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Expression pattern of CD2AP in the intact and collaterally sprouting nervous system.

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EXPRESSION PATTERN OF CD2AP IN THE INTACT AND
COLLATERALLY SPROUTING NERVOUS SYSTEM

By

Sarah Elizabeth Claypool Couch
B.S., Centre College, 2009

A Thesis
Submitted to the Faculty of the
School of Medicine of the University of Louisville
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for the Degree of

Master of Science

Department of Anatomical Sciences and Neurobiology
University of Louisville
Louisville, Kentucky

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A Thesis Approved on

July 19, 2011

By the following Thesis Committee:

Jeffrey Petruska, Ph.D.

Theo Hagg, M.D., Ph.D.

Robin Krimm, Ph.D.

DEDICATION

This thesis is dedicated to my parents, Cheri and Walter Johnson, for their support throughout the graduate school road as well as their encouragement to pursue learning to the fullest.

ACKNOWLEDGEMENTS

Scientific work is never the product of an individual, but rather a collaboration of ideas, techniques, shared best practices and preliminary data. I would like to thank first and foremost my mentor Dr. Jeffrey Petruska for his unending help and overall enthusiasm and excitement for science. His desire to really find out what was going on and method of attacking scientific questions will forever stay influence how I carry on in my professional career. I would also like to thank Dr. Ben Harrison and Dr. Kris Rau for their assistance willingness to discuss any question. I would also like to thank my fellow master's student, Sean Trusty, as completing this process with a peer made it such much more enjoyable. Finally I would like to thank the two departments I worked with, the Kentucky Spinal Cord Injury Research Center for the use of phenomenal core equipment and wisdom available around every corner, and the Anatomical Sciences and Neurobiology.

ABSTRACT

EXPRESSION PATTERN OF CD2AP IN THE INTACT AND
COLLATERALLY SPROUTING NERVOUS SYSTEM

Sarah E. Couch

July 19, 2011

Collateral sprouting (CS) occurs when uninjured axons respond to denervated tissue surrounding the axon by growing branches of the axon to reinnervate the denervated zone. This process is signaled by factors released by the denervated tissue. Nerve growth factor (NGF) has been shown to be required and sufficient for the initiation and maintenance of CS. Previous work showed CD2 associated protein (CD2AP) mRNA increased following a spared dermatome procedure to model collateral sprouting. CD2AP links the cytoskeleton to tyrosine kinase receptors such as TrkA (the NGF receptor), where it is involved in the cellular response to growth factor signaling. Here we provided a novel characterization of CD2AP protein in naïve sensory neurons and assessed whether it was regulated during CS. CD2AP immunostaining was observed in a variety of staining intensities and populations of cells but was not regulated in collateral sprouting as assessed by immunohistochemistry.

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INTRODUCTION

Neurons respond to changes in the innervation status of surrounding tissues differently depending on their unique characteristics including their sensitivity to external signals. Some non-injured neurons are able to collaterally sprout, sending axon projections into the surrounding tissue where the original nerves have been depleted. In the spared dermatome model of sprouting the naïve cutaneous afferent sensory neurons responsible for mechanoreception and heat nociception, the A δ and C fibers respectively, are a population able to collaterally sprout (Diamond et al., 1976). The large diameter axons with low stimulus threshold neurons are not able to sprout (Horch, 1981, Jackson and Diamond, 1984). The process of collateral sprouting begins when a region of tissue is denervated but requires 10-12 days for the measured expansion of the nociceptive field to be detected behaviorally by stimulating the mechanoreceptors by pinch (Diamond et al., 1992).

Collateral sprouting has not only been studied through sprouting of nociceptors in the periphery of rats but also in other systems. In cats the sympathetic nervous system supplying the superior cervical ganglion has been studied and identified as able to collaterally as well as measured expansion of rammifications within the spinal cord (Murray and Thompson, 1957, Liu and Chambers, 1958). Neurons of the central nervous

system have also been identified as able to collaterally sprout, most notably in the hippocampus participating in memory formation(Guth and Windle, 1970, Cooke and Bliss, 2006).

In the rat model of collateral sprouting nerve growth factor (NGF) has been shown to regulate the process. When NGF is administered to a naïve adult rat, precocious collateral sprouting of nociceptive afferent neurons can be induced. Also when anti-NGF antibodies are given to a rat with undamaged nociceptive afferent fibers surrounded by a denervated region, the expected collateral sprouting phenomenon is halted (Van der Zee et al., 1992). Under the same conditions if extra NGF is administered the time needed for collateral sprouting is reduced (Diamond et al., 1992). As NGF has been shown to be an integral part of the collateral sprouting process those neurons with receptors to NGF are presumed to be able to undergo collateral sprouting. It is the model developed by Diamond and colleagues, using the sensory neurons as a model for the collateral sprouting process that was used here.

The peripheral nervous system can be divided into efferent neurons, responsible for relaying information to the periphery and afferent neurons, which carry information from the periphery to the central nervous system. The majority of the cell bodies of the afferent pseudounipolar sensory neurons are held in the dorsal root ganglia (DRG) located along the periphery of the spinal cord. Information received from peripheral axons travel through the central branch and synapse on neurons in the spinal cord and brainstem. The cell body provides support for the axon to effectively transmit sensory information and chemical signals.

DRG neurons can be characterized by morphological differences such as cell body size, axon size, and myelination status that determine conduction velocity, all of which co-vary (Lawson, 1992). There are also physiological differences including which type of stimulus (such as temperature or vibration) produces an action potential within a population. Anatomical termination locations both in the periphery and in the central nervous system also differ by sensory neuron type. Each cell body can be identified by expressed molecules such as receptors on the cell membrane, neurotransmitters, binding molecules, cytoskeletal structures all which can be identified through immunohistochemical assays (Lawson, 1992, Koerber and Woodbury, 2002, Albrecht and Rice, 2010).

Neurons can also be classified by their expression of neuropeptides. Peptidergic sensory neurons express neuropeptides such as substance P or calcitonin gene-related peptide (CGRP) and contain TrkA receptors (Bennett et al., 1998, Petruska et al., 2000, Albrecht and Rice, 2010). Nonpeptidergic neurons can be identified immunohistochemically by the lectin glycoprotein isolectin B4 (IB4), isolated from the legume *Griffonia simplicifolia*, which binds specifically to cell surface glycoconjugates (Bennett et al., 1998). As a stain IB4 labels small cell unmyelinated primary afferent fibers with an overlap into CGRP positive staining cells (Wang et al., 1994, Petruska et al., 2000).

Expressed neurotrophin and neurotrophic-factor receptors are also used to characterize populations of cell bodies. Nerve growth factor receptors include tropomyosin related kinase (Trk) A as well as p75 receptors (Wehrman et al., 2007, Castaneda-Corral et al., 2011) and brain-derived neurotrophic factor (BDNF) receptors

are TrkB and p75 as well. Two other neurotrophins expressed and used by neurons are neurotrophin-3 (NT3) binding to the TrkA, Trk B, TrkC, and the p75 receptors (Farinas et al., 1998) and neurotrophin-4 (NT4) acts on cells through TrkB receptors. An important neurotrophic factor of a different family is glial cell line derived neurotrophic factor (GDNF) which is utilized in development of the nervous system and binds to the receptor GDNF family receptor alpha 1 and 2 (GFR α 1 and GFR α 2) (Chao, 2003, Albrecht and Rice, 2010). The GDNF family of ligands transmits the signal by first binding to the receptor, GFR- α that then forms a complex with Ret (Jing et al., 1996). After Ret has been activated by the formation of the ligand, receptor and Ret complex it is able to activate either the phosphatidylinositol-3 kinase (PI-3K) Akt pathway or the extracellular signal-related kinases (erk) pathway to affect the growth of podocytes (Figure 1) (Zhou et al., 2009). CD2 associated protein (CD2AP) has been identified to co-localize with Ret in podocytes and act as regulator of the half-life of Ret affecting the potency of the GDNF ligands (Tsui and Pierchala, 2008).

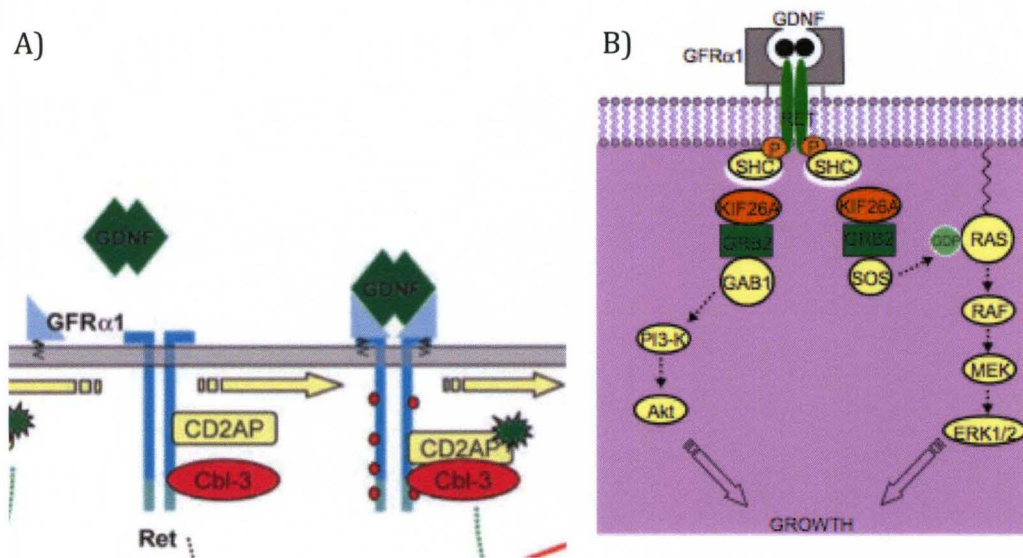


Figure 1: Schematic of GFR α 1 Interacting with Ret (Zhou et al., 2009).

A) Binding of GDNF ligand to the receptor GFR α 1 forming a complex with Ret and CD2AP B) Following the binding of GDNF to its receptor activation of the PI-3 Kinase/Akt pathway and the ERK1/2 pathway to effect cell growth.

