Experimental Neurology 293 (2017) 62–73

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Bilateral tactile hypersensitivity and neuroimmune responses after spared nerve injury in mice lacking vasoactive intestinal peptide

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ARTICLE INFO

Article history:
Received 10 November 2016
Received in revised form 21 March 2017
Accepted 24 March 2017
Available online 27 March 2017

Keywords:
Neuropathic pain
Chronic pain
Central sensitization
Mirror-image pain
Cytokines
Microglia
Astrocytes

ABSTRACT

Vasoactive intestinal peptide (VIP) is one of the neuropeptides showing the strongest up-regulation in the nociceptive pathway after peripheral nerve injury and has been proposed to support neuropathic pain. Nevertheless, the story may be more complicated considering the known suppressive effects of the peptide on the immune reactivity of microglial cells, which have been heavily implicated in the onset and maintenance of pain after nerve injury. We here used mice deficient in VIP and the model of spared nerve injury, characterized by persistent tactile hypersensitivity. While tactile hypersensitivity developed similarly to wild type mice for the ipsilateral hindpaw, only transgenic mice showed a mirror-image tactile hypersensitivity in the contralateral hindpaw. This exacerbated neuropathic pain phenotype appeared to be mediated through a local mechanism acting at the level of the lumbar spinal cord as a distant nerve lesion in the front limb did not lead to hindpaw hypersensitivity in VIP-deficient mice. Innocuous tactile hindpaw stimulation was found to increase a neuronal activation marker in the bilateral superficial laminae of the lumbar dorsal horn of VIP-deficient, but not wild type mice, after SNI. A deeper study into the immune responsiveness to the nerve lesion also proved that VIP-deficient mice had a stronger early pro-inflammatory cytokine response and a more pronounced microglial reactivity compared to wild type controls. The latter was also observed at four weeks after spared nerve injury, a time at which bilateral tactile hypersensitivity persisted in VIP-deficient mice. These data suggest an action of VIP in neuropathic states that is more complicated than previously assumed. Future research is now needed for a deeper understanding of the relative contribution of receptors and fiber populations involved in the VIP-neuropathic pain link.

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1. Introduction

Vasoactive intestinal peptide (VIP) is a widely expressed 28-amino acid peptide with functions that reach well beyond blood vessel dilation and gastrointestinal tract motility (Delgado and Ganea, 2013; Delgado et al., 2004). During the last decades, VIP has been implicated in the development of neuropathic pain (Dickinson and Fleetwood-Walker, 1999). This condition is frequently caused by peripheral nerve damage and associated with a variety of symptoms of which tactile hypersensitivity has been considered as one of the most debilitating (Meldrum, 2000). Previous research has shown that peripheral nerve injury triggers an up-regulation of VIP in neurons of the dorsal root ganglia and leads to increased VIP expression in the superficial dorsal horn of the spinal cord (Hokfelt et al., 1994; Shehab, 2014; Villar et al., 1989). Within the dorsal horn, which is a critical site for nerve injury-induced neuroplasticity that underlies persistent pain states (Berger et al., 2011), the gene expression of the two VIP receptors, i.e. VPAC1 and VPAC2 has been reported to undergo a down-regulation and up-regulation, respectively (Dickinson et al., 1999). Spinal neurons express both types of receptors and their binding by specific agonists can induce neuronal activation. Pharmacological studies on VIP and pain have thus far focused on VPAC2, demonstrating that a specific antagonist effectively reduced tactile and heat hypersensitivity in a rat model of peripheral nerve injury (Garry et al., 2005). These data suggest that nerve injury causes modification in the VIP system, which promote neuropathic pain symptomatology.

Within the central nervous system (CNS), VIP does not exclusively target neuronal cells as VIP receptors are also found on glial cells such as astrocytes and microglia (Ashur-Fabian et al., 1997; Cholewinski and Wilkin, 1988; Delgado et al., 2002). VIP has been found to exert a strong suppressant effect on immune-challenged response through

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http://dx.doi.org/10.1016/j.expneurol.2017.03.019
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binding of the VPAC1 receptor, reducing the production of immune mediators such as pro-inflammatory cytokines and chemokines (Delgado et al., 2002; Delgado et al., 2003). Considering the importance of these immune mediators together with microglial reactivity in the development of neuropathic pain (Aldskogius and Kozlova, 2013; Austin and Moalem-Taylor, 2010; Gao and Ji, 2010), it might be speculated that VIP, through immunomodulation, negatively influences neuropathic pain symptomatology through immunomodulation.

While a role of VIP in neuropathic pain is well established, the nature of this role remains unclear. We here aimed to understand the effect of a full depletion of VIP in a mouse model of spared nerve injury, which is characterized by persistent tactile hypersensitivity (Bourquin et al., 2006). Previous work had already shown that mice lacking pituitary adenylate cyclase-activating peptide (PACAP), a neuropeptide structurally related to VIP, had normal nociception, but reduced inflammatory and neuropathic pain (Mabuchi et al., 2004). Even though PACAP can bind both VPAC1 and VPAC2, the latter effects are likely mediated through its high-affinity PAC1 receptor as PAC1 knock-out mice were found to show normal nociception (Jongsma et al., 2001) as well as reduced pathological pain (Jongsma et al., 2001; Martin et al., 2003).

In order to study putative effects of VIP depletion on tactile hypersensitivity after peripheral nerve injury we here used genetically modified mice lacking VIP. The mice have been generated through homologous recombination disrupting the VIP gene (Colwell et al., 2003) and leading to absence of VIP as well as peptide histidine-isoleucine, another neuropeptide encoded by the same gene (Girard et al., 2006).

2. Materials and methods

2.1. Animals

In this study we used male and female adult homozygous $VIP^{-/-}$ mice (at start of experiments: about 3 months old and 24 ± 2 g), bred on a C57BL/6 background and kindly donated by prof Vincent Lelièvre of the University of Strasbourg. $VIP^{+/+}$ mice served as their wild type controls. For algesimetric experiments, we further used female $VIP^{-/-}$ mice and $VIP^{+/+}$ mice, which were studied separately from male mice. Mice were genotyped after birth with genomic DNA extracted from a tail biopsy using PCR (data not shown) as described elsewhere (Colwell et al., 2003). All experiments were performed in strict adherence with the EU directive of 22/09/2010 (2010/63/EU) and approved by the ethical committee on animal experimentation (2010/UCL/MD/023). Our animal laboratory received the number LA2230419 from the Belgian Ministry of Agriculture. Mice were kept in groups of 5–10 animals in makrolon cages (43 × 26 × 15.5 cm) with ad libitum access to water and food, and at a regular 12 h/12 h light/dark cycle.

2.2. Surgery

Animals were subjected to three different groups with respect to nerve injuries; spared nerve injury (SNI), median nerve injury (MNI), or sham surgery as previously described (Bourquin et al., 2006; Tos et al., 2008). Additionally, hindpaw incision (PI) was used as a model of transient tactile hypersensitivity (Brennan et al., 1996; Pogatzki and Raja, 2003). In brief, anesthesia was induced and maintained using 2.5–3% sevoflurane in oxygen. For SNI, the left sciatic nerve was exposed at its trifurcation at the mid-thigh region through blunt dissection. The tibial and common peroneal branches were gently freed from surrounding connective tissues and were then transected at approximately 2 mm distal to the point of trifurcation. The nerve stumps were oriented in opposite directions, followed by closing of the skin with 6/0 prolene sutures. Sham-operated mice underwent the same procedure with the exception that no nerve injury was inflicted. For MNI, the left median nerve was exposed in the forelimb region between the axilla and elbow. The median nerve was gently isolated from surrounding tissues at the level of the pectoralis major muscle and then transected using spring scissors (Fine Science Tools GmbH, Heidelberg, Germany). The nerve stumps were oriented in opposite directions and the skin was closed using 6/0 prolene sutures (Ethicon, Livingston, Scotland). For PI, a 5-mm longitudinal incision was made through the skin, fascia and muscle of the plantar hindpaw using a surgical scalpel blade no. 11 (Swann Morton, Sheffield, England). The incision extended from 2 mm proximal to the heel toward the toes. The wound was closed with two single knot sutures of 6/0 nylon (Ethicon, Livingston, Scotland).

