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Distribution and chemical coding of sensory neurons innervating the skin of the porcine hindlimb

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ABSTRACT

The aim of the present study was to establish the origin and chemical phenotyping of neurons involved in skin innervation of the porcine hind leg. The dorsal root ganglia (DRGs) of the lumbar (L_4–L_6) and sacral (S_1–S_3) spinal nerves were visualized using the fluorescent tracer Fast Blue (FB). The morphometric analysis of FB-positive (FB+) neurons showed that in the L_4, L_5, S_1 and S_2 DRGs, the small-sized perikarya constituted the major population, whereas in the L_6 and S_3 DRGs the medium-sized cells made up the major population. In all these ganglia, large-sized FB+ perikarya constituted only a small percentage of all FB+ neurons. Immunohistochemistry revealed that small- and medium-sized FB+ perikarya contained sensory markers such as: substance P (SP), calcitonin gene related peptide (CGRP) and galanin (GAL); as well as various other factors such as somatostatin (SOM), calbindin-D28k (CB), pituitary adenylate cyclase-activating polypeptide (PACAP) and neuronal nitric oxide synthase (nNOS). Meanwhile large-sized FB+ perikarya usually expressed SP, CGRP or PACAP. In the lumbar DRGs, some large cells also contained SOM and CB. Double-labeling immunohistochemistry showed that SP-positive neurons co-expressed CGRP, GAL or PACAP; while PACAP-positive cells co-expressed GAL or nNOS. Neurons stained for SOM were also immunoreactive for CB or GAL, while neurons stained for nNOS were also immunoreactive for GAL. In conclusion, the present data has indicated that the distribution and chemical phenotyping of the porcine skin-projecting neurons are different within DRGs of the lumbar (forming a femoral nerve) and sacral (forming a sciatic nerve) spinal nerves.

1. Introduction

Pseudounipolar afferent neurons localized in dorsal root ganglia (DRGs) have two branches extending from the common trunk: a peripheral process innervating the somatic or visceral target and the central branch conveying information to the spinal cord or brainstem (Gilbert, 2000; Rowe and Iwamura, 2001). In humans, as well as in other species that have been studied so far (rat, mouse and cat), skin-projecting neurons supplying the lower leg/hind paw were distributed in a distinct set of DRGs. For example, in humans such neurons were found in L_1–S_3 DRGs, in rodents they were found within the L_4–L_6 DRGs while in the cat they were localized within the L_6–L_7 and S_1 DRGs (Barabas and Stucky, 2013; Mclachlan and Jänig, 1983; Minett et al., 2014; Royden et al., 2013; Takahashi et al., 2003).

From a physiological point of view, skin-projecting DRGs neurons may be divided into four main subtypes: (i) nociceptors, sensing painful (nociceptive) stimuli, (ii) pruriceptors, conveying itch sensations, (iii) thermoceptors, registering temperature information and (iv) a huge, quite heterogeneous group of low-threshold mechanoreceptors (LTMRs), encoding touch – i.e., a wide variety of non-painful mechanical stimuli (McGlone and Reilly, 2010). However, each of these physiologically-defined neuronal subsets could be further separated into different functional subtypes on the basis of various histological as well as physiological parameters such as their soma size, axon diameter, degree of myelination as well as conduction velocity (for a review see Abraira and Ginty, 2013). For example, pig DRG cells were divided into small (<30 μm) to medium-sized (<50 μm) and large perikarya (>50 μm; Bossowska et al., 2009) according to the diameter of their soma, while their axons were classified as A and C fibers (Harper and Lawson, 1985). However, it should be stressed that each of the populations of neuronal processes should, in fact, be considered as a set of terminals emerging from functionally distinct heterogeneous groups of DRG neurons. For example, the population of "Aβ fibers" (an anatomical substrate for LTMRs) could be further divided into at least four functional subtypes, distinguished primarily by their ability to adapt to sustained mechanical stimulation (rapidly-adapting vs. slowly-adapting – RA vs. SA, respectively). These four functional subtypes would be: (i) Merkel

