Drosophila as a model to study the blood-brain barrier

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Running Head: *Drosophila* blood brain barrier

i. Summary

The *Drosophila* blood brain barrier (bbb) has been shown to be largely analogous in structure and function to the vertebrate bbb. Thanks to the genetic tools available for this organism, *Drosophila* is uniquely suited to study bbb physiology and function, with high relevance for mammalian function. In this chapter we discuss targeting strategies to specifically mark and manipulate bbb cells, how to test bbb barrier integrity, and methods to isolate single bbb cells.

ii. Key Words

Drosophila, blood brain barrier, Subperineurial (SPG) cells, Gal4/UAS/Gal80^{ts}, cell dissociation

1. Introduction

Like in vertebrates, the insect blood brain barrier protects the brain from components of the circulating fluid and allows selective uptake of nutrients and other important molecules. A big difference to vertebrates lies in the fact that insects do not have blood vessels but an open circulatory system. The hemolymph, the circulating fluid, is moved through the body of the animal by the pumping action of the heart and bathes all organs – except for the nervous system that is protected by the bbb. The insect hemolymph contains particularly high K+ concentrations that would be detrimental to neuronal function. Due to the absence of blood vessels, the blood brain barrier surrounds the entire brain like a tight "cap" (Figure 1). The insect bbb is best studied in *Drosophila* where it has been shown to be analogous in structure and function to the vertebrate bbb. In contrast to vertebrates, the insect bbb is formed by two layers of glial cells, an outer layer called Perineurial glial (PG) cells and the inner layer, the subperineurial glial cells (SPG) with the septate junctions that form the tight barrier. The SPG barrier forms early in development and its cells do not divide in later stages anymore. Therefore, the number of SPG cells is low. To adapt to the growing brain, the cells flatten and become polyploid [1]. Despite differences between the insect and the mammalian bbb, it has been shown that components that form the barrier (for example neurexins and forms of claudins), as well as many of the functional properties are shared between species. Several excellent reviews and recent papers discuss these aspects in detail [2-7]. Given the unparalleled genetic tools available in *Drosophila*, this tractable organism is uniquely suited to study bbb physiology and function, with high relevance for mammalian function [8]. These studies go beyond mere barrier function, with novel insights starting to emerge about physiological processes inside bbb cells that influence neuronal development, adult neuronal function and sex-specific behavior [9-12].

In this article we will describe several methods that are being used to examine and manipulate *Drosophila* bbb function. We will discuss the specific labeling of bbb cells

using the Gal4/UAS/Gal80^{ts} system, the dissociation and selection of bbb cells, and a method to examine bbb integrity.

2. Bbb labeling and manipulation using the *Gal4/UAS* system

One of the most powerful tools in *Drosophila* research, the binary *Gal4/UAS* system allows the manipulation of cells in a tissue-specific and temporally restricted manner (Figure 2 A) [13,14]. It allows the expression of desired molecules in the cells of choice and is efficiently used to label cells with fluorescent molecules, or to manipulate cells by expression of interfering/silencing RNAs (RNAi) or any transgene of interest. The *Gal4/UAS* system makes use of the yeast transcription factor *Gal4* that binds to the *UAS* sequence (upstream-activating-sequence) and activates transcription of sequences downstream of *UAS*. *Gal4* and the *UAS* constructs are introduced into the flies as independent transgenes, and strains containing either element can be crossed as desired. The system allows expression of sequences of choice (such as fluorescent proteins, or interfering RNAs) in any cells of choice, as long as a specific promoter sequence is known that directs expression to the targeted cells. Temporal control of expression is achieved by the simultaneous presence of a transgene that ubiquitously expresses a temperature-sensitive inhibitor of Gal4, *Gal80^{ts}* [14] (Figure 2B). When the animals are kept at 18^{o}C, *Gal80^{ts}* represses Gal4 and no expression occurs from the UAS target. Upon shifting of the flies to temperatures between 29 – 32^{o}C, *Gal80^{ts}* is inactivated and *Gal4* can begin transcription of the sequences downstream of UAS. The use of a conditional expression system to manipulate transcript levels at defined times allows the study of the temporal requirement of a gene. This is also valuable for genes that might have functions in both development and adulthood.

