NJCBIR Final Report.

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2. Name of Organization/Institution: Rutgers University-University of Pennsylvania.

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1. Project Aims:

Traumatic brain injury (TBI) is the leading cause of death and disability among people less than 45 years old. However, effective TBI therapies are still elusive. TBI also can initiate insidious neurodegeneration that is associated with a greater risk of Alzheimer's disease, and many TBI survivors have seriously disabling cognitive and/or neurobehavioral problems. A major TBI component thought to contribute to the neurologic deficits found in most TBI survivors is diffuse axonal injury (DAI), which results from damaged or dysfunctional axons throughout the white matter. In addition to axons, brain neurons also grow out dendrites, which appear to be particularly important for cognition, memory and plasticity in the brain. Our work aims to increase our understanding of the effect of TBI, and in particular DAI, on dendrite structure and integrity. Cognitive deficits, including memory loss are frequent in TBI survivors, including those with mild TBI. Since dendritic functions are important in establishing long-term memory, we hypothesized that DAI may induce pathological alterations of dendritic structure and function.

For this project, our Specific Aims were:
1) Characterize dendrite alterations after axonal stretch injury.
2) Determine the effect of stretch injury on myelin and the myelinated axons.
We used a well-established *in vitro* model of stretch-induced axonal injury on rat cortical neurons to reproduce the deformation experienced by axons in TBI, then analyzed dendritic alterations (Figure 1).

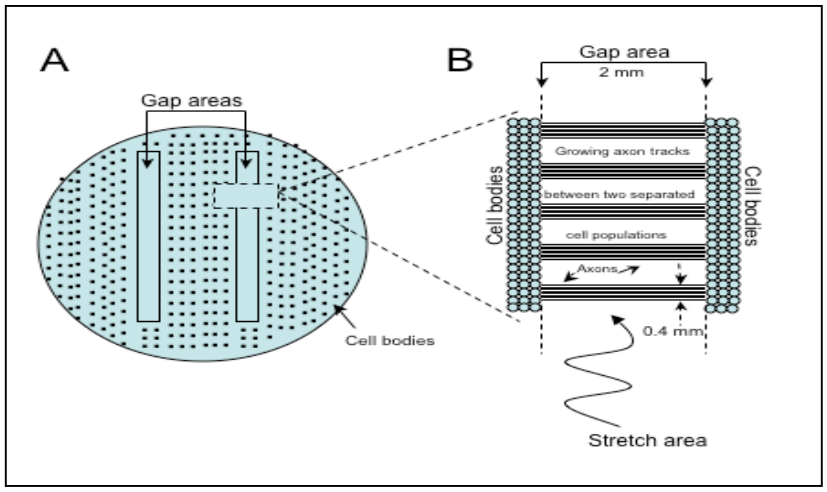

Figure1: Schematic representation of the steel well (A) in which two silicon barriers are placed over the elastic membrane to generate two cell-free gaps. Each barrier contains laddering microchannels (B) that allow axon growth on 2 mm longitudinal tracks.

2. Project Successes:

Stretch-induced axonal injury causes transient dendritic beading:

Following axonal stretch, dendrite morphology is altered. Dendritic alterations manifested as periodic swellings (or beads) along the dendritic shaft (Figure 2). Beads formed within minutes after stretch and lasted for several hours. It was maximal immediately after stretch (up to 50% of dendritic processes could be affected within an area of approximately 130-160 μm width along each injured gap's side), and decreased over time (5-10% of dendritic processes remained beaded 5 h after stretch). Examination of dendritic beading revealed several interesting characteristics:

1) Bead number per dendrite Length Unit (100 μm) was greatest 5 minutes after stretch, then gradually decreased over time and by 15 h very few cells displayed dendritic beading.

2) We analyzed bead distribution along the dendrite shaft by measuring inter-bead distances. We found that 70-90% of beads were separated by 0-10 μm regardless of which time-point was examined (between 5 min and 5 h after stretch) and that their distribution pattern along the dendrite shaft was similar. In addition, over time beads were more spaced apart, consistent with the observation that there were fewer beads per dendrite Length Unit.
3) Bead size (perimeter) was similar when examined between 5 min and 5 h after stretch. There was a large range in bead perimeter, with greater than 60% of beads having a perimeter between 3 and 7 μm, regardless of the time-point examined. Dendritic beads were generally spherical, although some displayed a more irregular appearance, particularly large beads (greater than 5 μm in length).