Understanding the mechanisms by which sensory neurons are able to collaterally sprout may provide information on how to manipulate process. For individuals with extensive damage to their nervous system finding ways to manipulate the renervation of surrounding tissue following a nerve injury can provide therapeutic options. The process of collateral sprouting of sensory neurons is not always an improvement for the animal; following experimental spinal cord injury studies it has been observed to contribute to the development of pathological conditions including autonomic dysreflexia (Marsh and Weaver, 2004, Hou et al., 2009). Autonomic dysreflexia is characterized by paroxysmal hypertension, profuse sweating, flushing of skin above the level of spinal cord injury lesion as well as potentially life threatening bradycardia.

In previous work the Petruska lab modified a spared dermatome surgical model developed by Diamond (Diamond et al., 1976, Doucette and Diamond, 1987, Diamond et al., 1992) to examine the transcriptional changes in the spared DRG. Two time-points were selected representing the initiation (7 days) and maintenance (14 days) phases of collateral sprouting as suggested by Diamond (Doucette and Diamond, 1987, Diamond et al., 1992). Assessment of transcription was done by AffyMetrix microarray and select results validated by qPCR. One of the most highly upregulated microarray probes was for the one for CD2AP, which was upregulated at 7 days but not at 14 days, perhaps representing a factor important for the initiation of collateral sprouting. This result was confirmed with qPCR (Figure 2).

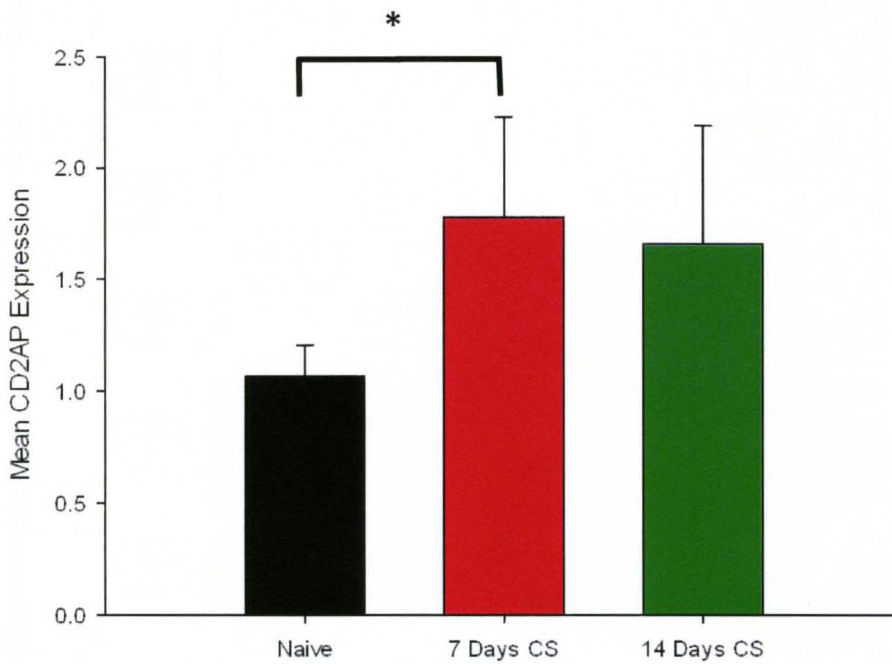


Figure 2: CD2AP qPCR Mean Expression for Naïve (n=4), 7 Day Collateral Sprouting (n=8) and 14 Day Collateral Sprouting (n=10) animals normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) $p < 0.05$.

The known function of CD2AP made it a rational target for investigation as a possible regulator of collateral sprouting. CD2AP was first studied for its role in T-cell activation and has since also been studied in kidney podocyte morphology. In T-cells, the membrane bound complex of differentiation 2 (CD2) adhesion molecule binds to the ligand of an antigen-presenting cell to activate the T-cell(Huppa and Davis, 2003). Binding of the antigen-presenting cell to CD2 requires the CD2 associated protein to initiate cytoskeletal polarization and clustering of CD2 protein at the point of contact (Dustin et al., 1998). In kidney podocytes, the cells making up the slit diaphragm of the glomerulus, or the filtering unit of the kidney, CD2AP is known to interact with nephrin, podocin, F-actin spots and the Arp2/3 complex responsible for new actin filaments and branch points (Welsch et al., 2005, Gigante et al., 2009, Lowik et al., 2009). CD2AP is able to link to the cytoskeleton of podocytes through an actin-binding site indicating a potential point of interaction with other types of cell cytoskeleton proteins (Kim et al., 2003, Welsch et al., 2005). The effective linking together of podocytes relies on the proper structure of the cell processes. In knockout studies, CD2AP mice died at the age of 6 – 7 weeks from protein urea caused by renal failure. CD2AP haploinsufficient humans suffer from focal segmental glomerulosclerosis (FSGS) while haploinsufficient mice developed FSGS at 9 months and were more susceptible to glomerular injury(Lowik et al., 2009).

The ability of CD2AP to act as an adaptor type protein in podocytes and T cells may provide an insight into how the protein works in other cell types. Where cytoskeletal reorganization is required in collateral sprouting for new processes to form and extend to the denervated tissue, an adaptor molecule connecting the cytoskeleton to elements

responsive to the signals involved in collateral sprouting would be used in appropriately extending the axon processes. While the Petruska lab was able to identify significant differences in the changes of mRNA levels of CD2AP in the initiation phase of collateral sprouting compared to naïve animals in the whole dorsal root ganglion, examination of protein levels within individual neuron cell bodies was the next step in analyzing CD2AP's role in the process. We hypothesized that collaterally sprouting neurons would produce more CD2AP to aid in the necessary cytoskeletal reorganization triggered by factors binding to receptors involved in axon elongation to new targets. The measured amount of CD2AP protein in individual cells undergoing collateral sprouting would therefore be used to identify the population of the DRG neurons participating in the collateral sprouting process. Given that the distribution of CD2AP in DRG neurons had not previously been described, it was possible that such a large change in expression might allow CD2AP to potentially serve as a biomarker to identify the collateral sprouting population.

METHODS AND MATERIALS

To determine if the previously characterized antibody provided by the A. Shaw lab (Washington University, St. Louis) would appropriately stain CD2AP in our studies, we tested staining in PC12 cells in which the levels of CD2AP were artificially modulated. Transfected PC12 cells manipulated with NGF were stained with the CD2AP antibody raised in rabbits and then fluorescently labeled with 594 anti rabbit (Invitrogen Corp, Carlsbad). Nuclei were labeled blue using DAPI. Using a Zeiss AxioScope microscope the image was captured with a Spot camera and software and processed using Photoshop.

For immunohistochemical analysis, naïve and experimental T11 DRGs post fixed with 4% paraformaldehyde solution and cryoprotected by storage in sucrose, were sectioned with a Lecia 3050s cryostat into 12 μm sections. Sections were laid out onto a series of 10 slides double-coated with gelatin and poly-L-lysine, two sections from each animal per slide. Three naïve and three experimental animals of the same condition, either 7 days or 14 days were represented, with each time point in its own row. In total three series of 10 slides were filled, each slide with 12 sections (Figure 3). On each slide the two representative sections from the same rat were 120 μm apart to avoid any single cell being represented in both sections. To validate that the T11 DRGs were not injured

during the surgery a slide for each time condition was stained for activating transcription factor 3 (ATF3)(Santa Cruz Biotechnology Inc., Santa Cruz) and 594 anti-rabbit (Invitrogen Corp., Carlsbad). ATF3 is used as a neuronal marker of injury within the nervous system as it is specifically induced in both sensory and motor neurons following nerve injury (Tsujino et al., 2000). A marker for central or peripheral neurons is the antibody against a protein, neuronal nuclei (NeuN), which labels the neuron cell nuclei exclusively in mixed cell samples such as DRGs (Mullen et al., 1992). Experimental slides were stained for NeuN (Millipore, Billerica) to identify whole neuron nuclei, CD2AP (A. Shaw, Washington University, St. Louis) and a third experimental counter stain, either IB4 lectin FITC conjugate(Sigma-Aldrich Corp., St. Louis)or CGRP (Fitzgerald Industries, Acton). Fluorescent secondary immunohistochemical stains were used to visualize the primary stains; pacific blue anti-mouse (Invitrogen Corp., Carlsbad) to visualize NeuN, 594 anti-rabbit (Invitrogen Corp., Carlsbad) to visualize CD2AP, and 488 anti-guinea pig (Invitrogen Corp., Carlsbad) for CGRP.

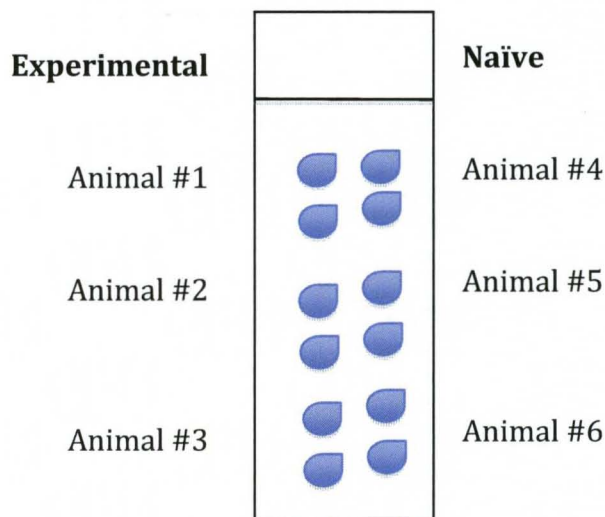


Figure 3: Schematic of Slide Layout

Images of the sections were captured on an inverted Nikon Eclipse Ti microscope using a Nikon camera and Elements software. Camera exposure settings were established and remained constant for capturing the entire slide and experiment. Sections were analyzed using the elements software to identify entire cells that had positively stained nuclei as regions of interest (ROIs). The cell size and staining intensity of each channel for each ROI was recorded and analyzed with Excel and Sigma Plot.

RESULTS

Validation of the antibody used was achieved by immunohistochemical staining of CD2AP in CD2AP-GFP transfected PC12 cells, Figure 4. The CD2AP antibody brightly stained the same cells, in the same places where the transfected GFP-CD2AP was visualized. All PC12 cells express CD2AP at low levels however only those cells transfected with CD2AP-GFP stained brighter with the A. Shaw CD2AP antibody. From this data the Shaw antibody was validated to correctly label CD2AP. This was consistent with previously published work using this antibody where it was found in podocytes (Shih et al., 2001, Huber et al., 2006, Marafioti et al., 2008).