2.3. Von Frey algesimetry

The animals were first habituated to the experimenter and then placed in the experimental setting, which consisted of transparent plastic chambers on top of an elevated wire mesh. The mice were allowed to acclimatize to the environment and explorative behaviors and major grooming typically reduced after about 20 min. Then, the von Frey hair filament test was performed. The filament set used for the assessment consisted of seven calibrated von Frey hair filaments; 0.03, 0.07, 0.17, 0.4, 0.7, 1.2, and 2.0 g (Stoelting, Wood Dale, IL, US). In this test, filaments are applied perpendicular to the sural nerve territory at the lateral aspect of the plantar hindpaw surface and maintained in a slightly buckled position for a maximum duration of 6 s. Only in case of a paw withdrawal with aversive behavioral signs (brisk withdrawal with postural change, attacking of filament, licking of stimulated paw, etc.), the response is considered as positive. The test is started at the 0.4 g filament and positive responses are followed by stimulation with the next-lower force filament; negative responses are followed by stimulation with the next-higher force filament in the set. This 'up-down' procedure is continued until maximally six responses have been obtained that started with a combination of 'no response-response'. In case of a positive response to the very first filament application, a total of maximally five further applications are performed. In case negative responses are obtained at the highest force filament or in case positive responses are obtained at the lowest force filament, no further applications are performed. On the basis of this assessment, the 50% paw withdrawal threshold (PWT) is calculated as described previously (Chaplan et al., 1994). Mice which consistently show positive responses to all filaments or negative responses to all filaments receive higher (2.0 g) or lower (0.04 g) cut-off scores, respectively.

2.4. Innocuous tactile hindpaw stimulation of SNI animals

The responsiveness of spinal neurons in the lumbar spinal cord toward innocuous tactile hindpaw stimulation was investigated. $VIP^{-/-}$ mice and $VIP^{+/+}$ mice that had undergone an SNI surgery 7 days earlier were subjected to tactile innocuous hindpaw stimulation. Briefly, the animals received one stroke of the plantar hindpaw surface every 2 s for a period of 10 min. Strokes were made with the flat portion of the investigator's thumb and toward the distal footpad. During the entire time, the animals were maintained under anesthesia using 2.5–3% sevoflurane in oxygen. For both genotypes, control animals that had undergone SNI 7 days earlier were kept under anesthesia for the same duration, but received no tactile hindpaw stimulation. At 1.5 h after stimulation, the animals were sacrificed and spinal cord tissue was processed according to the same procedures as described in Section 2.6.

2.5. qRT-PCR

Quantitative PCR was performed on ipsilateral and contralateral quadrants of the lumbar spinal cord of mice at 3 d and 7 d after SNI or sham surgery. Briefly, animals were asphyxiated using CO_2 and the spinal cord was extracted through hydro-extrusion. The lumbar enlargement was selected for further processing and divided in four quadrants. Only the ipsilateral and contralateral dorsal quadrants

were used in this investigation. These were snap-frozen using liquid nitrogen and stored at −80 °C until further use. Before mRNA extraction, tissues were weighed (6.7 ± 1.6 mg for 3 d tissues and 6.8 ± 1.6 mg for 7 d tissues). mRNA extraction was performed by spin column-based nucleic acid purification (Boom et al., 1990) and using the RNeasy® mini kit (Qiagen, Antwerp, Belgium). Tissues from two animals, with same genotype and surgery group, were pooled during the RNA extraction and considered as one sample (n). In brief, tissues were disrupted in a glass homogenizer using lysate buffer and β-mercaptoethanol. After a series of centrifugation steps, RNA was eluted in RNAse-free water. The amount of RNA was measured by spectrophotometry. A DNAse step was performed and mRNA was retrotranscribed using iScript cDNA synthesis kit (Qiagen, Antwerp, Belgium). A 3-step protocol was used for DNA amplification, performed with the CFX Connect ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Nazareth, Belgium). The initialization step was performed at 95 °C for 3 min. This was followed by the denaturation step at 95 °C for 10 s. The annealing step of primers was performed at 60 °C for 10 s for all genes considered, finally followed by the elongation step at 72 °C for 30 s. The denaturation, annealing, and elongation cycle was run 35 times. In order to check for primer specificity/amplicon products, a melting curve was created after the amplifications. This curve was created after 10 s at 95 °C by increasing the temperature from 65 °C to 90 °C. The SQ values were used to compare the expression of target genes, each normalized against the expression of GAPDH and βactin. The following primer sequences were used; TNFα forward primer: 5′ ccaccacgctcttctgtct3′, reverse primer: 5′tccagctgctcctccactt3′; IL1β forward primer: 5′tcgctcagggtcacaagaaa3′, reverse primer: 5′ catcagaggcaaggaggaaaac3′; GAPDH forward primer: 5′catggccttccgtgttccta3′; reverse primer: 5′ccctcagatgcctgcttca3′; βactin forward primer: 5′ctgatccacatctgctggaag3′, reverse primer: 5′tcctgagcgcaagtactctgt3′.

2.6. Immunohistochemistry

At 7 d and 28 d after SNI or sham surgery animals from both genotypes were sacrificed by CO_2 asphyxiation followed by transcardial perfusion with first phosphate buffered saline (PBS, 0.1 M, pH 7.4) and then 4% paraformaldehyde in PBS. Spinal cords were isolated through hydro-extrusion and the lumbar enlargement was then immersion fixed overnight in the same fixative. Hereafter the tissue was cryoprotected by overnight incubation in 10% sucrose (in PBS) and a three-night incubation in 25% sucrose (in PBS). Subsequently, the tissues were frozen using powdered dry-ice and stored at −80 °C until further use. For cryosectioning, the L4–5 lumbar spinal cords were embedded in tissue glue (Tissue Tek Optimal Cutting Temperature solution, O.C.T., Sakura Finetek, Antwerp, Belgium) and cut transversally at −30 °C using a Leica cryostat. Sections of 30 μm thickness were collected serially on Superfrost®Plus object glass slides (ThermoFisher Scientific, Braunschweig, Germany) respecting a 360 μm-intersection distance for sections on the same glass slides (with 10 sections per slide). Slides were stored at −80 °C until the time of the staining. The same tissue extraction procedure and processing was performed with the second cohort of animals 7 d after SNI surgery and 1 h and half after receiving (or not receiving) the non-nociceptive stimulation. Then, glass slides were thawed for at least 30 min followed by three washing steps of each 10 min in PBS containing 0.5% Triton X-100 (PBS-T), PBS, and PBS-T. Sections were then stained for either the macrophage/microglial marker ionized calcium-binding adapter molecule-1 (Iba1), for the astrocytic marker glial fibrillary acidic protein (GFAP) or for the neuronal activation marker (cFos). For Iba1 staining, sections were incubated for 1 h in PBS-T containing 5% normal goat serum (NGS), followed by an overnight incubation in primary antibody solution at 4 °C (rabbit anti-Iba1 from Wako, cat. no. 019/19741; 1:1000 in PBS-T containing 1% NGS). For GFAP staining, sections were immediately incubated overnight in primary antibody solution at 4 °C (rabbit anti-GFAP from Dako, cat. no. IS524; 1:1000 in PBS-T). For cFos staining, sections were incubated for at least 12 h at room temperature (rabbit anti-cFos from Santa Cruz Biotechnology, cat. no. sc-52; 1:2000 in PBS-T containing 10% NGS). The next day, sections were washed with PBS-T, PBS, PBS-T, each for 10 min and then incubated for 1 h in secondary antibody solution at room temperature (goat anti-rabbit Alexa-594 or goat anti-rabbit Alexa-555 at 1:100 in PBS-T; for Iba1 staining this solution also contained 1% NGS). Hereafter, sections were washed three times with PBS and coverslipped using 80% glycerol in PBS.

2.7. Image analysis

The sections stained for Iba1, GFAP and cFos were examined under a digital inverted EVOS microscope (Advanced Microscopy Group, Mill Creek, WA, US) that uses a light-emitting diode (LED) illumination system and was equipped with a Texas Red light cube. Within the first 72 h after completion of each staining, a total of 5 photomicrographs were taken from the ipsilateral and contralateral dorsal horns at the L4-L5 level. Image analysis was performed using the NIH ImageJ analysis software (version 1.48v). The dorsal horn was delineated as region of interest according to the Paxinos and Watson atlas (Kew and Kemp, 2005). After background signal subtraction, the fraction of the dorsal horn that was covered by immunoreactivity (IR) for Iba1 or GFAP was determined as area fraction (AF). In order to investigate the glial responsiveness to SNI in the animals, the AF of immune reactivity (IR) of SNI mice was expressed relative to the average AF of IR in sham-operated mice. For the cFos experiment, the number of cFos-IR cells was manually counted in the ipsilateral and contralateral superficial laminae (laminae I and II), according to the Paxinos and Watson atlas (Kew and Kemp, 2005). For both genotypes, the responsiveness to tactile hindpaw stimulation was expressed as fold-change over sham-stimulated control animals.

2.8. Data presentation and statistics

Behavioral data are presented as means ± Standard-error of the mean and qRT-PCR and immunohistochemical data are presented as means + Standard-error of the mean. The GraphPad Prism 5.0 software was used for statistical analysis. Algesimetric data over time were compared using a 2-way analysis-of-variance (ANOVA) for time, genotype and interaction. A Bonferroni post-hoc analysis was performed to compare genotype data at individual time points. All other analyses (baseline PWT of genotypes; qRT-PCR results of genotypes; immunohistochemical data of genotypes) were compared using unpaired Student's *t*-tests after controlling data for normality. A *p*-value of 0.05 was regarded as the level of statistical significance.

