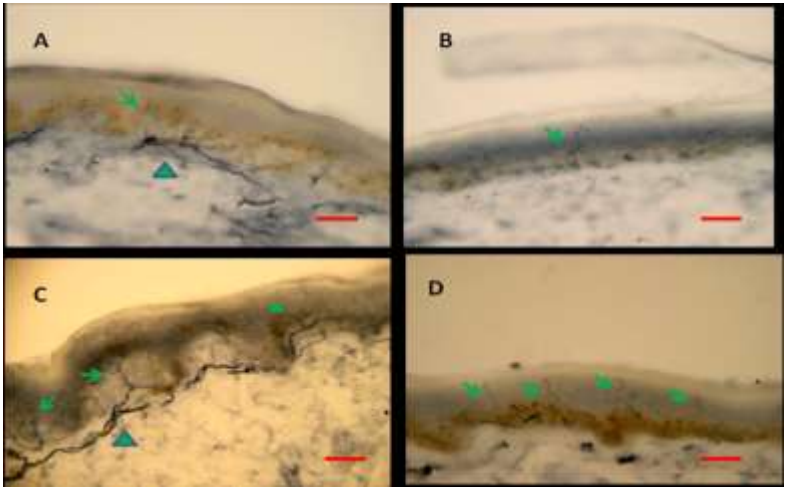

Fig .3 **A** and **B** skin biopsies images (X40) (from thigh and ankle respectively) in patient with distal symmetric neuropathy showing lower IENF and subepidermal plexus than the control (**C,D**). Green arrows show single nerve fibers arising from subepidermal neural plexus bundles (green arrowshead). Red s**cale bar = 70 µm.**

	'ainful	**Non-painful**
	Neuropathy (23)	**Neuropathy (7)**
Clinical data		
Age (year)	65.4±8.9	57.7 ± 13
NCS data		
Sural SNAP (µV)	2.6±5	3.5±8.8
Ulnar SNAP (µV)	7±38	10±5.4
SEP data		
N1 hand (µV)	*).78±2.5*	..96±2.6*
N1 hand (ms)	243±72	209±27
N2-P2 hand (µV)	3.1 ±7.5	16.4±12*
N2-P2 hand (ms)	264.5±28	247±31
N1 foot (µV)	3±7	3.1±3.4*
N1foot (ms)	186±21	163±20

N2-P2 foot (μV)	11±11.1	28±8.9*
N2-P2 foot (ms)	320±35	211±30
IENF (f/mm)		
THIGH	5.7±3	6.5±2.8
ANKLE	1.9±1.75	3.2±1.75

Table 3. Clinical, neurophysiological and morphological data in pts with painful and non-painful neuropathy. NCS, nerve conduction study; SNAP, sensory nerve action; LEP, laser evoked potential; IENF, intraepidermal fibers. *P<0.0001

Whereas LEP amplitude (both the N1 component and the N2–P2 complex) significantly differed between patients with and without pain ($P < 0.0001$), NCS and IENF density data did not ($P = 0.6$). as assessed by skin biopsy (Fig. 3) (Table 3).

4. Discussion

In this prospective (although evaluated at one time point only, the patients were sequentially examined and recruited) clinical and neurophysiological study in a cohort of patients with cryoglobulinemia, we collected all patients with distal symmetric sensory neuropathy. Although in cryoglobulinemia nerve damage is due to the vasculitis, and thus multiple mononeuropathy should be expected, no-one presented mononeuropathy. This feature is probably due to the low temperature at which cryoglobulins precipitate. The extremity temperature is usually lower that the rest of the body, thus increasing the possibility of cryoglobulin precipitation causing vascular occlusion and nerve damage. Moreover, our patients

predominantly showed sensory polyneuropathy. The possible mechanism may be in the pathogenesis of neuropathy vasculitis-related: the small vessels of the sensory nerves fascicles are more sensitive than those of motor fascicles [16].

We found that whereas LEP amplitude significantly differed between patients with and without pain, NCS data did not. We provide neurophysiological evidence that non-nociceptive Aβ-fibre injury has no role in the development of neuropathic pain. Moreover, the correlation between ongoing burning pain with LEP suppression indicates that this type of pain is associated with axonal damage of nociceptive fibers, as assessed previously [17]. Although IENF density technique assesses nociceptive fibers as LEPs recordings, we found that only LEPs data have correlations with the severity of neuropathic pain. This could be explained by the fact that skin biopsy selectively shows ENFs arising from subepidermal neural plexus bundles and penetrating the basement membrane, losing their Schwann cell sheath. Whereas laser radiant-heat pulses excite the free nerve

endings both Aδ and C in the superficial skin layers. In clinical practice, their main limitation is that ultralate LEPs (related to C-fibre activation) are technically more difficult to record. They are usually recorded after laser stimuli (biggers diameter and longer duration than Aδ stimuli setting) applied in trigeminal regions, where the density of full C fiber is higher and the distancy from the central nervous system is lower than any other region in the body . It is much more difficult their recording after stimuli applied in extra-trigeminal areas. Intraepidermal density involves just nerve fibers crossing the derma-epidermal junction, but this allows to obtain important informations about unmyelinated fibers of extra-trigeminal areas. We observed that the density of intraepidermal fibres correlated with the duration of the disease: the longer cryoglobulinemia lasts a worse intraepidermal innervations , as assessed by skin biopsy. Our finding agree to previous studies showing that the incidence of neuropathic pain conditions peaks in the elderly [18]. These reported data probably merely reflect the higher frequency of peripheral nerve diseases in the elderly (e.g.

the prevalence of peripheral neuropathy rises from 2.4% in the general population to 8% in subjects older than 55 years) [19]. The age-related changes in the somatosensory system, reported in many clinical studies [20], have influence on the development of pain in distal symmetric peripheral neuropathy.

