

Text S1: Culturing whole brain explants on culture plate inserts

All manipulations were performed using disinfected materials and working in a clean environment to prevent bacterial or fungal contamination.

Coating the inserts

Millicell low height culture plate inserts (Milipore, Cat No: PICMORG50) were placed in a sterile Petri dish (Falcon N° 35 3001) containing 1 ml of sterile PBS Dulbecco's (D-PBS). On top of the membrane a freshly prepared coating solution of Laminin (3.3 $\mu\text{g}/\text{mL}$; BD Biosciences Cat No: 354232) and Polylysine (33.3 $\mu\text{g}/\text{mL}$; BD Biosciences Cat No: 354210) in sterile D-PBS was added. The culture plate inserts were incubated overnight at 37°C. The next day they were washed extensively with sterile D-PBS and were stored at 4°C for up to three weeks. Just before use the coated inserts were transferred to an empty sterile Petri dish.

Preparing the explants

To explant their brains, adult female flies of the desired genotype were collected within 4 days after eclosion. After CO₂ anaesthesia the flies were placed in a 1.5 ml centrifuge tube on ice, keeping them alive but immobile. Before dissection a fly was washed in 70% Ethanol for a few seconds and placed in a sterile Petri dish containing ice cold Schneider's *Drosophila* Medium (GIBCO). The brains were dissected out in this medium as careful and as fast as possible (< 3 min). Parts of the eyes and medulla were left

attached when they were difficult to remove. Damage to the brain or delay in the speed of the dissection reduced the quality of the brain culture. Damaged brains were discarded. This procedure was repeated for up to twelve brains (dissected within ~30 min). The dissected brains were then washed in ice cold dissection medium. For larval brains the same procedure was followed. The eye/antennal disc complex was left attached to the brains.

Loading the culture plate insert

After dissection and washing, the brains were placed in a drop of medium on the membrane of the culture plate insert, using small forceps. Up to twelve brains were placed on the same insert. When all brains were in place, their antero-posterior orientation was verified and corrected if necessary. Excess medium was removed using a clean tissue, leaving only a thin film of medium covering each brain. 1.1 ml of culture medium was then added to the Petri dish containing the insert. The culture dishes with the explants were kept in a plastic box in a humidified incubator at 25°C. The culture medium was refreshed every two days.

Culture medium

The standard culture medium (Gibbs and Truman, 1998) consisted of 1% antibiotic/antimycotic solution (10 000 U/ml penicillin, 10 mg/ml streptomycin, 250 µg amphotericin B (SIGMA-ALDRICH A 5955)), 10% Foetal Bovine Serum and 10 µg/ml insulin in Schneider's *Drosophila* Medium (GIBCO). For optimization of this medium (Figure S1A) the antibiotics and/or antimycotics were removed. In the subsequent

experiments penicillin and streptomycin were still added to the medium, amphotericin B was not. For the culture of larval brains 1 μ g/ml of Ecdysone was added.

Injury of sLNv axonal tracts in cultured brains

To injure sLNv tracts, the brains of flies of the genotype *PDF-Gal4, UAS-CD8-GFP; UAS-CD8-GFP* were explanted as described. The GFP signal in the sLNv axons of the brain explant was visualized at 100X with a Leica MZ FLIII stereo microscope. A micromanipulator was used to position the tip of an Eppendorf Microdissector (Eppendorf, Germany Cat No: 920 00 401-6) on the axonal tract (Figure 4A). The Piezo Power MicroDissection device was activated, causing a rapid vibration of the sharp microchisel tip (frequency between 30-60 Hz, and amplitude 75-100%=1.5 μ m) which cut the axons. The injury (an interruption in the GFP pattern) was verified at 200X. In case of unsuccessful injury, the brain was discarded. Axons were only injured on one side of the brain. The other sLNv tract was later used to ascertain the health of the preparation when analyzing regeneration. For cohorts of injury that were compared at different time points, the brains of female sibling flies were dissected, cultured and injured on the same day and fixed and stained at the appropriate time. Experiments were performed in duplicate.

Immunohistochemistry of cultured brains

Brains cultured on culture plate inserts were fixed in a first phase by replacing the culture medium in the Petri dish with fixation solution (3.7% formaldehyde in PBS) for 30 minutes. Then 1 ml of fixation solution was added carefully drop by drop on top of the filter. The pre-fixed brains attached well to the membrane. Brains that partially or

completely detached from the membrane were excluded from further analysis. After fixation for at least another 2 hours, the fixation solution both on and below the membrane was replaced by PBS. The membrane was subsequently cut out of the plastic insert and processed as described for immunohistochemistry. Antibodies used were: mouse or rabbit anti-GFP (1/500; Molecular Probes); anti-PDH from P. Taghert (1/40000); rat anti-TIM (UPR-42, 1/1000) from A. Sehgal. After the staining procedure the membrane with the brains was placed on a microscope slide, immersed in Vectashield mounting medium for fluorescence and covered by a coverslip.

