Human-like chronic pain pathologies induced in porcine skin innervation and epidermal keratinocytes by proximal sciatic nerve insult: A translational platform to develop therapeutics for neuropathic pain

***P. ALBRECHT[1], I. SABBAG[2], D. CASTEL[3], E. RUGGIERO[1], M. DOCKUM[1], S. MEILIN[4], F. RICE[1]**

[1]Integrated Tissue Dynamics, LLC, Rensselaer, NY; [2]Lahav Research Institute, Kibutz Lahav, Negev, Israel; [3]Neufeld Cardiac Research Institute, Sheba Medical Centre, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; [4]MD Biosciences, Ness Ziona, Israel; Oakdale, MN, USA

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Mechanisms of Peripheral Neuropathic Pain I

Introduction

Safe and effective treatment of chronic pain associated with a wide variety of peripheral neuropathies (i.e., neuropathic pain, NP) remains one of the most common and debilitating health care challenges, despite an enormous investment in research. Current therapeutics typically provide limited, unpredictable relief with a high incidence of deleterious side effects, while promising therapeutics primarily developed and tested in animal models have repeatedly failed to translate to success in human NP [1-4]. Among these afflictions are neuropathies associated with various types of nerve traumas ranging from overt transections and crushes, to insidious compression, irritation, and inflammation, to inexplicably minor soft tissue damage. NP in animals, as in humans, is a complex phenomenon composed of physiologic, social, emotional, and behavior components. Pain research with rodent models over the years has contributed greatly to our understanding of the pathophysiology of NP, however ***rodent models are proving to be limited in their contribution of translating preclinical pharmacology results to the clinic***. Rodent models of traumatic nerve injury are commonly used for NP research because of their relative simplicity, reproducibility, and low cost. These models include sciatic nerve crush, partial sciatic nerve ligation, spinal nerve ligation, spared nerve injury, and loose-suture irritation also referred to chronic constriction injury (CCI). Of these, the CCI is among the least invasive, yet causes robust pain behaviors, and is perhaps most representative of human peripheral neuritis due to nerve irritation, inflammation, and constriction.

To address the disappointment of such rodent models to translate to successful human applications, we have developed a modified CCI peripheral neuritis trauma (PNT) model in pigs, an animal which has significantly more anatomical, physiological, and neurological similarity to humans. The structure and innervation of pig skin is especially similar and more relevant to that of humans, providing a platform for assessments of skin biopsies, a technology which has been increasingly used to discover pathologies associated with a variety of painful peripheral neuropathies in humans. As a large animal model, pigs are also a more cost effective and ethically acceptable alternative to non-human primates.

Two particularly important discoveries have been made using skin biopsies from humans with a variety of painful peripheral neuropathies. First, a seemingly paradoxical reduction typically occurs among small caliber unmyelinated and lightly myelinated innervation (C and Aδ fibers, respectively), especially in the epidermis, which are a presumed source of PN. However, electrophysiological assessments indicate that remaining innervation has become hyperactive. Second, increases and decreases can occur, respectively, among excitatory and inhibitory neural signaling properties that are expressed in differentially stratified patterns among the multilayered keratinocytes of the epidermis. These neurosignaling patterns have been implicated in modulating the normal sensitivity of cutaneous innervation, particularly small fibers terminating in or near the epidermis. Importantly, both the normal and pathological stratified patterns are dynamic, being recapitulated across generations of replenished keratinocytes. Furthermore, animal models demonstrate neurochemical pathologies among epidermal keratinocytes may be induced by proximal nerve traumas, indicative of a cross-talk between the innervation and keratinocytes. The purpose of this study was to further validate the pig sciatic PNT model in comparison to overt nerve crush injuries using the INTiDYN ChemoMorphometric Analysis (CMA™) platform [5-10] with skin biopsies to profile the impact on cutaneous innervation and neurochemical properties of the epidermal keratinocytes relative to that observed in humans with NP afflictions.