In conclusion our findings indicate that neuropathic pain reflects damage to nociceptive axons, as showed by the correlation between the intensity of ongoing pain and LEP attenuation; moreover loss of nociceptive axons is a biomarker of the duration and the severity of neuropathy as assessed by skin biopsy.

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Chapter 9: Hyperalgesic activity of kisspeptin in mice.

Chapter based on: Hyperalgesic activity of kisspeptin in mice. Molecular Pain 2011, 7:90 doi:10.1186/1744-8069-7. Spampinato S, Trabucco A, Biasiotta A, Biagioni F, Cruccu G, Copani A, Colledge WH, Sortino MA, Nicoletti F, Chiechio S

Background

Kisspeptin is a 54-amino acid peptide originally discovered for its activity as metastasis-suppressor [1]. It is encoded by the Kiss1 gene as a 145-amino acid precursor protein and cleaved to a 54- amino acid protein as well as into shorter products (kisspeptin-10,-13,-14) known to play a critical role in the neuroendocrine regulation of reproduction [2-5]. In the brain, kisspeptin is localized not only in areas involved in gonadotropin secretion, but also in other regions such as the amygdala, hippocampus, and the periacqueductal gray [6,7]. Its action is mediated by a 7-TM receptor named GPR54, also known as KISS1R, which is coupled to polyphosphoinositide hydrolysis *via* a Gq/11

GTP binding protein [2, 8]. Loss-of-function mutations of GPR54 cause a non-Kallman variant of hypogonadotropic/hypogonadism in humans (i.e. hypogonadotropic/hypogonadism without anosmia) [2, 9]. Interestingly, the expression of kisspeptin and GPR54 is not restricted to the hypothalamus. Relatively high levels of kisspeptin and GPR54 are found in forebrain regions, such as the hippocampus and amygdala, as well as in the periacqueductal grey [10]. The investigation of the extrahypothalamic functions of kisspeptin is still at its infancy. Treatment with kainic acid increases kisspeptin mRNA levels in the hippocampus, and kisspeptin enhances the amplitude of excitatory postsynaptic currents in granule cells of the hippocampal dentate gyrus [6, 7]. This suggests a potential role for kisspeptin in the regulation of synaptic plasticity in the CNS. Recent findings have shown an intense kisspeptin and GPR54 immunostaining in dorsal root ganglia (DRG) neurons and in lamina I and II of the dorsal horns of the spinal cord [11, 12]. The transcripts of kisspeptin and GPR54 are up-regulated in DRG and dorsal horn neurons in the complete

Freund adjuvant (CFA) model of chronic inflammatory pain [12], suggesting that kisspeptin may play a role in mechanisms of nociceptive sensitization. However, how precisely kisspeptin regulates pain sensitivity is obscure at present. We now report that peripheral or intrathecal injection of kisspeptin causes hyperalgesia and induces biochemical changes that are consistent with mechanisms of peripheral and central nociceptive sensitization.

Methods

Animals Adult male CD1 mice (Charles River, Calco, CO, Italy), 129S6/Sv/Ev wild-type, and 129S6/Sv/Ev *Gpr54*-knock-out mice [13] aged between 8 and 9 weeks were used in these experiments. Mice were housed 10 animals per cage with food and water *ad libitum* in standard 12/12 h light/dark cycle, for a period of 2 weeks before testing. All experiments were carried out according to the recommendations of Institutional Animal Care and Use Committee (IACUC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug administration Kisspeptin (Calbiochem Merck KGaA, Darmstadt, Germany) was dissolved in 5% DMSO and injected intrathecally (3 µl) or subcutaneously (5 µl) into the plantar surface of the right hind paw using a 10 µl luertip-syringe (Hamilton) fitted with a 30-gauge needle. p234 (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and injected in a volume of 3 µl for intrathecal administration or 5 µl for intraplantar administration. Behavioral experiments *Hot plate test.* The hot plate test (Ugo Basile, Italy) was used to asses thermal sensitivity. CD1 mice were placed onto the hot plate at the temperature of 55 ± 0.1 °C. Paw withdrawal thresholds were determined in the hind paws of ipsilateral hind limb. Animals were kept on the plate until the first sign of ipsilateral paw lift or lick was recorded as basal withdrawal latency (pre-drug latency). A maximum cut-off paw withdrawal latency of 20 seconds was chosen to prevent tissue damage (cutoff time). Post-dose thresholds were taken at 5, 15, 30, and 60 minutes after drug administration (post-drug latency). For each animal, results were expressed as the percentage maximum possible effect

(%MPE) calculated using the following formula: [(post-drug latency – pre-drug latency)/(cutoff time – pre-drug latency)] x 100.

Formalin test. Inflammatory pain was assessed using the formalin test. Ten µl of a 5% formalin solution was injected subcutaneously into the plantar surface of the right hind paw of CD1 mice. After the injection, mice were immediately placed in a plexiglas box (20 × 15 × 15 cm) surrounded by mirrors to allow the observation of nociceptive responses that include licking, lifting and shaking of the injected paw. Tests were performed between 08:00 h and 12:00 h to minimize variability.

