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Research Report

Bcl-x_L increases axonal numbers but not axonal elongation from rat retinal explants

Gunnar P.H. Dietz*, Birgit Dietz, & Mathias Bähr

Neurologische Universitätsklinik, Waldweg 33, 37073 Göttingen, Germany

DFG Research Center for Molecular Physiology of the Brain (CMPB)

**Corresponding author*

Address for manuscript correspondence

Gunnar P. H. Dietz, Ph.D.

Neurologische Universitätsklinik

Waldweg 33

D-37073 Göttingen

Germany

e-mail: gdietz@gwdg.de

Tel: +49-551-39 12836

Fax: +49-551-39 14302

Homepage: http://www.mi.med.uni-goettingen.de/baehr-lab/Staff___Positions/Dietz/dietz.html

Running Head: Tat-Bcl-x_L and axonal regeneration

Abstract

The Bcl-2 family of proteins has been characterized as a key regulator of cell death programs. In addition, these proteins also play important roles in cellular differentiation, such as axonal growth. The role of Bcl-2 family members on axonal regeneration and neurite extension has been controversial so far. Here, we examine the influence of Bcl-x_L on axonal regeneration from adult retina explants *in vitro*. We delivered recombinant Bcl-x_L into retinal tissue, mediated by the Tat-protein transduction domain, and observed its effect on retinal axon extension. We found that Bcl-x_L increased the number of regenerating neurites, but did not increase their length. Our results indicate that Bcl-x_L stimulates axonal initiation but not axonal elongation after crush injury to retinal explants, without altering the number of surviving neurons.

Keywords: Nerve trauma, Cell penetrating peptide (CPP), Protein transduction domain (PTD), regeneration, optic nerve crush, Bcl-2 family, apoptosis, retinal ganglion cell.

1 Introduction

About 80 years ago, Ramon y Cajal stated that in the central nervous system (CNS) “this defective capacity for regeneration … [depends on] the absence in the surroundings of catalytic agents able to overcome the osmotic equilibria of the cones of growth, to provoke their vigorous nutrition, and to direct the path they must follow” [57]. Work in the following decades showed that the loss of adult CNS neurons’ capability to regenerate when their axon is severed is due to extrinsic signals from glial cells and extracellular matrix molecules formed in the glial scar [7, for a few recent examples, 58, 65]. However, part of the decline in regenerative capacity is also due to changes within the neurons themselves [24]. These intrinsic influences have also been shown for retinal ganglion cells [29, 55], which are often used as a model system to examine axonal degeneration and regeneration [37, 66]. It is not known how these internal changes may occur.

The role of Bcl-2 family members in promoting or inhibiting apoptosis is widely known[49, for review]. However, less work has been published on other functions of these genes, for example on synaptic transmission [34], proliferation and cell cycle regulation [6, 12, 15], or sensory [50] or dopaminergic [47] neuronal differentiation. Of particular interest in our context is that experiments investigating *bcl-2* overexpression or null mutant on retinal ganglion cells [10, 11, 14], primary embryonic sensory neurons [31], a neural-crest-derived cell line [68], a dopaminergic cell line [53], or PC-12 cells [35,

45, 63] suggest that Bcl-2 promotes axonal or neurite growth or regeneration, independently of its ability to promote neuronal survival. However, after intracranial nerve crush, *bcl-2* overexpression did not improve axonal regeneration [13, 48], and the earlier findings of Chen et al. could not be reproduced [48]. *Bcl–2* overexpression also did not enhance optic nerve regeneration stimulated by a peripheral nerve transplant [32]. Moreover, in highly purified postnatal RGC, adenoviral *bcl-2* overexpression itself did not induce axonal growth [28, 29]. In the entorhinal-hippocampal formation, *bcl-2* overexpression did not increase the regenerative potential of axotomized neurons [61]. In other studies, the enhanced recovery of facial nerve injury in *bcl-2*-overexpressing-mice might be attributed to an improved survival of the axotomized neurons [51].

The *bcl-x* gene has been isolated by low-stringency hybridization of a cDNA library with a *bcl-2* probe [5]. Bcl-2 and Bcl-x_L are similar in structure, share common pathways, and are partially redundant in their anti-apoptotic activity [9, 62]. They are both expressed in the adult and in the developing brain [30]. A major difference, however, is their subcellular localization: while Bcl-x_L acts primarily on the mitochondrial membrane, Bcl-2 is localized to other membranes as well, such as the ER [36]. The *bcl-x_L*-deficient mouse is the only targeted deletion of a *bcl-2* family member that shows a profound neuronal phenotype [46, for review], suggesting thatBcl-x_L plays a particularly important role in nervous system development. This role is not only to promote cellular survival, but also to influence neuronal differentiation, of dopaminergic neurons for example [47, 60]. The results of some experiments using viral vectors suggest that Bcl-x_L inhibits neuritogenesis in retinal

ganglion neurons [54], while others indicate that Bcl-x_L inhibits axonal growth [42].

