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COMMITTEE FOR AID AND EDUCATION IN NEUROCHEMISTRY (CAEN)
CATEGORY 1B: Research supplies for use in the applicant's home laboratory
International Society for Neurochemistry
RESEARCHER: Vinicius de Toledo Ribas, PhD

REPORT

PROPOSED PROJECT

Combinatorial strategy to enhance axonal regeneration of corticospinal neurons after spinal cord injury

INTRODUCTION

Spinal cord injury (SCI) is a serious condition that can result in permanent sensory and/or motor deficits (Yip & Malaspina, 2012) and currently cannot be sufficiently repaired by any therapy. One of the major challenges to achieve functional recovery after SCI is the promotion of robust axon regeneration. After a lesion to the mature central nervous system (CNS), there is essentially no re-growth of axons beyond the point of injury. This lack of regeneration in the adult mammalian CNS is in part due to the presence of extrinsic inhibitory molecules at the injury site, but also because adult CNS neurons possess an intrinsically low capacity for axon growth compared to the developing CNS (Yang & Yang, 2012; Yiu & He, 2006). This high capacity of axon regeneration of developing CNS neurons is attributed to the expression of key transcription factors that orchestrate molecular programs important for axonal growth. Thus, the identification of transcription factors, which recapitulate these pro-regenerative mechanisms, is a pivotal strategy to enhance the regenerative capacity of adult CNS neurons. The transcription factor Fezf2 is a proneural gene and play different roles during CNS development (Shimizu & Hibi, 2009). In the cerebral cortex, Fezf2 is essential for differentiation of projection neurons in layer V, including corticospinal neurons (CSN) (Eckler & Chen, 2014). It has been shown that CSN deficient for Fezf2 fail to project axons towards the spinal cord (Chen et al., 2005a; Chen et al., 2005b, Molyneux et al., 2005), indicating that Fezf2 is involved in the regulation of axon outgrowth of CSN. Moreover, increased neural activity could also contribute to enhance axon regeneration. Different studies suggest that electrical activity can enhance neuronal survival and axon regeneration (Morimoto, 2012). Recently, it has been shown that increased neural activity promotes robust axon regeneration of optic nerve axons after lesion (Lim et al., 2016). Therefore, the combination of Fezf2 overexpression and increased neural activity could be a promising combinatorial strategy to induce robust axon regeneration after adult CNS lesion.

HYPOTHESIS

Our hypothesis is that a combinatorial strategy based on the expression of Fezf2 and

increased neural activity promotes robust axon regeneration after traumatic lesion to the CNS.

SPECIFIC AIMS

1. Test if expression of Fezf2 and/or enhanced neural activity increases axon regeneration after axotomy of primary cortical neurons cultured in a microfluidic device *in vitro*.

2. Test if expression of Fezf2 and/or enhanced neural activity improves corticospinal tract (CST) axon regeneration and promote synaptic reconnection of regenerating axons after spinal cord injury.

PRELIMINARY RESULTS

In order to overexpress the transcription factor Fezf2 and to increase neural activity we used adeno-associated virus (AAV) vectors expressing Fezf2 (AAV.Fezf2) and the DREADD (Designer Receptors Exclusively Activated by Designer Drugs) M3-mutant muscarinic receptor (AAV.hM3Dq), respectively. As control we used the AAV vector expressing only mCherry (AAV.mCherry) (figure 1).