Mice were observed for 1 hour. Formalin scores were separated into two phases, phase I (0-10 min) and phase II (15-45 min). The mean behavioural score was calculated in blocks of 5 min for each of the two phases. A mean response was then calculated for each phase.

Spontaneous pain. CD1 mice that received intraplantar injection of kisspeptin or vehicle were placed in a cage immediately after the injection, and the duration of hind paw lifting and licking during the first 5 minutes were

measured. All behavioral tests were analyzed by observers blind to the treatment of the animals.

Immunohistochemistry:

Skin biopsies. Animals were euthanized with chloral hydrate (320 mg/kg i.p.). 2.5-mm punch skin biopsies from the plantar surface of the hind paws were performed and fixed in Zamboni fixative (2% paraformaldehide, 15% picric acid saturated aqueous solution, 0.1 M phosphate buffer pH 7.4) for 24 hours. Biopsies were cryoprotected with 20% sucrose in PBS overnight at 4°C. Sections of 10 µm were cut at the cryostat and mounted on glass slides for immunohistochemical analysis. Immunohistochemistry procedures were performed as previously described [14].

Double immunofluorescence was performed in skin biopsies from CD1 male mice incubating sections overnight with polyclonal rabbit anti-human PGP 9.5 (1:1000; AbD Serotec, Kidlington, UK) and goat polyclonal anti-GPR54 (1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and then for 1 h with secondary fluorescein anti-rabbit

(1:100; Vector Laboratories, Burlingame, CA) and Cy3 antigoat (1:400; Chemicon, Billerica, MA) antibodies. Control staining was performed without the primary antibodies. Immunostaining was performed in skin biopsies from male 129S6/Sv/Ev wildtype and 129S6/Sv/Ev *Gpr54*- knock-out mice [13] to test the specificity of the anti-GPR54 antibody. Tissue sections were incubated overnight with goat polyclonal anti-GPR54 (1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and then for 1 h with secondary biotin-coupled anti-goat (1:100; Vector Laboratories, Burlingame, CA). SG (SG substrate kit; Vector Laboratories, Burlingame, CA, USA) chromogen was used for detection. *Spinal cord*. CD1 mice (n=5 per group) were used. 3 min after kisspeptin (3 nmol) or vehicle (DMSO) were co-injected with formalin in the right hind paw and lumbar spinal cords were removed and fixed in formalin (4%) overnight, transferred in 70% ethanol and included in paraffin.

Ten serial sections were cut and used for immunohistochemical analysis. Deparaffinized sections were treated with 10 mmol/L citrate buffer, pH 6.0, and

heated by microwave for 10 minutes for antigen unmasking. Sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were incubated overnight with monoclonal rabbit antibody anti phospho-p44/42 (Erk1/2) (Thr202/Tyr204) (D13.14.4E)XPTM (1:200; Cell Signaling Technology, Denver, MA, USA) and then for 1 h with secondary biotin-coupled anti-rabbit (1:200; Vector Laboratories). 3,3-Diaminobenzidine tetrachloride was used for detection. Control staining was performed without the primary antibodies.

Densitometric analysis of p-ERK immunoreactivity Intensity of p-ERK immunoreactivity was quantified by measuring the optical densities of the outer laminae of the dorsal horn in the stained sections relative to the background (ventral horn) . Images were acquired using a computer-based microdensitometer (NIH Image Software, Bethesda, MD, USA). Values were the mean of measurements made on ten sections (10 µm) sampled 1 into a 3 series spanning the extent of the L4-L5 spinal cord. Western blot analysis CD1 Mice were sacrificed 3 min following treatment and skin

lysates of all groups were processed in western blot. Skin homogenates were obtained as previously described [15]. Ten µg of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto protein-sensitive nitrocellulose membranes (Criterion blotter; Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in Odyssey blocker (LI-COR Biosciences, Lincoln, NE) for 1 h, and the following primary antibodies were used: anti-TRPV1 (phospho S800) polyclonal antibody (1:400, Abnova, Aachen, Germany); anti-actin monoclonal antibody (1:1000, Sigma). Secondary antibodies were: goat anti-rabbit (IRD800CW) and goat antimouse (Alexa 680, LI-COR, Bioscience) antibodies. Proteins were detected with the Odyssey Infrared Fluorescence Imaging System (LI-COR).

Results

Knowing that the kisspeptin receptor, GPR54 (KISS1R), is present in DRG neurons [12], we performed immunofluorescent analysis to examine whether the receptor was also present in peripheral nociceptors. We

focused on the peripheral role of kisspeptin in the modulation of acute and inflammatory pain. First we examined the specificity of the GPR54 antibody in skin biopsies from GPR54 KO mice. No immunostaining was seen in sensory nerve terminals of GPR54 KO mice (Fig. 1). The nature of the nonspecific staining seen in the outer portion of the skin of GPR54 KO mice is unknown. In punch skin biopsies from the mouse hind paw, sensory fibers ascending vertically between the keratinocytes to reach the stratum corneum of the epidermis were identified by fluorescent immunostaining for the neuron-specific ubiquitin hydrolase, PGP9.5 [14] (Fig. 2A). These fibers also expressed GPR54, as shown by double fluorescence immunostaining (Fig. 2B,C). Behavioral experiments were performed after peripheral (intraplantar) and central (intrathecal) administration of kisspeptin at doses ranging from 0.1 to 3 nmol [16]. We first examined the effect of intraplantar injection of kisspeptin on nocifensive behavior in naïve mice. Nocifensive behavior consisting of licking, flinching and shaking of the injected paw was evaluated after a single injection of kisspeptin (3