Materials and Methods

Study design

38 male Danish Landrace x Large White crossbred pigs (*Sus domestica*) from the domestic herd at Lahav Laboratories, Negev, Israel. Pigs were 9-weeks old, weaned, and weighed 15 ± 1 kg at study initiation. All pigs were kept under conventional production conditions, housed in open pens (1.4 x 2.4 m) in groups of 2-3 on a 12 hr light-dark cycle for the7 days prior to study initiation. Feeding occurred three times daily using specific pig food (Dry Sows; Ct # 5420; Milobar, 7880, Oshrat, Israel), and pigs were provided opportunities to root and chew for enrichment. Fresh water was provided *ad libitum* by an automated system. The animal procedures consisted of four phases: (1) habituation (Pre-Op Day -5 to -1); (2) surgery (Day 0); (3) follow-up on Post-Operative Days (POD) ranging from 1 to 28; and (4) euthanasia and skin biopsy ranging from POD1 to 28. All procedures and experiments were approved by the M.D. Biosciences Institutional Animal Care and Use Committee (IACUC), and were designed to reduce numbers and undue suffering in accordance with the International Association for the Study of Pain (IASP).

The study consisted of two parts.

1. *Comparison of sciatic nerve traumas.* In a first series of experiments, a comparison was conducted on three types of unilateral sciatic nerve insults in pigs. As described below, unilateral sciatic nerve was fully crushed (FC) in four pigs, partially crushed (PC) in seven, and CFA-soaked ligature induced PNT in eight. Three additional pigs underwent sham surgeries consisting of sciatic nerve exposure but no injury (n=22 total pigs). Following pain and motor assessments (as described below), FC pigs were euthanized by pentobarbital overdose on POD 18 and three of the PC pigs were euthanized on POD 10. The remaining PC and all PNT pigs were euthanized at POD 28. Immediately following euthanasia, a 1x1cm skin biopsy was excised from the dorsum of the ipsilateral hindfoot and prepared for subsequent immunochemical analyses of innervation and epidermal keratinocytes (as described below).
2. *Time-course of induced sciatic nerve PNT pathologies.* Following analysis of the various pathologies among the innervation and epidermal keratinocytes caused by the different sciatic nerve injury models, a second set of experiments was conducted to assess the time-course of the robust PNT pathologies. PNT was performed on a unilateral sciatic nerve and pigs were analyzed for pain and motor symptoms. Four pigs each were euthanized on POD 1, 7, 14, and 21 (n=16 total pigs) and a 1x1cm biopsy was immediately excised from mirror image sites on the dorsum of both the operated (ipsilateral) and unoperated (contralateral) hind feet, and prepared for immunochemical analyses.

Anesthesia and Surgery

On the day of surgery (Day 0), pigs walked freely to the preparation room. Pigs were anesthetized by inhalation of 3% isoflurane/100% O2 mixture using a face mask, and the entire duration of the surgery procedures was approximately 30 minutes.
Detailed surgical methods for the various models and have been previously described [11,12].

Three different types of sciatic nerve trauma and no trauma exposure were tested in these studies:

1. *Full Length Nerve Crush (FC)*: Following sciatic nerve exposure, a hemostat was applied on the entire nerve bundle width for a period of 30 seconds to create a 10mm long crush injury. The hemostat was removed and the wound was closed.
2. *Partial Length Nerve Crush (PC)*: Following sciatic nerve exposure, a hemostat was applied on the lateral portion of the sciatic nerve; a conveniently-sized hook was used for placement between the nerve bundles to capture only part of the sciatic nerve. The nerve was crushed for a period of 30 seconds to create a 5mm long crush injury. The hemostat was removed and the wound was closed.
3. *Peripheral Neuritis Trauma (PNT)*: Three 3-0 silk threads (Assut-UK), each 3 cm in length, were immersed in complete Freund's adjuvant (CFA; 1 mg/ml) overnight. Following sciatic nerve exposure, the pre-soaked threads were used to create 3 loose ligations (1-2 mm apart) surrounding the lateral half of the sciatic nerve bundle.
4. *Sham:* A control group of pigs underwent anesthesia, skin and muscle incision/retraction, and sciatic nerve exposure, but the nerve was unmanipulated (i.e., nerve remained fully intact), and the wound was closed as described.