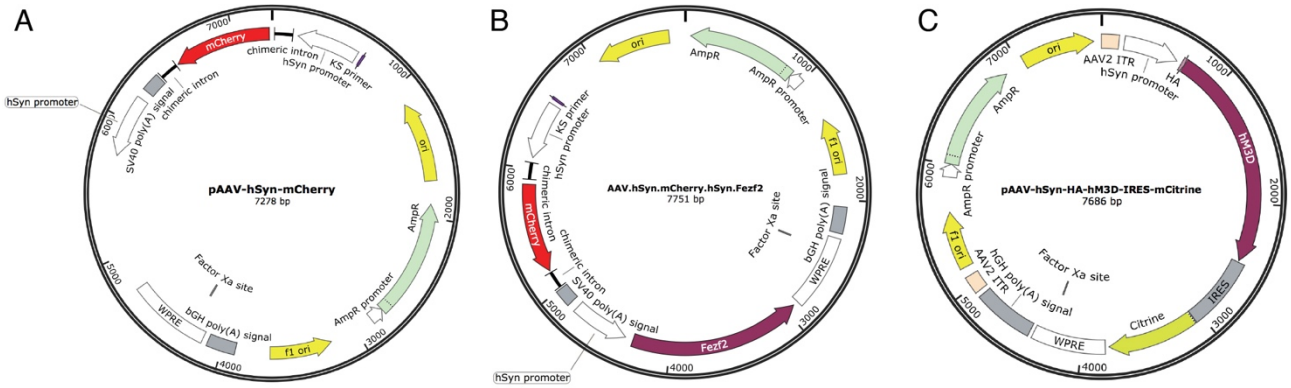

Figure 1 - Maps of the AAV vectors constructs. (**A**) AAV.mCherry, (**B**) AAV.Fezf2, (**C**) AAV.hM3D.

First we tested the ability of the AAV vectors to transduce neurons *in vitro*. In order to do that we cultured primary cortical neurons in a 24 well plate. Immediately after seeding we added the AAV vectors and four days later pictures were taken using a fluorescent microscope. We could verify that all vectors were able to transduce efficiently the cortical neurons, as seem by the expression of the fluorescent reporter genes (figure 2).

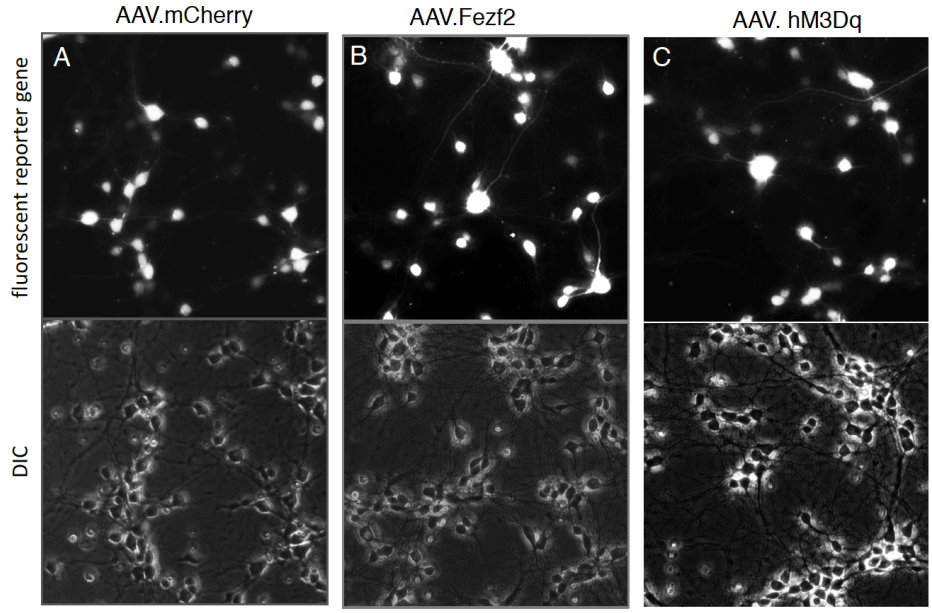

Figure 2 – Representative pictures of primary cortical neuron culture 4 days after transduction with the following AAV vectors: (**A**) AAV.mCherry, (**B**) AAV.Fezf2 and (**C**) AAV.hM3Dq. The pictures show the transduced neurons expressing the fluorescent reporter protein (**A** and **B**: mCherry; **C**: mCitrine) in the upper panels and all neurons in the bottom panels (DIC).

After confirm that the viral vectors are working *in vitro*, we, then, evaluate if overexpression of Fezf2 is able to promote neurite outgrowth *in vitro*. For that we prepared primary cortical neurons, transduced with AAV vectors expressing, either, Fezf2 or mCherry and four days later we quantified the neurite length. The result shows that in the Fezf2 overexpressing neurons the mean neurite length per neuron is higher compared to the control (figure 3). This preliminary data suggests that Fezf2 might promote neurite outgrowth, however, additional experiments are needed. The follow up experiment will be to evaluate if overexpression of Fezf2 and/or enhanced neural activity increases axon regeneration *in vitro* after axotomy of primary cortical neurons cultured in a microfluidic chamber that separates axons from soma and dendrite.