Naïve cell bodies, Figure 5, show robust CD2AP staining in small area neurons as well as lightly stained small neurons. All cells within the DRG are stained with a range of intensities from weak to strong. The large and medium cell bodies for the most part are weakly stained with some medium intensely stained medium sized cell bodies. CD2AP antibodies clearly label cell bodies in the cytoplasm and axoplasm while the nucleus appears devoid of signal. At higher magnification, using confocal microscopy, Figure 5C, shows clear punctate staining throughout the cytoplasm leaving the nucleus unlabeled, different cell bodies with individual staining intensities, and punctate staining in the axoplasm indicating specific staining.

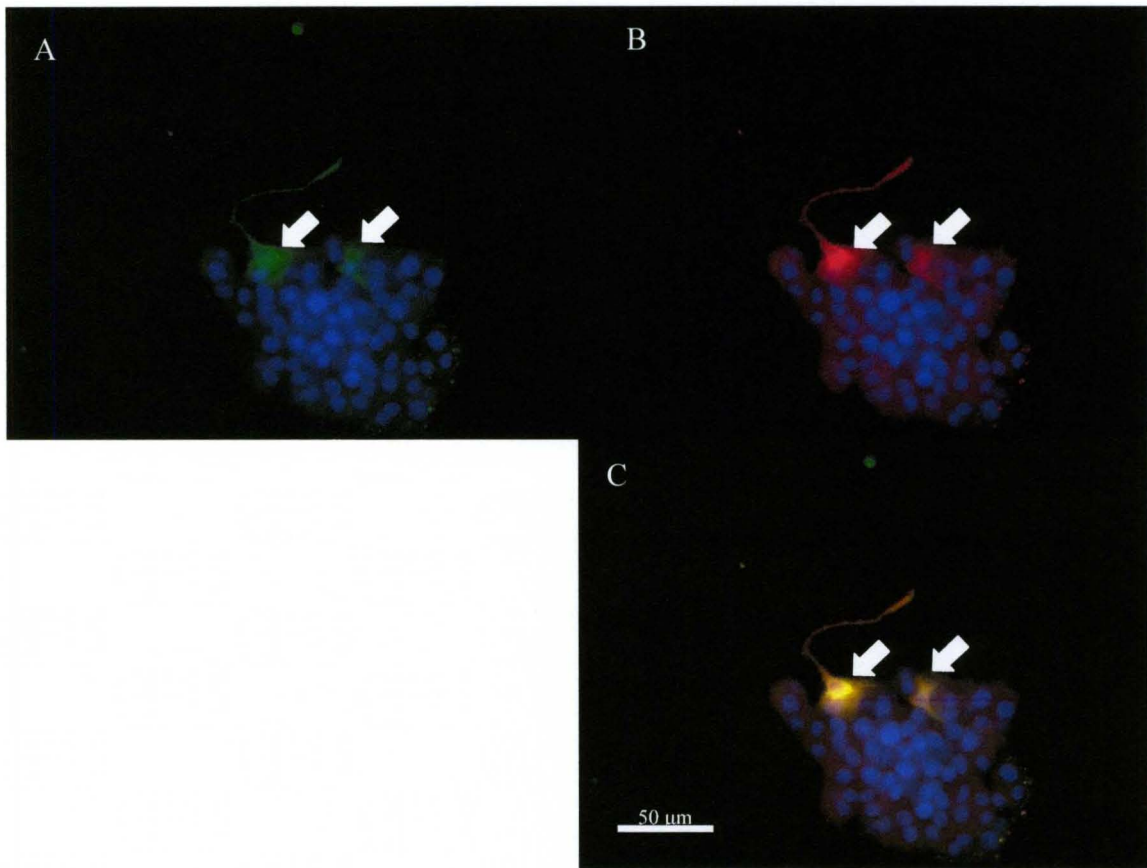


Figure 4: CD2AP Transfected PC12 Cells.

PC12 cells in culture transfected with CGRP-GFP conjugated protein and treated with NGF, cell nuclei are stained blue with DAPI. A) CD2AP-GFP transfected PC12 cells indicated by arrows B) CD2AP visualized in all cells consistent with constitutive expression and bright staining in those cells with transfected PC12 cells C) Overlay showing antibody selectively labels CD2AP

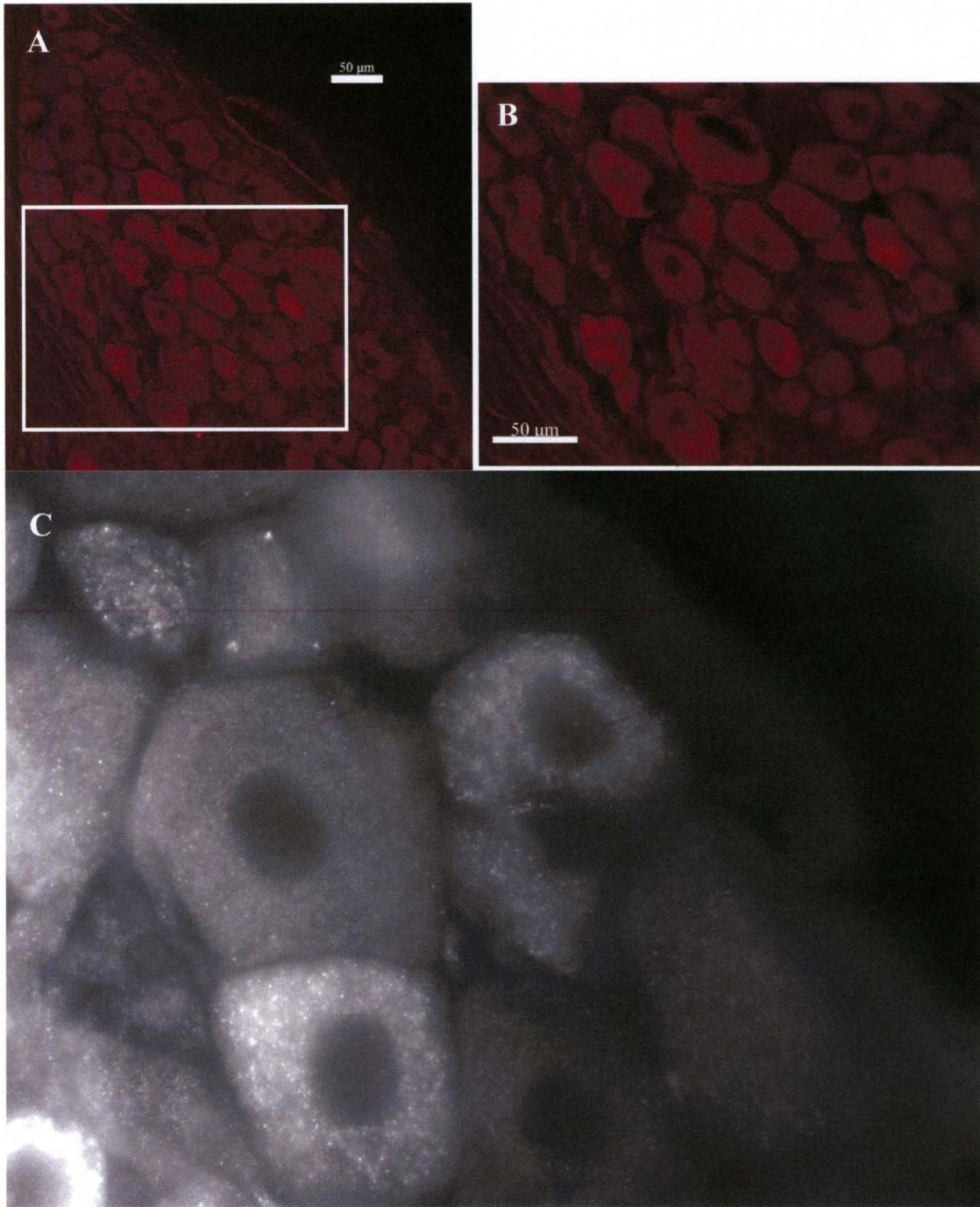


Figure 5: Naïve DRG Cell Bodies labeled with CD2AP A) DRG image captured at 20x B) DRG cell bodies captured at 40x. C) Confocal image of naïve DRG cell bodies at 63x

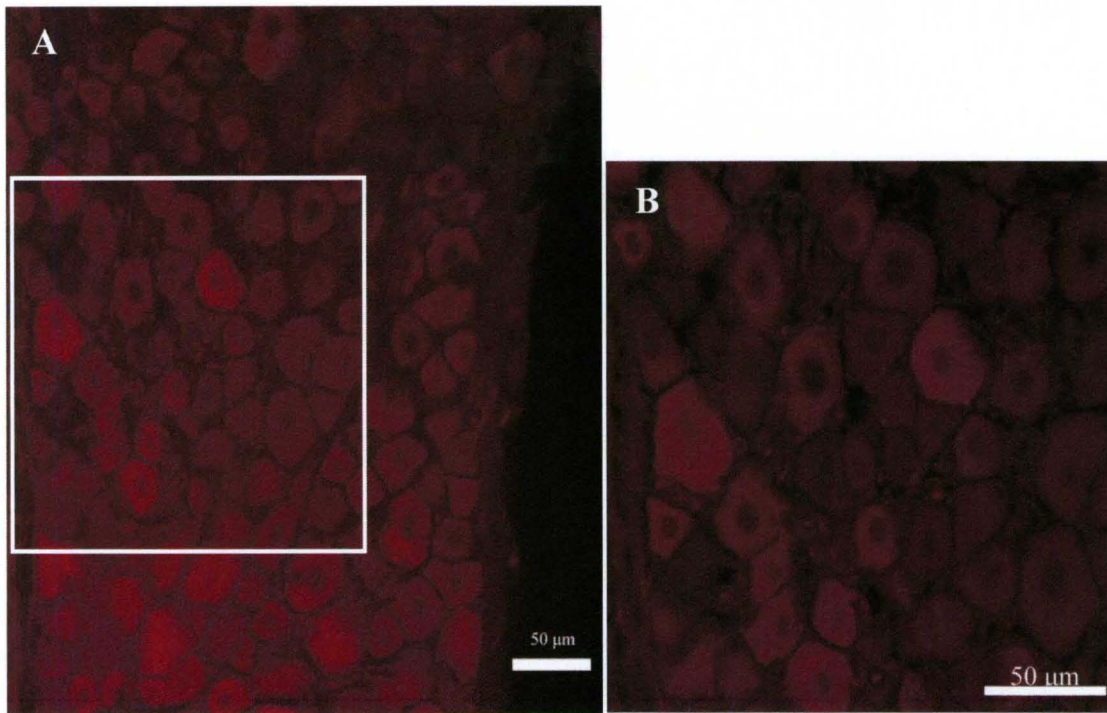


Figure 6: 7 Day Collateral Sprouting DRG Cell Bodies Labeled with CD2AP, animal AHI. A) DRG image captured at 20x B) DRG cell bodies captured at 40x.

