Adapted Primary Literature for Teaching

Cell-level Effects of Concussion

Original research report: M. A. Hemphill, et al. 2011. A Possible Role for Integrin Signaling in Diffuse Axonal Injury. PLoS One. 6 (&) e22899. Retrieved from http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0022899

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Abstract

Scientists don't know much about what concussion does to nerve cells. Prior studies suggest that the major effect is on the protein "channels" (pores) in the cell membrane through which ions flow during nerve cell electrical activity.[1] The hypothesis of this study was that a key part of the response to concussion could be protein "integrins" that help neurons stick to each other. To mimic the effect of combat blasts on neurons, they grew rat-brain neurons in a bath of nutrient fluid on a plastic sheet that they could stretch, and in the process stretch the neurons attached to the sheet. They also tested the damage caused by the force of magnetic micro-tweezers acting on neurons that had magnetic beads stuck on their surface. These methods revealed that local damage depended on membrane integrins. Damage could be reduced by treating the neurons with a drug that that helps make intracellular structural proteins[2] more stable.

Introduction

Previous studies had shown that the most frequent wound of U.S. troops in Iraq and Afghanistan is blast-induced trauma, We do not know just how the concussion-like trauma is produced from the explosion energy when flying metal (shrapnel) does not damage tissue. Brain scans (magnetic resonance imaging, MRI) of blast victims indicate that blast-affected troops have altered structure of the brain "white matter" (the large fiber tracts of nerve cell processes that connect different parts of the brain and spinal cord). Just what happens at the cell level in this white-matter damage is not known.

One unique way to study this issue would be to culture nerve cells and subject them to mechanical force, observing what happens to certain cellular structures. The authors hypothesized that one target of blast injury could be the chemicals that help neurons stick together. One class of such chemicals are "integrins," which are membrane-bound proteins that act like glue to hold membranes of adjacent neuron cell bodies and processes (neurites) together. These not only hold cell membranes together but they also link to structural proteins inside the neuron so that concussion damage to integrins could disrupt structures inside the neurons. Altering internal structural proteins would disrupt cellular synthesis processes and ionic currents that underlie the electrical activity of neurons.

Methods

Neurons were grown in a culture of fluid that contained nutrients and a balance of salt known to keep neurons alive and well. Neurons were stretched by a high-speed stretcher that could pull on both ends of an elastic plastic-like sheet on which neurons had settled (Fig. 1). Since the neurons were stuck to the sheet, pulling on the sheet had the effect of pulling on the neurons.[3]

Neurons were grown for five days before tests to allow time for them to grow processes and to get firmly attached to the plastic sheet.

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[1] The flow of ions across nerve membrane is an electrical current, similar to the flow of electrons in a metal conductor. In living tissue, the fluids are the conductor.

[2] Proteins either have function (like enzymes) or form skeleton-like structure inside the cell (like tubules).

[3] They didn't use rubber bands, because rubber would leak chemicals into the culture medium that might have been toxic to neurons.

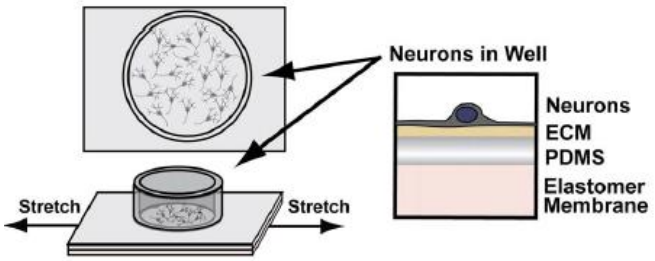

Fig. 1. Diagram of apparatus used to culture the neurons in a way to allow them to be stretched by pulling on the stretchable (elastomer) membrane. Right: cross-section view.

To measure effects on the neuronal proteins, they used an antibody to tubulin, which is a protein that sticks to the small protein tubes inside of neurons. These microtubules are essential for transporting chemicals throughout a nerve cell and its large network of axons and dendrites. When viewed under a microscope that uses a higher-energy light than visible light, the antibody shines (fluoresces), showing the proteins to which it is bound.