nmol/5 µl) or vehicle into the plantar surface of the right hind paw. The time spent in nocifensive behavior was recorded for 5 min after the injection. Intraplantar injection of kisspeptin (3 nmol/5 µl) induced brief nocifensive behavior that lasted for about 5-15 seconds, whereas no signs of pain were seen in vehicle-injected mice (Fig. 3A). We then assessed the effect of kisspeptin on acute thermal pain using the hot plate test. Intraplantar injection of kisspeptin (3 nmol/5 µl) significantly reduced paw withdrawal latency in response to heat as compared to intraplantar injection of vehicle (Fig. 3B), whereas no differences were observed after p234 injection (0.1 nmol/5 µl) (Fig. 3C).

For the assessment of inflammatory pain, mice were subjected to the formalin test, 15 min after intraplantar (0.1, 1 and 3 nmol/5 µl) or intrathecal (0.1, 0.5 and 1 nmol/3 µl) injection of kisspeptin. Intraplantar injection of formalin elicits a biphasic nocifensive response characterized by licking, lifting and shaking of the injected paw. The first phase of the formalin test, starting immediately after formalin injection and lasting for about 10 min, represents

a form of acute pain elicited by direct activation of nociceptors. The second phase of the test (occurring approximately 15-45 min after formalin injection) reflects the development of nociceptive sensitization in the dorsal horns of the spinal cord [17, 18]. Intraplantar injection of both 1 and 3 nmol/5 µl of kisspeptin (15 min prior to formalin injection) caused hyperalgesia in the first and second phases of the formalin test whereas no effects were observed at the lower dose of 0.1 nmol/5 µl (Fig. 4). We also assessed the effect of the selective GPR54 antagonist, peptide 234 (p234) [19] in the formalin test. As opposed to kisspeptin, intraplantar injection of p234 (1 nmol/5 µl; 15 min prior to formalin) significantly reduced nocifensive behavior (Fig. 4B). A lower dose of p234 (0.1 nmol/5 µl) induced a trend to an analgesic effect, which was not statistically significant (Fig. 4B). We also examined whether intrathecal injection of kisspeptin or p234 could affect nocifensive behavior in the formalin test. Kisspeptin injected intrathecally at the dose of 1 nmol/3 µl, 10 min prior to intraplantar injection of formalin, significantly increased nocifensive behavior in the first and second

phases of the formalin test. A lower dose of kisspeptin (1 nmol/3 µl) caused hyperalgesia in the first phase, and a non significant trend to hyperalgesia in the second phase of the test (Fig. 4C). When injected intrathecally, compound p234 was analgesic at doses of 0.1 and 1 nmol/3 µl in both phases of the formalin test (Fig. 4D). The hyperalgesic activity of kisspeptin in both phases of the formalin test led us to investigate whether the peptide could induce biochemical changes that were consistent with mechanisms of peripheral and central sensitization. We therefore examined TRPV1 channel phosphorylation in the skin of the hind paw, and activation of ERK1/2 in the dorsal horns of the spinal cord in mice subjected to intraplantar injection of formalin preceded by kisspeptin or vehicle. Immunoblot analysis with anti-phosphorylated TRPV1 antibodies showed a single band at the expected molecular size of 95 kDa. We observed that in mice pretreated with vehicle, intraplantar injection of formalin slightly increased the levels of phosphorylated TRPV1 in the ipsilateral hind paw as compared to naïve mice. This effect was largely amplified in mice pretreated with

kisspeptin (3 nmol/5 µl, 15 min prior to formalin injection) (Fig. 5). Activation of the mitogen activated protein kinase (MAPK) pathway was examined by immunohistochemical analysis of phosphorylated ERK1/2 in the dorsal horns of the spinal cord after intraplantar injection of formalin preceded by vehicle or kisspeptin. Formalin injection preceded by vehicle slightly enhanced phosphorylated ERK1/2 immunostaining in the dorsal horn ipsilateral to the injection side as compared to the contralateral dorsal horn or the dorsal horns of naïve mice (Fig. 6). Pretreatment with kisspeptin (3 nmol/µl) dramatically enhanced the expression of phosphorylated ERK1/2 in the ipsilateral dorsal horn (Fig. 6).

Discussion

These data offer the first demonstration that kisspeptin, a peptide known for its role in the regulation of the hypothalamic-pituitary-gonadal axis, lowers pain threshold and enhances nocifensive behavior in mice. Immunohistochemical analysis showed the presence of the kisspeptin receptor, GPR54, in peripheral sensory fibers, a

finding that is consistent with the detection of GPR54 mRNA and protein in DRG neurons [11, 12]. The lack of staining in GPR54 KO mice indicates that GPR54 is present in peripheral nociceptors explaining the hyperalgesia caused by intraplantar injection of kisspeptin in the hot plate and formalin test. We wish to highlight that intraplantar kisspeptin induced only a small nocifensive response on its own, suggesting that a main action of kisspeptin is to amplify pain sensitivity in response to noxious stimuli. Intraplantar injection of the GPR54 antagonist, p234, caused a robust analgesia in the formalin test, suggesting that endogenous kisspeptin acts extracellularly to activate GPR54 receptors during inflammatory pain. Kisspeptin is present in DRG neurons, where it co-localizes with isolectin B4 and calcitonin gene-related peptide, and its expression is up-regulated by chronic inflammatory pain [12]. It is likely that kisspeptin is released from peripheral nociceptors in response to noxious stimuli, therefore behaving as an autocrine/paracrine factor to promote peripheral nociceptive sensitization.