CD2AP staining intensity of T11 DRG cell bodies was compared between sections from either 7 days (Figure 6) or 14 days (Figure 9), following the spared dermatome procedure and the naïve sections (Figure 5) on the same section.

Compared to the naïve population of DRG cell bodies, the 7-day animal population had a significantly higher CD2AP intensity compared to naïve cell bodies throughout the cell area distribution (Figure 7A). Analysis by the Mann-Whitney U rank sum test of the difference in median values of CD2AP intensity indicated significantly different median intensity values between naïve and 7-day collateral sprouting samples ($p < 0.001$). The small area cell bodies of the 7-day animals appear to have the largest CD2AP intensity. In 7-day collateral sprouting animals, 75% of the population of cell bodies (the 75% with highest CD2AP intensity) appeared to have higher CD2AP

intensity than naïve animals. Note that the curves in Figure 7B cross, with the 7-day CS curve shifting to the right for the top 75% of the population.

The data were also considered on a per-animal basis, Figure 8. The naïve animals grouped close together with similar CD2AP intensity. The 7-day experimental animals however were very different in distribution of CD2AP intensity. Animal ASI had the lowest overall CD2AP intensity while the AHI animal had the highest CD2AP intensities. When analyzed as means of individual animals, the grand mean showed no significant differences between naïve and 7-day collateral sprouting groups ($p=0.645$).

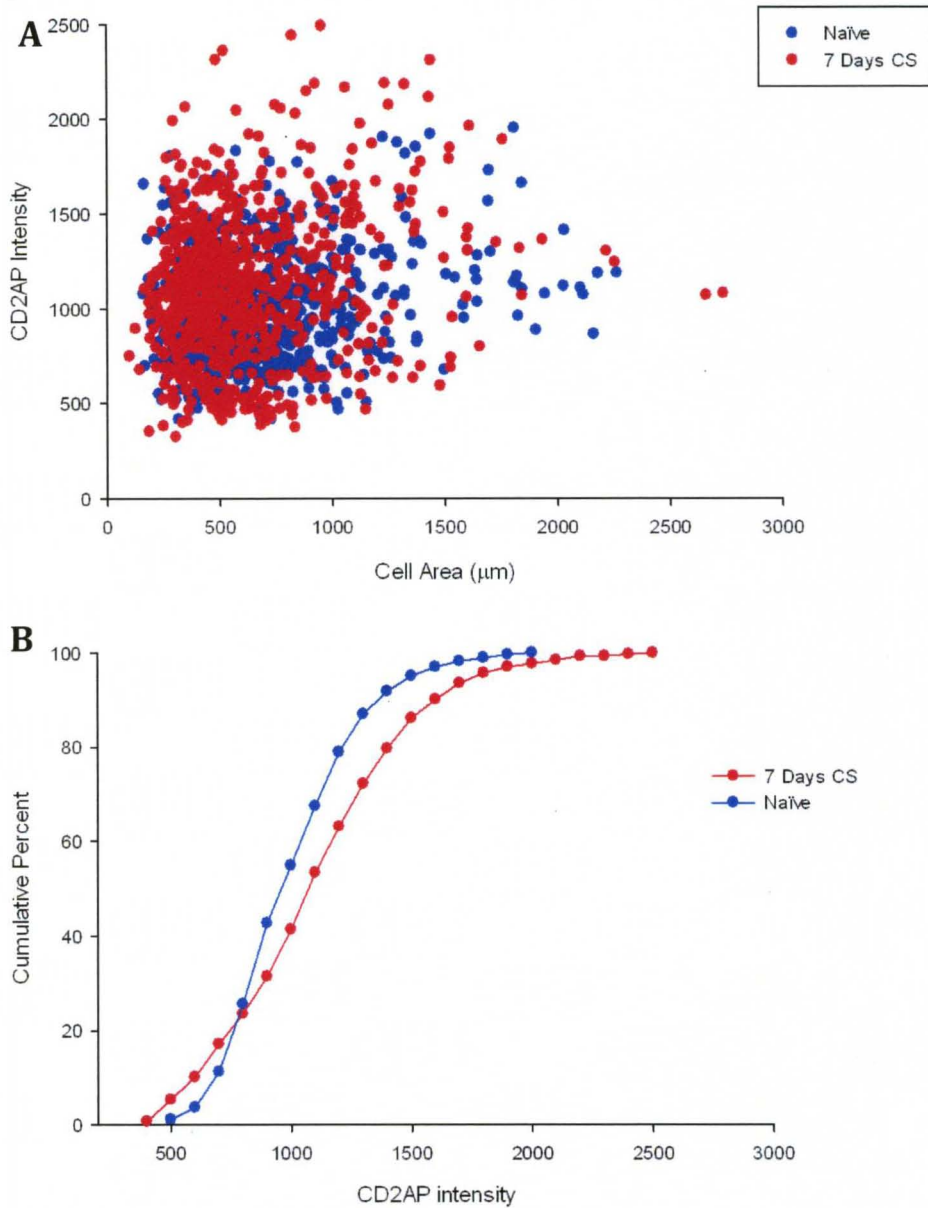


Figure 7: CD2AP Intensity in 7 Day Collateral Sprouting and Naïve Cell Bodies.

A) Scatterplot of CD2AP intensity compared to the cell area of each individual cell body B) Cumulative Sum Histogram displays the population distribution change in the CD2AP intensity staining of the naïve population compared to 7-day collateral sprouting animals.

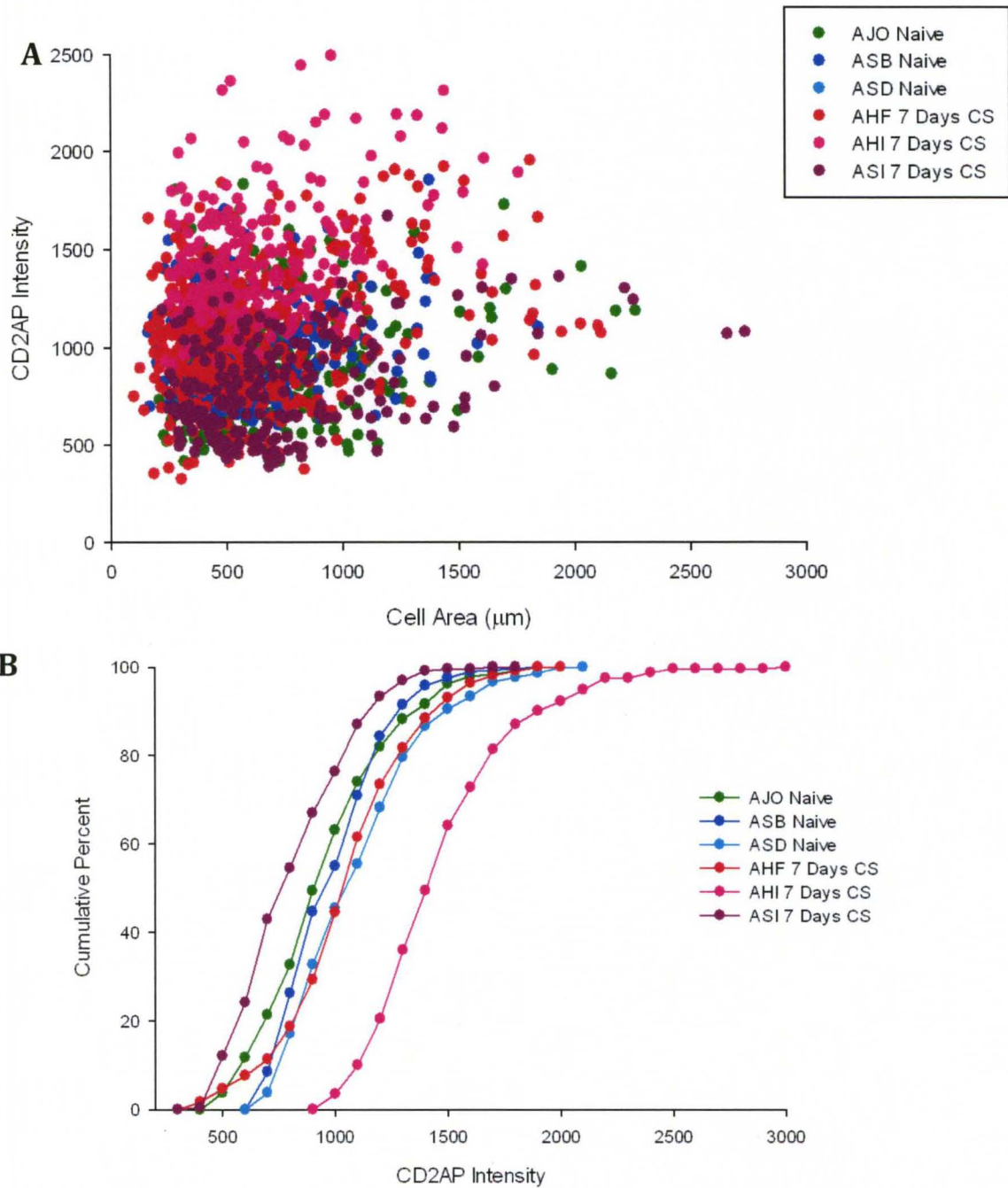


Figure 8: CD2AP Intensity by Individual 7 Day Collateral Sprouting and Naïve Cell Bodies A) Scatterplot of CD2AP intensity compared to the cell area of each individual cell body in Naïve and 7 day collaterally sprouting animals B) Cumulative Sum Histogram displays the population distribution change in the CD2AP intensity staining each animal.