3. Results

3.1. $VIP^{-/-}$ mice show no alteration in tactile hindpaw sensitivity

Although the role of VIP in nociception is not fully understood, the literature suggests that VIP directly influences neuronal activity in the superficial dorsal horn (Dickinson et al., 1997). We therefore aimed to determine the baseline paw withdrawal threshold for tactile hindpaw stimulation in $VIP^{-/-}$ mice and compare it with that of wild type controls. Adult male $VIP^{-/-}$ mice showed a PWT of 1.25 ± 0.04 g, which was similar to the 1.29 ± 0.04 g value of male $VIP^{+/+}$ mice (Fig. 1). Even though we focused on male mice in this investigation, we also verified baseline tactile hindpaw sensitivity for the female gender and noticed no significant difference between $VIP^{-/-}$ mice and $VIP^{+/+}$ mice (PWT of 1.13 ± 0.07 g and 1.27 ± 0.06 g, respectively).

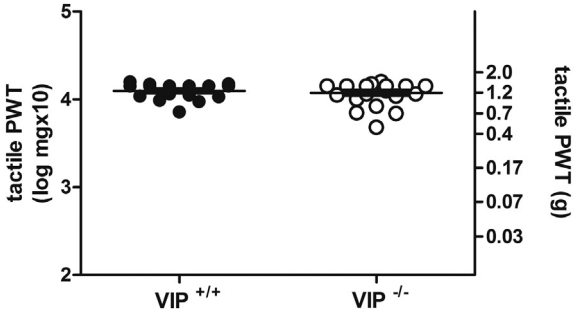

Fig. 1. Baseline tactile hindpaw sensitivity in the two genotypes. PWT are shown for naïve adult male VIP$^{+/+}$ ($n = 19$) and VIP$^{-/-}$ ($n = 24$) mice. The right y-axis shows the PWT in gram values and the left y-axis shows the PWT in the log units equivalent to the same gram-value multiplied by a factor 10,000 as previously described (Chaplan et al., 1994). *PWT*, paw withdrawal threshold; *g*, grams; *mg*, milligrams; *VIP*, vasoactive intestinal peptide.

3.2. Tactile hypersensitivity after SNI is not restricted to the side of the injured nerve in VIP$^{-/-}$ mice

Peripheral nerve injury is known to increase the expression of VIP in dorsal root ganglia and in the dorsal horn of the spinal cord (Shehab, 2014). The involvement of this neuropeptide in pathological pain states has been previously suggested (Dickinson and Fleetwood-Walker, 1999; Dickinson et al., 1999), but never tested in mice lacking VIP. We here chose for the frequently used model of SNI (Bourquin et al., 2006) in which tactile hypersensitivity has been reported to be highly persistent. Similar to wild type controls, mice lacking VIP showed a statistically significant drop in the tactile PWT of the ipsilateral hindpaw (Fig. 2A). This drop reached lower cut-off values of 0.41 g for both genotypes from the first day after injury onwards and these low PWT scores were maintained for the entire 4 week observation period. In contrast to wild type controls, VIP$^{-/-}$ mice showed an SNI-induced tactile hypersensitivity in the contralateral hindpaw to an extent that was similar to that of the ipsilateral hindpaw (Fig. 2B). Also this contralateral tactile hypersensitivity was maintained for the full observation period. The post-operative hypersensitivity of VIP$^{-/-}$ mice was not a consequence of the surgical intervention per se as it was absent after sham-surgery (Fig. 2C, D). We then wondered whether a nerve injury may be sufficient to induce, in VIP$^{-/-}$ mice, a systemic pathological state characterized by diffuse, wide-spread hypersensitivity. In order to test this hypothesis, we assessed hindpaw tactile sensitivity before and after an injury of the median nerve in the mouse forelimb. For both genotypes, the PWT was not statistically significantly affected by this surgical intervention (Fig. 2E, F). The abovementioned experiments were repeated for VIP$^{-/-}$ and VIP$^{+/+}$ mice of the female gender and we obtained similar data as for males in all cases (SNI, sham, and MNT; Fig. 3).

3.3. More pronounced neuronal activation within the superficial dorsal horn upon innocuous tactile hindpaw stimulation in VIP$^{-/-}$ mice compared to VIP$^{+/+}$ mice

After having observed the bilateral hypersensitivity to (innocuous) tactile hindpaw stimulation in nerve lesioned VIP$^{-/-}$ mice, an experiment was designed in which innocuous tactile stimulation was

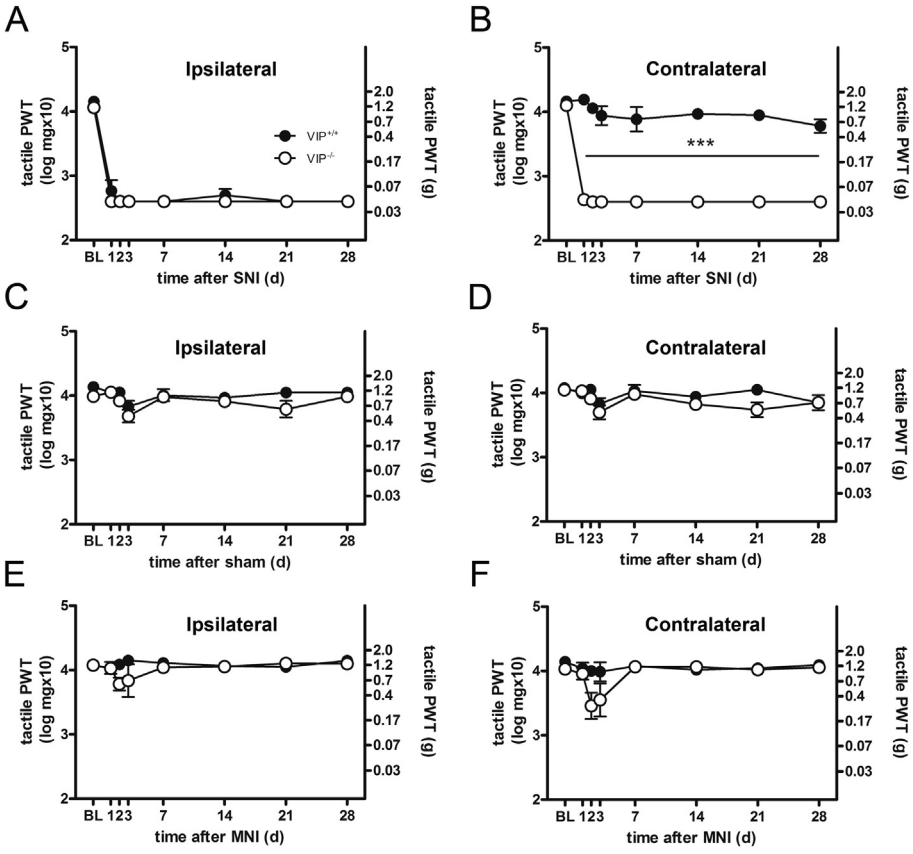

Fig. 2. Effect of nerve injuries on tactile hindpaw sensitivity in male mice of both genotypes. Tactile PWT were determined for the ipsilateral and contralateral hindpaw before and after SNI (**A,B**; $n = 4$ VIP$^{+/+}$ and $n = 7$ VIP$^{-/-}$), sham-surgery (**C,D**; $n = 4$ VIP$^{+/+}$ and $n = 4$ VIP$^{-/-}$), and MNI (**E,F**; $n = 5$ VIP$^{+/+}$ and $n = 7$ VIP$^{-/-}$). The right y-axis shows the PWT in gram values and the left y-axis shows the PWT in the log units equivalent to the same gram-value multiplied by a factor 10,000 as previously described (Chaplan et al., 1994). Note that only SNI evokes tactile hypersensitivity, which is specific for the ipsilateral hindpaw of VIP$^{+/+}$, but of similar intensity in the ipsilateral and contralateral hindpaws of VIP$^{-/-}$. While decreases in PWT were also observed in the first days after sham and MNI, these did not reach statistical significance. *PWT*, paw withdrawal threshold; *g*, grams; *mg*, milligrams; *d*, days; *BL*, baseline; *SNI*, spared nerve injury; *MNI*, median nerve injury; *VIP*, vasoactive intestinal peptide; ***, $p < 0.001$ (VIP$^{+/+}$ vs VIP$^{-/-}$).