Pain Assessments

Mechanical sensitivity. Various thickness nylon filaments (Touch Test (von Frey) Sensory Evaluator Kit, model 58011, Stoelting Co., Wood Dale, IL, USA). The tests were performed in the home pens of each pig, using a modification of the up/down methods previously described for rodents (Chapman 1994). Various filaments with exertion to bend ranging from a minimum of 1 g (diameter = 0.229 mm; force = 9.804 mN) to a maximum of 60 g (diameter = 0.711 mm; force = 588.253 mN) were used. The filaments were applied on the dorsal area of the foot and on the external side of the knee three times each with a 5-10 second interval between applications. If withdrawal response was not achieved, the next thicker filament was applied, continuing until 60 g cutoff was achieved (most all uninjured pigs). If a withdrawal response was achieved, the next lower force filament was reapplied. Following six filament application tests alternating the filaments (up/down), the force required to achieve a withdrawal reaction was determined. Mechanical sensitivity testing was conducted on Day -1, and on POD ranging from 7 to 28 (Table 1).

Tactile sensitivity. The tactile stimulus consisted of a 12.5 cm pigeon feather, which delivered light tactile stimulation upon gentle brush stroke across the dorsal area of the foot. Responder pigs expressed all three of the following behaviors: moving away, shaking and keeping the leg up, and guarding the leg for a period of 5 seconds. Tactile sensitivity testing was conducted on Day -1, and on POD ranging from 7 to 28, and the percentage of pigs that responded was recorded (Table 1).

Spontaneous pain behavior. The solitary performance and social behavior for each pig was scored during a 10-minute observation period. Seven behavioral parameters were observed and recorded: 3 for solitary performance and 4 for social behavior, including standing posture/weight bearing, appearance (leg guarding, leg shaking), vocalization, and social behaviors (restlessness, agitation, aggression, isolation). Each behavioral parameter was graded (0-2) and the sum of all 7 parameters was considered the final score [11]. Higher scores are indicative of more spontaneous pain behaviors, recorded on Day -1, and on POD ranging from 7 to 28 (see Table 1).

Motor Function Assessment

The ability of the pigs to use their leg properly was assessed by observing standing posture and ability to walk properly [11]. Motor function was graded from 0 to 2 points: 0 = normal; 1 = occasional flip of the foot; 2 = not able to keep the foot in the normal position. This grading system was used to assess foot position when standing and walking, thus the maximum possible score was 4 (severe motor dysfunction). Any pigs experiencing secondary wounds (e.g., to the toe nails or to the dorsal area of the foot) due to a loss of motor function and foot drag were culled for ethical reasons. All FC pigs were culled at POD 18 to prevent the development of secondary wounds, therefore, no data was recorded on POD 28. Motor function assessments were performed on POD 7 to 28 (Table 1).

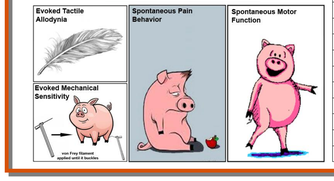
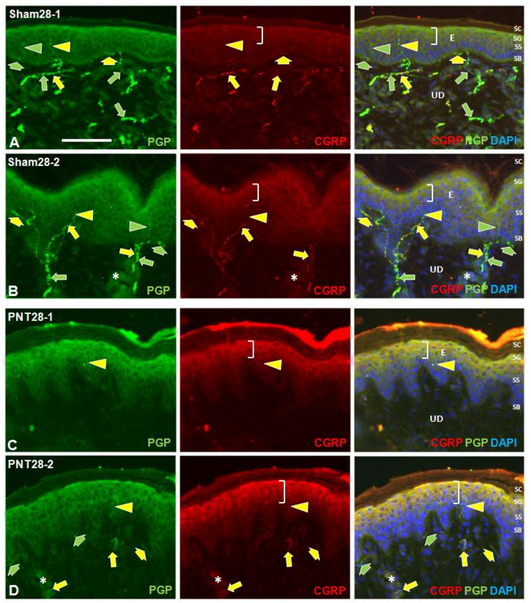