To examine the function of Bcl-x_L [20, 38] or neurotrophins [21, 39, 40] in more detail, we coupled it to the so-called protein transduction domain derived from the HIV Tat protein. This 11 amino acid sequence allows the delivery of proteins and other substances across cellular membranes and the blood-brain barrier [59]. We showed in earlier studies that Tat-Bcl-x_L prevents apoptosis in models for neurodegenerative diseases, e.g. potassium and serum deprivation [20]; nerve trauma [20]; ischemia [38]; a model for bacterial meningitis [22]; a model for multiple sclerosis [18], β-amyloid toxicity [56] and other models (Dietz et al., in preparation).

The only neurons that project their axon through the optic nerve are the ganglion cells of the retina. When retinal explants, preconditioned by crush lesion or axotomy before dissection, are placed on a permissive substrate like laminin, some retinal ganglion cells regenerate their axon[3]. This system makes it possible to test growth factors or conditioned medium [64] or other substances [2] for their ability to promote neurite extension. To clarify whether Bcl-x_L influences neurite extension, we delivered the protein via the Tat protein transduction domain into retinal tissue, and tested it for its effect in the *in vitro* assay for neurite outgrowth from retinal ganglion cells.

2 Materials and Methods

2.1 Purification of Tat fusion proteins

The *tat-bcl-x_L* construct was cloned and the protein purified as described [20, 52]. The pTAT-HA vector [59] used to generate the Tat domain control protein, which had also been used to clone the *Bcl-x_L* expression construct, was kindly provided by S. F. Dowdy (San Diego, CA). *Tat-bcl-x_L* was expressed in *E. coli* strain BL21(DE3)pLysS (Novagen, Madison, WI). Inclusion bodies were disrupted by sonication in 8 M urea. Bacterial debris were pelleted and supernatant subjected to metal-affinity chromatography using a Ni–NTA matrix (Qiagen, Hilden, Germany). We removed salt by gel filtration on Sephadex G-25M (Amersham Biosciences, Uppsala, Sweden). We confirmed the identity of proteins by PAGE and Western blotting (Fig. 2 A). Anti-hemagglutinin antibodies were purchased from BAbCO (Richmond, CA).

2.2 Surgical procedure, tissue explants, and image recordings for neurite growth assay

Rats were treated according to German guidelines for the care and use of laboratory animals and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Male Wistar rats (bred from rats purchased from Harlan Winkelmann GmbH, Borchen, Germany)

weighing 200 – 250 g were anesthetized by ip injection of 420mg/kg chloral hydrate. The eyes of the animals were carefully mobilized by slight pressure near the optic cavity. The optic nerve was crushed by tightly squeezing it between the (bent) prongs of watchmaker's tweezers for 5 sec. Three days after this preconditioning crush, the retina was dissected and freed from connective, lens and vitreal tissue, spread on a cellulose nitrate filter, pore size 0.45µm (Sartorius, Göttingen, Germany) using aspiration as described [1] and chopped into 500 µm strips(Fig. 1 A). The 6 middle membrane-tissue strips were transferred onto poly-L-lysine and laminin-coated cell culture dishes with a gas-permeable base (petriPERM culture dish, VIVASCIENCE AG), with the tissue facing the substrate. To keep the strips in place, they were weighed down with small steel blocks (Fig. 1 B). Three strips were explanted per 5.5 cm dish with 4 ml of medium.

The retinal explants were incubated in a medium as described [3] in 70% O_2 to enhance the number of outgrowing fibers, 5% CO_2, at 37°C, with the test protein at 100nM, 300nM, 1µM or 3µM concentrations. At day 0 and day 3, vitamin C was added to a final concentration of 10 µg/ml. After 5 d in culture, the tissue was fixed and stained for neurofilament using the SMI 31 monoclonal antibody (1:1000 in TBS) against neurofilament (Sternberger Monoclonals Inc., Lutherville, Maryland).

