

Bergmann et al.:

An Intact Insect Embryo for Developmental Neurotoxicity Testing of Directed Axonal Elongation

Supplementary Data

Methods

Locust embryos

Locust eggs (*Locusta migratoria*) were collected in batches from the same egg pod (50-60 siblings of the same age) from our crowded laboratory culture at the day of egg deposition, and kept horizontally in moist petri dishes at 30°C until dissection. On day 3 after egg deposition, embryos were carefully staged to 32.5% of completed embryogenesis according to Bentley et al. (1979), surface sterilized in 70% ethanol, and dissected in serum-free L15 media with 1% penicillin/streptomycin (Invitrogen). At this stage (comparable to a three day chicken embryo), the open dorsal side of the embryo is exposed to the yolk mass and the closed ventral side with the developing central nervous system and limb buds is covered by a membrane, analogous to the vertebrate amnion. Dorsal closure of both entoderm and ectoderm, and formation of the primary cuticle, takes place much later, at 60% of development. After removal of amnion membrane and yolk, embryonic tissue is freely accessible to chemicals from both sides.

Incubation and exposure to test compounds

For an individual experiment, embryos of a single egg pod were collected in pairs in 200 µl L15 (with solvent, as appropriate) in 48 well plates, in rows of 5 wells per concentration (5 technical replicates). One group of start controls was fixed immediately after dissection and kept in PBS at 4°C until the next day. One group of 10 embryos received L15 media only (with DMSO, if appropriate), the other rows received test solutions prepared freshly from frozen stocks of chemicals (see Tab. S1). When necessary DMSO concentrations were 0.1% or higher, the appropriate amount of DMSO was added to all solutions, including media controls. In preliminary experiments, we confirmed that DMSO alone had no effect on general viability or neurite elongation at concentrations up to 1%. Depending on the number of intact embryos recovered from an individual egg pod, between 2 and 4 different concentrations could be tested in each experiment. Embryos were briefly washed in 200 µl test solution, and incubated in fresh test solution in 48 well plates at 30°C for 24 h.

Viability assay and immunofluorescence labeling

After washing for 5 min in L15, a resazurin reduction viability assay was performed: Embryos were incubated in 5% Alamar Blue (Invitrogen) in L15 for 2 h at 30°C, including a row of blanks (without embryos). 150 µl of the supernatant from each well was transferred to a 96 well plate, and fluorescence was measured at 530 nm excitation/590 nm emission using a Tecan Infinite 200 reader. Subsequently, embryos were fixed in 4% paraformaldehyde for 45 min. Washing steps and dilution of immunolabeling reagents were carried out in phosphate buffered saline with 0.1% Triton X-100 as a detergent (PBS-T). Leg bud pioneer neurons were immunolabeled for a neuronal cell surface marker (anti-HRP, Dianova, 1:2000 preceded by permeabilisation in 0.3% saponin and blocking in 5% normal rabbit serum for 45 min each). Labeled neurons were visualized by a biotinylated rabbit anti-goat antiserum (1:250, Dianova) and streptavidin-CY3 (1:250, Sigma) plus 0.1 µg/ml DAPI as a nuclear marker. The anti-HRP antibody recognizes a carbohydrate moiety on cell surface proteins of neurons in a large number of ecdysozoan invertebrate species (Jan and Jan, 1976; Haase et al., 2001), but not in non-ecdysozoan invertebrates, or in vertebrates.

Measurement and evaluation

Defects in axonal outgrowth and navigation of pioneer axons were detected via conventional fluorescence microscopy using a Zeiss AxioScope with an HXP 120 light source, AxioCam 506 color camera and ZEN lite software. Selected preparations were subjected to confocal microscopy using a Leica TCS SP5 and Leica LAS AF software. For each leg bud, elongation of pioneer axons along their predefined pathway was scored between 0 and 100 according to the scheme depicted in Figure 1A. Pioneer neurons of both hindleg buds of each embryo develop independently. Thus, the axon elongation score of the pioneer axons of each leg bud is considered an individual measurement. From each individual measurement, the average elongation score of the start controls of this experiment was subtracted, to receive the elongation between start and end of the experiment. Negative values were counted as zero elongation. In a next step, each elongation value was normalized to the average elongation of the media (solvent) controls of this experiment. This resulted in individual values between 0% and 100% (or even above 100%, when a neuron had

grown farther than the controls). Embryo viability measurements were also normalized to the average viabilities of the media controls (after subtraction of the average blank values). Normalization allowed for pooling of data from different separate experiments to reflect biological variability. Biological variability is also reflected in the absolute measurements (viabilities and elongation scores) of all control embryos (see Fig. S1).