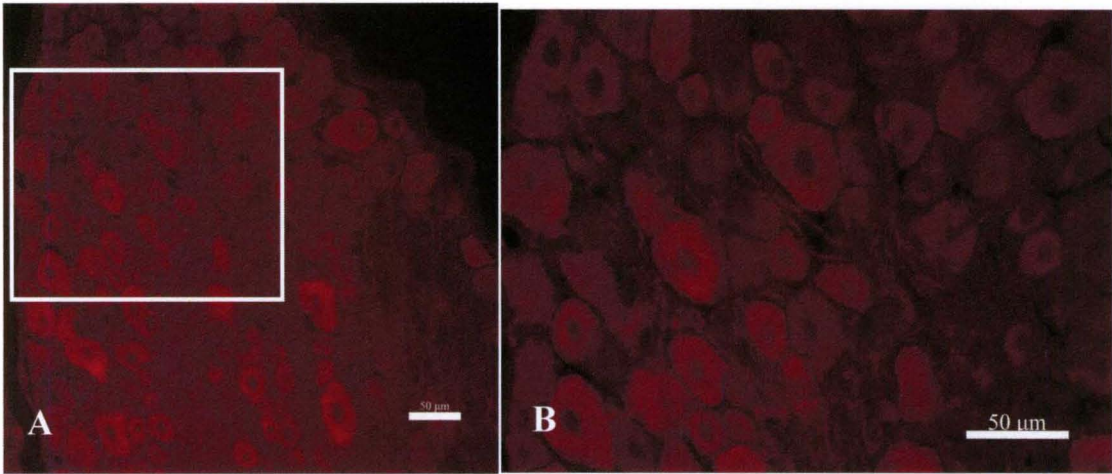


Figure 9: 14 Day Collateral Sprouting DRG Cell Bodies Labeled with CD2AP. A) DRG image captured at 20x B) DRG cell bodies captured at 40x.

Both the naïve and 14-day collateral sprouting cell bodies have a similar cell body area distribution in the DRGs, CD2AP expression is significantly different ($p < 0.001$, Mann-Whitney U rank sum test: Figure 10A). The cumulative sum histograms of CD2AP intensity demonstrate that the 14-day collateral sprouting population had less intense CD2AP staining compared to naïve levels (Figure 10B).

Looking at the differences in CD2AP intensity compared to the cell area between the individual animals shows similar overlaps in the scatter plot (Figure 11A) considering the experimental condition, naïve or 14 day collateral sprouting. CD2AP intensity cumulative sum graph (Figure 11B) indicates the individuals within each group were similar. Analyzing the group differences with group means derived from mean values of each individual animal showed significant differences between the two conditions, Student's T-Test $p < 0.05$.

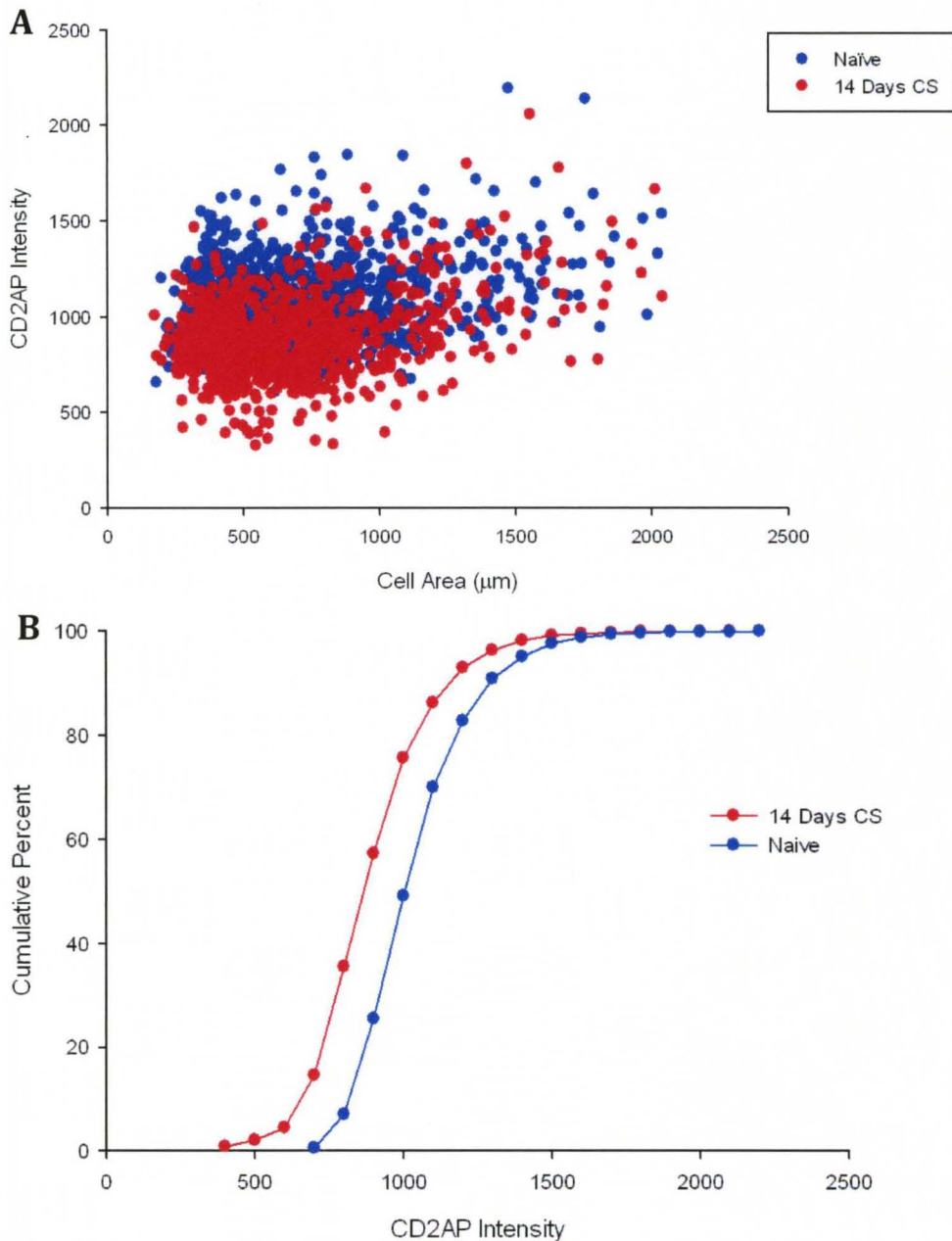


Figure 10: CD2AP Intensity in 14 Day Collateral Sprouting and Naïve Cell Bodies. A) Scatterplot of CD2AP intensity compared to the cell area of each individual cell body B) Cumulative Sum Histogram displays the population distribution change in the CD2AP intensity staining of the naïve population compared to 14-day collateral sprouting animals.

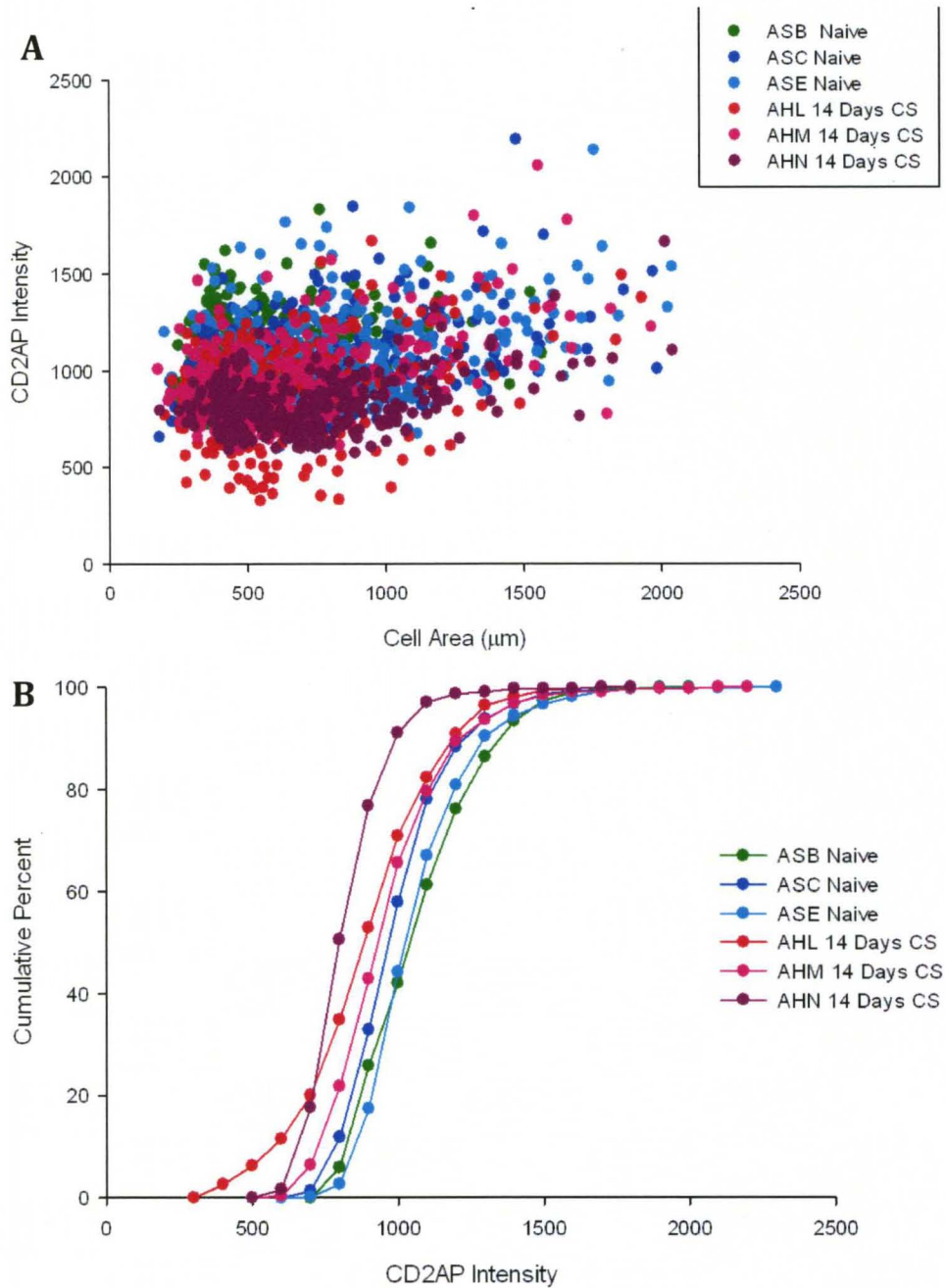


Figure 11: CD2AP Intensity by Individual in 14-day Collateral Sprouting and Naïve Cell Bodies. A) Scatterplot of CD2AP intensity to the cell area of each individual cell body of the individual animals within each condition, naïve and 14 day collateral sprouting
 B) Individual CD2AP histogram cumulative sum showing the population distribution of three naïve and three 14-day animals following spared dermatome surgery. $p < 0.05$.