Table 1: Evoked and Behavioral Pain Analysis

	Day -1	POD 7	POD 10	POD 18	POD 28
Mechanical Sensitivity Response (von Frey; g force)					
SHAM	46.4 ± 8.3	60.0 ± 0.0	60.0 ± 0.0	53.2 ± 6.8	60.0 ± 0.0
FC	60.0 ± 0.0	60.0 ± 0.0	54.3 ± 13.9	1.7 ± 0.4**#	n/d
PC	57.6 ± 9.1	39.7 ± 28.2	50.3 ± 16.6	5.5 ± 3.4**#	6.5 ± 0.5**#
PNT	60.0 ± 0.0	3.7 ± 1.4*#	1.0 ± 0.0**#	5.1 ± 3.2*#	2.6 ± 2.3**#
Feather Allodynia (% responders)					
PNT	0.0	83.3	100	100	83.3
Spontaneous Pain Behavior (composite score)					
FC	0.0	5.7 ± 1.2	7.7 ± 1.5	6.3 ± 1.6	6.0 ± 1.0
PC	0.0	5.9 ± 1.2	7.3 ± 1.9	5.3 ± 1.5	5.3 ± 0.3
PNT	0.0	7.8 ± 2.1	6.4 ± 1.6	6.5 ± 1.0	7.0 ± 0.6
Motor Function Scale (composite score)					
FC	0.0	2.8 ± 0.6	3.0 ± 1.0	1.3 ± 1.6	n/d
PC	0.0	2.3 ± 1.0	1.4 ± 1.4	0.6 ± 0.4	0.8 ± 0.6
PNT	0.0	0.33 ± 0.82	0.0	0.0	0.0

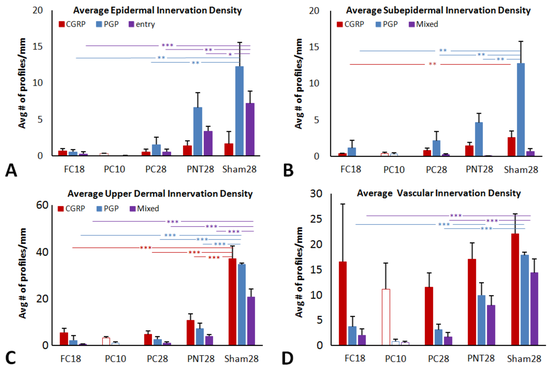
*p<0.05 vs. Day-1; **p<0.01 vs. Day-1. #p<0.05 vs. sham

Figure 1: Cutaneous Innervation and CGRP IR Alterations



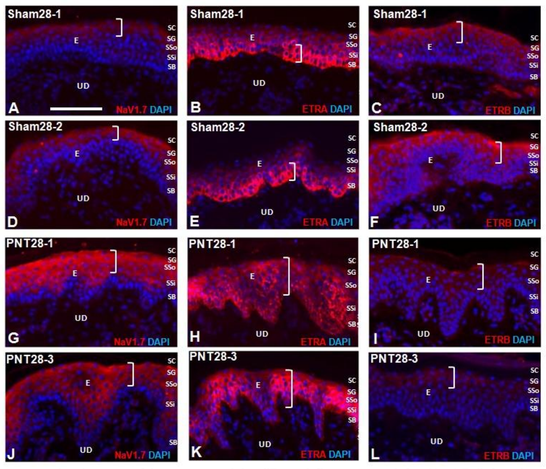
Representative immunoreactivity (IR) for PGP (green fluorescence, left column) and CGRP (red fluorescence, middle column) in ipsilateral biopsies from the dorsal hindfoot of two Sham28 (A,B) and two PNT28 pigs (C,D). Merged images with DAPI nuclear staining (blue fluorescence) are shown in the right column. E, epidermis; UD, upper dermis; SB, Stratum Basalis; SS, stratum spinosum; SG, Stratum Granulosum (SG); SC, Stratum Corneum. Scale bar = 100m. Arrowheads indicate immunolabeled neural profiles in the epidermis which are sensory endings. Broad arrows indicate immunolabeled neural profiles in the subepidermis, immediately subjacent to the epidermal basement membrane, which are a mix of individual fibers or two or more fibers within small nerves. Long arrows indicate immunolabeled neural profiles in the upper dermis which are a mix of individual fibers and two or more fibers within small nerves, some of which are affiliated with small upper dermal blood vessels seen with DAPI labeling of cells in the vessel walls (asterisks). Nonpeptidergic neural profiles only labeled for PGP (green arrowheads and arrows), and peptidergic neural profiles double labeled for CGRP and PGP (yellow arrowheads and arrows). Innervation at all levels was depleted following PNT28 injury compared with Sham28 biopsies. Middle panel brackets indicate the epidermal keratinocyte strata labeled for CGRP, which was more intense following PNT28 injury compared with Sham28 biopsies.