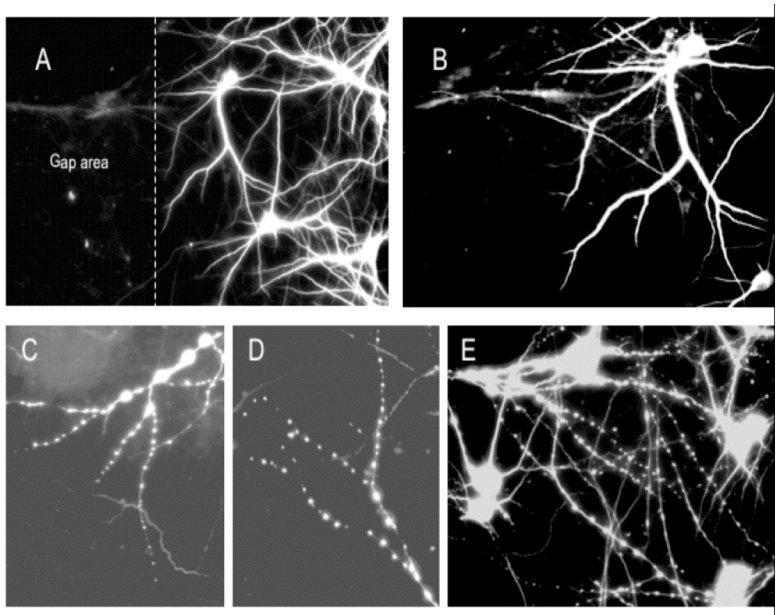

Figure 2. Fluorescent photomicrographs of control (A, B) neurons stained with the dendritic marker MAP2, and beaded dendrites 5 minutes after axonal stretch (C, D, E). Magnification 200X (A), 400X (B, C, D, E). Dotted line in (A) indicates the limit between the gap area (left) and the growing dendrites.

Our results established that dendritic beading was consistently observed after stretch. In addition, beads formed rapidly (within 5 min) and remained for several hours. Whereas their number slowly decreased over time (between 5 min and 5 h), the percentage of cells displaying dendritic beading declined more rapidly during the same period (affecting up to 50% of the cells at 5 min, but only 5-10%, 5 h after stretch). In contrast, bead size varied little over time, and there did not seem to be a progressive regression of their size. Transient changes in dendritic morphology and structure, as observed in our *in vitro* model of axonal stretch injury, may play a role in the neurological deficits long observed after TBI.

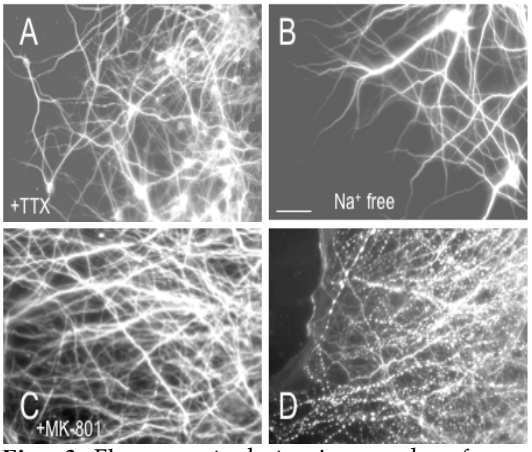

Fig. 3. Fluorescent photomicrographs of axon-stretched neurons stained with the dendritic marker MAP2 and fixed 5 min after stretch. Cells were pre-incubated: (A) with the VGSC TTX, (B) in absence of extracellular sodium, (C) with the NMDA receptor antagonist MK-801. In (D), cells were axon-stretched only. All 3 pharmacological treatments prevented dendritic beading (compare A, B, C with D). Magnification 400X.

Ionic basis of stretch-induced axonal injury.

Ionic disturbances, and in particular abnormal sodium influx, have been implicated in dendritic bead formation after glutamate exposure (excitotoxicity) or oxygen-glucose deprivation. When cells were pre-treated with the voltage-gated sodium channel (VGSC) blocker tetrodotoxin (TTX, 1 μM) before axonal stretch, dendritic beading was almost completely prevented (Figure 3A). Furthermore, pre-incubating cells in absence of sodium (replaced with equimolar N-methyl-D-glucamine) before axonal stretch injury also

prevented dendritic beading, confirming sodium influx is involved in DAI-induced dendritic bead formation (Figure 3B).

N-methyl-D-aspartate receptors (NMDAR), a class of glutamate receptors, have been implicated in excitotoxicity-induced dendritic beading. We found that pre-treatment with the selective NMDAR blocker MK-801 (20 μM) before axonal stretch injury suppressed dendritic beading, suggesting that glutamate receptors are involved in stretch-induced dendritic beading (Figure 3C). We conducted experiments to determine whether a change in NMDAR expression occurred following axonal stretch. Cell extracts were collected before and 5 min and 1 h after stretch, then subjected to Western blot analysis using an antibody against the NMDAR subunit NR1, which is essential to the receptor's function. No significant change in NR1 subunit's expression was observed between control (unstretched) and stretched cultures.