Co-staining with IB4 or CGRP was completed with the intention of determining if CD2AP was preferentially expressed in the nonpeptidergic or peptidergic neurons respectively (Figure 12). Analysis of the intensity distributions of CD2AP compared to IB4, labeling mostly non-peptidergic cutaneous nociceptors, or with CGRP labeling peptidergic neurons, in naïve tissue or either collaterally sprouting can be analyzed. While there was no consistent overlap in increase IB4 staining to CD2AP staining (Figure 13) there was a wide distribution of CD2AP staining in those cells with “low level” CGRP staining while medium and high level CGRP staining did not show increased or decreased levels of CD2AP staining (Figure 14). CGRP staining has a range of expression but it is not constitutively expressed by all neurons. Therefore neurons with low levels of staining intensity need to be further examined for those that are negative versus those that have low intensity staining for accurate characterization of the identified cell bodies.

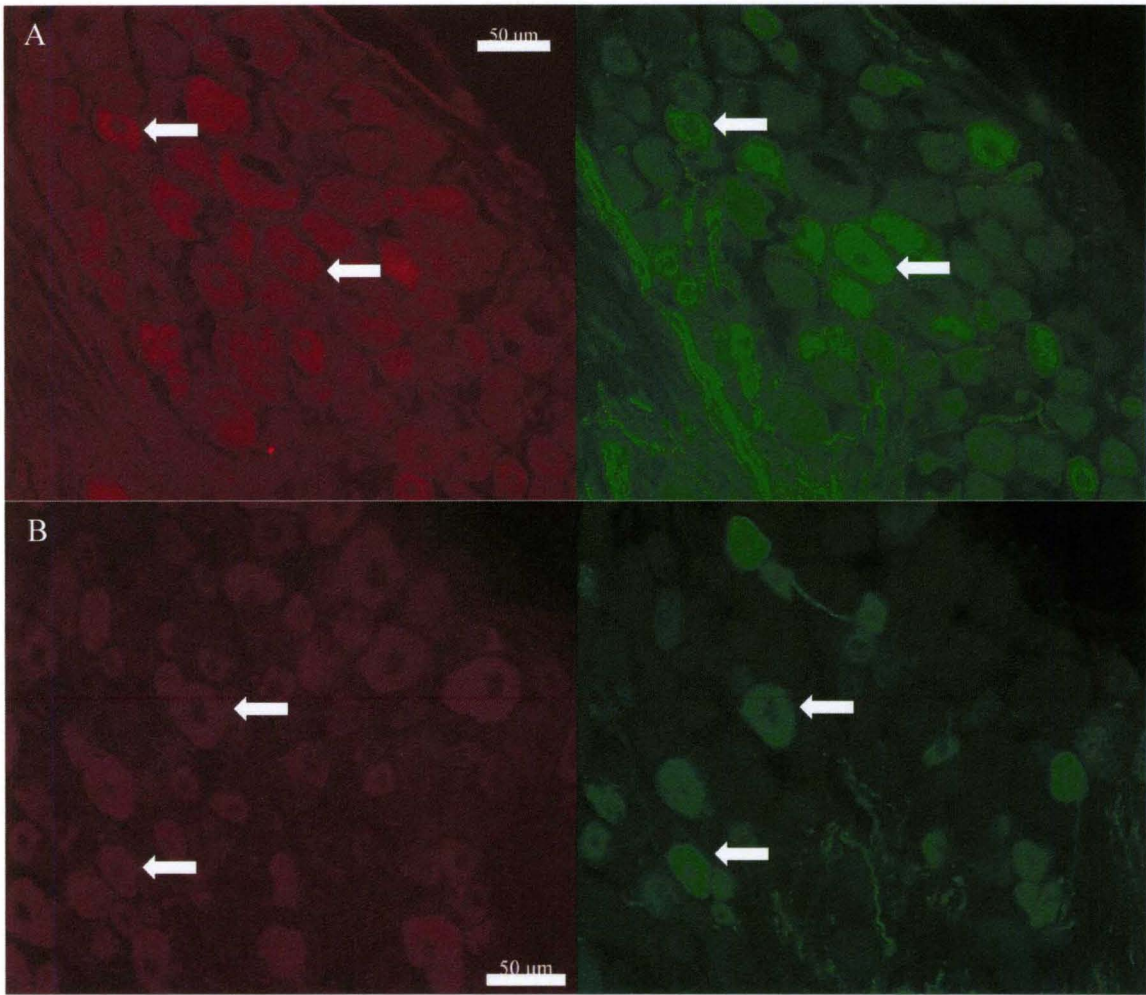


Figure 12: Sections of naïve DRG stained with CD2AP (red) and either IB4 (green in A) or CGRP (green in B). Arrows indicate some of the cells double labeling for CD2AP and either co-stain, IB4 or CGRP.

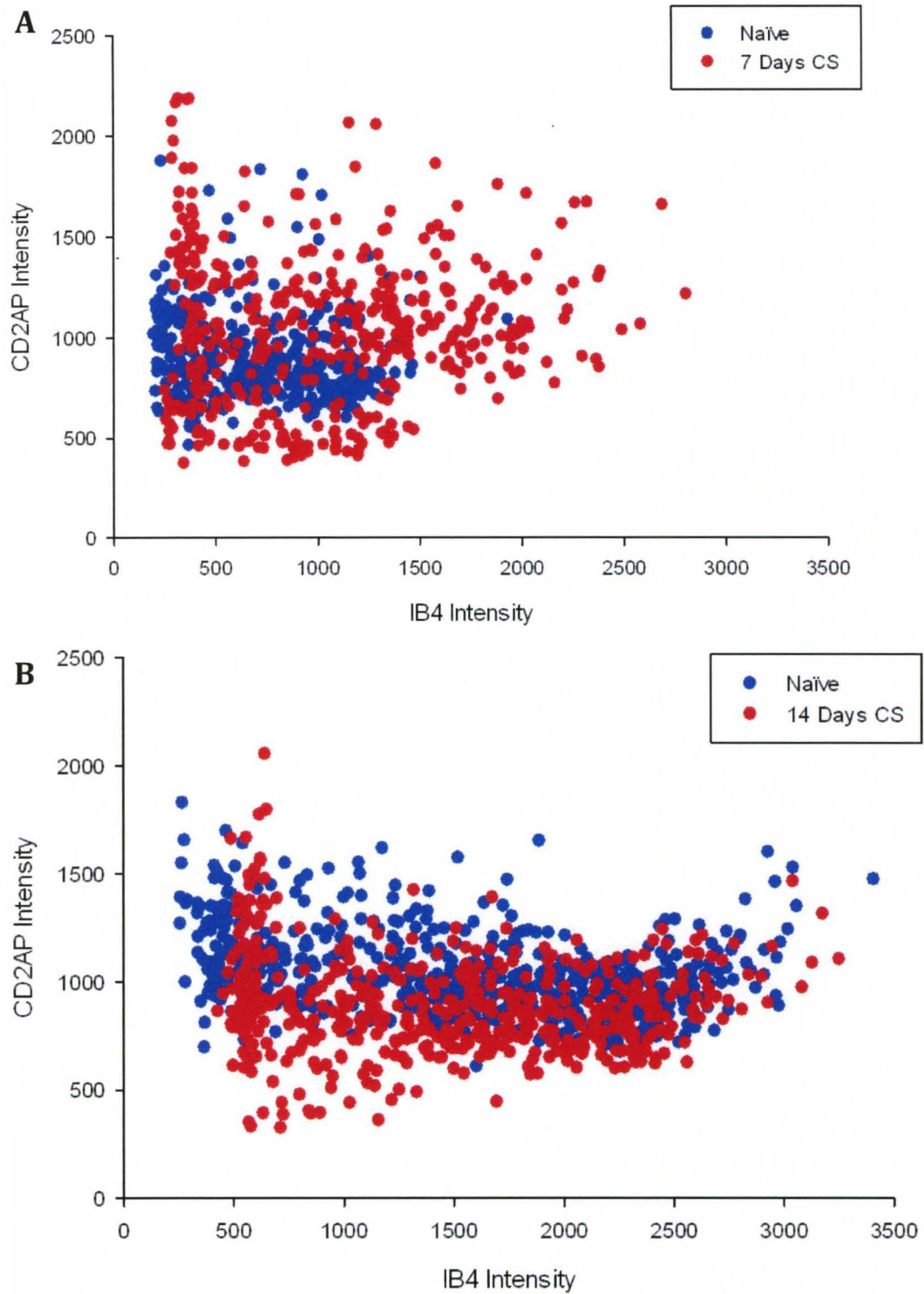


Figure 13: Scatterplot of CD2AP Intensity Compared to IB4 Staining Intensity for naïve versus A) 7 days collateral sprouting B) 14 days collateral sprouting