Whether other cells can produce and secrete kisspeptin during inflammation is unknown at present. Phosphorylation of the TRPV1 ion channel is a key event in mechanisms of peripheral nociceptive sensitization [20-22]. The TRPV1 receptor can be phosphorylated by multiple protein kinases, including protein kinase A, protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II, and SRC [23-34]. In, particular, PKC phosphorylates TRPV1 at Ser-502 and Ser-800, thus amplifying ion channel activity [31, 35-37]. Intraplantar kisspeptin caused a robust increase in (Ser800)-TRPV1 phosphorylation, an effect that was likely mediated by the activation of the GPR54 receptor, with ensuing stimulation of inositol phospholipid hydrolysis, diacylglycerol formation, and PKC activation [2, 8]. Thus, kisspeptin might act similarly to other hyperalgesic molecules that activate Gq-coupled receptors and phosphorylate TRPV1 channels in peripheral nociceptors, such as bradykinin, group-I mGlu receptor agonists, P2Y2 receptor agonists, EP1 receptor, and prokineticin [28, 38-48]. Hyperalgesia by kisspeptin and analgesia by p234 were also seen in the second phase of the

formalin test, which reflects the development of central nociceptive sensitization in the dorsal horns of the spinal cord [17, 18]. Central nociceptive sensitization is mediated by a series of mechanisms that ultimately lead to an enhancement of excitatory transmission at the synapses between primary afferent fibers and second order sensory neurons in the dorsal horns of the spinal cord [24]. The relevance of the MAPK pathway in the development of central sensitization has been highlighted in a recent review [48]. Intraplantar injection of formalin is known to induce a rapid phosphorylation of ERK1/2 in the spinal cord, which has been causally related to the increase in nocifensive behavior seen in the second phase of the formalin test [49]. Pharmacological activation of mGlu1 and mGlu5 receptors, which also couple to the Gq protein just like GPR54 [50], can also enhance ERK1/2 phosphorylation in the spinal cord [51]. Activation of GPR54 by kisspeptin has been shown to stimulate the ERK/MAPK pathway both in recombinant expression systems and hypothalamic explants [52, 53]. Intraplantar injection of kisspeptin markedly amplified ERK1/2

phosphorylation induced by formalin in the ipsilateral dorsal horn, evidence that nicely supports the behavioral data obtained with kisspeptin in the second phase of the formalin test. Interestingly, kisspeptin retained the hyperalgesic activity (and p234 the analgesic activity) when injected by the intrathecal route. Thus, it is likely that the modulation of pain sensitivity by GPR54 extends beyond 10 peripheral nociceptors. Effects of kisspeptin on different receptors cannot be excluded. In particular it has been reported that kisspeptin can also bind neuropeptide FF (NPFF) receptors [54]. However in our hands intrathecal injection of kisspeptin lowers pain threshold, whereas intrathecal injection of NPFF is known to cause analgesia [55], thus the effect of kisspeptin in the spinal cord is likely mediated by the activation of the GPR54 receptor excluding an interaction of kisspeptin with NPFF receptors. The presence of GPR54 receptor in the amygdala [56] may suggest that kisspeptin acts also at higher brain centers that control the affective components of pain and contributes to the top-down regulation of pain threshold.

Conclusions

In conclusion, our data disclose a new aspect in the physiology of kisspeptin and suggest that peripheral GPR54 receptor antagonists (lacking potential hypothalamic side effects) can be developed as new drugs for the treatment of inflammatory pain. In addition, it will be interesting to explore whether individuals with hypogonadotropic hypogonadism due to inactivating mutations of GPR54 show alterations in the sensitivity to pain.

Figures

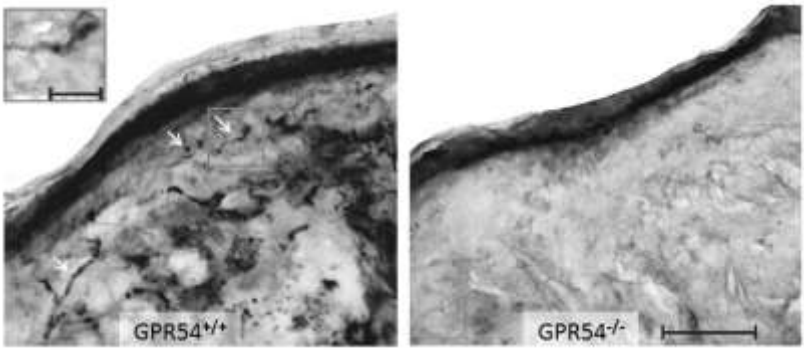

Figure 1 – Immunostaining for the kisspeptin receptor, GPR54, in the mouse skin of GPR54 WT and KO mice. Representative immunostaining showing the specificity of the GPR54 antibody in the peripheral nerve endings of the mouse skin of GPR54+/+ mice (left panel). No mmunostaining is observed in GPR54-/- mice (right panel). Scale bar 100 µm. The insert shows an immunopositive fiber at higher magnification (scale bar = 20 µm).