Concentration-response curves and statistics

Values of the same concentrations from at least three different experiments (from different egg pods, biological replicates) were averaged and plotted in GraphPad Prism 8.0 as means \pm S.E.M. Concentration-response curves were generated by fitting four-parameter sigmoidal functions. IC₅₀ values were determined from the curve, unless values below 50% were not reached. In these cases, the highest used concentrations were used to determine IC₅₀ instead, according to Krug et al. (2013). For statistical analysis of the effect of Y27632 on neurite elongation impaired by 40 nM rotenone, we performed a Kruskal-Wallis test on the normalized data, as described above, followed by Dunn's post hoc test with correction for multiple comparisons, using GraphPad Prism 8.0. Significances are expressed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Tab. S1: Specifications of test compounds used

Test compound	CAS No.	Order No. Sigma	Solvent	Solvent conc.	Stock conc.	Max conc.
Diltiazem hydrochloride	33286-22-5	D2521	aqua dest	0.5%	1 M	5 mM
Verapamil hydrochloride	152-11-4	V4629	DMSO	0.5%	500 mM	2.5 mM
Cytochalasin D	22144-77-0	C8273	DMSO	0.033%	1 mM	0.333 μ M
Colchicine	64-86-8	C9754	aqua dest	0.5%	10 mM	50 μ M
Rotenone	83-79-4	R8875	DMSO	0.1%	400 μ M	400 nM
Y-27632 dihydrochloride	129830-38-2	Y0503	aqua dest	0.5%	10 mM	50 μ M

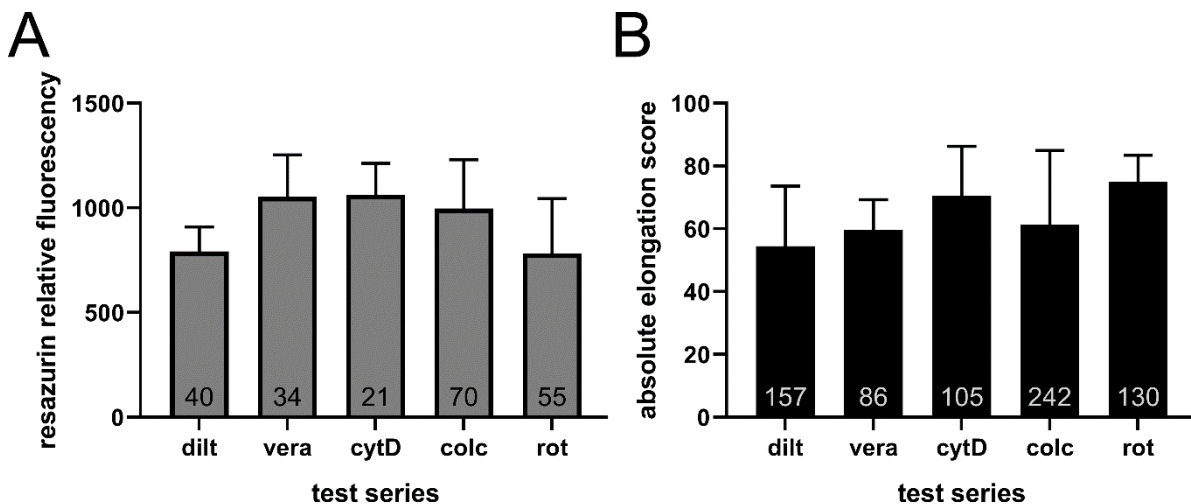


Fig. S1: Variation of absolute measurements of all solvent controls for the 5 test series

Each bar is the average \pm SD of all solvent controls in each experimental series (numbers in the bars denominate numbers of controls in each series).

References

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