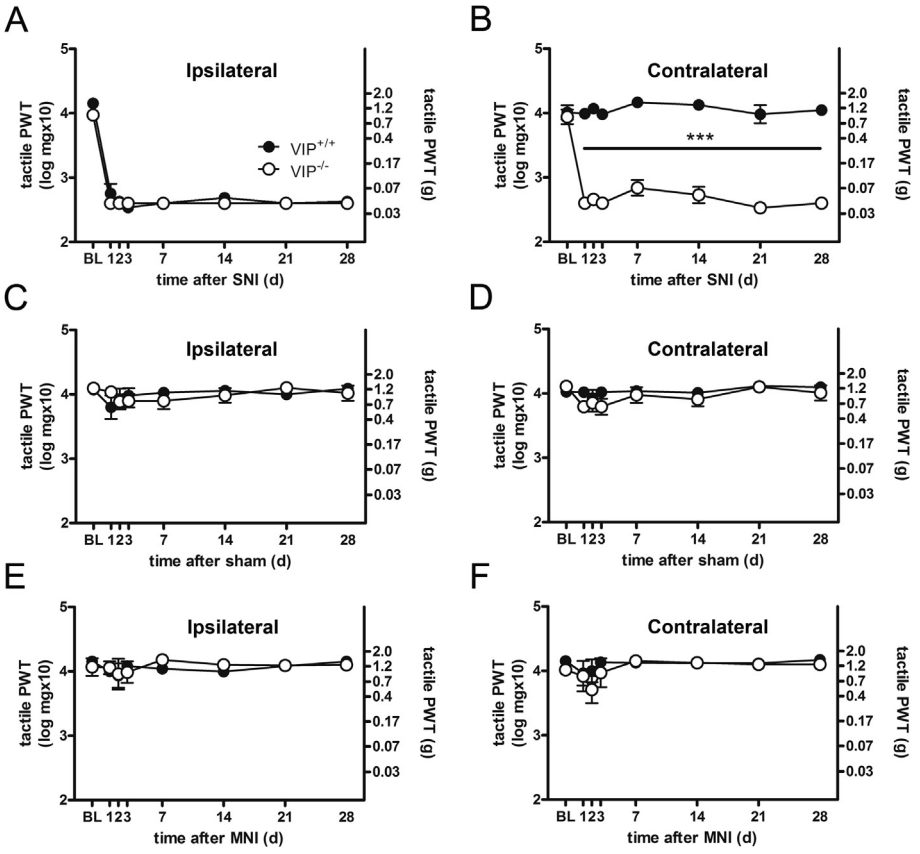

Fig. 3. Effect of nerve injuries on tactile hindpaw sensitivity in female mice of both genotypes. Tactile PWT were determined for the ipsilateral and contralateral hindpaw before and after SNI in female mice (**A**,**B**; $n = 4$ VIP$^{+/+}$ and $n = 4$ VIP$^{-/-}$), sham-surgery (**C**,**D**; $n = 4$ VIP$^{+/+}$ and $n = 4$ VIP$^{-/-}$), and MNI (**E**,**F**; $n = 3$ VIP$^{+/+}$ and $n = 2$ VIP$^{-/-}$). The right y-axis shows the PWT in gram values and the left y-axis shows the PWT in the log units equivalent to the same gram value multiplied by a factor 10,000 as previously described (Chaplan et al., 1994). Note that the tactile hypersensitivity following SNI in female VIP$^{+/+}$ is restricted to the ipsilateral hindpaw, while VIP$^{-/-}$ mice show a tactile hypersensitivity of similar intensity in both hindpaws. The drop in PWT following MNT in female mice was observed in the first days after sham and MNI, but never reached statistical significance. *PWT*, paw withdrawal threshold; *g*, grams; *mg*, milligrams; *d*, days; *BL*, baseline; *SNI*, spared nerve injury; *MNI*, median nerve injury; *VIP*, vasoactive intestinal peptide; ***, $p < 0.001$ (VIP$^{+/+}$ vs VIP$^{-/-}$).

provided for 10 min simultaneously to both hindpaws of mice at 7 days after SNI. Since tactile hypersensitivity has been previously associated with neuronal sensitization in the superficial dorsal horn (laminae I and II) (Jaken et al., 2011; Woolf et al., 1992), we studied the number of neurons expressing the immediate early activation marker cFos in this region of the lumbar spinal cord and related this number to the number of cFos-IR cells found in genotype-matched SNI mice that had not received the 10 min of tactile hindpaw stimulation. While both genotypes showed an increase in cFos cell numbers within the bilateral dorsal horn, the increases in VIP$^{-/-}$ mice were substantial (Fig. 4A) and appeared higher than those in VIP$^{+/+}$ mice. Quantification showed that the spinal neuronal responsiveness to tactile hindpaw in the superficial dorsal horn of VIP$^{-/-}$ mice was ±0.69 and ±1.78-fold greater than that of VIP$^{+/+}$ mice for the ipsilateral and contralateral sides, respectively (Fig. 4B).

3.4. A higher immune responsiveness to SNI in VIP$^{-/-}$ mice

The immune system has become increasingly implicated in the inception of chronic pain (Marchand et al., 2005). Moreover, VIP is known to have an inhibitory influence on the expression of immune mediators such as cytokines and chemokines by cells from both the adaptive and innate immune system (Ganea et al., 2003) that contribute to neuroimmune signalling in the dorsal horn after peripheral nerve injury (Costigan et al., 2009; Zhang et al., 2007). We therefore chose here to investigate the gene expression of two canonical pro-inflammatory cytokines, i.e. tumor necrosis factor-α (TNFα) and interleukin-1β (IL1β) in the dorsal quadrant of the lumbar spinal cord. At three days after surgery, the responsiveness of VIP$^{-/-}$ mice to SNI was found to be significantly higher than the responsiveness of VIP$^{+/+}$ mice for both cytokines (Fig. 5A–D). Notably, this higher responsiveness was detected at both the ipsilateral (Fig. 5A, C) and contralateral (Fig. 5B, D) sides. At seven days after surgery, the responsiveness to SNI had largely disappeared (Fig. 5E–H) and only the relative expression of the IL1β gene was still slightly higher in VIP$^{-/-}$ mice as compared to VIP$^{+/+}$ mice (Fig. 5G), even though this difference did not reach statistical significance.

3.5. VIP$^{-/-}$ mice show altered bilateral microglial reactivity in the dorsal horn after (unilateral) SNI

Microglial cells in the dorsal horn of the spinal cord have been heavily implicated in the persistent pain-like behaviors that can be observed in animal models of peripheral nerve injury (Aldskogius and Kozlova, 2013). Moreover, the immune suppressant effect of VIP has been largely described in microglial cell cultures (Delgado et al., 2002; Delgado et al., 2003). In order to look into microglial responsiveness to SNI under the influence of VIP depletion, we analysed the expression of the microglial marker Iba1 in the lumbar dorsal horn of animals of both genotypes and compared SNI and sham conditions. At seven days after SNI both genotypes showed a similar increase in Iba1 IR in the ipsilateral dorsal horn compared to sham-operated animals (Fig. 6A). The increased signal was particularly visible in the superficial dorsal horn and seemed to be more pronounced at the medial aspect (Fig. 6A). When looking at higher magnification (Fig. 6 insets), the increased signal seemed to be related to a higher density of Iba1 IR cells. In order to test whether there was a genotype effect on microglial reactivity upon peripheral nerve injury, we performed a quantitative analysis of Iba1 IR in the entire lumbar dorsal