2.1. Materials: A critical feature for specificity of expression is the promoter used to drive expression of Gal4. Several *bbb-Gal4* transgenic lines have been generated and described. Among them, the *moody-Gal4* (also called *SNG-Gal4* or *SPG-Gal4*) lines generated by Bainton et al. [9] have been widely used [9,2,3,6,7]. Several sublines exist that contain the *SNG-Gal4* insertion at different chromosomal location and that may vary in the degree to which they express in a few other cells outside of the SPG. As a standard procedure *Gal4* lines should be crossed to a *UAS-fluorescent protein* to examine the expression pattern of a particular *Gal4* line. Our lab has recently generated a *Mdr65-Gal4* line using the promoter of the SPG-specifically expressed *Mdr65* gene [6], a P-glycoprotein homolog (Figure 2A; unpublished). In comparison, a genomic fusion construct that leads to the expression of the *indy* protein fused to GFP (*indy-GFP*) is expressed in both SPG and PG cells (as shown in Figure 1B). Line *9-137*-GAL4 (Ulrike Heberlein, Janelia Farm Research Campus,VA) is expressed in both layers of the bbb [2]. A different transgenic line carrying *indy-Gal4* has recently been described that is

specifically expressed in PG cells [15]. These fly lines can be obtained from the labs that created them.

3: Assessment of barrier integrity

Like in mammalian systems, the integrity of the bbb is tested by injection of small-molecular-weight molecules into the circulatory system and assessment of their exclusion from the brain. 10 KD Dextran coupled to fluorescent Texas Red (Dextran-TR) is effectively excluded from the brain in flies with an intact bbb. Since flies have an open circulatory system, the dye can be injected into the fly's abdomen. Following injection the dye circulates throughout the body of the animal and will accumulate at the bbb, excluded from the brain, where its accumulation can be visualized following brain dissection. This system has also successfully been used to screen for new mutants that affect bbb integrity, be it in development or in adults. When the bbb is leaky, the dye will not be excluded and enter the brain and fluorescence can be seen accumulating in the eyes of intact flies when flies with un-pigmented eyes (white eyes) are used [6]. Protocols for this assay have been developed by Schwabe et al. and Bainton et al. [9,16].
Figure 3A shows accumulation of 10 kD Dextran-TR at the bbb surrounding the brain in wildtype flies. In contrast, flies with a leaky bbb (such as *moody* mutant flies [9]) have a defective barrier and the dye diffuses through the bbb as shown in Figure 3B.

3.1 Materials:

- 2.5 mM 10 kDa Texas-Red conjugated Dextran, fixable (Invitrogen D-1863) in H_2O.
- Ice
- 4% Paraformaldehyde (EM grade, Polysciences Inc. #00380-250)
- Microinjector
- Razor blades
- Microscope slides and cover slips
- Double-sided tape
- Vectashield Antifade Mounting Media with DAPI (Vector Laboratories # H-1200)

Brain dissections: Several youtube videos are available with good instructions on how to dissect brains from fly heads. We like https://www.youtube.com/watch?v=j4rVa7JCzdg [17]. It is important to clean the brains up as thoroughly as possible (i.e. remove fat body and trachea). The secret to intact and cleanly dissected brains is a LOT of practice!

3.2 Approach:

1. Anesthetize adult flies on ice
2. Micro-inject a small amount (20-50 nl) of 2.5 mM 10 kDa Texas-Red conjugated Dextran in H_2O in between sternites or under the scutellum
3. After injection allow flies to recover in regular food vials overnight
4. Anesthetize flies on ice
5. Remove fly heads with a razor blade and drop them into 4% Paraformaldehyde in PBS. Fix heads for 30 minutes at room temperature (RT).
6. Remove the proboscis (mouth part) for enhanced penetration of the fixative and incubate for an additional 5 minutes at RT.
7. Dissect out the brain. Wash in 1X PBS 3 times for 30 minutes each.
8. Mount the brains on a slide with Vectashield mounting media containing DAPI to stain DNA. After a few hours, seal the coverslip with nail polish.
9. View under a confocal microscope. DAPI- stained cell nuclei are visualized at 405nm, Texas Red Dextran at 633 nm.