To evaluate axonal growth, each tissue strip and the stained axons were digitally photographed in their entirety using a Zeiss Axioplan 2 fluorescent microscope and Zeiss Axiovision Software (Zeiss Jena, Germany) (Fig. 1 C). Images of whole strips (up to a few mm in length) were assembled from single pictures taken with a 10X objective (Fig. 1 D). A total of 144 strips were

analyzed. To measure strip circumference, neurite numbers and neurite length observed under different Tat protein concentrations, a macro was programmed using the KS400 image analysis package (Zeiss, Jena, Germany). To determine the length distribution shown in Fig. 3 C, the sum of neurite length measurements of the Tat-HA control condition was set to 100. Neurite lengths of Tat-Bcl-x_L-treated cultures were normalized against the control condition according to their neurite density relative to Tat-HA control. Neurite lengths measured at different protein concentrations were divided into intervals of 0-50%; 50-100%; 100-150%; 150-200%; 200-250% and larger than 250% of the average of Tat-HA control condition. The total of the intervals determined in the Tat-HA control condition was set to 100%, and the sum of the interval counts for each Tat-Bcl-x_L concentration were normalized. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni-Dunn's post hoc test.

2.3 Determination of retinal ganglion cell survival

RGC were pre-labeled 3 d before optic nerve crush by injection of the retrograde tracer FluoroGold (Biotium, Hayward, CA) into the superior colliculus. To that end, rats were anesthetized with chloral hydrate as above, an incision was made along the midline of the scalp, and two holes were drilled 6.04 mm posterior of the bregma and 1.4 mm lateral to the left side of the saggital suture. 2 µl of a 5% FluoroGold solution in PBS were injected 4.2 mm deep from the skull surface over 5 min. Afterwards, the needle was left in

place for another minute to avoid leakage of the dye along the needle track. Subsequently, the scalp was closed by a suture. 5 days later, the optic nerve was crushed as described above. 3 d after this surgery, the retinas were dissected and cultured for 5 d with recombinant proteins. Explants were fixed with 4% PFA and the FluoroGold-labeled retinal ganglion cells were counted in 9 fields of each explant.

2.4 Immunohistochemistry

To determine transduction of retinal explants in culture, they were treated with Tat-Bcl-x_L immediately after preparation, allowed to incubate over night, washed 5 times with PBS, fixed in 4% PFA, blocked with 2% BSA, and incubated with mouse monoclonal anti-HA antibody (BAbCO, Richmond, CA, 1:200 in PBS with 2% BSA) for 1 hour at 37°C. The secondary antibody was conjugated with Cy™3 (Dianova, West Grove, PA, 1:500 in PBS/2% BSA). No retinal outgrowth was detectable yet at this stage of the preparation.

3 Results

To determine whether Tat-Bcl-x_L transduces retinal tissue *in vitro*, we treated rat retinal explants immediately after their preparation with the recombinant protein and performed immunohistochemistry using an antibody against its hemagglutinin tag. We detected no staining in untreated tissue (Fig. 2 B, D).

Tissue treated with the recombinant protein revealed staining throughout the explant (Fig. 2 C, E), with the strongest labeling at its edge. The weaker staining is probably because the inner part of the explant is thicker than its marginal zone, and thus displays a lower signal when stained with antibodies and viewed under a fluorescent microscope. In conclusion, Bcl-x_L can be delivered into retinal tissue *in vitro* when fused to the Tat-CPP.

We next examined the effect of Tat-Bcl-x_L on axonal regeneration.3 d after mechanically crushing the optic nerve *in vivo*, retinae were dissected and tissue strips cultured for another 5 d, in the presence of Tat-Bcl-x_L or Tat-only control protein. We then counted the number of neurites that had regrown during the culture period. The number of neurites increased to over 170 % of control after treatment with 100nM, 300 nM, 1μM or 3 μM Tat-Bcl-x_L (Fig. 3 A). On the other hand, the average length of the neurites significantly decreased under Tat-Bcl-x_L treatment (Fig. 3 B). However, it was not clear whether Bcl-x_L inhibits neurite elongation, or whether the additional neurites it stimulated to grow might have remained shorter, decreasing the average length of all neurites measured. Therefore, we also examined the size distribution of the neurites (Fig. 3 C). Tat-Bcl-x_L treatment did not diminish the number of neurites longer than 150% of control. Rather, the decrease in the average neurite length in cultures with Tat-Bcl-x_L treatment was due to the increased number of short neurites.