Since NMDAR are highly permeable to calcium ions, we examined whether calcium influx played a role in axonal stretch injury-induced dendritic beading. Removal of extracellular calcium before axonal stretch significantly increased the number of beaded dendrites (> 90% in calcium-free medium versus 40-50% in calcium-containing medium) (Figure 4). However, this beading was qualitatively different from cultures in which calcium was present during axonal stretch injury since the majority of beads appeared larger and were spaced further apart along the dendritic shafts. Examination of dendritic beading in calcium-free medium revealed the following characteristics:

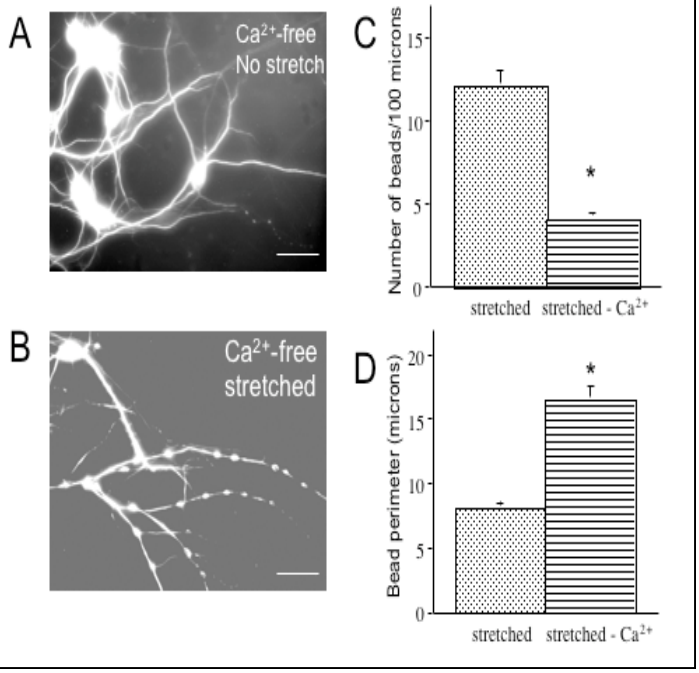

Figure4. Fluorescent photomicrographs of dendritic processes immunostained for MAP2 demonstrate smooth processes of uninjured cultures pre-incubated in calcium-free medium (A). However, 5 min after axon stretch, dendrites displayed extensive beading (B). Histograms (C) and (D) illustrate quantitative analysis of dendritic beading and show a decreased number of beads (C) and an increased bead perimeter (D) in calcium-free medium after stretch, compared to calcium-containing medium. Bead number and perimeter were determined at X400 and X800 magnification, respectively. Values represent mean ± S.E.M. from 3 independent experiments. * $p < 0.0001$ vs. calcium-containing medium. Scale bar = 25 μm in (A, B).

1) Bead number per dendrite Length Unit (100 μm) was decreased compared to calcium-containing medium.

2) Bead distribution along the dendrite shaft (inter-bead distances) was fairly equal over a wide range (> 20 μm), as opposed to beads generated in calcium-containing medium which inter-bead distances were principally distributed over a narrow range (0-10 μm).

3) Bead size (perimeter) was significantly different whether calcium was present or absent during stretch. We observed a shift in distribution pattern from smaller beads in calcium-containing medium to bigger beads in calcium-free medium. Indeed, greater than 65% of the beads had a perimeter comprised between 0 and 9 μm in calcium-containing medium, as opposed to only 10% of the beads in calcium-free medium. In contrast, 50% of the beads in

calcium-free medium had a perimeter comprised between 17-26 μm, as opposed to less than 10% of the beads in calcium-containing medium.

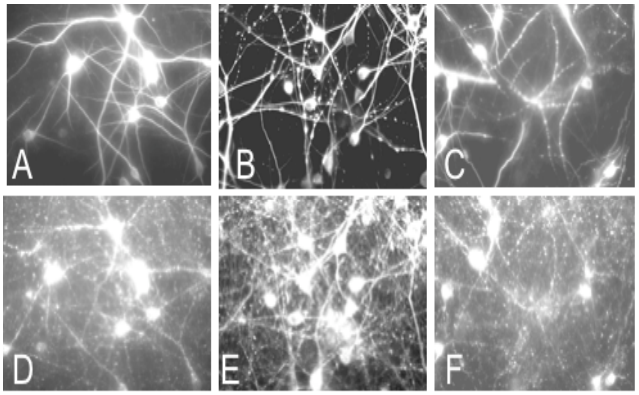

Fig. 5: Fluorescent photomicrographs of control (unstretched, A,D) and axon-stretched neurons (B,C,E,F) stained with MAP2 (A,B,C) and the actin marker phalloidin (D,E,F) and fixed 5 min after stretch. In C and F, cells were pre-incubated with cytochalasine D. Cyt D did not prevent dendritic beading after stretch. (compare C with B). No difference in actin staining was observed between control and stretched cultures (compare D and E). Magnification 400X