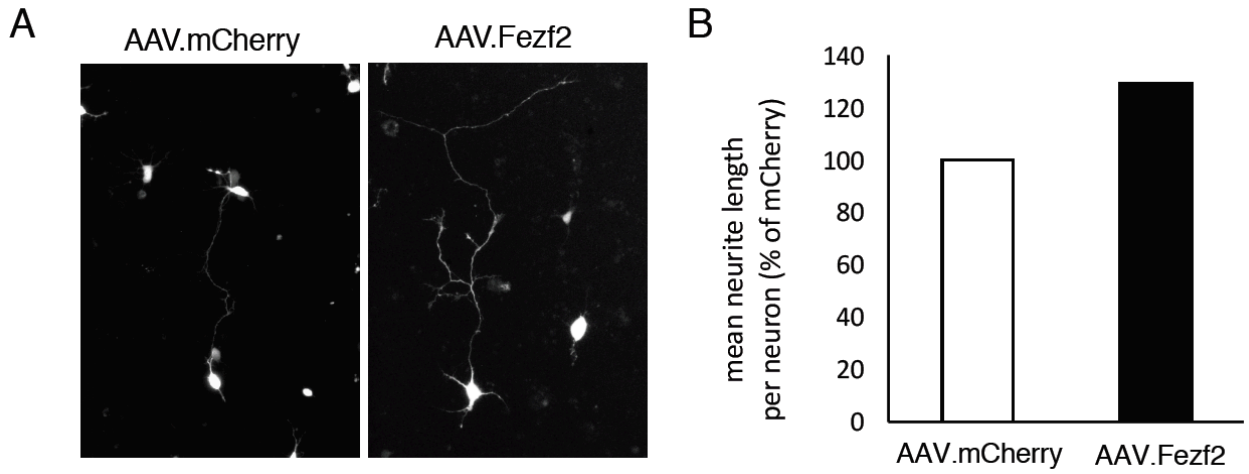

Figure 3 – (**A**) Representative pictures of primary cortical neurons 4 days after transduction with the vectors AAV.mCherry and AAV.Fezf2, showing the expression of the fluorescent reporter protein mCherry in the cell soma and neurites. (**B**) Quantification of mean neurite length per neuron, normalized to the control group AAV.mCherry (n=1).

To examine if overexpression of Fezf2 and/or enhanced neural activity improves axon regeneration after spinal cord injury, we, first, tested if the AAV vectors are able to transduce efficiently the corticospinal neurons *in vivo*. For that we injected the AAV vectors (AAV.Fezf2, the control AAV.mCherry or AAV.hM3Dq) into the sensorimotor cortex to allow expression of the transgenes in the corticospinal neurons. Four weeks later we performed coronal cryosections of the brain and pictures were taken using a fluorescent microscope. We found that the viral vectors AAV.mCherry and AAV.Fezf2 efficiently transduced pyramidal neurons in the sensorimotor cortex, as seen by the expression of mCherry (figure 4A and 4B). However, the viral vector AAV.hM3D was able to transduce only a few cortical neurons, as seen by low number of pyramidal neurons expressing mCitrine (figure 4C). This suggest that the AAV.mCherry and AAV.Fezf2 are working efficiently *in vivo*, however, the AAV.hM3D needs to be tested again in order to confirm the vector efficacy *in vivo*. We are currently performing an experiment using a new aliquot of AAV.hM3D to confirm the efficiency of this viral vector. Confirming the efficiency of AAV.hM3Dq, the next experiment will be to evaluate if overexpression of Fezf2 and/or increased neural activity promotes axon regeneration after spinal cord injury.