To examine whether the increased number of neurites was related to an enhanced *in vitro* survival of retinal ganglion cells after treatment with the anti-apoptotic protein, we retrogradely labeled the cells *in vivo* before surgery by injection of a tracer into the superior colliculus. 3 d after this injection, the

optic nerve was crushed. After another 3 d, the retinae were dissected and cultured *in vitro* for either 4 h (Control day 0) or for another 5 d. During the 5 d culturing period, retinal ganglion cell densities remained constant, both when treated with Tat-Bcl-x_L or Tat domain control protein (Fig. 4).

4 Discussion

The results of our experiments showed that Bcl-x_L, independent of its anti-apoptotic effect, increases the number of neurites in an *in vitro* axonal regeneration model, while decreasing their *average* length. The decrease in the average length is probably due to the fact that the additional grown neurites remain shorter, rather than an overall reduction in neurite growth rate.

These results are reminiscent of data obtained for Bcl-2, which is similar in structure to Bcl-x_L [17, for review] and acts as a regulatory switch for the growth of CNS axons: overexpression of *bcl-2* increased the number of axons, while *bcl-2* deletion decreased it [10]. In that publication, the *length* of the axons was not quantified. In a follow-up study, the effect of Bcl-2 was compared to the effect of Bcl-x_L. *bcl-x_L* overexpression doubled the number of neurites regenerating after optic nerve crush [33, Fig. 1D], although this increase did not reach statistical significance in their paper. In isolated retinal ganglion cells in culture, Bcl-x_L again increased the number of RGCs with axons by approximately 60 % [33, Fig. 2E]. Although this is again in good agreement with our findings, the authors concluded, due to the lack of statistical significance, that “Bcl-x_L does not promote axon regeneration”. The

length of the neurites was slightly increased by Bcl-x_L overexpression, which, however, was not statistically significant [33].

On the other hand, retinal ganglion cells, when treated with a *bcl-x_L*-expressing adenovirus, showed increased survival, but decreased neurite outgrowth [54]. Oshitari et al. used a different method to assess the extent axonal growth, i.e. number of neurites/mm^2, which is not immediately comparable to our quantification. Other authors have claimed that adenovirally expressed *bcl-x_L* enhances axonal growth [42]. In contrast to Oshitari et al., a neuron-specific promoter was used, which apparently has a different effect on axonal regeneration as compared to ubiquitous expression. As Tat fusion proteins transduce most cell types tested *in vitro* [19], it may also act on glial cells, for instance, enhancing their survival in culture, or stimulating them to release factors that in turn affect retinal ganglion cell regeneration. Consistent with findings using adenovirally delivered Bcl-x_L controlled by a strong, unspecific promoter [54], Tat-Bcl-x_L decreased average neurite length. The use of Tat fusion proteins does not distinguish between cell autonomous and non-cell autonomous effects. The close Bcl-x_L-relative, Bcl-2, also acts non-autonomously on axonal regeneration in retinal ganglion cells [41], which might also be the case for Tat-Bcl-x_L.

Considering our immunohistochemical data (Fig. 2) and earlier published work using the Tat cell penetrating peptide, Tat-Bcl-x_L is likely to efficiently and quickly penetrate the whole explant. Nevertheless, Tat-Bcl-x_L might enhance the initiation of neurite formation with a delay, so that those additional neurites would have less time to elongate until the preparations are fixed and analyzed after the culture period. As we did not observe a lower

number of long neurites (Fig. 3 C), an inhibition of axon elongation by Bcl-x_L seems unlikely. For future experiments, we might consider pretreating the retinas *in vivo* with Tat-Bcl-x_L or with a viral vector, to increase the time Bcl-x_L would have to act on signal transduction pathways in the retina. However, such a strategy also has its pitfalls. Viruses, proteins, or lens injury can activate macrophages, which in turn release factors that indirectly enhance retinal ganglion cell survival and regeneration [25, 26, 43, 67]. Keeping the retinal explants in culture for a longer time might reveal whether the recombinant protein promotes or inhibits axon elongation. However, for technical reasons, higher neurite density and length would not be desirable, as this would make it virtually impossible to separate individual neurites from one another when tracing them on digital images for quantification.