In separate studies, they used magnetic micro tweezers to pull on different parts of a neuron that had been coated with micro-beads. Thus, they could measure how tightly the neurons remained attached to the plastic sheet.

Results

Stretch tears the cell membrane and breaks down the microtubules inside (Fig. 2).

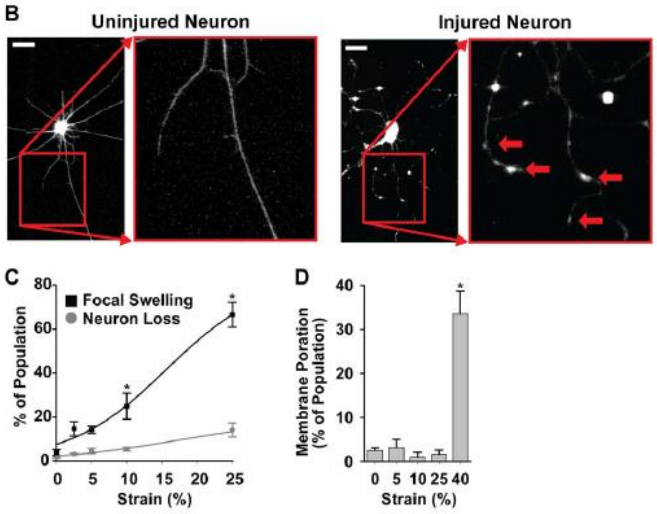

Fig. 2. **B** Before stretching, the upper left photo taken through a fluorescent microscope shows a neuron as a smooth white blob. The red enlarged area in the next image shows two neuron processes with a smooth distribution of the antibody, indicating normal structure of the tubules. But in the upper right frame, we see the difference when neurons have been stretched. This visual field shows several neurons as white blobs, but the enlarged view of their processes reveals uneven antibody distribution. Areas where antibody accumulates (red arrows) indicate that the underlying tubulin has been broken in several places and reformed as clumps.

C Graph showing how increasing stretch increased the damage. With increasing stretch (Strain %), some neurons actually died, and a large percentage of them became swollen. Data averaged over four tests. **D.** Effects of stretch on the movement of a dye into neurons. At low levels of stretch the dye did not enter easily, but at the highest level of stretch, a large percentage of the neurons took up the dye. In C and D, error bars show the variation among different tests. The little "T" above the bars represents the "standard error" variation around the mean of all tests. Bars labeled with * indicate that the odds that this difference could have occurred by chance alone is less than 5%.

Increased movement of dye into neurons indicates tears or holes in the membrane. Because this only occurred at the highest level of strain produced, the next tests focused on strains of 0-10% since they caused signs of damage that could not be explained by tears in the membrane.

The next tests examined how tightly neurons remained stuck to the stretchable plastic sheet. Other studies had shown that such binding is created by a mixture of different proteins on the neuron membrane. In order to direct the growth of neurites, they used stretchable sheets with micro-contact printed lines (10 µm wide), coated with one of two chemicals (PLL or FN) to promote attachment of the neurites to the sheet. The total area of bound neurite was measured with a fluorescent chemical marker, vinculin, an intracellular structural protein linked to the surface-binding protein complex (see diagram A in Fig. 3).

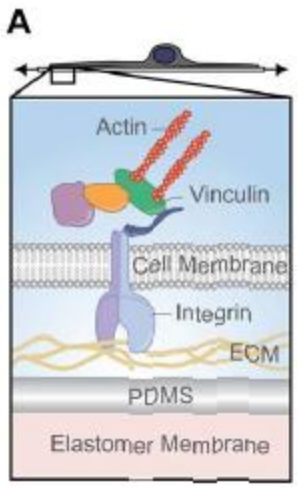

Fig. 3 A. Diagram showing how intracellular proteins (actin and vinculin) are coupled to the surface binding by way of integrin, which in turn binds to the layer of extracellular matrix (ECM) deposited by the serum in the bathing fluid. ECM sticks to the printable lines on the substrate (labeled here as PDMS).