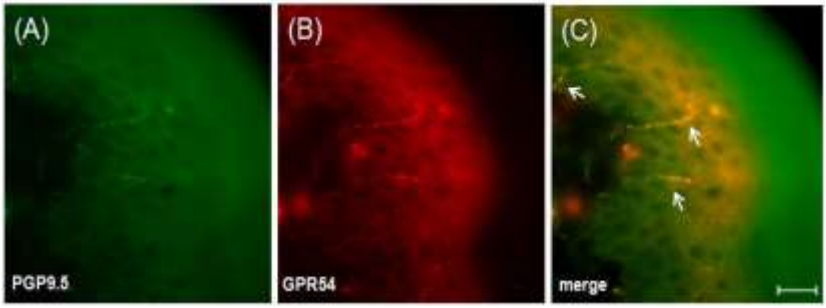

Figure 2 - Double immunofluorescent staining for the kisspeptin receptor, GPR54, and PGP9.5 in the mouse skin. Immunofluorescent staining of PGP9.5 and GPR54 is shown in (A) and (B), respectively. Coimmunolocalization is shown in (C) (see arrowheads). Scale bar 20 μm.

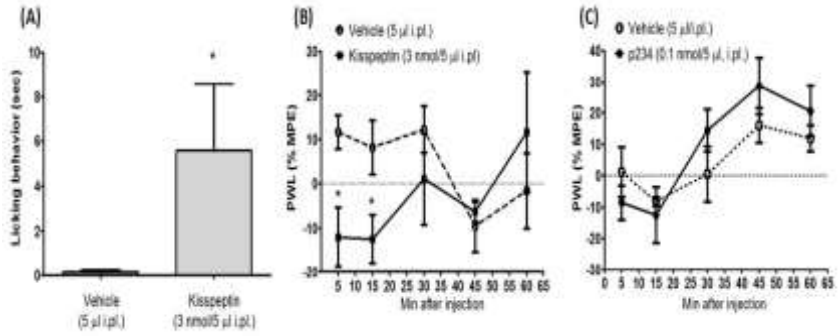

Figure 3 – Intraplantar injection of kisspeptin lowers pain threshold in the hot plate. The nocifensive response to intraplantar injection of kisspeptin (3 nmol/5 µl) in naïve mice is shown in (A). Data are means ± S.E.M of 6 mice, and refer to the number of sec spent in licking behavior in the first 5 min following injection. *p<0.05 (Student's t test) vs. mice injected with vehicle. Data obtained in the hot plate test are shown in (B). For each animal, the percentage maximum possible effect (%MPE) was calculated using the following formula: [(post-drug latency) - (pre-drug latency)/(cutoff time) - (pre-drug latency)] x 100. Data are means ± S.E.M. of 6 to 8 mice. *p<0.05, two-way ANOVA followed by Fisher's *post hoc* test. PWL, Paw-withdrawal latency.

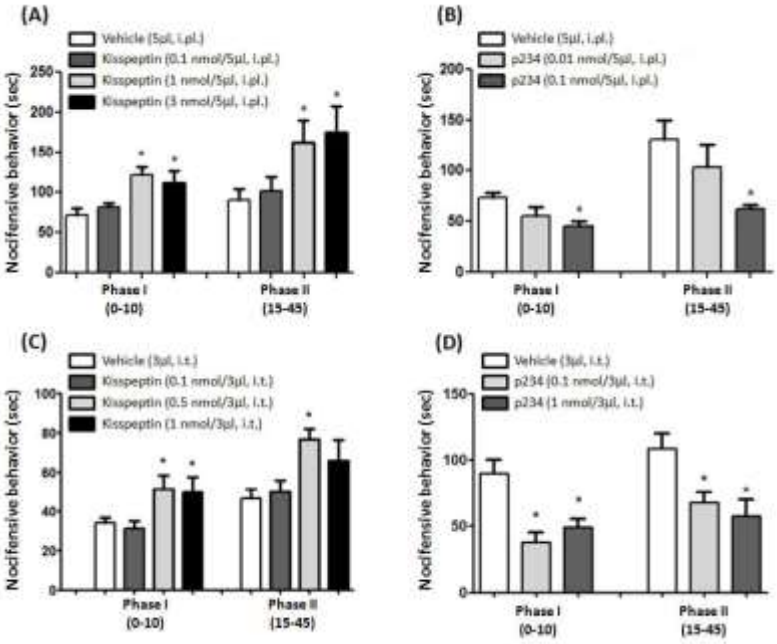

Figure 4 – Effect of intraplantar or intrathecal injection of kisspeptin or the GPR54 antagonist, p234, in the formalin test. Data obtained with intraplantar (i.pl.) injection of kisspeptin (1 or 3 nmol/5 µl) or p234 (0.01 or 0.1 nmol /5 µl) on the first (0-10 min) and second (15-45 min) phases of the formalin test are shown in (A) and (B), respectively. Drugs were injected 15 min prior to the intraplantar injection of formalin. Data obtained with intrathecal (i.t.) injection of kisspeptin (0.5 or 1 nmol /3 µl) or p234 (0.1 or 1 nmol/3 µl) are shown in (C) and (D), respectively. Data are means + S.E.M. of 8-12 mice per group. *p<0.05 vs. the respective groups of mice injected with vehicle (one-way ANOVA followed by Fisher's *post hoc* test).

