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Multi-molecular and structural profiles of the skin and cutaneous innervation that validate a pig proximal nerve injury models for translational research on human peripheral neuropathic pain

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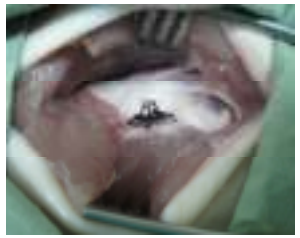
Introduction:

Nerve trauma resulted from either mechanical injury such as accident, amputation or inflammatory/immune disease may give rise to neuropathic pain, which are often resisting to treatment. This pain in animals as in human is a complex phenomenon composed of physiological components as well social, emotional and behavior components. Although, working with rodent models over the years contributed to our understanding of the pathophysiology of chronic neuropathic pain, these models are somewhat limited in their contribution of translating preclinical pharmacology results to the clinic. Herein, we document a new pig model for chronic pain induced by proximal peripheral nerve trauma. Multi-molecular immunolabel show pathological profiles of the innervation and neural signaling properties of keratinocytes like those observed in human patients afflicted by PHN and CRPS (Albrecht et al,. 2006; Hou et al., 2011; Ibraham et al. (2003); Khodorova et al 2003; Petersen et al., 2001, 2010; Zhou et al, 2008).

Surgical Method:

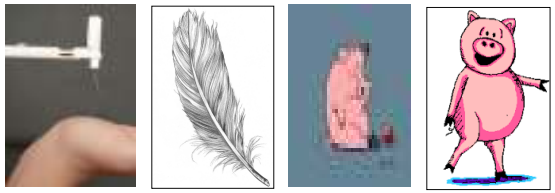
Induction of trauma: an incision of 8-10 cm was made through the skin and fascia on the left side of the lower back towards the caudal end, approximately 1.5 cm lateral and parallel to the spine line of the pig. Then the muscles were retracted and the sciatic nerve was exposed. Three 3-0 silk threads were each 3 cm length were immersed in CFA (1 mg/ml; MD Biosciences) overnight prior to surgery. Then, once the sciatic bundle was exposed, these pre immersed threads were used to create 3 loose ligation surrounding the lateral half of the sciatic nerve bundle in 1-2 mm apart.



Pain assessment:

A. Withdrawal response

B. Spontaneous changes



Von Frey Test. (Castel et al., EJP 2013).

Feather Test

composite behavioural Scale (CBS)

Motor Function

IHC Method:

Tissue: Following the behavioral assessments, the pigs were euthanized and skin biopsies were collected from the dorsal area of the foot. The biopsies were immersion fixed in 4% paraformaldehyde (in 0.1M phosphate buffered saline; pH7.4) for 4 hours at 4°C, rinsed in PBS at 4°C, cryoprotected o/n in 30% sucrose/PBS, frozen, and cryostat sectioned at 14µm thickness perpendicular to the epidermal surface. Sections were thaw mounted in serial order, alternating across at least 20 slides such that each slide contained sections from equally spaced intervals throughout the biopsy.

Immunochemistry: Biopsy specimen were processed following established protocols for integrated multi-molecular immunofluorescence assessments and were evaluated utilizing the ChemoMorphometric platform developed by Intergrated Tissue Dynamics, LLC. The innervation was characterized by double labeling with combinations of antibodies against PGP9.5 (rabbit polyclonal,1:800 UltraClone) and against CGRP (sheep polyclonal, 1:800, AbCam). As shown in previous studies of human postherpetic neuralgia (PHN) and complex regional pain syndromes type 1 (CRPS), epidermal keratinocytes also express neural neuromodulatory properties implicated in algesia that can increase in PHN and CRPS patients, as well as properties implicated in analgesia, that can decrease. Among algesic modulators, immunolabeling in keratinocytes was assessed for CGRP, NaV1.7 (rabbit polyclonal, 1:500, Alomone), and endothelin-1 receptor A (ETRA, rabbit polyclonal, 1:500, Abcam). Among analgesic modulators, immunolabeling in keratinocytes was assessed for endothelin-1 receptor B (ETRB) . Secondary antibodies were conjugated with Cy3 (red) or Alexa 488 (green). Cell nuclei were counterstained for DAPI (blue)

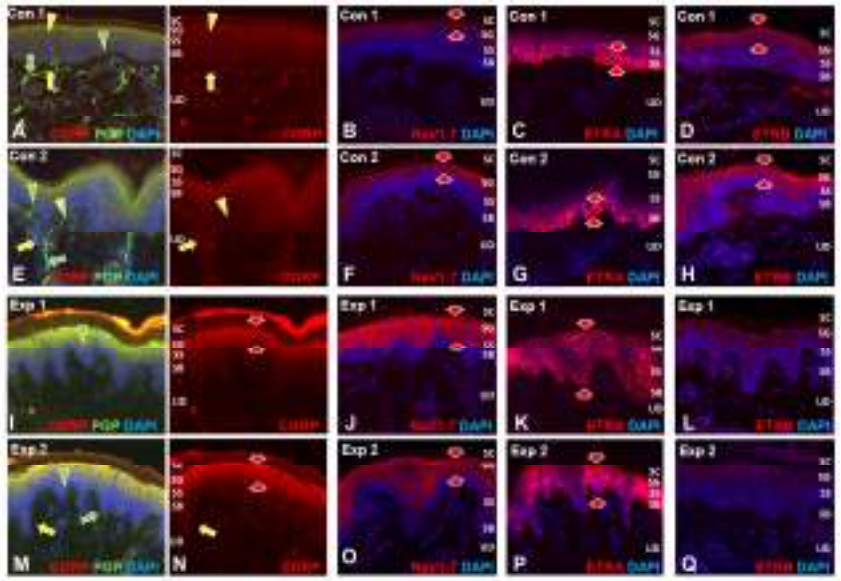
Analysis: Epifluorescence Images were captured utilizing an Olympus BX51-WI microscope equipped with conventional fluorescence filters (Cy3: 528-553 nm excitation, 590-650 nm emission; Cy2/Alexa488: 460-500 nm excitation, 510-560 nm emission), a Hamamatsu ER, DVC high-speed cameras, and 3 axis motorized stage system interfaced with Neurolucida software (MBF Bioscience, Essex, VT).