Figure 2: Quantification of Innervation Alterations



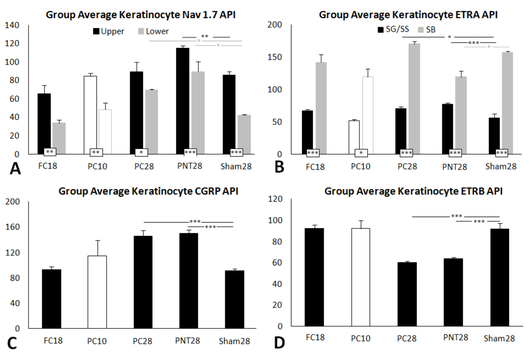
Quantitative analysis of innervation densities in ipsilateral biopsies following different sciatic nerve injury models. Data collected from 3 sections/pig double-labeled for CGRP and PGP. FC18 = full sciatic nerve crush POD 18 survival (4 pigs); PC10 = partial sciatic nerve crush POD 10 survival (3 pigs); PC28 = partial sciatic nerve crush POD 28 survival (4 pigs); PNT28 = CFA soaked loose sciatic sutures POD 28 survival (8 pigs); Sham28 = shame control POD 28 (3 pigs). Significant differences across groups of sciatic nerve injury models are shown (note that the data from PC10 pigs (open bars) were not included in statistical comparisons). A. Average epidermal innervation that was peptidergic (CGRP, red bars) was not significantly altered across any model, whereas nonpeptidergic innervation (PGP, blue bars) was significantly reduced among FC18 and PC28 compared with Sham28. IENF in contact with the basement membrane (entry, purple bars) were also significantly reduced among FC18, PC28, and PNT28 models compared with Sham28. B. Average subepidermal innervation that was peptidergic (CGRP, red bars) was reduced among FC18, and nonpeptidergic (PGP, blue bars) was reduced among FC18, PC28, and PNT28 compared with Sham28. C. Average upper dermal nonvascular innervation that was peptidergic (CGRP, red bars), nonpeptidergic (PGP, blue bars), and mixed (purple bars) were all significantly reduced among FC18, PC28, and PNT28 compared with Sham28. D. Average upper dermal vascular innervation that was peptidergic (CGRP, red bars) was not significantly altered across any model, whereas nonpeptidergic (PGP, blue bars), and mixed (purple bars) vascular innervation was reduced among FC18 and PC28 biopsies compared with Sham28. *p≤0.05; **p≤0.01; ***p≤0.005.

Figure 3: Keratinocyte Nav1.7, ETA, ETB IR Alterations



Representative images of immunoreactivity (IR; red fluorescence) for Nav1.7 (left column), ETRA (middle column), and ETRB (right column) in ipsilateral biopsies from the dorsal hindfoot of two Sham28 (A-F) and two PNT28 (G-L) pigs, with DAPI nuclear staining (blue fluorescence). Normal epidermal keratinocytes form a psudostratified epithelium with differential structural strata as described in Figure 1, and here the stratum Spinosum was further delineated as inner (SSi) and outer (SSo). Brackets indicate the strata where immunolabeling was detected. Nav1.7 was primarily expressed among SG and SSo keratinocytes in Sham28 biopsies (A,D). Following PBT28 injury, Nav1.7 IR was more intense and extended into the deeper SSi strata (G,J). ETRA IR was intense and primarily restricted to SB and SSi keratinocytes in Sham28 biopsies (B,E). Following PBT28 injury, ETRA expression was detected throughout SB, SSi, SSo, and SG (H,K). ETRB was primarily expressed in Sham28 biopsies (C,F). Following PBT28 injury, ETRB expression among SG and SSo keratinocytes was decreased to near absent (I,L).