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cells-innervating Aβ SA1-LTMRs, responsible for the recognition of the static nature of the touch stimuli, (ii) Ruffini's corpuscles-related Aβ SA2-LTMRs, sensing the stretching of skin, (iii) Meissner's corpuscles-supplying Aβ RA1-LTMR fibers, sensitive to movements across the skin and (iv) Aβ RA2-LTMRs, terminating in Pacinian corpuscles and sensing high-frequency vibrations (Zimmerman et al., 2014). However, these subtypes do not convey nociceptive stimuli (Johnson, 2008). Aδ terminals located within the skin are mainly responsible for sensing mechanical (especially pinprick or pinching), thermal and chemical stimuli; these afferents have also been shown to be involved in hyperalgetic states in mice (Byers and Bonica, 2001; Yeomans et al., 2004). Small-sized neurons that form the source of intradermal C fibers constitute the major population of skin-supplying DRG cells (Smith and Lewin, 2009). Since these neurons respond to thermal, chemical (acute and chronic itch) as well as nociceptive stimuli, they were therefore termed polymodal cells (Johnson, 2008). Furthermore, although it is now generally accepted that virtually all touch sensations are mediated by the above-described fast conducting Aβ LTMRs, there is a growing body of evidence that a population of C fibers (termed "C-tactile afferents") play a crucial role in conveying the so-called "pleasure touch stimuli" generated during grooming or nurturing behaviors (McGlone and Reilly, 2010; Zimmerman et al., 2014). Thus, according to the above-mentioned data, it is widely accepted that the vast majority of small-sized (and at least part of the medium-sized) sensory neurons participate in nociception/pruriception as well as thermal stimuli sensing (Djouhri et al., 2003; Petruska et al., 2000). Meanwhile, large-sized perikarya are mainly involved in mechanoreceptive and/or proprioceptive information transmission (Le Pichon and Chesler, 2014; Li et al., 2016).

Immunohistochemical studies have demonstrated that the small-sized skin-projecting DRG neurons may be roughly divided into two groups. The first group is the "non-peptidergic" population, which binds *Bandeiraea simplicifolia* lectin (I-B4) and utilizes glutamate as its main transmitter (Plenderleith and Snow, 1993). This group is most likely involved in itch processing (for a review see Ma, 2010). The second group is the "peptidergic" population, which exhibits numerous bioactive molecules that are widely accepted as markers of nociceptive cells (Ye et al., 2013). These substances include, among others: (i) substance P (SP), a peptide responsible not only for neurogenic dermatitis, but also for wound healing (for a review see Blais et al., 2014), (ii) calcitonin gene-related peptide (CGRP; Taddese et al., 1995), a peptide that apart from its involvement in sensory transmission, appears to act as a very prominent regulatory agent of the immunological and inflammatory reactions of the skin, interacting with the antigen-presenting capability of Langerhans cells (Granstein et al., 2015), (iii) somatostatin (SOM; Molander et al., 1987) which is most likely expressed by a subset of pruriceptors (Stantcheva et al., 2016) as well as nociceptors (Shi et al., 2014), (iv) galanin (GAL; Kashiba et al., 1994) responsible not only for nociceptive (mainly thermal) transmission, but also for the regenerative abilities of sensory cells, especially the peripheral, skin-projecting terminals of them (Hill et al., 2010), (v) nitric oxide synthase (NOS; Wang et al., 2013), a marker for NO synthesizing afferent cells, contributing to mechanical hypersensitivity during the maintenance phase of neuropathic pain (Kim et al., 2011), (vi) pituitary adenylate cyclase-activating polypeptide (PACAP; Moller et al., 1993), involved not only in pain processing, but also in the neuroprotection of challenged DRG neurons (McIlvain et al., 2006) or (vii) calbindin-D28k (CB; Duc et al., 1994), a calcium-binding protein being a specific marker to a distinct population of capsaicin-resistant skin-projecting neurons (Kashiba et al., 1990).