Tests showed that the vinculin fluorescence was more numerous and dense with the FN coating (data not shown here).

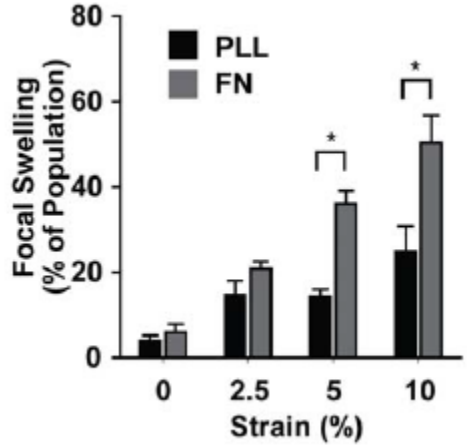

Fig. 3 continued. **E.** Effects of different degrees of stretch on the vinculin tagged membrane binding complex under the two coating conditions of PLL and FN. The percentage of neurons with widespread patches of swelling after stretch was greater on a FN coated sheet than on one coated with PLL. Data averaged over 4 trials for PLL and 8 for FN. Error bars as in Fig. 2.

Next the researchers wanted to see if stretching caused an especially damaging effect on certain parts of a neuron (cell body, dendrites, axons). They predicted that the cell body, which has larger and more surface binding patches would protect it from stretch. So, they used their magnetic micro-tweezer tool to pull on neurons that had been coated with magnetic micro-bead chemical applied to different regions of the neuron. By increasing the magnetic force, they could peel neurons off of the PLL or FN coated surfaces. The speed at which a neuron was pulled off in response to increase strain was a straight-line relationship on the PLL surface but resulted in an S-shaped curve from the FN surface. They believe this difference was due to the fact that FN binding was stronger.

They then tested binding strength at different parts of a neuron, again using the magnetic tweezers to see how much force was needed to pull the neuron off at the cell body versus the neurites. As expected, the binding was stronger at the cell body. In other words, strain damage was greater on the neurites (their Fig. 3, data not shown here).

Since integrins form a bridge between the intracellular proteins and the binding complex on the sheet (recall Fig. 3A), they wanted to see if damage to integrins would spread throughout the cell interior. For this they focused on FN binding, because it specifically involves integrins, while PLL does not. A quick pull on FN-coated beads consistently induced formation of local swellings on neurites extending from the opposite side of the cell body (100% of injured neurons) showing that damage spread throughout the neuron, Pull on PLL coated beads also injured neurons, but only in local regions, not throughout the whole cell. In other words, stretch damage, even if applied in only one spot, spreads throughout the cell from disturbance of integrin. No signs of injury were seen with magnetic field alone nor attached beads alone, or (their Fig. 3, data not shown here).

Since integrin seems to be crucial, the next question was whether integrin spread the damage specifically or through some other

secondary mechanisms, which could include a cascade of reactions inside the neuron.

To test this question, they thought about protein-breaking enzymes in cells called "calpains" which others had shown can break down the protein structure (called "cytoskeleton") inside the cell. If they could bathe neurons with some drug or chemical known to inhibit calpains, they might be able to prevent strain-induced damage if that damage were due to secondary, intracellular consequences of strain on integrins. In their test with one calpain inhibitor on PLL-bound cells they found no protection against stretching the sheet. (their Fig. 4A, data not shown). Studies by others had shown that an intracellular protein known as ROCK normally breaks down cytoskeleton, in particular neurotubules. ROCK is a target in current medical research aimed at finding a treatment for nerve system diseases caused by degeneration of nervous tissue.

One of these chemicals being studied by others is called HA-1077, a drug that inhibits ROCK. This drug, when added to the cell bath in this stretch system immediately after a stretch, caused a progressive ("dose-dependent") decrease in the percentage of neurons that had local swellings after stretch (Fig. 4B). This apparent protection occurred with both 5 and 10% stretch (Fig. 4C).