At this point we can only speculate on the mechanisms by which proteins of the Bcl-2 family might affect axonal growth. Bcl-2 reduces ER Ca^{2+} uptake in neurons, thereby increasing the intracellular Ca^{2+} response to nerve injury and *CREB* and *Erk* expression, which stimulate axon growth and regeneration [33]. Chen et al. have suggested that Bcl-2 might affect neuritogenesis via the Ras/Raf pathway [10]. On the other hand, both Raf-1 [4] and Erk1/2 [16], have been implicated in Bcl-2 phosphorylation in some instances, which might provide a feed-back loop for its regulation. Although Bcl-2 and Bcl-x_L share many structural and functional similarities [9, 62], including phosphorylation sites within the loop domain [8], Bcl-x_L is primarily located in the mitochondrial membrane [36] and thus, in contrast to Bcl-2, is not involved in Ca^{2+} homeostasis. Likewise, only Bcl-2, but not Bcl-x_L, acts to increase neurite extension via c-Jun N-terminal kinase activation [23]. Thus,

the signal transduction pathways mediating neurite extension are even more elusive for Bcl-x_L than for Bcl-2.

bcl-x_L is expressed at higher levels in the adult rat retina than *bcl–2* [44]. So far, it is not clear whether Bcl-2 family members contribute to the failure of RGC to regenerate after lesion. As the effect caused by Tat-Bcl-x_L is small, certainly many other factors are involved [27, for review], and their individual contribution remains to be determined.

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FIGURE LEGENDS

Fig. 1 Preparation of tissue explants and evaluation of axonal growth as described in the Methods section (2.2). Retina spread onto a nitrocellulose filter and cut into 500 µm strips (A). Filter-attached retina strips in a cell culture dish with a gas-permeable base (B). Representative tissue area of a retinal explant recorded with a 10 x objective, after 5 d in culture, stained for neurofilament. Bar = 220 µm (C). Complete retinal control explant strip, not treated with protein, with axonal growth after 5 d in culture (D); assembled from images similar to (C). White arrows point to neurites that were counted and measured. Bar =415 µm.

Fig. 2 Tat-Bcl-x_L transduces retinal tissue *in vitro*. A. Western analysis of purified Tat-Bcl-x_L confirming identity of the protein and Coomassie staining of polyacrylamide gel to demonstrate the purity of the protein preparation. B-E: Immunohistochemistry using an antibody against the hemagglutinin tag of the recombinant protein showing transduction of retinal tissue *in vitro*. Retinal strips treated with no protein (B, D) or with Tat-Bcl-x_L (C,E), Phase (B, C) and images under fluorescent light (D, E, Cy3 filter). Bar = 130 µm.

Fig. 3 Tat-Bcl-x_L stimulates neurite initiation, but not their elongation. A) The neurites per strip circumference incubated with 100nM, 300 nM, 1 µM, or 3 µM Tat-Bcl-x_L, normalized against control conditions (with Tat-HA treatment set to 100%). B) Average length of neurites from retinal explants under Tat-

Bcl-x_L treatment, normalized against control condition (Tat-HA treatment set to 100%) (±SEM), $p<0.05$ (*) or $p<0.001$ (**), (±SEM). We did not detect a significant difference between the 100 nM and the 300 nM or between the 1 µM and the 3µM condition. C) Length distribution of axons under different protein treatments in 50% intervals with Tat-Bcl-xL concentrations between 100 nM and 3 µM. The sum of the numbers of all length intervals in the control condition was set to 100.

Fig. 4 Retinal ganglion cells survive in explants after 5 d in culture and are not influenced by Tat protein treatment. The graph shows neuronal densities in a retina after 4 h in culture (control day 0) or after 5 d in culture and treated with 1 µM Tat control protein or with Tat-Bcl-x_L ($p>0.5$) (±SEM).