It must be stressed that there is still a lack of data concerning both the distribution pattern as well as the neurochemical features of porcine skin-projecting DRGs neurons supplying the hindlimb. Such data could be of great importance because the pig, as opposed to rodents (rats, mouse and guinea pig) or carnivores (cat), shows distinct anatomical, physiological, biochemical and immunological similarities to humans (Avon and Wood, 2005). Therefore, the pig should be considered to be an especially valuable species for studying human skin diseases, such as dermal melanoma, hypertrophic scarring, bullous pemphigoid and allergic and contact dermatitis among others (Herron, 2009; Hruban et al., 2004; Olivry et al., 2000; Vana and Meingassner, 2000; Zhu et al., 2003). Furthermore, although the organization of the dermal innervation in the pig is still not fully elucidated, porcine skin has also been used as a model to study mechanism(s) of cutaneous pain, wound and burn lesion healing as well as radiation and laser exposure (Di Giminiani et al., 2013; Kim et al., 2013; Levi et al., 2011; Sullivan et al., 2001). Therefore, the present study was aimed at (i) revealing the sources of origin of afferent fibers involved in skin innervation of the porcine hindlimb by the use of retrograde tract-tracing, (ii) defining of the chemical phenotypes of traced neurons by means of single- and double-immunofluorescence labeling and (iii) comparing the inter- and intraganglionic distribution pattern, as well as the neurochemical features of retrogradely-labeled neurons that have sent their processes to cutaneous branches of the femoral and sciatic nerves.

2. Materials and methods

2.1. Animals

Four juvenile female crossbred gilts (Pietrain x Duroc), aged 8–12 weeks and weighing 15–20 kg were used. All animals were housed and treated in accordance with the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). All experimental procedures were approved by the Local Ethics Commission of the University of Warmia and Mazury in Olsztyn (no. 70/2012). During the entire experiment, all efforts were made to minimize both the number of animals used as well as their suffering. Seven days before the beginning of the experiment, the animals were transported from a farm to the local animal facility where they were individually housed in stalls under conditions of natural light and room temperature. All animals were fed with a commercial grain mixture and tap water ad libitum. 24 h before surgery feeding was stopped.

2.2. Anesthesia, surgery and tissue processing

Thirty minutes before the main anesthetic (sodium thiobarbital; Thiopenthal, Sandoz, Austria; 20 mg/kg b.w., i.v., in a fractionated infusion) was given, the animals were pretreated with atropine sulfate (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and azaperone (Stressnil, Janssen Pharmaceutica, Belgium; 2.0 mg/kg b.w., i.m). After induction of the surgical anesthesia, the skin of the left hindlimb was gently shaved, disinfected with a 1% water-alcohol solution of iodine tincture and then a chessboard-style grid was drawn on it with a dermatologically neutral, permanent marker; the sides of the resulting squares measured approximately 1 cm. By the use of a Hamilton syringe equipped with a 26 gauge needle, 1 μl of 5% aqueous solution of fluorescing retrograde neuronal tracer Fast Blue (FB; EMS-Chemie GmbH, Groß-Umstadt, Germany) was injected intradermally into the center of each square. For example, 50 injections was made in the area innervated by the cutenous branches of the femoral nerve (50 μl) and for the sciatic nerve another 50 (50 μl). This procedure allowed for the complete coverage of the skin regions innervated by cutaneous branches of the femoral and sciatic nerves, respectively (Fig. 1a). Four weeks later all animals were euthanized by an overdose of sodium thiobarbital and, after the cessation of breathing and heart beat, transcardially perfused with an initial flush of a preperfusion solution containing 0.9% NaCl (POCH, Poland), 2.5% polyvinylpyrrolidone (Sigma, Germany), 0.05% procaine hydrochloride (Polfa, Poland) and 20,000 IU of heparine (Polfa, Poland; added just before the perfusion started), followed by 4% paraformaldehyde (Merck, Germany) in 0.1 M phosphate buffer (pH 7.4). Th_{12}–S_4 DRGs ipsilateral to the tracer injection sites were collected from all studied animals and then postfixed by immersion in the

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