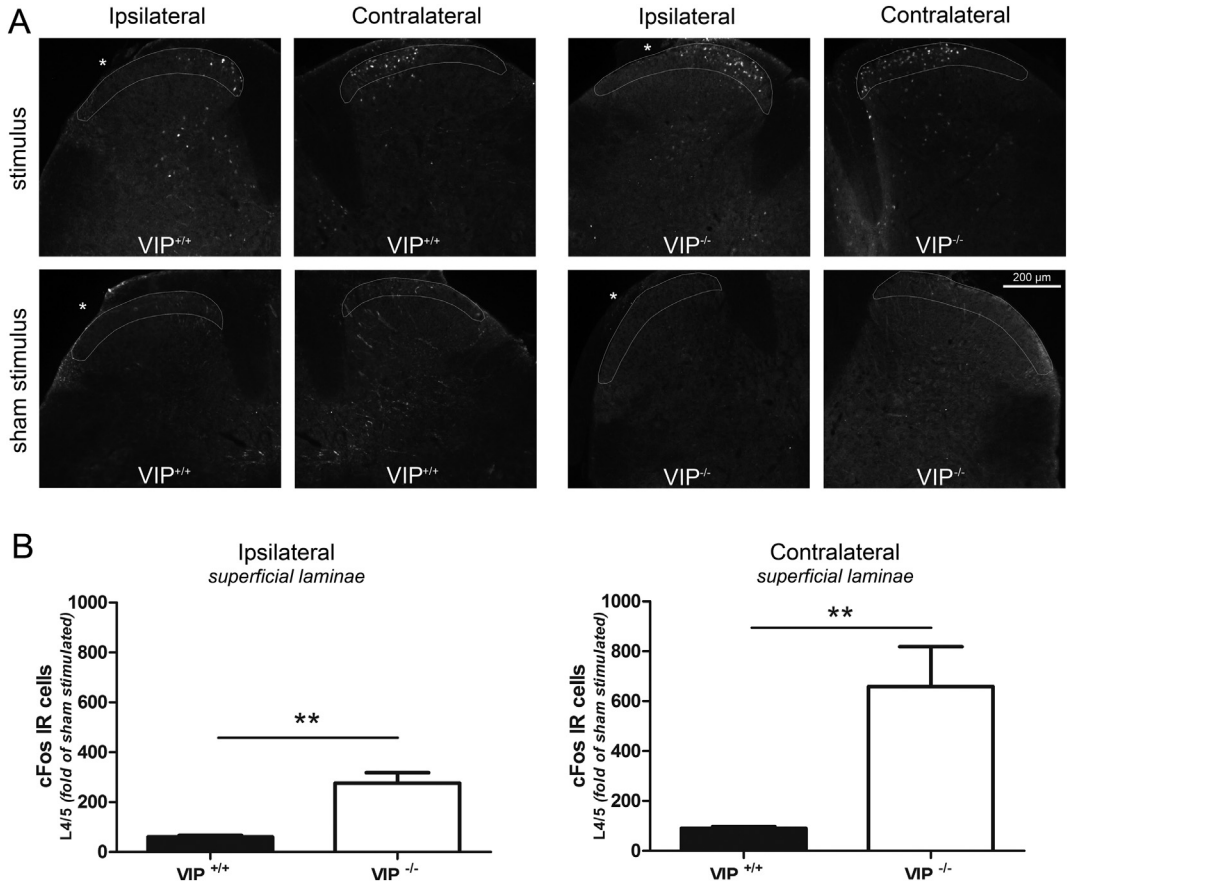

Fig. 4. Effect of innocuous tactile hindpaw stimulation on the expression of the early neuronal activation marker cFos in the superficial laminae of nerve injured mice. cFos immunohistochemistry of L4–5 spinal cord of mice one week after SNI shows increased numbers of positive cells in the superficial laminae upon innocuous tactile stimulation both hindpaws (**A**). On the side of the nerve lesion (indicated with asterisks in **A**) as well as on the contralateral side, the innocuous tactile stimulus triggered cFos expression in both the deeper and the superficial dorsal horn (the latter is in **A** marked within white lines). This observation applies to both mice strains (upper panels in **A**). Note that animals which had received SNI 7 days prior to sham paw stimulation (no stimulation, but same anesthesia event) showed only few, if any cFos-IR cells. **B**: The number of cFos-IR cells was quantified after background signal subtraction. Quantification on cFos-IR cells are, for both genotypes, expressed for SNI mice ($n = 6$ per genotype) who received innocuous hindpaw stimulation as a fold-change over SNI mice who received no hindpaw stimulation ($n = 5$ for VIP$^{-/-}$ and $n = 4$ for VIP$^{+/+}$). A significantly greater fold-increase in cFos cell number can be observed in both the ipsilateral and contralateral superficial dorsal horns of VIP$^{-/-}$ mice compared to VIP$^{+/+}$ mice; *IR*, immune reactivity; ***, $p < 0.01$; **, $p < 0.01$.

horn, thus including all six laminae of Rexed (Kew and Kemp, 2005). We found that, compared to wild type controls, VIP$^{-/-}$ mice showed a clearly higher Iba1 IR responsiveness to nerve injury than VIP$^{+/+}$ mice (Fig. 6B). This higher responsiveness was observed for both ipsilateral and contralateral dorsal horns. As bilateral tactile hypersensitivity in VIP$^{-/-}$ mice persisted for at least the four weeks following surgery, we also performed Iba1 immunohistochemical analyses at this latest observation time point. At four weeks after surgery, the Iba1 IR responsiveness to SNI was again found to be higher for VIP$^{-/-}$ mice than for VIP$^{+/+}$ mice (Fig. 7A), and this effect proved to be statistically significant (Fig. 7B). Similar immunohistochemical analyses were performed for the astroglial marker glial fibrillary acidic protein (GFAP) and while a clear GFAP IR responsiveness was detected in the dorsal horn, this effect was similar for VIP$^{-/-}$ mice and VIP$^{+/+}$ mice (data not shown).

3.6. Evidence that central sensitization is key to altered pain phenotypes in VIP$^{-/-}$ mice

Central sensitization has been the major interest for pain researchers since the pioneering work by Clifford Woolf (Woolf, 1983). The data presented thus far seem to suggest that the pathological pain phenotype in VIP$^{-/-}$ is causally related to central sensitization. As we have used full-body knockout mice in this investigation, an additional experiment was performed to gain insights into the relevance VIP action in the central nervous system may hold to pain phenotypes. With an increasing awareness on the key role of central sensitization in the chronification of pain syndromes (Deumens et al., 2013), we reasoned that normally transient pain conditions should become persistent if central sensitization persists. We therefore selected a model of plantar hindpaw incision, in which tactile hypersensitivity develops rapidly but gradually resolves over the course of about a week (Banik et al., 2006). When subjected to PI, VIP$^{+/+}$ mice indeed showed a rapid-onset ipsilateral tactile hypersensitivity (within a day), which started to recover after four to five days in both genders (Fig. 8A, C). Contrarily, VIP$^{-/-}$ again developed a contralateral tactile hypersensitivity, but also a persistence of bilateral tactile hypersensitivity for at least 10 days during which no sign of recovery could be detected (Fig. 8).

4. Discussion

In this study we described a bilateral tactile hypersensitivity in nerve injured mice lacking VIP. These mice also showed an increased neuronal activation to innocuous tactile paw stimulation in the bilateral superficial spinal cord. Also an early increase in the production of pro-inflammatory cytokines and an enhanced responsiveness of microglial cells to the nerve injury was observed on both sides of the lumbar spinal cord. The latter was evidenced for both early and late stages after nerve injury. The importance of central plasticity in the pain phenotype of VIP knock-out mice was further evidenced by data showing that VIP deficiency caused a chronification of tactile hypersensitivity. The