Actin microfilaments are not involved in axonal stretch-induced dendritic beading:

Actin is an important cytoskeletal component of dendritic spines that are dynamic structures that provide a structural basis for synaptic plasticity and memory. We observed that pre-treating cultures with the actin depolymerizing agent cytochalasine D (10 μM) before axonal stretch did not prevent dendritic beading (Figure 5). Conversely, using an actin-stabilizing agent, jasplakinolid (1 μM), before stretch also did not protect against stretch-induced dendritic beading. Furthermore, no change in actin's staining pattern was observed between stretched and unstretched (control) cultures at any time point examined (5 min-5 h), when cells were immunostained with the actin-labeling agent phalloidin. These experiments suggest that the actin cytoskeleton does not participate in axonal stretch-induced dendritic beading.

Microtubule-associated protein 2 (MAP2)'s expression does not change after axonal stretch:

Disruption of cytoskeletal proteins including MAP2 occurs in dendrites after TBI and DAI *in vivo*. We observed by immunofluorescence of MAP2-stained dendrites that the dendrite region separating beads often shrank in diameter, occasionally becoming undetectable, thus revealing a fragmented MAP2 staining. However, when analyzing cell extracts collected at 5 min and 1 h after axonal stretch by Western blotting using anti-MAP2, no significant change in MAP2 expression was observed (Figure 6). It is possible that a redistribution rather than a change in expression of MAP2 occurs after stretch in beaded dendrites.

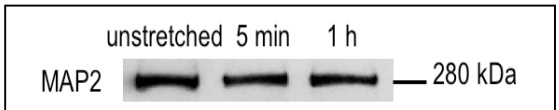

Figure 6

Analysis of injury response of myelinating glial cells: We had difficulties establishing myelinating co-culture system using the in vitro DAI model. Instead, we utilized both in vitro and in vivo nerve injury (axotomy) model to study myelin response to axon damage and its effect on nerve regeneration. The study has been published in Molecular and Cellular Neuroscience.

3. Project challenges:

The experimental *in vitro* model of stretch-induced axonal injury requires a high cell density to establish well-defined axon tracks at regular intervals across two neuronal cell populations. While this system is highly reliable and well-adapted to study axon-only injury, it proved difficult to analyze the effect of axon stretch on synaptic contacts in individual dendrites. In addition, our results have established a direct link between the injured axons and their associated dendrites, however, the high culture density and interconnectivity of neurites did not allow to determine whether the morphological alteration of dendrites we observed was from postsynaptic neurons or only from neurons whose axons

were mechanically injured. A similar challenge arose with the analysis of myelin. We will continue to improve the culture system to combine myelinating co-cultures with the in vitro DAI injury model.

4. Implications for future research:

We have shown that dynamic mechanical deformation of isolated axons induces indirect pathologic changes of associated dendrites. In addition to replicating the deformation experienced by axons during inertial brain injury, the present results obtained with this model also are important because converging evidence suggests that altered dendrite plasticity may underlie many of the deficits observed after TBI. Furthermore, impaired learning and memory, for which dendrites play a major role, and behavioral dysfunctions are common sequelae after TBI. Thus, understanding the effect of DAI on dendrites may lead to novel strategies for intervention in TBI. Future research should determine how dendritic beading affects dendrite integrity and function since proper dendrite growth and morphology are key determinants of neuron and so central nervous system (CNS) function, and any alteration in dendrite growth and/or structure can impair brain function and recovery. In particular, whether postsynaptic transmission is altered after axonal stretch and whether it is a short-term (dependent upon bead formation) or long-lasting (as opposed to the transient nature of beading) phenomenon could provide useful information. Along this line, examining the physiological correlates of *in vivo* models of TBI will be necessary since results obtained *in vitro* may differ from the more complex in vivo environment.

5. Plans to continue the research:

This project has generated important data that support our original hypothesis that axonal injury leads to dendritic alterations. We intend to continue this important research in the following years by using these results to apply for NIH funding.

6. Additional federal or other support for brain injury research:

We do not currently have additional funding to continue our project on TBI.

7. List of publications and presentations:

Hubert Monnerie, Min D. Tang-Schomer, Akira Iwata, Douglas H. Smith, Haesun. A. Kim, Peter D. Le Roux. Dendritic alterations after dynamic axonal stretch injury *in vitro*. Exp Neurol 224:415-423, 2010.

Yang, D. P., D. P. Zhang, K. S. Mak, D. E. Bonder, S. L. Pomeroy and H. A. Kim Schwann cell proliferation during Wallerian degeneration is not necessary for regeneration and remyelination of the peripheral nerves: axon-dependent removal of newly generataed Schwann cells by apoptosis. Mol. Cell. Neurosci. 38:80-88, 2008