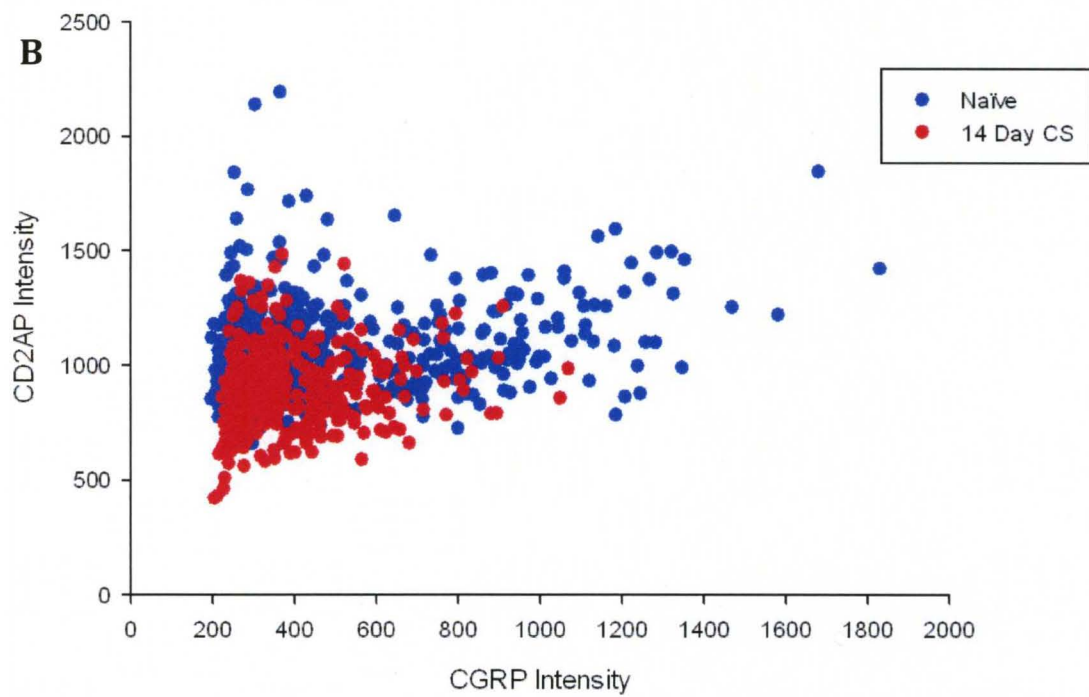
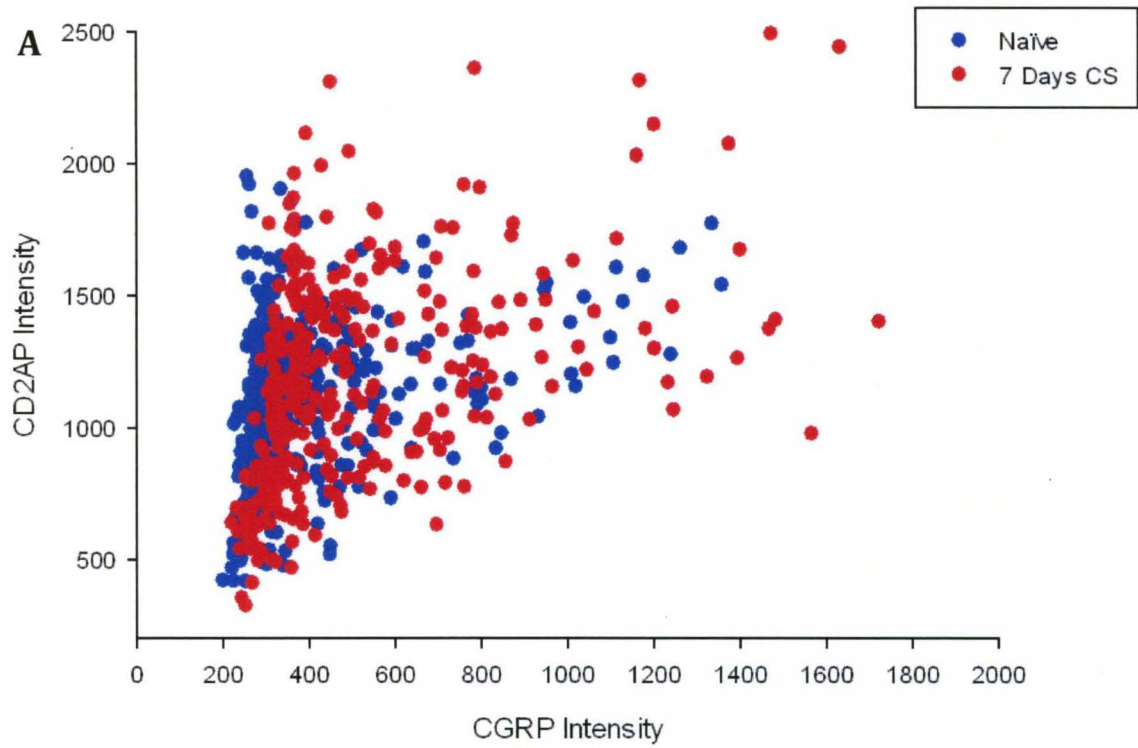


Figure 14: Scatterplot of CD2AP Intensity compared to CGRP staining intensity for naïve versus A) 7 days collateral sprouting B) 14 days collateral sprouting

Analysis of the cell area of naïve and experimental conditions was also performed. The cumulative sum histogram of the cell area of naïve animals compared to 7-day collateral sprouting animals, Figure 15A, appeared similar however there was a significant difference between the conditions when considering all cells on a per-animal basis for each group (Figure 15B), Mann-Whitney U rank sum test $p < 0.05$.

Analysis of the cell area of naïve and 14 day collateral sprouting DRG cell bodies using cumulative sum histograms, Figure 16A, again appeared similar when entire population. Examination of the individual cell body area, Figure 16B, showed, like the comparison between 7 day and naïve, no significant differences between individual animals.

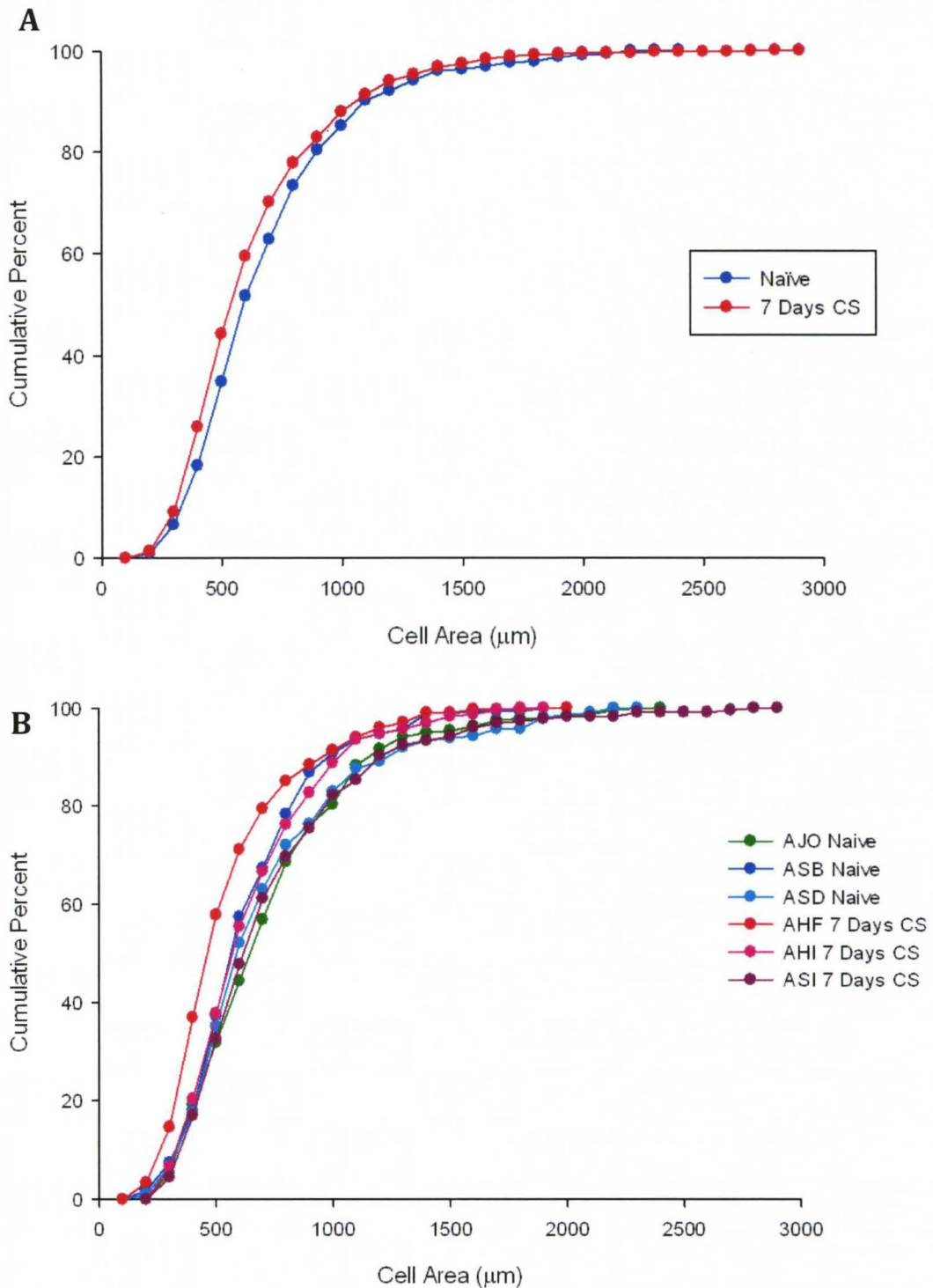


Figure 15: Cumulative Sum Histogram Displays the Population Distribution Change in the 7 Day Collateral Sprouting Cell Body Area
 A) Differences between naïve and 7 days collateral sprouting animals
 B) Individual animals graphed independently $p < 0.05$