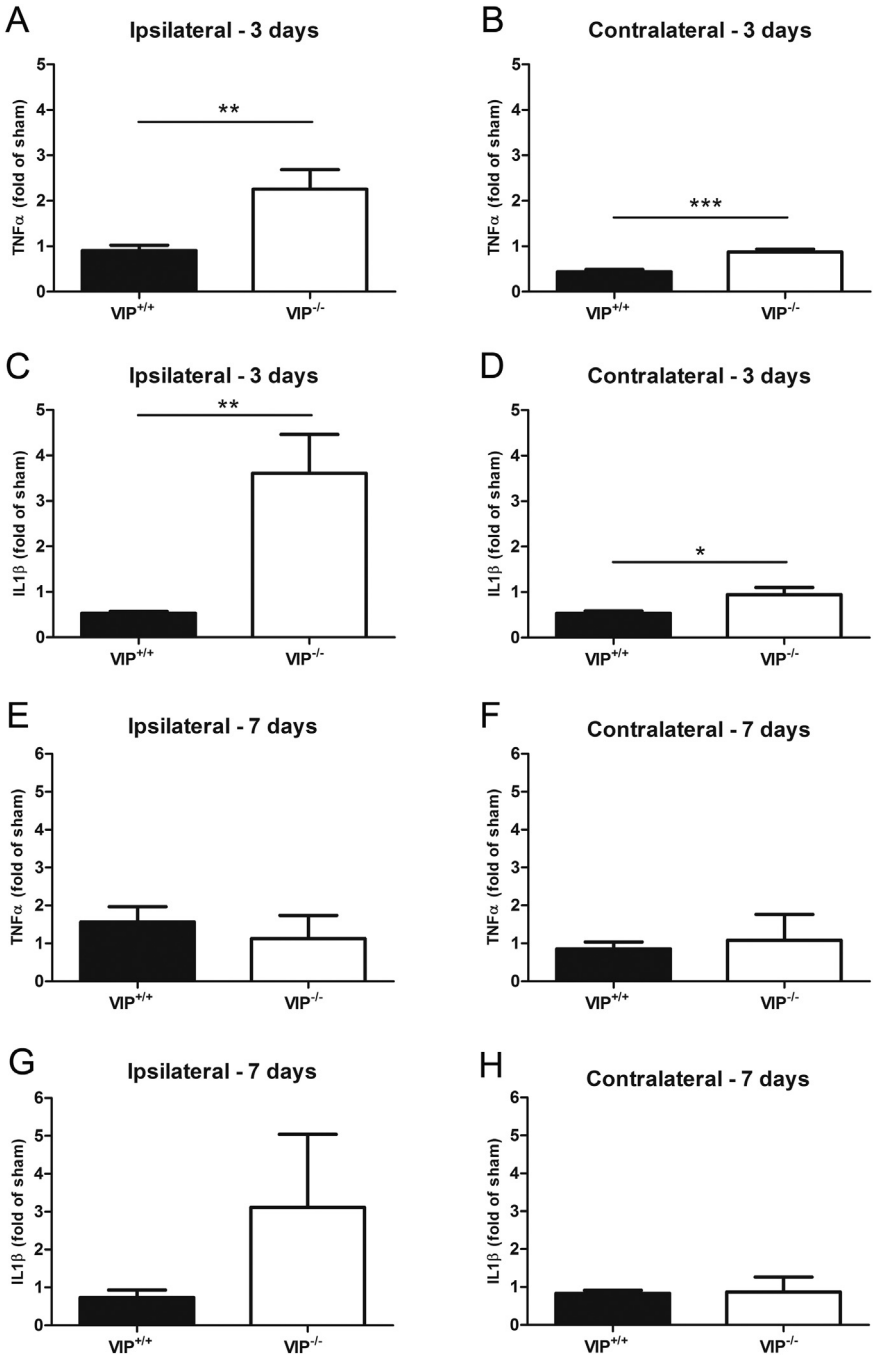

Fig. 5. Early cytokine responsiveness to SNI in the ipsilateral and contralateral dorsal lumbar spinal cord. At 3 and 7 days after SNI, qRT-PCR for the two canonical pro-inflammatory cytokines TNFα and IL1β was performed. Hereto, lumbar quadrants of $VIP^{+/+}$ ($n = 6$) and $VIP^{-/-}$ ($n = 5$) spinal cord tissue were obtained. The relative amplification for the cytokines in the samples was normalized to the relative amplification of GAPDH and βactin. In order to study the immune responsiveness to nerve injury, values were then expressed relative to those obtained with tissues of the sham-operated control animals of the respective genotype. At 3 days (**A–D**), but not 7 days (**E–H**), the cytokine responsiveness of $VIP^{-/-}$ was significantly higher than that of $VIP^{+/+}$. *TNFα*, tumor necrosis factor-α; *IL1β*, interleukin-1β; *VIP*, vasoactive intestinal peptide; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (all: $VIP^{+/+}$ vs $VIP^{-/-}$).

transition from acute to chronic pain typically requires modification in the CNS (Deumens et al., 2013).

VIP has been assigned with neuroprotective and immunomodulatory properties in neurological diseases and neurons modify the expression of VIP and its receptors upon injury and inflammation (Waschek, 2013). Injury to peripheral nerves has been found to increase the expression of VIP in primary afferent neurons of the dorsal root ganglia, particularly those which are small- or medium-sized (Shehab, 2014). The VIP signal in the spinal cord of nerve injured animals is also increased, possibly resulting from the central projections of primary afferents increasing their VIP release. A role of VIP in pain after nerve injury has been previously suggested (Dickinson and Fleetwood-Walker, 1999), but if anything, this role appeared to concern a maintenance of pain states rather than a protective role against pain. Our data, obtained in a unilateral nerve injury model, suggest that the story is more complicated than that. Tactile hypersensitivity, one of the most debilitating symptoms of neuropathic pain (Meldrum, 2000) was, in fact, exacerbated by VIP deficiency, affecting both the ipsilateral and the contralateral hindpaw with no gender differences. This observation somehow resembles the mirror-image pain that has been reported for a small fraction of

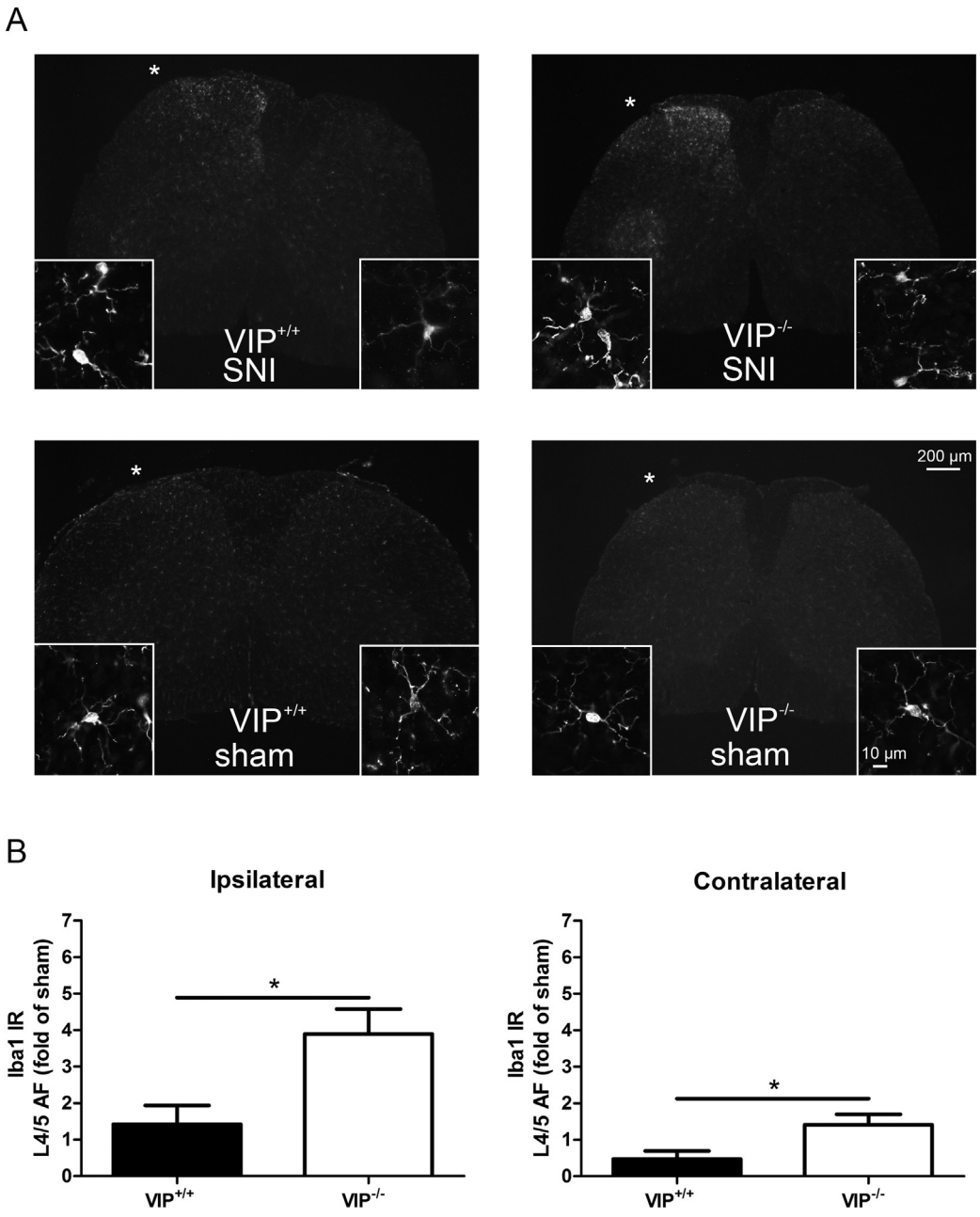

Fig. 6. Early microglial responsiveness to SNI in the ipsilateral and contralateral lumbar spinal cord. Iba1 immunohistochemistry of the L4–5 spinal cord of SNI mice at seven days showed an increased signal in the dorsal and ventral horns as compared to sham-operated mice (**A**). The effect was observed mainly at the side ipsilateral to the injured nerve (indicated with asterisks in **A**) and particularly pronounced in $VIP^{-/-}$ mice. The insets in the subfigures of **A** show magnifications of a representative part of the superficial dorsal horn. The scale bars in the down-right subfigure and inset apply to all other subfigures and insets as well. Quantification of the Iba1 responsiveness to SNI was performed by measuring the area fraction of dorsal horn occupied by Iba1 IR in SNI animals relative to sham-operated control animals of the same genotype. $VIP^{-/-}$ mice ($n = 6$) showed an increased responsiveness in the dorsal horn on both sides of the lumbar spinal cord compared to $VIP^{+/+}$ mice ($n = 5$; **B**). *SNI*, spared nerve injury; *VIP*, vasoactive intestinal peptide; *Iba1*, ionized calcium-binding adapter molecule-1; *AF*, area fraction; *IR*, immune reactivity; *, $p < 0.05$ ($VIP^{+/+}$ vs $VIP^{-/-}$).

chronic pain patients (Veldman and Goris, 1996). Moreover, it is not the first time that bilateral tactile hypersensitivity is reported in an animal model. In fact, it has been observed after a variety of insults (Huang and Yu, 2010), ranging from animal models of tissue inflammation and ischemia to cancer and nerve injuries including the spared nerve injury used in the present investigation. While the mechanisms that explain the contralateral hypersensitivity after a unilateral insult remain largely unclear, both neuronal and immune theories have been proposed (Huang and Yu, 2010).