3.3 Notes:

To mount fly brains on a slide without them being 'smashed' by the coverslip, put a small square of double-sided tape onto the slide. With a razor blade cut a small window into the tape into which the brains will be put like in a basket. Add mounting media to the "window", add the brains, and cover with a coverslip. A little 'canal' can be cut from the square so that extra mounting media can drain. The tape will allow the brains some room while the height of the slide plus coverslip is still compatible with imaging under an upright microscope.

4. Isolation of bbb cells

SPG cells can be isolated either manually or by FACS sorting. Both approaches require that the bbb cells are labeled by fluorescence (by using the *Gal4/UAS* expression system, for example, as described above) and that brains are dissected prior to isolation of the cells. It is important that surrounding tissues are removed carefully. Below we will describe the sorting of SPG cells following a protocol that was developed by DeSalvo et al. (2014) [2], the most efficient protocol developed to date. Figure 4 illustrates the progression from SPG cells on the brain to isolated cells. It is worth noting that since SPG cells are large and very flat, once removed from their neighbors, their shape changes.

4.1 Isolation of fluorescently marked bbb cells by FACS sorting

Cells can be sorted when marked with GFP or dsRed (or other fluorescent proteins). The marker protein can either be cytoplasmic, nuclear or membrane-bound, depending on the choice of the UAS line and the protein localization signals attached to the protein. A large variety of fly strains with *UAS-fluorescent-protein* transgenes are available from the

Bloomington stock center (https://bdsc.indiana.edu/). Figure 3 illustrates the removal of bbb cells from brains in which bbb cells are labeled either by whole cell and membrane-bound expression of GFP, or by nuclear expression of dsRed.

4.1.1 Materials:

- 50 mg/ml Collagenase A in ddH_2O (Millipore Sigma # 10103578001)
- 50 mg/ml DNase I in ddH_2O (NEB, #M0303S)
- Schneider's culture medium (BD Biosciences)
- BSA (5 or 10%)
- 0.5 M EDTA
- Thermomixer R (Eppendorf)
- Dissecting forceps
- Eppendorf tubes
- Ice
- 100 um filter unit that fits on top of 50 ml Falcon tube (Falcon Filters, # 352360)

4.1.2 Approach:

1. Prepare the collagenase solution on the day of use.
2. Pre-heat thermomixer to 37°C: Fill slots with water, check the temperature with a thermometer
3. Dissect fly brains in cold filtered Schneider's medium containing 1% BSA in batches of 10 to 15 per Eppendorf tube and keep on ice. Dissect for 2 hours or less.
4. Coat 50 ml Falcon tubes with Schneider's/BSA solution by adding 1 ml to the bottom of the tube, swirl around to coat the bottom and remove. Continue with the same solution to coat all required 50 ml Falcons (1 per sample). Place a 100 µm filter onto the Falcon tube.
5. Pre-coat a 1 ml tip with Schneider's/BSA. Use it to remove the medium from the brain sample by holding the tube up to light to ensure you don't remove the brains from the bottom (be aware of any floating brains).
6. Add 1 ml Schneider's/BSA to wash the samples. Remove solution and replace with 220 µl Schneider's/BSA.
7. Add 10 µl collagenase and 5 µl DNase to the side of the tube and flick gently to mix.
8. Immediately insert tubes into the 37°C thermomixer and shake at 500 rpm for 5 minutes. Return samples to ice immediately.
9. Add 2.5 µl 0.5 M EDTA to each sample to inactivate the enzymes. Mix, then remove the Schneider's/BSA + brains and add to the appropriate 50 ml Falcon filter. Pipette any drops on the underside of the filter and add to the filtrate (be careful to not add bubbles).

10. Keep the tubes on ice until ready for FACS sorting. We have found it ok to keep cells on ice for transport to FACS sorting facility for 1-2 hours after dissociation.
11. Prepare one sample with non-labeled bbb cells as a control for FACS sorting.
12. Coat the FACS tube and all pipettes to be used with Schneider's Medium/1% BSA. FACS sort the cells with a 100 μm nozzle into Medium/1% BSA or RNA isolation buffer.
13. Non-fluorescent cells are sorted first to determine the window in which they appear. A fair amount of autofluorescence was observed in the control calibration experiment. This control was used to define the window for the collection of GFP positive cells.
14. Samples can be processed for RNA extraction immediately or stored for later use at -80°C.

