Aims. We aimed at comparing the effect of dual on intravitreal calcium channel inhibition on retinal ganglion cells (RGC) survival and axonal regeneration in a model of optic nerve crush (ONC) lesion and analyzing axon regenerate past a lesion site. Recently we addressed this question by inhibition of calcium channels. JNK is a serine/threonine kinase that regulates blocking of acute axonal degeneration by calcium channel inhibition facilitates subsequent axonal regeneration until the lesion site was missing. We now showed that axonal stabilization by calcium channel inhibition significantly increases axon regeneration up to 2-fold distal to the lesion site, thus confirming this hypothesis. However, the increase in axonal regeneration was limited to the area close to the crush site, at larger distances from the crush site (≥400 μm), the treatment was not effective. In the adult CNS, a lesion to the axons results in axonal degeneration and the axons fail to regenerate past the point of the original injury. The failure in the regenerative response of adult CNS neurons is predominantly caused by the weak intrinsic growth capacity of adult neurons and the presence of growth-repressing molecules in the CNS environment. In our study we did not target the inhibitory environment neither the intrinsic capabilities for axonal outgrowth. Thus, the effect we observed on axonal regeneration is only due to the increased axonal stabilization. In conclusion, our proof-of-principle study showed that axonal stabilization by inhibition of calcium channels facilitates axonal regeneration and it could be combined with additional strategies in order to elicit a more robust effect.

In addition to axonal protection, promoting cell survival is essential for successful regeneration. We therefore also evaluated neuronal survival and found that inhibition of calcium channels increases RGC survival after ONC. We targeted AMPA receptors and these receptors have been linked to excitotoxic neuronal death which involves increased calcium influx. In addition, previous studies also found that inhibition of calcium channels increased RGC survival. Now, our study showed that in addition to the effect on RGC survival, calcium channel inhibition decreases axonal degeneration and improves axonal regeneration. These effects mediated by calcium channel inhibition seem to be specific and were not observed in previous studies targeting other mechanisms involved in axonal degeneration. For example, the Wallerian degeneration (Wlds) mutation, which protects axons from degeneration through a completely different mechanism, does not increase RGC survival (Beirovski et al., 2008). Thus, our study points to calcium channels as the key targets, which in addition to axonal protection also regulate RGC survival.

We next evaluated the molecular downstream cascade involved in the effects of calcium channel inhibition. Here we found that inhibition of calcium channels reduces calpain activity in the lesioned optic nerve. Calpain is a calcium-dependent protease, which degrades a variety of vital cellular components. Calpain inhibitors protect axons from degeneration, indicating that calpain activity is important for axonal degeneration. Moreover, calpain inhibition has a neuroprotective effect against axonal damage-induced RGC death. Thus, the reduction of calpain activity by calcium channel inhibition could provide a mechanistic link to the effect on axonal degeneration and RGC survival. We also found that the activity of the c-Jun N-terminal kinase (JNK)/c-Jun signaling pathway was not affected by inhibition of calcium channels. JNK is a serine/threonine kinase that regulates RGC death and axon degeneration after optic nerve injury. The tran...
The activation of JNK/c-Jun signaling on RGC cell body and axons after optic nerve crush (ONC). (A) In untreated animals, ONC lesion leads to a rapid influx of calcium through different calcium channels, which induces axonal degeneration and retinal ganglion cells (RGC) death. These effects were accompanied by an increase in the activity of calpain and the JNK/c-Jun signaling pathway that most likely contribute to axonal degeneration and cell death. (B) Application of calcium channel inhibitors (amlodipine, NBQX, and amiloride) decreased axonal degeneration, increased RGC survival and improved axonal regeneration. Mechanistically, calcium channel inhibition decreased the activities of calpain, JNK/c-Jun signaling pathway and increased the activity of Akt, which suggest that these mechanisms could be involved in the effects of calcium channel inhibitors.

Several previous studies focused on axonal stabilization after traumatic injury to the CNS. For example, ONC induces activation of JNK signaling pathway in RGC via TNFα. Our study now points to calcium influx as an additional mechanism involved in activation of the JNK/c-Jun signaling pathway. Therefore, decreased activity of JNK/c-Jun by calcium channel inhibition could be an additional mechanism contributing to attenuated axonal degeneration and RGC death. Finally, we showed that the activation (phosphorylation) of the pro-survival serine/threonine protein kinase Akt was increased in the ganglion cell layer of retinas treated with calcium channel inhibitors. Akt has a pivotal function in mediating survival signaling in neuronal cells, as well as, axonal outgrowth, including in RGC. Thus, the increase in Akt activation induced by calcium channel inhibition could explain the effects on RGC survival and axonal regeneration (Figure 1).

A recoverable state of axon injury persists after a contusive spinal cord injury (Williams et al., 2014). Thus, our study contributes to an improved understanding of the value of calcium influx blockers in the limitation of axonal degeneration and neuronal death, as well as improved axonal regeneration. In a translational approach, additional studies will be required to titrate the optimal dosage, the exact timing, the best localization to apply the treatment and any to implement combinatorial strategies.

Taken together, in our study, using a rat ONC model, we found that application of calcium channel inhibitors preserved axonal integrity from acute degeneration and consecutively increased survival of RGCs and improved axonal regeneration. Moreover, we showed that calcium channel inhibitors decreased lesion-induced calpain activation, attenuated the activation of the JNK/c-Jun signaling pathway and increased the activation of the pro-survival kinase Akt, suggesting that these mechanisms could be involved in the effects of calcium channel inhibitors. In conclusion, our study shows that an intervention targeting axonal integrity could be an important step in a combinatorial therapeutic strategy to promote functional recovery after traumatic injury to the CNS and points to calcium channel inhibitors as valuable therapeutic agents in CNS trauma.

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