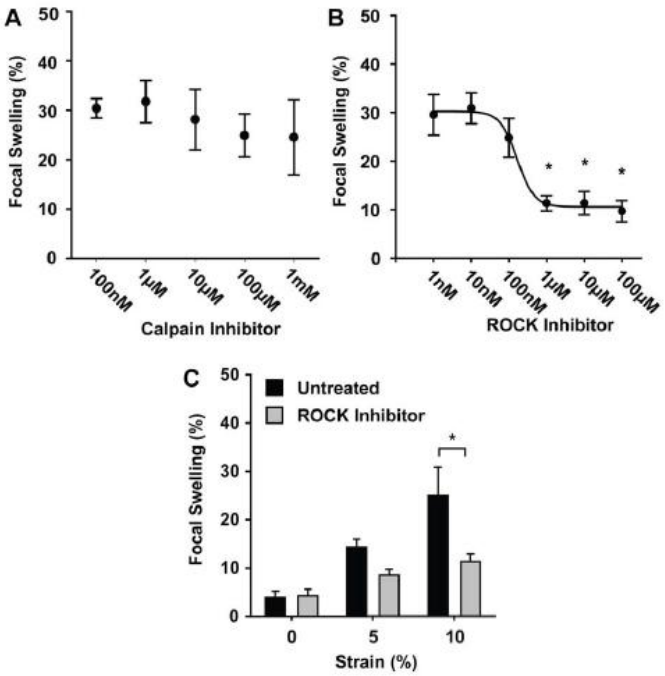

Fig. 4.B. Tests of stretch effects on different parts of a neuron. **A.** Adding a chemical that inhibits calpain, an intracellular enzyme that breaks up proteins at certain points in amino acid sequence. Increasing concentrations (left to right) of the chemical was unable to protect stretched neurons from swelling. B. But adding a ROCK inhibitor had a conspicuous protective effect at concentrations of 1 micromole and greater. C. The ROCK protective effect was seen against both %5 and 10% strain magnitude. The bracket with the * indicates that the difference at the 10% strain was not due to chance, but a statistically reliable indicator of the chemical's protective effect.

Discussion

The authors believe that the experiments tested the effects on mechanical stretching on neuronal membrane integrins and indirectly on the extracellular binding of the membranes and intracellular protein structures. The force applied caused localized spots of neuronal swelling, which is similar to what happens in brain that has been exposed to concussion. Even pulls too weak to cause tears in the membrane disrupted the coupling of cytoskeleton proteins via integrins to the extracellular binding complex.

The authors think these findings are especially significant because reports of many others have shown integrins to be so crucial to normal cell function at synapses, ion channels, growth

of neurites, and even at behavioral levels such as memory formation (which is stored in changes in synapse structure).

Because adhesion failed more easily in neurites than along the cell body, authors concluded that concussion damage preferentially occurs in neurites. Moreover, the data indicated that local damage often spreads through wide areas inside the neuron because integrins are coupled to intracellular structural proteins. Authors cite studies by others who used different methods but arrived at similar conclusions.

The authors noted that although reports by others suggested calpains were involved in force damage to cells, the results in this present study showed that inhibiting calpain had no protective effect. Others had shown that the intracellular protein RhoA promotes cytoskeleton breakdown by way of another linked protein called ROCK. Thus, they reasoned that a chemical that inhibits ROCK might protect against strain damage. They were able to reduce damage to neurites with a ROCK inhibitor. ROCK protein is part of the chain of proteins that can cause many changes in cytoskeleton structure. Especially important is the damage it does to microtubules and the transport of materials along neuritis.

These studies suggest that concussion might cause damage by way of an integrin-mediated signaling cascade involving a ROCK-mediated pathway. Thus, key chemical systems causing concussion damage have been identified, making these targets for drug development that might reduce concussion damage.