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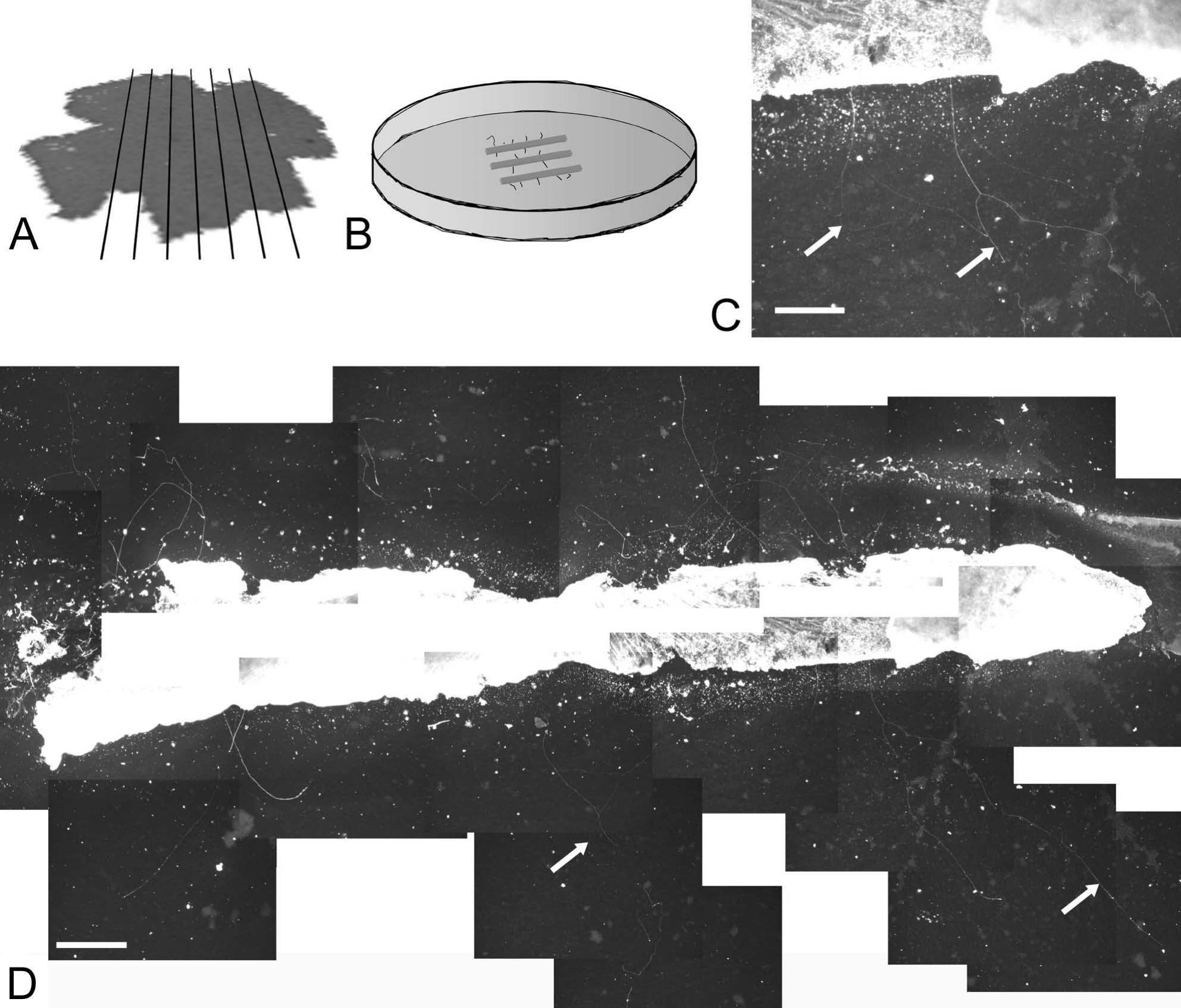
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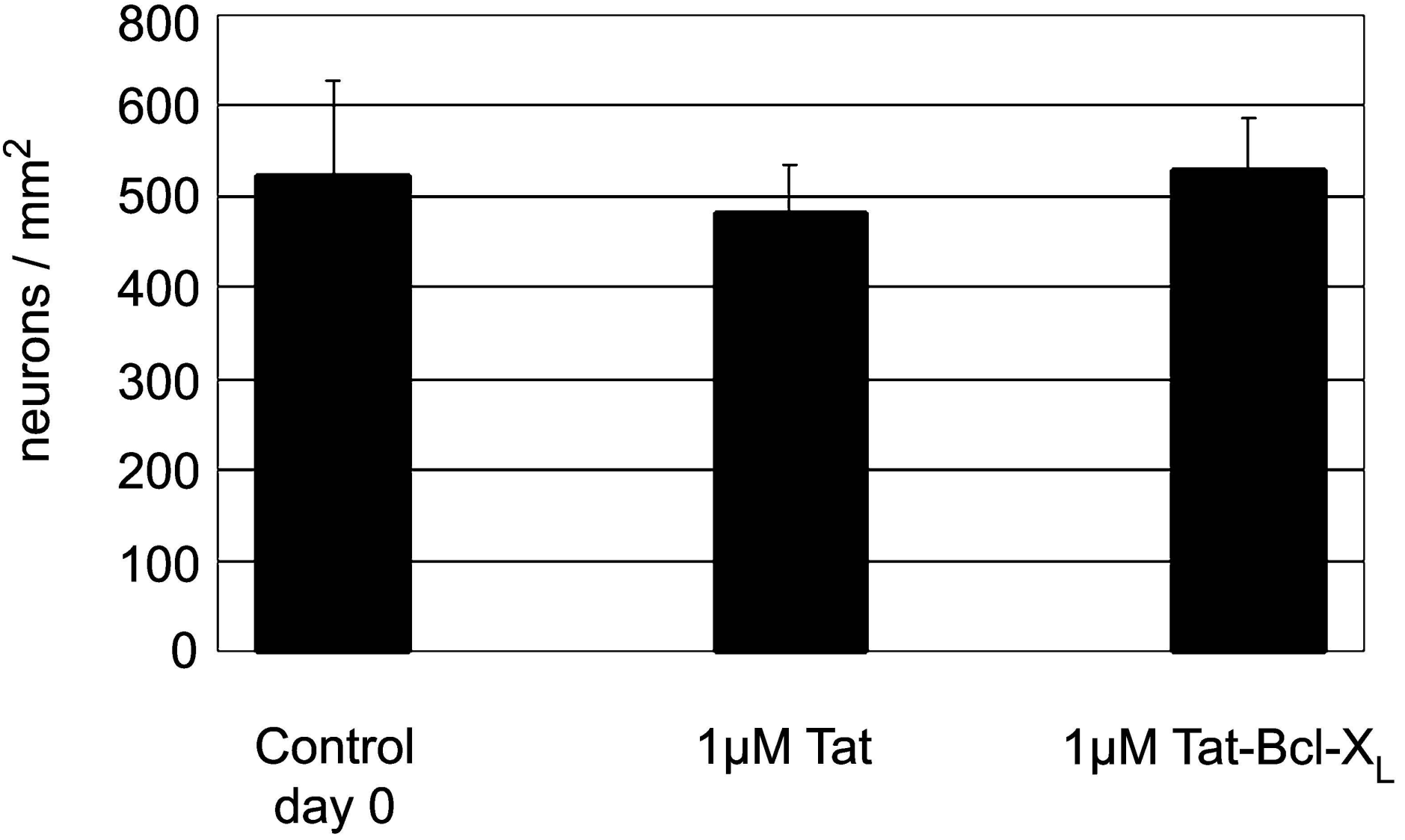
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