4.1.3 Notes:

- We recommend following the procedures recommended by your FACS core for sorting.
- It is helpful to have a dissecting scope with a UV source for dissection to check progress, but not necessary.
- In our experience, there is a fairly large number of auto-fluorescing cells and some cell debris. We have set a stringent cutoff for fluorescent positive cells.
- Yield from about 30-50 brains has been around 500-700 SPG cells. It is possible that this number can be increased when cells can be sorted sooner after dissociation.
- Collagenase A: We have found that different lots of collagenases can vary widely in their efficiency to dissociate bbb cells (even when ordered from the same supplier under the same order number). Therefore, new batches need to be optimized. Incubate samples at 750 rpm for 5 minutes if collagenase is ineffective at 500 rpm for 5 minutes. We found one batch that was unable to remove the cells. A comparison of the composition of different lots with the help of the supplier showed that while collagenase amounts were similar, the preparation contains other proteases whose amounts can vary widely. It is possible that they contribute to the dissociation of the cells and the variability among lots.
- If cells are not sorted, but the goal is to just enrich for SPG cells, following dissociation and filtration the cells can be pelleted and dissolved in the desired solution. For example, the cells can be placed on a slide for visualization. If the volume containing the isolated cells is larger than desired, the cells can be pelleted by centrifugation at 4^{o}C 5000 rpm for 10 minutes and resuspended in the volume and medium of choice.

4.2 Remove cells by dissection and forceps

We have found that due to the coherence of bbb cells (due to their septate junctions) it is possible to remove the cells in clusters under a dissecting miscroscope with a UV light source using fine forceps. These collections will contain some non-bbb cells and will not be as clean as FACS sorted preparations.

4.2.1 Materials:

- Flies in which SPG cells have been marked by expression of a fluorescent protein such as GFP or dsRed.
- Dissecting microscope with UV light
- Small petri-dish filled half with 1.5% agarose, covered with 1x PBS, for dissection
- Ice
- Forceps: Either ultra-fine (Dumont #5SF Forceps, order# 11252-00) or fine (Dumont#5 fine forceps for dissection ,straight, # 11254-20). Both can be used.
- Dry Ice

4.2.2 Methods:

1. Anesthetize flies on ice
2. Dissect flies on ice in a small petri dish half-filled with agarose covered with cold PBS
3. Dissect fluorescent cells under a stereomicroscope with a UV source
4. To transfer the cells to a solution such as Trizol, we have found it useful to freeze a droplet of a couple of microliters of Trizol in a weigh boat on top of dry ice. Touching of the little 'frozen ball' with the forceps while the cells are still attached to the dissecting forceps causes the cells to "jump over" to the ice droplet. Several batches of cells can be accumulated on one droplet which can subsequently been frozen at -80 degree Celsius for later processing.

Acknowledgements: This research has been supported by grants from the National Science Foundation (NSF) to B.D.

5. References

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Figure legends

Figure 1

Isolated adult *Drosophila* brains with bbb visualized. Dorsal is on top. (A) Nuclei of SPG cells are labeled by expression of dsred. Genotype: *Mdr-Gal4/+; UAS-dsRed/+*. SPG cells are large and flat, and their number is low. (B) Both layers of the bbb (PG and SPG cells) express a genomically encoded indy-GFP fusion protein, visualized by immunohistochemistry (green). DNA is shown in blue (the majority of the nuclei seen are neuronal). An optical confocal section is shown to illustrate the tight barrier layer that surrounds the entire brain. The opening inside the brain with a bbb layer is the oesophageal foramen.

Figure 2

The Gal4/UAS/Gal80^{ts} system allows temporally controlled expression of sequences of choice in the cells of choice. (A) Gal4 expression is directed to the bbb by a bbb-specific promoter. There, Gal4 binds to UAS and leads to the transcription of the downstream sequence. However, at the same time, a ubiquitous tubulin promoter guides direction of the temperature-sensitive Gal80^{ts}, an inhibitor of Gal4. At 18^{o}C, Gal80^{ts} is active and inhibits Gal4 activity and transcription is blocked. To release this block, animals are shifted to 32^{o}C. At this temperature Gal80^{ts} becomes inactive, and Gal4 can initiate transcription form the UAS promoter.
(B) Protocol to manipulate bbb cells specifically in mature adult flies using the approach described in (A).