Integrity of LNv axons in cultured Drosophila brains

Having established that the overall morphology of the brain is preserved in culture, we next examined in more detail the morphology of LNv axonal tracts. The axonal tracts are only suitable for an axonal lesion model if the tracts did not spontaneously degenerate in *uninjured* cultured brains. Using the medium described in Gibbs and Truman, 1998 (See materials and methods for details), we observed spontaneous degenerative changes in the ILNv axonal tracts at four days in culture (Figure S1), however a remarkably high percentage (~70%) of the sLNv axon bundles remained intact (Figure S1A). This may be explained by its relative confinement to a small part, posteriorly in the brain whereas the long ILNv axons traverse the whole brain and terminate on the anterior face of the optic lobes (Figure 1H, I). When cultured, this part of the axonal tree was in direct physical contact with the insert membrane. This contact may have caused increased degeneration as suggested by the reduction of these degenerative changes when the brains were cultured with the anterior side up (Figure S1A).

To improve further the health of the sLNv axons we made some small changes to the composition of the medium (Figure S1A). We found that removal of all antibiotics and the antimycotic Amphotericin B from the medium increased the percentage of intact sLNv axons at 4 days to at least ~94%, without effects on bacterial or fungal contamination. To further corroborate these findings, we cultured brains for different times ranging from 2 hours to 8 days using the optimized medium (Figure S2). These experiments first of all confirmed the low rate of axonal degeneration (maximum ~6%) in the sLNv up to 6 days in culture (Figure S2A). We also found that defasciculation of axonal bundles progressively increases at longer times in culture. This may be due to the overall flattening of the brains on the membrane. On the other hand, small axonal branches progressively form on both the axonal shafts as well as their terminal arborescences (Figure S2D, E; arrows). sLNv axons in the explants therefore not only remain intact, they form new axonal branches.

In conclusion, the overwhelming majority of sLNv axonal tracts, while undergoing a number of morphological adaptations including defasciculation and sprouting, remain intact in explanted, cultured brains.

Text S2: Quantifying *de novo* axonal growth

This text accompanies figure 6. To allow numerical quantifications of the newly grown axons, it is crucial to determine the point of novel axon growth. (A) To know precisely where the novel growth starts, we imaged the injured brains approximately 6 hours after injury. (B) We then kept the brains in culture at 25°C for a total of 3 days after which we fix the brains and imaged the injured axon stump again. (C) By comparing the

morphology of the cut axon stump six hours and three days after injury, we can identify the point where the novel axons started to regrow (yellow circles). After identifying the newly grown axons and determining their starting point of regrowth (yellow circles) we performed numerical quantifications. As no models to study axonal regeneration in flies exist yet, quantitative criteria to measure novel growth in our injury model were yet to be defined. To compare the effect of different genetic manipulations on the regenerative capacity of damaged CNS neurons, the following criteria were carefully defined to measure more objectively the observed regrowth after injury.

Percentage of brains showing regrowth after injury.

To calculate the percentage of brains showing regrowth, we counted the number of brains that sprouted at least one novel axon from the cut tip and quantified their percentage on the total number of brains examined. For example, in control experiments, 20 brains were analysed of which seven grew at least one novel axon after injury. This results in 35% of brains showing regrowth after injury. This measurement allows us to assess how potent a genetic manipulation is in increasing the number of brains capable of sprouting new axons after injury.

Percentage of axons showing regrowth after injury.

To calculate the percentage of axons regrowing after injury, we counted the total number of newly sprouted axons from the cut tip and quantified their percentage relative to the total number of axons injured. To calculate the total number of injured axons, we multiplied the number of injured brains by four, which is the average number of sLNv

neurons in one brain hemisphere. For control brains, the number of examined brains is 20. The average axons cut per brain is four (only LNV in one hemisphere are injured) so the total number of cut axons for control brains is 80 (20 x 4). The number of novel sprouts after injury is 11, so the percentage of axonal regrowth after injury is 12.4% [11/80]. This measurement allows us to calculate how many of the cut axons are regrowing after injury.

Extent of regrowth.

To measure the extent of axonal regrowth, we measured the length by freehand tracing of each newly grown axon extension and express the length of a novel axon in μm . This quantification allows us to measure how lengthy the novel axons are. In addition, we also compute the direct distance a novel axon can cover from its start point of regrowth till the tip of the novel axon. This measurement allows us to assess how far the novel axons can reach in a straight line. The length of the novel axons and their computed distance were measured using ImageJ software.