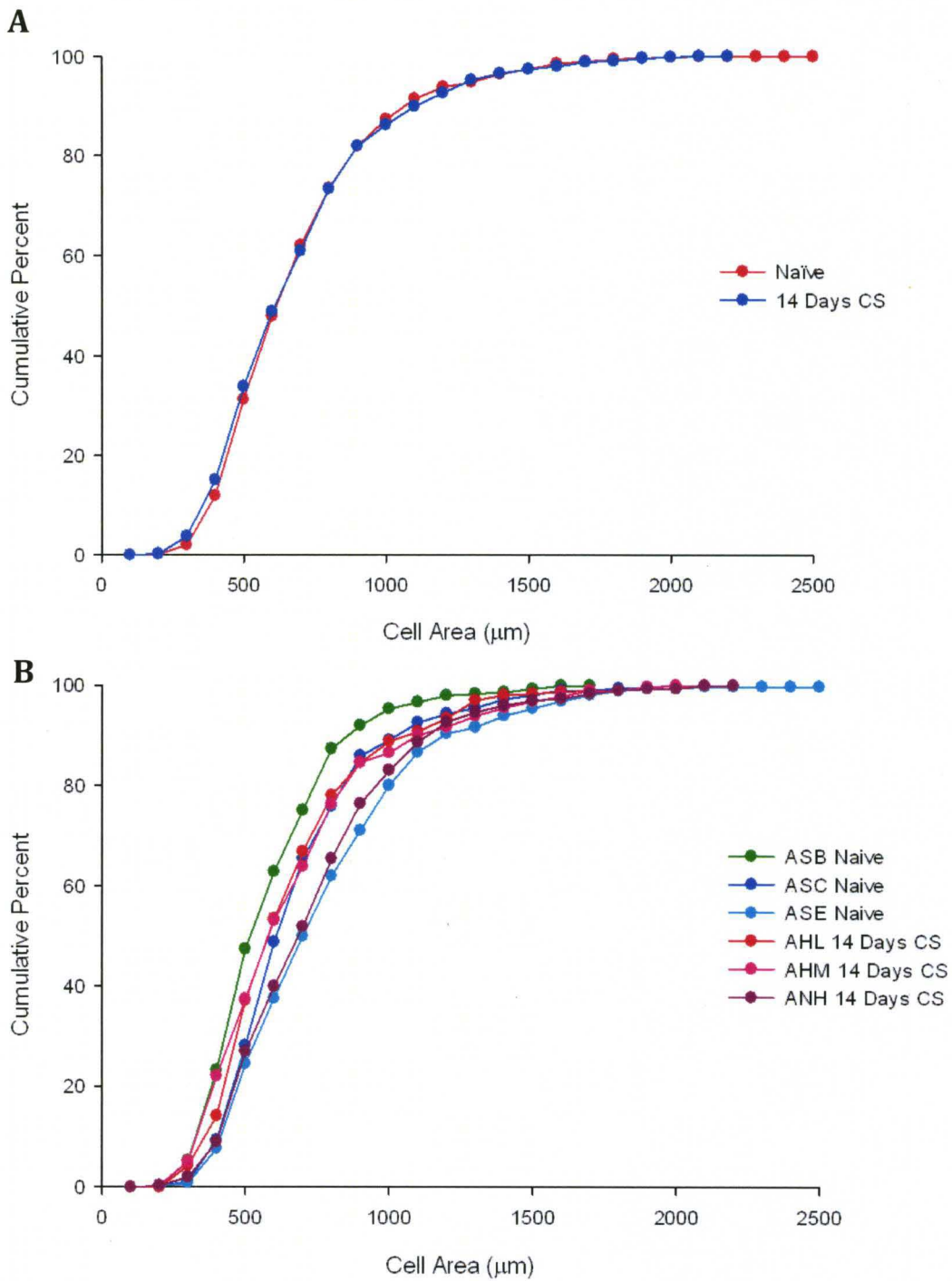


Figure 16: Cumulative Sum Histogram Displays the Population Distribution Change in the 14 Day Collateral Sprouting Cell Body Area A) Differences between naïve and 14 day animals B) Individual animals graphed independently

DISCUSSION

Significant differences seen in CD2AP mRNA expression by the AffyMetrix gene chip and qPCR were not reflected at the protein level in DRG neuron cell bodies by immunohistochemical analysis. A significant increase in the CD2AP intensity in the 7-day collateral sprouting group was indicated by the Mann-Whitney U test of all neurons in each condition considered together. However upon further analysis of the 7-day animals and naïve conditions considering individual animals using grand mean analysis did not indicate a significant difference between the groups.

Measurements within the early phase of early collaterally sprouting time points are inherently limited. The expansion of the spared dermatome through collateral sprouting is not detectable by the CTM reflex before 10 days. Therefore there is no way to detect collateral sprouting that fails for whatever reason before retrieval of the tissue for analysis. We cannot trace those neurons undergoing collateral sprouting at early stages as they have not yet grown into the denervated skin. There is also no known biomarker for collateral sprouting in individual neurons again limiting identification of collateral sprouting prior to behavioral validation or available tracing of successful collateral sprouting.

One of the animals used for the 7-day collateral sprouting population, ASI, was indeed slightly different. ASI was not operated on at the same time as the two other animals within the group. A different anesthesia, ketamine instead of phenobarbital, was used and a different individual completed the spared dermatome procedure. ASI had the lowest CD2AP intensity levels not only compared to 7-day experimental animals but also to the naïve animals. One method of quality control is the assessment of ATF3 expression, an indicator of nerve injury that is a separate process, which might interfere with collateral sprouting. It is possible that some of the 7-day collaterally sprouting animals may not have been undergoing collateral sprouting in the same way as other collateral sprouting neurons. It is also possible the quality control parameters established for acceptable levels of ATF3 positive neurons is too high so some DRGs with damaged neurons undergoing regeneration were included in the analysis of collateral sprouting. ASI also had the highest number of ATF3 positive neurons though it passed the threshold level set by our lab, which has been established more by experience than empirical testing. It also showed negative immunostaining for another collateral sprouting target, which may emerge as a biomarker. Such differences between the experimental values created too much variance within the 7-day individual animals to see significance in the combined CD2AP measured intensity compared to naïve levels. However, the higher measured intensity of CD2AP in the two other 7-day experimental animals, AHF and AHI, suggest there was effective translation of mRNA to protein in the cell bodies of DRGs where collateral sprouting was in the initiation phase. However, statistical analysis of the animals using the mean expression of CD2AP showed no difference between the naïve and 7 day collateral sprouting population even when ASI was removed.

Comparison between 3 naïve animals and 2 collaterally sprouting animals does not provide a large pool for analysis when considering n as the number of animals.

In the initiation phase of collateral sprouting the neurons are in the process of responding to the denervation of the surrounding tissue. The formation of new proteins or increased levels of constitutively expressed proteins is likely required for the neuron to respond. The range of CD2AP staining intensity indicates protein within the cell bodies vary depending on the individual cell body. Apparently increased levels seen in the cell bodies of two of the three 7 day collateral sprouting animals suggest the increased production of CD2AP, potentially in response to the denervation status of the surrounding dermatomes. However the lack of statistical difference may be rooted in the possibility that the population of neurons with an increase of CD2AP protein within the cell body was too small to be identified by analyzing the entire population of cell bodies.

The co-stains, CGRP and IB4, were selected to identify CD2AP changes within previously characterized populations. Naïve animals and at 7 days following the spared dermatome procedure, there is not a discernable relationship between increased CGRP or IB4 staining with increased CD2AP staining intensity. Examining the maintenance phase of collateral sprouting (14 days), there was again no apparent sign of correlation between increase CD2AP staining and IB4 or CGRP positive staining. This indicates the range of CD2AP intensity within a population is not directly linked to identified CGRP or IB4 populations, peptidergic or non-peptidergic respectively.

The measure of CD2AP intensity suggested a decrease in 14-day animals compared to naïve according to both statistical comparisons, between the populations as a whole using the Mann-Whitney U test and between the two conditions based on the

individual animals in the grand mean analysis. One explanation of a lower staining intensity could be the function of CD2AP is in the periphery and the protein is sent out of the cell body following the production of mRNA. If the cell body is responding to denervation of surrounding dermatomes releasing trophic factors and the role of CD2AP is to act as an adaptor protein between the cytoskeleton and trophic factor tyrosine kinase receptors (as in podocytes(Welsch et al., 2005, Gigante et al., 2009, Lowik et al., 2009) and sympathetic neurons(Tsui and Pierchala, 2008)), it stands to reason the protein might be transported to the sites required for action at 14 days, the periphery of the sprouting axons. Behavioral tests of the CTM reflex showed effective collateral sprouting at 14 days as the mapped responsive region had expanded. Some of the CD2AP produced by the sensory neuron cell bodies participating in collateral sprouting was potentially already in place participating in the cytoskeletal reorganization while other molecules of CD2AP were still on their way to participate in the process.

Gene chip and qPCR analysis showed CD2AP was significantly increased only at 7 days following the spared dermatome procedure compared to naïve conditions. The probe and primer for each matched to the 3' untranslated region (3'-UTR) of CD2AP a region known to regulate the mRNA in many ways. Bound proteins and regulatory RNAs such as microRNAs provide regulation directed to the 3'-UTR. The methods of control include determining the overall stability and half-life of mRNA, allowing translation proteins to bind or not, and trafficking the mRNA to various parts of the cell(Evans et al., 1994, Mazumder et al., 2003). Ribosomes located in the periphery of the axons in neurons have been identified to locally control mRNA translation to protein (Zheng et al., 2001). Considering local translation of mRNA, the increased levels of one specific

mRNA of CD2AP at seven days not seen at fourteen days and not consistently identified by immunostaining could be based in the transport of mRNA to the periphery where CD2AP is translated into functional protein.

The data at 14 days collaterally sprouting might provide insights in the data provided by the 7-day time-points. While there was measured significance at 7 days between the two populations, treatment and naïve, when considered as a whole the analysis only measured the shift between the median of each group. We expect changes only in a small percentage of each dorsal root ganglion population. Changes seen might be the result of a localized increase in CD2AP protein translation, but the lack of significance when examining the differences between the mean CD2AP intensity of each animal is still a relevant factor. At 7-days following the spared dermatome procedure, increased production of CD2AP mRNA might be transported out of the cell to the region of the axon undergoing collateral sprouting as well as a slight but inconsistent increase in CD2AP translation in the cell body prior to shipment out of the cell body.

It is also of note that cell area distribution did not change on the whole at 7 days (the initiation phase) or at 14 days (the maintenance phase) compared to naïve levels. Studies of soma size in neuron regeneration showed a decrease in the overall size by one week post axotomy and did not return to naïve size until four weeks post surgery when regenerated axons reached their target (Murphy et al., 1990). The change of cell soma size in regeneration is a part of the response to nerve injury and a unique aspect of process while in our collateral sprouting model there was no observed change, increase or decrease in cell size.

Our hypothesis noted an expected increase in CD2AP protein production from the measured increase in CD2AP mRNA in the cell bodies at 7 days following the spared dermatome procedure. While the increase in protein level was not seen at 7 days or 14 days following the procedure, there is still the possibility that expressed CD2AP mRNA produced in the initiation phase is being exported to locations outside the cell body for localized translation to functional protein. CD2AP interactions between the cytoskeleton and tyrosine receptor kinases still provide a point of further analysis as the axons are responding to changes in trophic factors supplied by the denervated tissue causing changes in cell morphology. If CD2AP is involved in collateral sprouting at the site of axon growth, then measuring the role of the protein at the periphery, at the site of collateral sprouting, is an important next step. Also considering the role of CD2AP in the periphery, it is possible that the protein mRNA is regulated by being transported to sites outside the cell body. Prevention of translation until it reaches the location where it is needed prevents early cellular energy expenditure making sure the protein is only used where it is needed.

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CURRICULUM VITAE

NAME: Sarah Elizabeth Claypool Couch

ADDRESS: University of Louisville
Department of Anatomical Sciences and Neurobiology
500 S Preston St
Louisville, KY 40292

DOB: Louisville, Kentucky – July 15, 1987

EDUCATION
& TRAINING: B.S., Biochemistry and Molecular Biology
Centre College
2005-2009