Results

Table 4. Pain Assessment During the Study Period

Method of Assessment	Study Days				
	-1 (prior to surgery)	7	10	18	28
	PNT animals				
vF Test Withdrawal Force (g)	60.00 ± 0.00	1.40 ± 0.49*	2.00 ± 1.20*	1.57 ± 1.20*	2.07 ± 1.51*
Feather Test (% responders)	0.00	83.33*	100.00*	100.00*	83.33*
CBS (mean group points)	0.00 ± 0.00	6.17 ± 2.14*	7.17 ± 2.64*	6.67 ± 2.66*	5.75 ± 1.41*
MF (mean group points)	0.00 ± 0.00	0.33 ± 0.82	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.41
	Sham animals				
vF Test Withdrawal Force (g)	60.00 ± 0.00	54.33±5.67	60.00 ± 0.00	60.00 ± 0.00	60.00 ± 0.00
Feather Test (% responders)	0.00	0.00	0.00	0.00	0.00
CBS (mean group points)	0.00 ± 0.00	0.3±0.2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MF (mean group points)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

vF: von Frey test; CBS: Composite Behavioral Scale; MF: Motor Function Scale.
*p<0.05 vs. Sham operated animals



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Summary

The trauma of the proximal sciatic nerve in the pig induced a variety of pain behaviors and thresholds like those that were consistent with symptoms of chronic pain symptoms associated with a variety of humans afflictions. This included: Tactile Allodynia expressed as withdrawal from vF filaments and light touch; changes in spontaneous daily behavior involving only minor to non excising motor dysfunction.

Multi-molecular immunofluorescence assessment of the skin revealed morphological and molecular pathologies like those in the skin of human patients afflicted with PHN and CRPS1 as well as other chronic pain conditions. This included a paradoxical reduction of small caliber innervation to the upper dermis and epidermis in which electrophysiological evidence indicates that the remaining innervation is hyperexcitable. The proximal nerve injury also induced an increase in the expression of CGRP, Nav1.7, and ETRA which have been implicated in algesic mechanisms, as well as a reduction in the expression of ETRB which has been implicated in analgesic mechanisms like that observed in human patients with PHN and CRPS. This shift in keratinocyte neurosignaling properties may contribute to the hyperactivation of remaining innervation.

These findings indicate that the pig model is a particular valuable platform for translational research on human chronic pain mechanism and potential therapeutic development.

FIGURE

Digital images of five immunolabel markers that reveal pathologies in the skin of 2 pigs with experimentally (Exp) induced pain as compared to the skin in 2 normal control pigs (Con). Layers of epidermal keratinocytes from deep to superficial are Stratum Basalis (SB), Stratum Spimosum (SS), and stratum granulosum (SG), which are composed of live keratinocytes, and Stratum Corneum (SC), which is composed of dead keratinocytes.

Panels A, C, E, and G: pairs of images from sections double labeled with anti-PGP9.5 (green), which reveals all known cutaneous innervation, and anti-CGRP (red), which shows subsets of peptidergic innervation as well as peptidergic keratinocytes. The right images of each pair show only the CGRP labeling. Arrowheads indicate sensory endings in the epidermis. Narrow arrows indicate axons and endings in the upper dermis (UD). CGRP-positive innervation is double labeled (yellow arrowheads and arrows) and CGRP-negative innervation is only labeled for PGP (green arrowheads and arrows). Both types of innervation are reduced in the experimental pigs (I & M left images) compared to that in control pigs (A & E left images). As shown in the right images, CGRP expression is increased among keratinocytes in SG and SS (between broad red arrows) in the painful skin.

Panels B, F, J, and O: Alternating sections labeled with anti-NaV1.7 (red), which is implicated in algesic mechanisms. In control pigs, NaV1.7 is expressed among keratinocytes in SG (B & F between red arrows). In experimental pigs, NaV1.7 expression is increased and expands to include keratinocytes of SG and SS (J & O between red arrows).

Panels C, G, K, and P: Alternating sections labeled with ETRA (red), which is implicated in algesic mechanisms. In control skin, ETRA immunolabeling is restricted to keratinocytes in SB (C & G between red arrows). ETRA expression spreads throughout SB, SS, and SG in the painful skin (K & P between red arrows).

Panels C, G, K, and P: Alternating sections labeled with ETRB (red), which is implicated in analgesic mechanisms. In control skin, ETRB immunolabeling is expressed in the keratinocytes of SG and into SS (D & H between red arrows). ETRB expression is substantially reduced in the painful skin (L & Q).