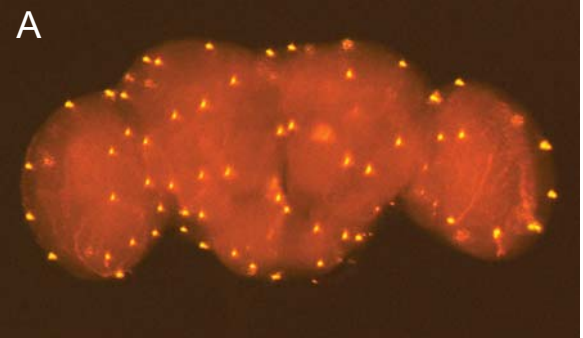
Figure 3

An intact bbb is not permeable to 10kD-TR. (A) Following injection of 10kD Dextran marked with the Texas-Red (TR) fluorophore, Dextran-TR circulates in the hemolymph and accumulates at SPG cells. The optical confocal section shows accumulation of Dextran-TR (red) at the barrier. Neuronal cell bodies inside the brain are marked with DAPI. (B) *moody* mutant flies have a leaky bbb [9] and Dextran-TR can be seen entering the brain (Picture reproduced with permission from Hoxha et al. PLoSGenetics, 2013 [10].)

Figure 4

Dissociation of SPG cells from isolated brains. SPG cells were labeled by SPG-specific expression of cytoplasmic GFP (A-C), or by nuclear dsRed (D-F) using the *Mdr-Gal4* driver. The whole-cell labeling in (A) illustrates the “cap-like” structure of the bbb. Isolated fly brains are shown (A, D). Following treatment with collagenase, the brains have lost most of the marked SPG cells (B, E). The brain in (B) is situated on the filter