Figure 4: Quantification of Keratinocyte IR Alterations



Immunolabeling average pixel intensity (API) for Nav1.7 (A, A'), ETRA (B, B'), CGRP (C), and ETRB (D) among epidermal keratinocytes in ipsilateral biopsies from each group of sciatic nerve injury model or sham surgery. Note that the data from PC10 pigs (open bars) were not included in statistical comparisons. A. Average epidermal Nav1.7 immunolabeling API among SG and SS keratinocytes (upper, black bars) was significantly increased in PNT28 biopsies, and among SB keratinocytes (lower, grey bars) in PC28 and PNT28 biopsies compared with Sham28. Significant Nav1.7 expression differences between upper and lower keratinocytes were detected in all groups. A'. The average API ratio of lower to upper keratinocyte strata Nav1.7 expression documents significant increases following PC28 and PNT28 sciatic nerve injury. B. Average epidermal ETRA immunolabeling API among upper keratinocytes (SG/SS, black bars) was significantly increased in PC28 and PNT28 biopsies, and significantly decreased among lower keratinocytes (SB, grey bars) in PNT28 biopsies compared with Sham28. Significant ETRA expression differences between SG/SS and SB keratinocytes was detected in all groups. B'. The average API ratio of upper (SG/SS) to lower (SB) keratinocyte strata ETRA expression documents significant increases inPNT28 biopsies compared with Sham28. C. Average epidermal CGRP immunolabeling API among vital keratinocytes was significantly increased in PC28 and PNT28 biopsies compared with Sham28. D. Average epidermal ETRB immunolabeling API among vital keratinocytes was significantly decreased in PC28 and PNT28 biopsies compared with Sham28. *p<0.05; **p<0.01; ***p<0.005.

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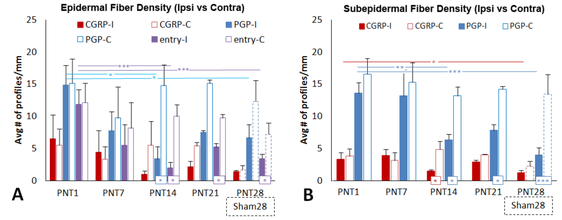
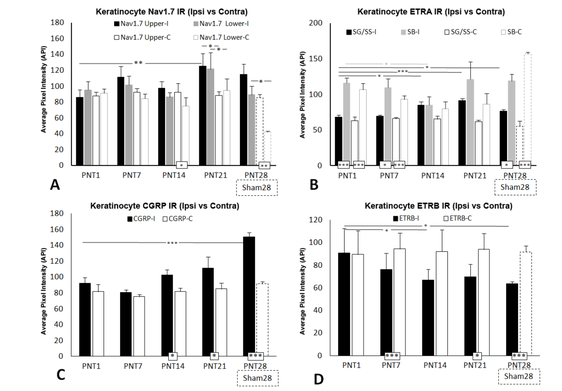
Figure 5: PNT Model Cutaneous Innervation Ratios