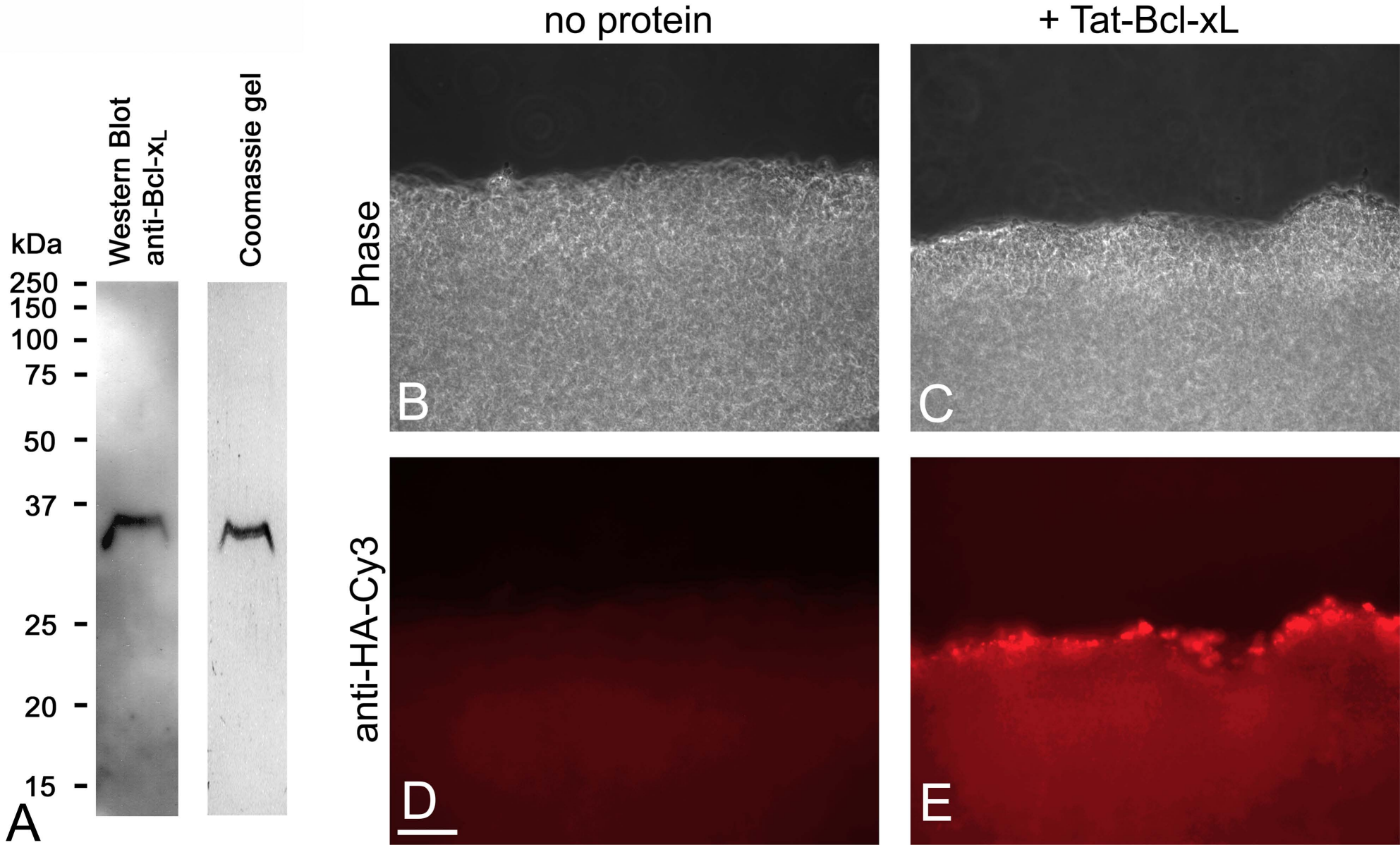
no protein
+ Tat-Bcl-xL
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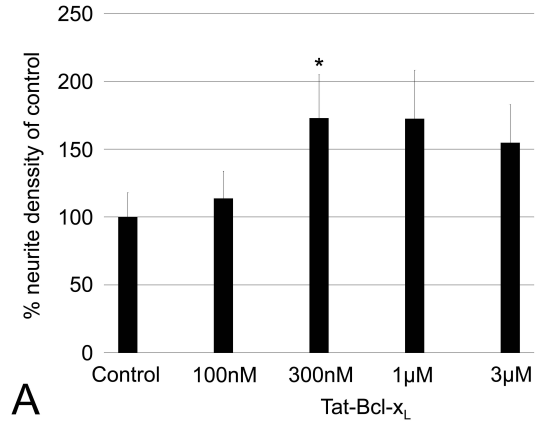
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Tat-Bcl-x$_L$