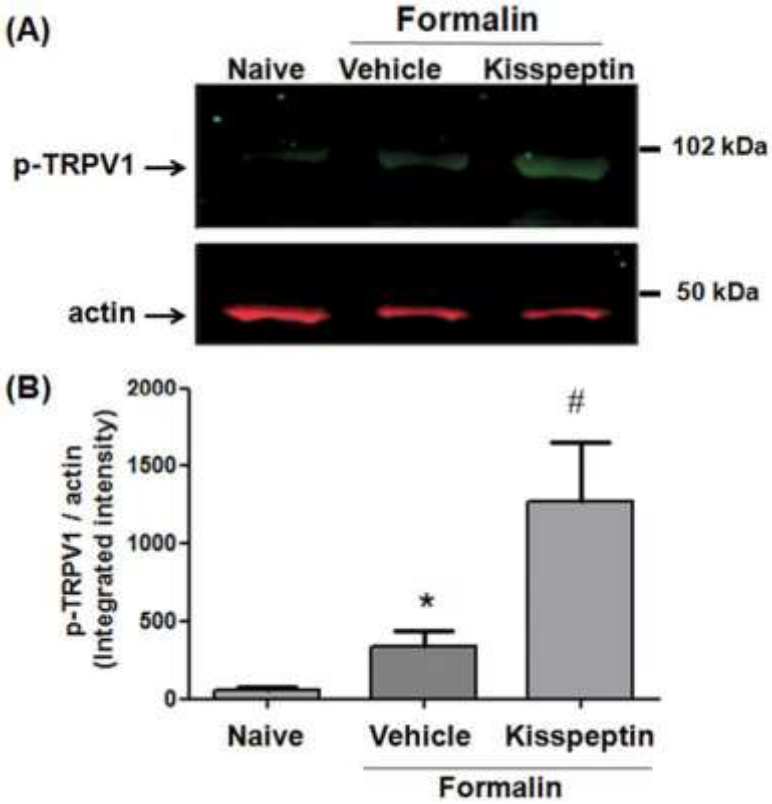

Figure 5 – Intraplantar injection of kisspeptin amplified the increase in TRPV1 phosphorylation in the skin of mice treated with formalin. A representative immunoblot of (Ser800)-phosphorylated TRPV1 in the skin of naïve mice and mice injected with formalin in the Absence or presence of kisspeptin (3 nmol/5 µl) is shown in (A). Densitometric analysis is shown in (B), where values are means + S.E.M. of 4 determinations. *p<0.05 vs. naïve mice, #p<0.05 or vs. mice treated with formalin alone (one-way ANOVA followed by Fisher's *post hoc* test).

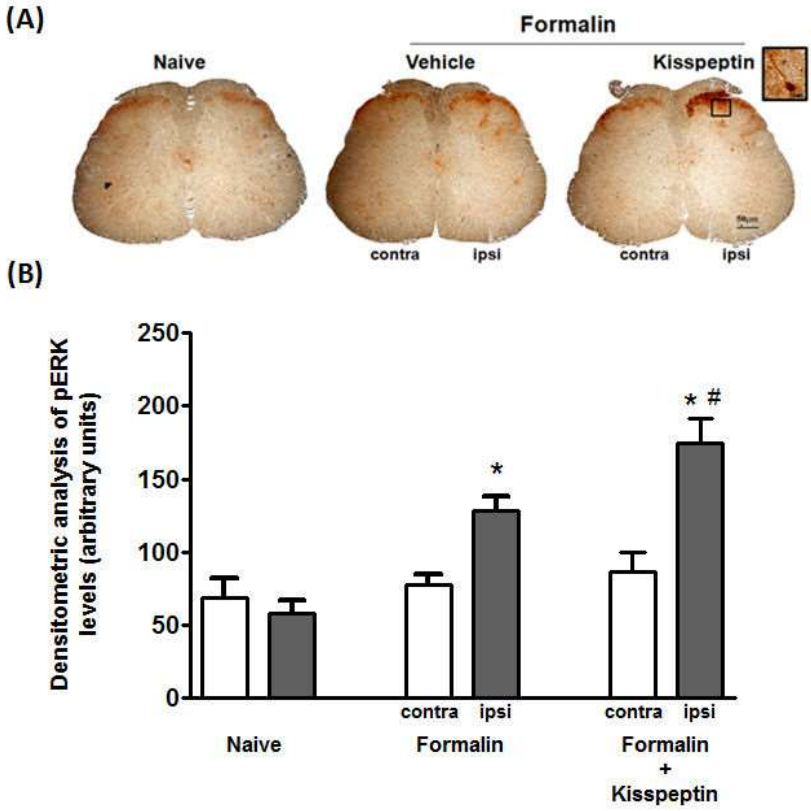

Figure 6 – Intraplantar injection of kisspeptin increased ERK phopshorylation in the ipsilateral dorsal horn of the spinal cord. (A) Immunohistochemical analysis of phosphorylated-ERK1/2 in the dorsal horns of the spinal cords of naïve mice and mice treated with formalin in the Absence or presence of kisspeptin (3 nmol/5 µl) is shown. Contra = contralateral; ipsi = ipsilateral. Scale bar = 50 µm. The insert shows an immunopositive neuron at higher magnification (scale bar = 10 µm). (B) Densitometric analysis of p-ERK immunoreactivity in the superficial laminae of the dorsal horn. *p< 0.05 vs. contralateral values; #p< 0.05 vs. formalin alone values (one-way ANOVA + Dunnett's Multiple Comparison Test).