As our data were obtained using full-body VIP knock out mice, a multitude of mechanisms may contribute to the observed neuropathic pain phenotype. For instance, compensatory plasticity might have occurred during the development of neural structures in $VIP^{-/-}$ mice. The most likely compensatory change upon VIP deficiency would concern the PACAP system. PACAP is structurally and functionally related to VIP and PACAP has been previously linked to both nociception and neuropathic pain (Davis-Taber et al., 2008; Mabuchi et al., 2004; Ohsawa et al., 2002; Sandor et al., 2010; Yokai et al., 2016). However, PACAP expression levels in the CNS of $VIP^{-/-}$ mice has been found to be comparable to those in wild type mice throughout development (Girard et al., 2006). From another perspective, VIP deficiency may cause behavioral phenotypes that are biologically related to pain. Previous work has for instance highlighted a disrupted circadian rhythm in these mice (Colwell et al., 2003), which was not very surprising

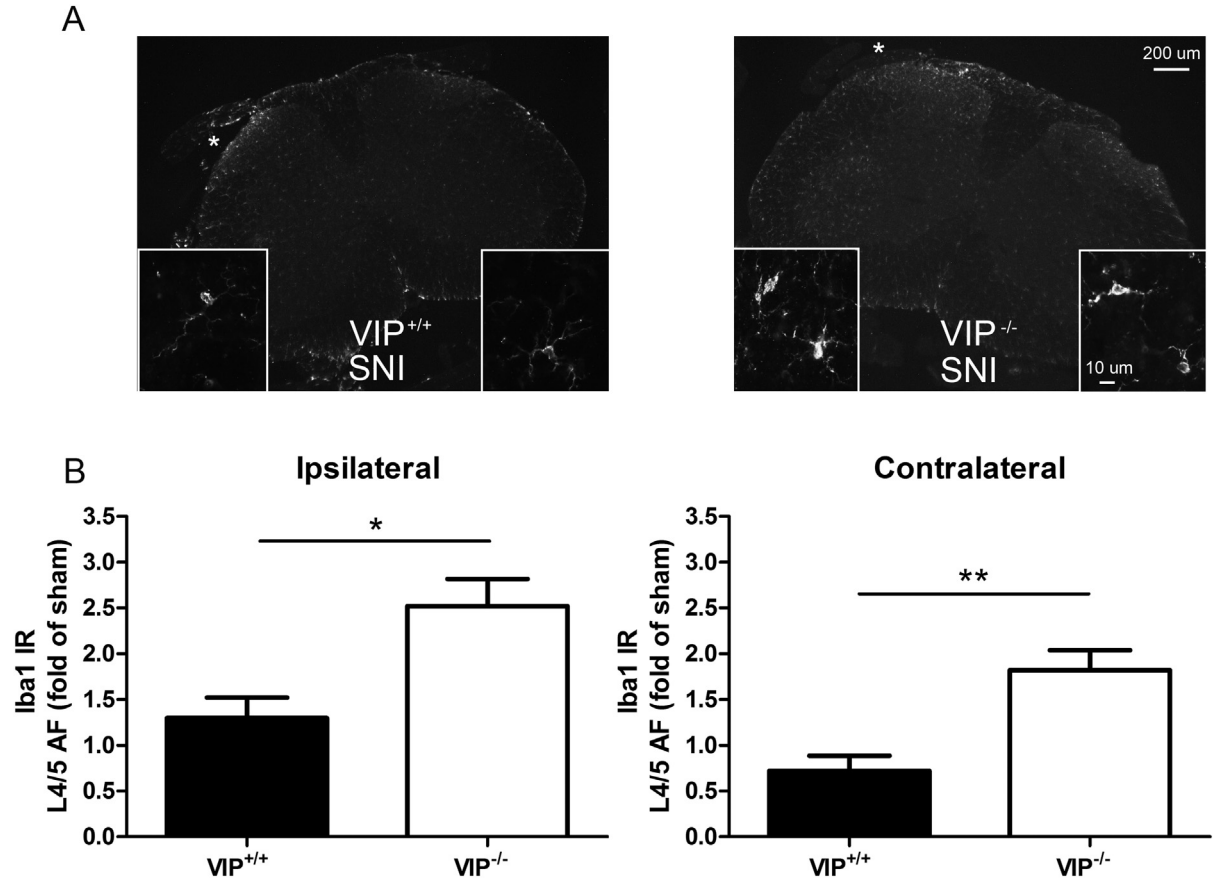

Fig. 7. Bilateral microglial responsiveness to SNI in VIP$^{-/-}$ mice persists at four weeks. At 4 weeks after SNI, the lumbar dorsal horn showed a high Iba1 IR (**A**) that was not as intense as at 7 days after SNI, but remained at levels above sham-operated controls (compare with Fig. 4**A**). The scale bars in the right subfigure and inset apply to the left subfigure and other insets as well. Quantification of the Iba1 responsiveness to SNI was done as described before and VIP$^{-/-}$ mice ($n = 4$) again showed an increased responsiveness in the dorsal horn on both sides of the lumbar spinal cord, compared to VIP$^{+/+}$ mice ($n = 4$; **B**). *SNI*, spared nerve injury; *VIP*, vasoactive intestinal peptide; *Iba1*, ionized calcium-binding adapter molecule-1; *AF*, area fraction; *IR*, immune reactivity; **, $p < 0.01$; *, $p < 0.05$ (both: VIP$^{+/+}$ vs VIP$^{-/-}$).

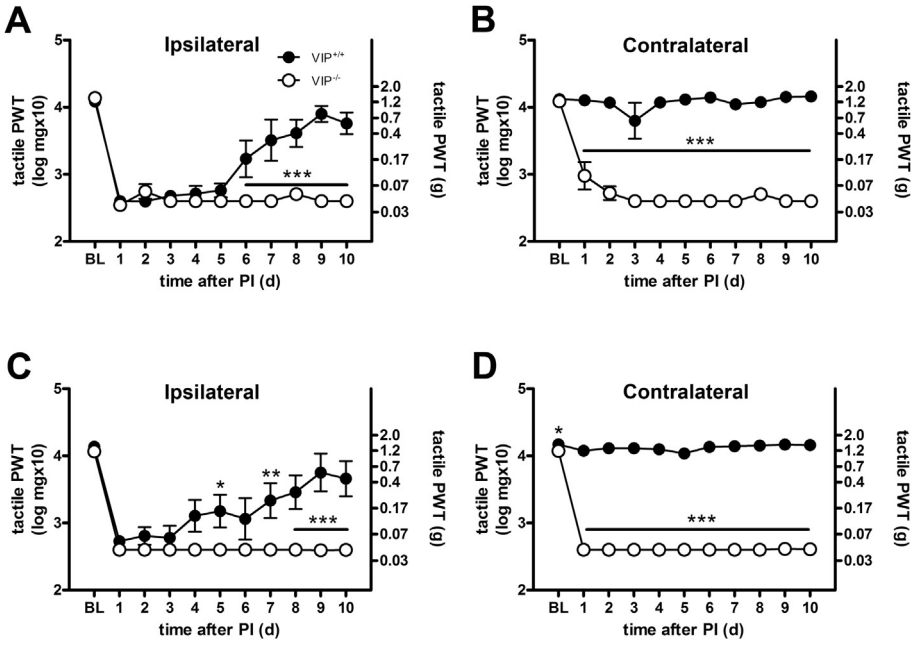

Fig. 8. Effect of plantar hindpaw incision on tactile hindpaw sensitivity in males and females of both genotypes. Tactile PWT were determined for the ipsilateral and contralateral hindpaw before and after PI in male (**A**, **B**; $n = 7$ VIP$^{+/+}$ and $n = 6$ VIP$^{-/-}$) and female (**C**, **D**; $n = 8$ VIP$^{+/+}$ and $n = 6$ VIP$^{-/-}$) mice. The right y-axis shows the PWT in gram values and the left y-axis shows the PWT in the log units equivalent to the same gram value multiplied by a factor 10,000 as previously described (Chaplan et al., 1994). *g*, grams; *mg*, milligrams; *d*, days; *BL*, baseline; *PI*, paw incision; *VIP*, vasoactive intestinal peptide; *, $p < 0.015$; ***, $p < 0.001$.

considering the strong expression of VIP by neurons in the suprachiasmatic nucleus that controls circadian oscillations and synchronization of these rhythms to light. Alterations in circadian rhythms may largely impact on pain perception (McEwen and Karatsoreos, 2015) and even occur in neuropathic states (Odo et al., 2014). It is, however, unlikely related to the pain data in the present investigation. First, VIP$^{-/-}$ mice did not show any alteration in normal tactile sensitivity. Second, the tactile hypersensitivity seemed to be related to a localized rather than systemic mechanism as a distant nerve injury (to the median nerve) did not cause tactile hindpaw hypersensitivity throughout the 4-weeks observation period. In our search for a mechanism to explain the persistent bilateral tactile hypersensitivity in nerve injured VIP$^{-/-}$ mice we therefore focused on the lumbar spinal cord.