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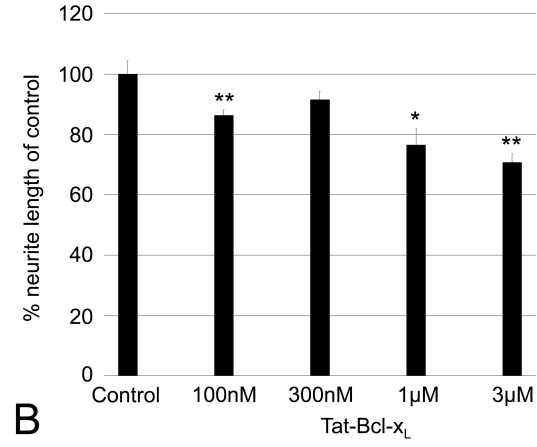
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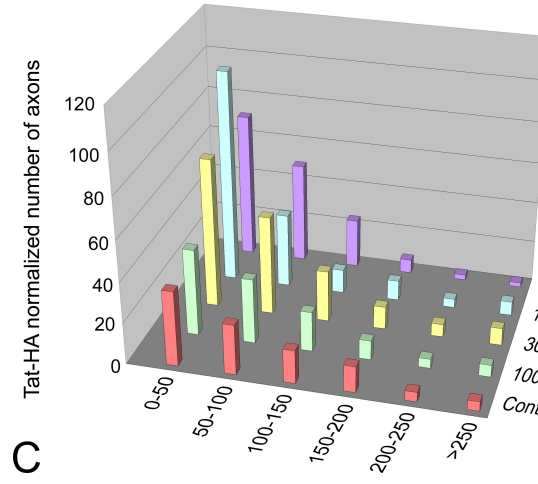
Tat-HA normalized number of axons
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3μM Tat-Bcl-x$_L$
1μM Tat-Bcl-x$_L$
300nM Tat-Bcl-x$_L$
100nM Tat-Bcl-x$_L$
Control

C