(F) The average gap size ($36\mu\text{m}$) was measured by drawing a straight line from the proximal stump to the closest distal stub in wild type samples fixed approximately 6 hours after injury (n=18). (G) Since activated JNK signaling was very potent in inducing lengthy axons well capable of bridging the lesion gap and entering the original target area, we defined additional criteria to allow numerical measurements of these observations too. To determine the direction of normal target area innervation, we first drew a line running with the axonal shaft of the LNV neurons (dotted blue line) which we

use as a reference line. Next, we drew a rectangle –parallel to the reference line– demarcating the area of the distal stub. Both the angle the rectangle encompasses ($b=62^\circ$) as well as the position of the rectangle ($a=23^\circ$) from the reference line was calculated to have a numerical approximation of the normal target area. All calculations were done as averages over 18 brains. Based on these criteria we calculated a) the percentage of brains regrowing at least one novel axon that grows towards the normal target area (Figure 8C light blue graphs and Figure 8D middle panel) and b) the percentage of brains regrowing at least one novel axon back into the normal target area (Figure 8C pink graphs and Figure 8D lower panel). (H and I) To quantify the direction of the novel axons in the injured brains, the same approach was used. (H) For each novel axon, a reference line was drawn running parallel with the axonal shaft of the LNvs. (I) The angle of the novel axons was calculated by first drawing a straight line connecting the origin of outgrowth with the tip of the novel axon and then measuring the angle of the end tip from the reference line. Using this approach, we measured the angle of the novel axons in reference to the axonal shaft and count the axons growing towards the target area (Figure 8C light blue graphs and Figure 8D middle panel) and back into the normal target area (Figure 8C pink graphs and Figure 8D lower panel).

Figure S1: sLNv axonal tracts remain intact in cultured brains.

(A) Table summarizing the different media compositions and their effect on axonal morphology in brains cultured for 4 days. The morphological characteristics defining the categories indicated above the columns are illustrated in C-E. (B) Overview image of half of an adult *Drosophila* brain stained for Nc82 (red) and GFP (green). Scale bar = $80\mu\text{m}$.

The white squares indicate the area of the brain shown in C-E. (C) Details of the sLNv axonal bundle in cultured brains. If all axons are intact the bundle is classified as Cat 2. Short axonal branches (arrows) can be observed. Cat 1 axonal bundles contain one fragmented axon (arrowheads). In Cat 0 bundles several axons are fragmented. Scale bar = $20\mu\text{m}$. (D) Details of the ILNv commissural axonal bundle in cultured brains. In Cat 2 bundles all axons are intact. Cat 1 axonal bundles contain one fragmented axon (arrowheads). In Cat 0 bundles several axons are fragmented. Scale bar = $20\mu\text{m}$. (E) Details of the axonal termini and synapses of the ILNv in the optic lobes. In Cat 2 innervation all terminal bulbs (arrows) are connected by fine axons. Cat 1 innervation shows non-connected terminal bulbs (arrowheads). In Cat 0 innervation all axons are fragmented. Scale bar = $20\mu\text{m}$. All images are projections of confocal stacks taken at $0.5\mu\text{m}$ distance. The genotype of the flies is *PDF-Gal4, UAS-CD8-GFP; UAS-CD8-GFP*

Figure S2: sLNv axons are remodelled in cultured brains.

(A) Table summarizing the frequency of morphological features of the axons at different times in culture. Group A and B represent duplicates of experiments performed independently of each other. (B-E) Representative images of sLNv axons at the indicated time in culture showing an overview (left panel), a detail of the axonal bundle (middle panel) and a detail of the terminal arbour (right panel). (B) At two hours in culture the axon bundle can appear defasciculated but mostly unbranched. (C) At two days in culture the axon bundle is often defasciculated; new branches are very rarely observed. (D) After four days in culture most axonal bundles are defasciculated and show small branches (arrows). Axonal branches develop also from the terminal arbour (arrows). (E) After six

days in culture most axonal bundles are defasciculated and branched (arrows). Small axonal branches on the terminal arbour (arrows) are observed.

All images are projections of confocal stacks taken at $0.5\mu\text{m}$ distance. The genotype of the flies is *PDF-Gal4, UAS-CD8-GFP; UAS-CD8-GFP*

Figure S3: (A-C) High magnifications of a regenerated axonal sprout growing from the proximal stump of an injured LNv axon, imaged 3 days after injury, showing GFP staining, PDH staining and merge, respectively. PDH expression can be detected in the new sprouts suggesting they are functionally connected to the rest of the axon. Genotype is *PDF-Gal4, UAS-CD8-GFP; tubPGAL80^{ts}/+; UAS-PKAc5.9F/+*

(D-E) Analysing the effect of hepCA on the regenerative capacity of cut axons.

Two approaches were used to describe the regenerative response of cut axons. (A) The effect of hepCA on the regenerative capacity expressed as the percentage of regrowing axons of the total number of axons studied (number of examined brains multiplied by four). (B) Alternatively, we also calculated the percentage of novel sprouts of the total number of novel sprouts growing towards and growing into the target area. For both methods of analysis, a significant enhancement of all criteria measured in the graphs was seen upon expression of hep^{CA}.