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Chapter 10: Conclusions

10.1 Neurophysiological and morphological assessment

Combining clinical, neurophysiological and morphological techniques, it is possible to objectively measure all nociceptive and non-nociceptive afferent systems (Aβ-, Aδ- and C-fibers) all over the body in each patient. Neurophysiological assessment of afferent function in patients with neuropathic pain is essential to increase our knowledge of the underlying pain-generating mechanisms. Conventional neurophysiological tests, such as nerve conduction studies or somatosensory-evoked potentials are often difficult to be applied in patients with some conditions (i.e trigeminal neuralgia or postherpetic neuralgia in thoracic areas); moreover, they are no able to investigate Aδ- and C- nociceptive fibres. In our studies we circumvented these two problems through the use of blink-reflex recordings in facial pain syndromes, and laser-evoked potentials to assess nociceptive pathway . Altough LEPs activate myelinated Aδ- and unmyelinated C-fibers,

does not allow differentiating the Aδ-nerves from the C-nerves in extratrigeminal areas. Moreover, laser stimuli activate the intraepidermal nociceptive terminals, rather than the nerve axons. So, we applied an additional technique: skin biopsy to take morphological informations of intraepidermal unmyelinated fibers and to calculate their density. Punch skin biopsy is easy to do, minimally invasive, and optimal for follow-up. Although low density of IENF is no correlated with the presence of neuropathic pain, it offers biomarkers of peripheral nerve damage, since the severe loss of IENF correlates with a more severe and longer neuropathy. Moreover, as discussed in the previous chapter (about kisspeptine), skin biopsy showed in cutaneous fibers the presence of GPR54 receptors, that could be targeted by novel analgesic drugs in the treatment of pain . Despite these advantages, it is available only in few research centres. Our group recommend its larger spreading.

10.2 Neuropathic pain phenotypes

Finally, we sought possible correlations between neurophysiological and morphological data and the various qualities of neuropathic pain as assessed by the NPSI. We found that LEP amplitude (in patients with painful neuropathy) correlated with ***spontaneous constant pain***, leading to an underling damage of nociceptive axons . Ongoing pain could be due to the abnormal spontaneous hyperactivity of damaged nociceptive fibres that have lost their intraepidermal terminals, as assessed by skin biopsy. We can not exclude the possibility that nociceptive pathway damage may provoke long-term changes in the central nervous system, including hyperactivity of the second order neurons of the nociceptive pathway (central sensitization), that may act as a current mechanism.

The delay in blink-reflex latency (i.e in patients with PHN), nerve sensory conduction velocity (i.e in patients with carpal tunnel syndrome), and somato-sensory evoked potential latency (i.e in patients with multiple sclerosis) correlated with paroxysmal pain and abnormal sensations. Our findings suggest that ***paroxysmal pain*** and abnormal

sensations reflect demyelination of non-nociceptive Aβ-fibres. A pathologic process in demyelinated Aβ-fibers is involved in the generation of pain. This is the case, even though Aβ-fibers normally do not convey noxious information. Consistently with previous animal studies describing spontaneous ectopic discharges recorded in Aβ-fibers after nerve injuries [1-3], we suggest that paroxysmal pain is related to high frequency bursts generated in demyelinated Aβ-fibers. It is still unclear, however, whether the abnormal activity in Aβ-fibers is sufficient to provoke pain per se, whether it arises after ephaptic transmission to neighboring C-fibers, or through a transmission to central multireceptive neurons (wide dynamic range neurons) [4].

While NCS data did not differ between patients with and without ***allodynia***, LEP amplitude was higher in patients with allodynia than in those without. We argue against a role of Aβ-fibres and central sensitization as the main mechanism for the development of allodynia in distal symmetric polyneuropathy. Our findings suggest, though do not prove, that *provoked pain* arises through still intact,

and sensitized nociceptive terminals. The partially preserved LEPs in patients with allodynia suggests that this type of pain might be related to the abnormal reduction of mechanical threshold of nociceptive terminals (peripheral sensitization).

We provided a clear evidence that neuropathic pain has different phenotypes, which very likely arise through a variety of distinct pathophysiological mechanisms. Pain should be classified and treated on mechanism-based grounds. Distinct neuropathic signs and symptoms are generated by abnormalities of specific primary afferent neurons (Aβ- vs. Aδ- and C-fibers). Moreover, sensory signs and symptoms are very heterogeneous in patients suffering from an identical disease entity. The different somatosensory abnormalities are considered to reflect different underlying pain-generating mechanisms. For this reason, the individual pattern of somatosensory abnormalities at the affected body area, may be a promising biomarker for the operating mechanisms. A comparison of objective neurophysiological and morphogical tests with the clinical phenotype is thus a

particularly appropriate approach to unravel the underlying pathophysiology, leading to detect a specific and positive treatment . Perhaps it is possible to extend the data analysis and correlate the neuropathic pain phenotype and the sensory neurophysiological and morphological testing with the treatment response obtained with certain drug classes. This effort would be invaluable for our understanding of pain.

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