Our data first highlight that neurons in the superficial dorsal horn, which are typically involved in relaying noxious signals up to higher brain centers (Mantyh and Hunt, 2004), show a stronger responsiveness to innocuous tactile hindpaw stimulation in VIP$^{-/-}$ mice than in VIP$^{+/+}$ mice. This effect was found to be bilateral and may contribute to the bilateral tactile hypersensitivity these mutant mice display after SNI. Nevertheless, we were particularly interested in the immune system because of its strong implication in chronic pain states (Marchand et al., 2005), which under neuropathic conditions particularly concerns microglial reactivity. Our data suggest that VIP deficiency exacerbates the immune responsiveness to nerve injury. Early after SNI, i.e. at three days, the increases in TNFα and IL1β were higher in the dorsal horn of VIP$^{-/-}$ mice compared to VIP$^{+/+}$ mice. Both pro-inflammatory factors are known to facilitate tactile hypersensitivity through CNS mechanisms (Reeve et al., 2000; Youn et al., 2008). Moreover, TNFα has been recently implicated in the development of contralateral tactile hypersensitivity after unilateral nerve injury (Cheng et al., 2014). The cytokine responsiveness in the dorsal horn after nerve injury in VIP$^{-/-}$ mice was, however, not persistent and could no longer be detected at 7 days after injury. At this late time point, however, the dorsal horn microglial reactivity to SNI was found to be higher for VIP$^{-/-}$ mice compared to their wild type controls. These data are in line with previous work demonstrating VIP's capacity to reduce microglial reactivity in models of neurodegeneration such as MPTP-induced dopaminergic cell death (Delgado and Ganea, 2003). Moreover, in vitro studies evidenced a broad immune suppressant effect of VIP on microglial reactivity to inflammatory insults (Delgado et al., 2002; Delgado et al., 2003). The microglial responsiveness to SNI was bilateral in VIP$^{-/-}$ mice and even found at four weeks after nerve injury, which likens the possibility of a microglial role as the contralateral tactile hypersensitivity persisted at this late time point and microglial reactivity has been highly implicated in persistent pain states (Aldskogius and Kozlova, 2013; Tsuda et al., 2005). Our data do warrant, however, a methodological remark. Wild type mice seemed to lack a clear spinal cytokine response and microglial reactivity upon nerve injury, which we suspect to be caused by our method of tissue collection. Spinal immune responses are typically restricted to the level of the spinal cord that receives direct innervation by the central projections of axotomized primary afferent fibers (Zhang et al., 2007). We here isolated the full lumbar enlargement for our tissue investigations, possibly diluting the signal of increased expression of cytokine or microglial markers. This, however, puts even more weight on the responsiveness in VIP$^{-/-}$ mice, the tissue of which was processed in exactly the same way.

The tactile hypersensitivity in nerve injured VIP$^{-/-}$ mice developed as rapid at the contralateral side as at the ipsilateral side. Many evidences in the literature show a delayed onset of contralateral hypersensitivity together with a lower intensity of this hypersensitivity compared to that of the ipsilateral side (Arguis et al., 2008; Schreiber et al., 2008). Nevertheless, rapid onset of contralateral hypersensitivity has been reported as well (Paulson et al., 2000; Seo et al., 2008; Spataro et al., 2004) and has been strongly linked to mechanisms acting at the level of the CNS, involving glial cells such as astrocytes (Seo et al., 2008; Spataro et al., 2004). In fact, astrocytes have been proposed to not only modulate, but also bridge neuronal pathways and networks, thereby functionally linking cells that are normally separated (Volterra and Meldolesi, 2005). While astrocytes express receptors for VIP (Ashur-Fabian et al., 1997; Cholewinski and Wilkin, 1988), we found no evidence for a different astrocytic GFAP reactivity to nerve injury between VIP$^{-/-}$ mice and VIP$^{+/+}$ mice. This lack of difference does not necessarily mean that astrocytes had no part in the onset and/or maintenance of mirror-image pain in our VIP$^{-/-}$ model. Expression levels of GFAP do not always reflect astrocyte functionality. The previously reported link between spinal astrocytes and contralateral tactile hypersensitivity in fact involved signalling through astrocytic gap junctions (Spataro et al., 2004). Interestingly, this type of signalling has also been linked to the reactivity of microglial cells upon trauma to CNS tissue (Davalos et al., 2005). Moreover, blocking astrocytic gap junctions can reduce CNS inflammatory reactions including IL1β expression (Spataro et al., 2004). We can therefore not rule out that spinal astrocytes, possibly operating as a coordinated network, have a role in the bilateralization of tactile hypersensitivity and immune reactivity in our nerve injured VIP$^{-/-}$ mice.

Although our data on dorsal horn neuronal sensitization and spinal immune modifications in nerve injured VIP$^{-/-}$ mice are suggestive of a role in the contralateral tactile hypersensitivity, a causal link cannot be made. VIP is a molecule with a large range of expression, encompassing both central and peripheral sites (Shehab and Atkinson, 1986). However, our experiments on the model of hindpaw incision are highly suggestive that modifications in the central nervous system of VIP$^{-/-}$ mice are most likely causally related to a pathological pain phenotype in these mice. The persistence of normally transient pain hypersensitivity has been linked to central sensitization in previous works (Alkaitis et al., 2010; Eijkelkamp et al., 2010). Even though we cannot rule out that also peripheral mechanisms might have been at work to contribute to the pain phenotype in VIP$^{-/-}$ mice, the persistent tactile hypersensitivity which developed bilaterally indicates the relevance of mechanisms acting at the CNS level. Follow-up experiments will need to be designed to study whether recombinant VIP or neutralizing antibodies against TNFα and IL1β may reverse the mirror-image tactile hypersensitivity in VIP$^{-/-}$ mice.

During the last years an increasing number of publications has addressed gender differences in pathological pain outcomes, particularly those related to immune mechanisms (Mogil, 2012; Rosen et al., 2017; Sorge et al., 2015). Even though we detected distinct immune profiles after nerve injury in VIP$^{-/-}$ mice, we could not evidence any proof of VIP's differential biological impact on pathological pain in males or females.

Even though our results support the concept that VIP plays a role in neuropathic pain and encourages further investigations into the exact mechanisms, which may importantly be immune-related, important questions remain unanswered. A challenge for future studies may reside in understanding which receptor proteins contribute to the neuropathic pain phenotype in VIP$^{-/-}$ mice. Of the two VIP receptors, VPAC2 receptor has been studied most intensively in relation to pain. Pharmacological activation of the spinal VPAC2 receptor in naïve rats has been found to induce phosphorylation of p38 mitogen-activated protein kinase (Garry et al., 2005), which is typically associated with microglial reactivity upon nerve injury (Ji and Suter, 2007). Moreover, when the spinal VPAC2 receptor was antagonized after peripheral nerve injury, p38 phosphorylation as well as established neuropathic pain-like behaviors were reduced (Garry et al., 2005). This data decreases the likelihood of a role of VPAC2 receptors in the bilateral tactile hypersensitivity of nerve injured VIP$^{-/-}$ mice. A role of VPAC1 is more likely as microglial cells exclusively express this receptor and it is through VPAC1 signalling that VIP had robust immune suppressive effects on immune-challenged microglial cells (Delgado et al., 2002; Kim et al., 2000). Nevertheless, a role of VPAC1 in the bilateral tactile hypersensitivity of VIP$^{-/-}$ mice remains to be confirmed in future investigations. Finally, even though this article focused on the CNS, the possibility of an involvement of

peripheral mechanisms cannot be ignored. Mirror-image pain has been previously documented after intradermal capsaicin treatment (Shenker et al., 2008), suggesting the involvement of primary afferent fibers of the C-type. Recent work also linked direct unilateral activation of tactile-specific nociceptors with the onset of robust and modality-specific bilateral sensitization (Osteen et al., 2016). Whether these or other primary afferent fiber populations contribute to the bilateral tactile hypersensitivity in VIP$^{-/-}$ mice remains to be determined.

In conclusion, we here found that VIP$^{-/-}$ mice show a bilateral tactile hypersensitivity after spared nerve injury and a bilateral increase in neuronal activation upon innocuous tactile paw stimulation. These observations may be related to a likewise bilateral cytokine and microglial reactivity in the spinal cord dorsal horns. Future investigations will need to deepen our mechanistic understanding of the requirement of specific primary afferent fiber populations and/or the receptors involved as well as their up-and down-stream signalling events.

Acknowledgements

The authors declare no competing financial interests. Professor Vincent Lelièvre (University of Strasbourg, France) is warmly thanked for providing the VIP$^{-/-}$ mice and professor Anne Des Rieux for primer sequences. This work was funded by the Ministry of Scientific Policy for financial support (ARC 10/15-026 to E.H.) and by the Fondation Louvain. A.G. was funded by the UCL (Projects FSR and Bourse de Patrimoine) and R.D. was funded by the UCL (FSR Incoming fellowship and Fonds de Recherche Clinique), and F.R.S.-FNRS.

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