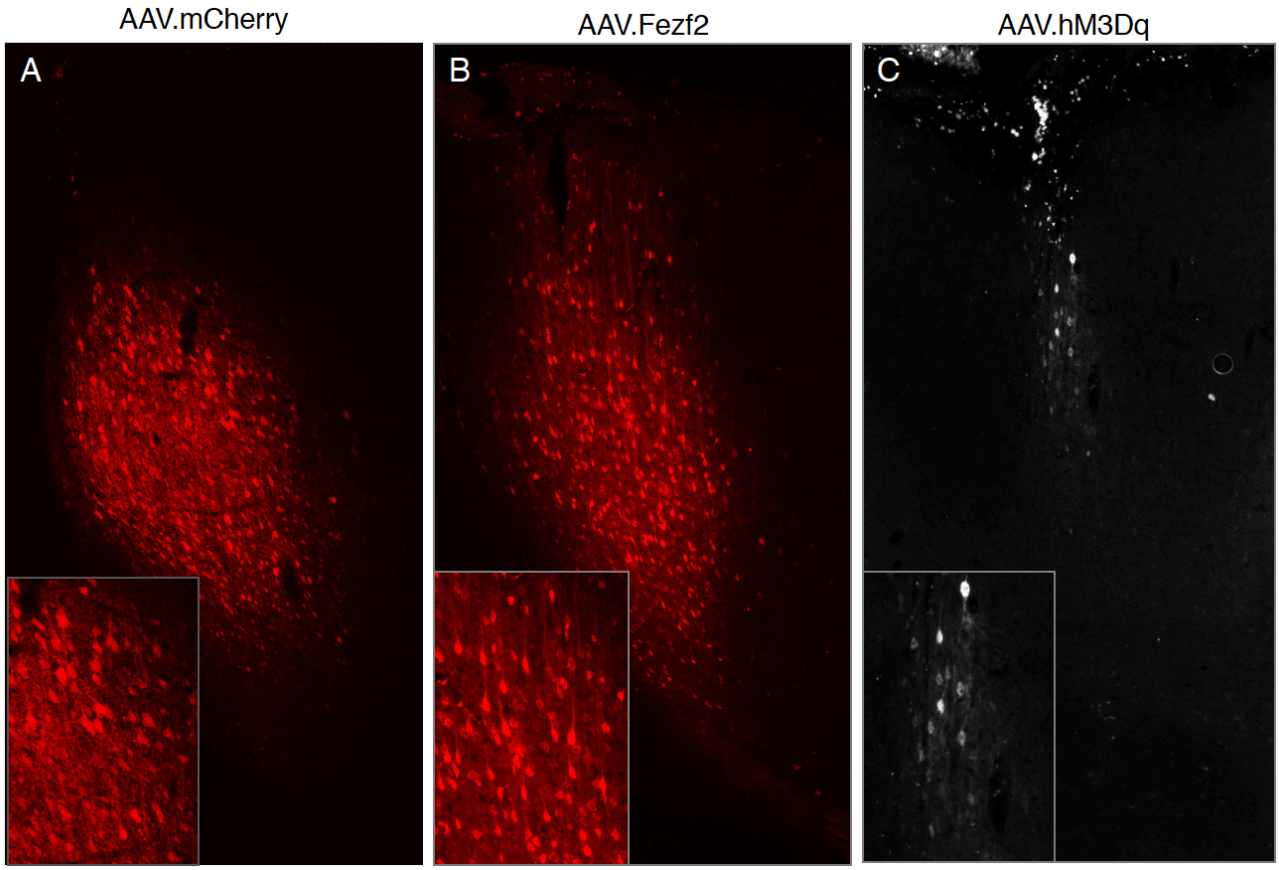

Figure 4 – Representative pictures of brain sections four weeks after (**A**) AAV.mCherry, (**B**) AAV.Fezf2 and (**C**) AAV.hM3Dq injection into the sensorimotor cortex, showing the expression of mCherry (**A** and **B**) and mCitrine (**C**) in cortical neurons of the sensorimotor cortex. Higher magnification of *Inset* demonstrates the labeling of pyramidal neurons and the transduction efficiency of each AAV vector.

CONCLUSION

Our preliminary results suggest that Fezf2 overexpression might increase neurite outgrowth, however, additional experiments are needed to confirm this result. This project is still ongoing and we expect to have a number of results in the following year. Taken together, this proposal could identify combinatorial strategies that allow CNS neurons to regenerate their axons after injury. Therefore, our proposal could be pivotal to develop new therapeutic treatment for patients with spinal cord lesions, a relevant neurological disorder with limited treatment options.

ACKNOWLEDGEMENTS

The ISN-CAEN grant for research supplies was very important for starting my laboratory at the Federal University of Minas Gerais in Brazil. Last year was my first year as an Assistant Professor and this grant assisted me to purchase a number of research supplies essentials for the initiation of this project, in particular the supplies needed for the implementation of the *in vitro* and *in vivo* models of CNS lesion. I am very grateful for this support and the ISN-CAEN will be acknowledged in future publications and presentations.

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FINANCIAL REPORT

We used the funds of the ISN-CAEN grant purchasing antibodies (anti-Fezf2, anti-Gap43, anti-GFAP, DyLight 488-Streptavidin, DyLight 649 anti-rabbit, Alexa Fluor 405 anti-mouse, Alexa Fluor 488 anti-rabbit); materials for surgery and animal care, clozapine-N-oxide (CNO), cell culture reagents, histology reagents and costs for multi user equipment.

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