Impact of sciatic nerve PNT insult on ipsilateral and unoperated contralateral cutaneous epidermal (A) and subepidermal (B) innervation subtypes from POD 1, 7, 14 and 21 biopsies (n=4/group). Results are compared to those from the ipsilateral biopsies of PNT28 (n=8) and sham28 (n=3, dashed bars) pigs (see Figure 2). A. Epidermal peptidergic neural profiles (CGRP, red bars), nonpeptidergic neural profiles (PGP, blues bars), and basement contact IENF (entry, purple bars) from ipsilateral (closed bars; -I) and contralateral (open bars; -C) biopsies. Decreased nonpeptidergic innervation (PGP) and decreased IENF (entry) was observed among PNT14 and PNT28 biopsies compared with Sham28. Significant ipsilateral (closed bars) decreases were detected among nonpeptidergic (PGP) innervation in PNT14 biopsies, and among IENF (entry) in PNT14, PNT21, and PNT28 biopsies compared with contralateral (open bars). B. Subepidermal peptidergic (CGRP, red bars) and nonpeptidergic (PGP, blues bars) neural profiles from ipsilateral (closed bars; -I) and contralateral (open bars; -C) biopsies. Decreased peptidergic innervation (CGRP) in PNT28 biopsies, and decreased nonpeptidergic innervation (PGP) in PNT14 and PNT28 biopsies compared with PNT1 was observed. Significant ipsilateral (closed bars) decreases were detected among peptidergic (CGRP, red bars) innervation in PNT14 biopsies, and among nonpeptidergic (PGP) innervation in PNT14, PNT21, and PNT28 biopsies compared with contralateral (closed bars; against Sham28 for PNT28). These data importantly indicate a relative preservation of peptidergic innervation. *p<0.05; **p<0.01; ***p<0.005.

Figure 6: PNT Model Keratinocyte IR Ratios



Impact of sciatic nerve PNT insult on ipsilateral and unoperated contralateral Nav1.7 (A), ETRA (B), CGRP (C), and ETRB (D) immunoreactivity (IR) API from POD 1, 7, 14 and 21 biopsies (n=4/group). Results are compared to those from the ipsilateral biopsies of PNT28 (n=8) and sham28 (n=3, dashed bars) pigs (see Figure 4). A. Epidermal Nav 1.7 API in SG/SS (upper, black bars) and SB (lower, grey bars) keratinocytes from ipsilateral (closed bars; -I) and contralateral (open bars; -C) biopsies. Increased Nav1.7 IR among upper keratinocytes was observed in PNT21 biopsies compared with PNT1, and PNT21 pigs also had increased upper and lower keratinocyte Nav1.7 IR ipsilateral compared with contralateral, and for lower keratinocytes in PNT28 compared with Sham28. Significant difference between upper and lower Nav1.7 IR was observed in Sham28 pigs, which was lost immediately and persisted after PNT insult, with a reestablished difference only seen on the contralateral side of PNT14 biopsies, indicating that PNT insult causes immediate ipsi- and contralateral changes in epidermal keratinocyte Nav1.7 expression. B. Epidermal ETRA IR documents increased upper keratinocyte (black) expression in PNT14, PNT21, and PNT28 biopsies, and increased lower keratinocyte (grey) expression in PNT14 biopsies compared with PNT1. Significant difference between upper and lower ETRA expression was observed in Sham28 pigs, which was lost in the PNT14 and PNT21 insults. C. Epidermal CGRP IR documents increased expression in PNT28 ipsilateral biopsies compared with PNT1. Ipsilateral to contralateral differences were detected for PNT14, PNT21, and for PNT28 compared with Sham28. D. Epidermal ETRB IR documents decreased expression in PNT14 and PNT28 ipsilateral biopsies compared with PNT1. Ipsilateral to contralateral differences were detected for PNT7, PNT21, and for PNT28 compared with Sham28. *p<0.05; **p<0.01; ***p<0.005.

CONCLUSIONS

- **Pig skin is highly similar to human skin and provides an excellent model to assess pre-treatment predictors and post-treatment outcomes, including topical compounded therapies.**
- **Pig PNT insult provides a large animal model with similar physical stressors and metabolic properties of humans.**
- **Pig PNT insult produces behavioral outcomes, decreased cutaneous innervation, and altered epidermal keratinocyte expression patterns that are consistent with pathologies among human NP patients.**
- **Pig PNT insult model has properties indicating an ideal translatable platform for discovery, development, pre-clinical safety, and efficacy testing of novel therapeutic strategies for treating human NP.**