that is used to collect dissociated cells. (C): Dissociated GFP labeled SPG cells after sorting. (F) An isolated SPG cell with dsRed expression in the nucleus.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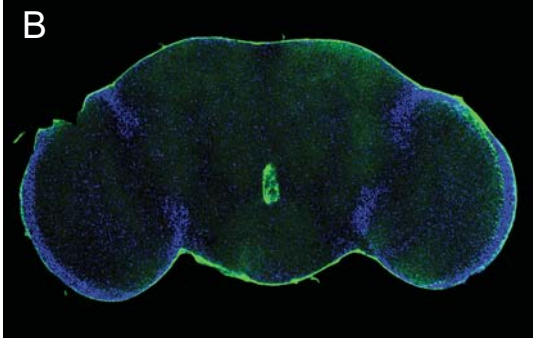
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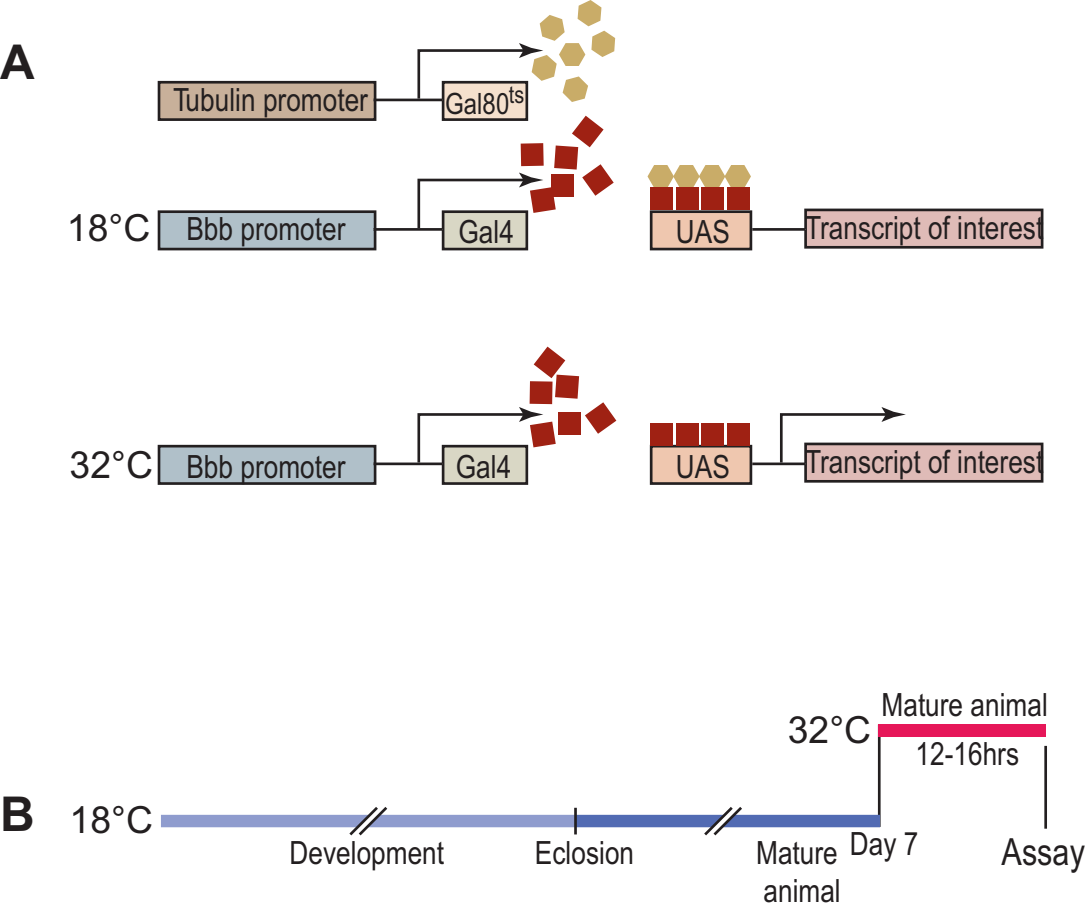
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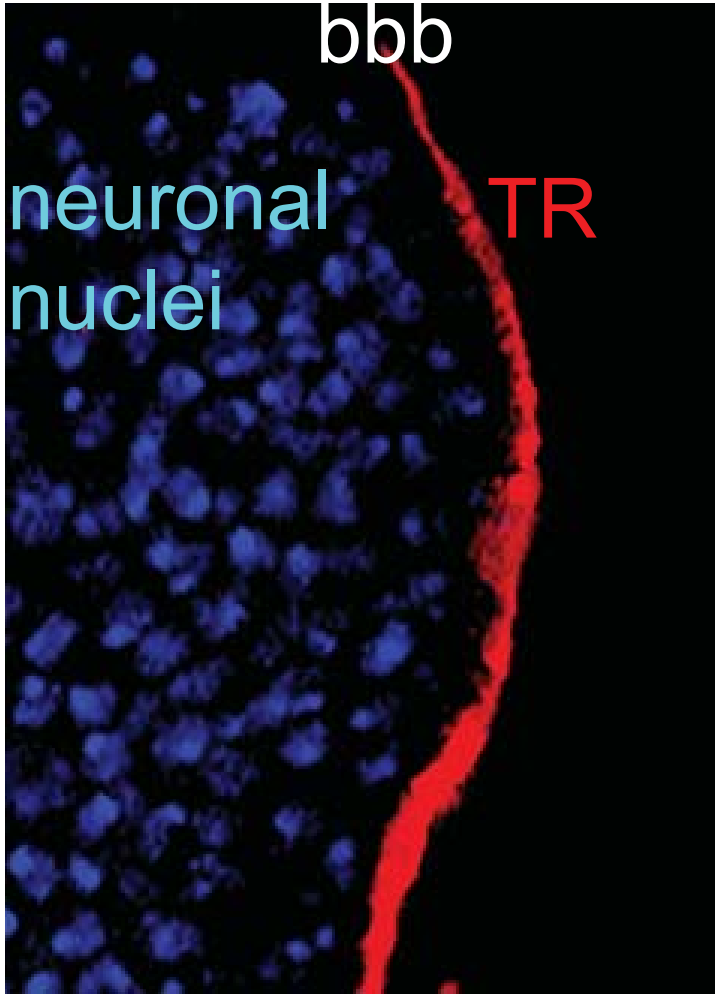

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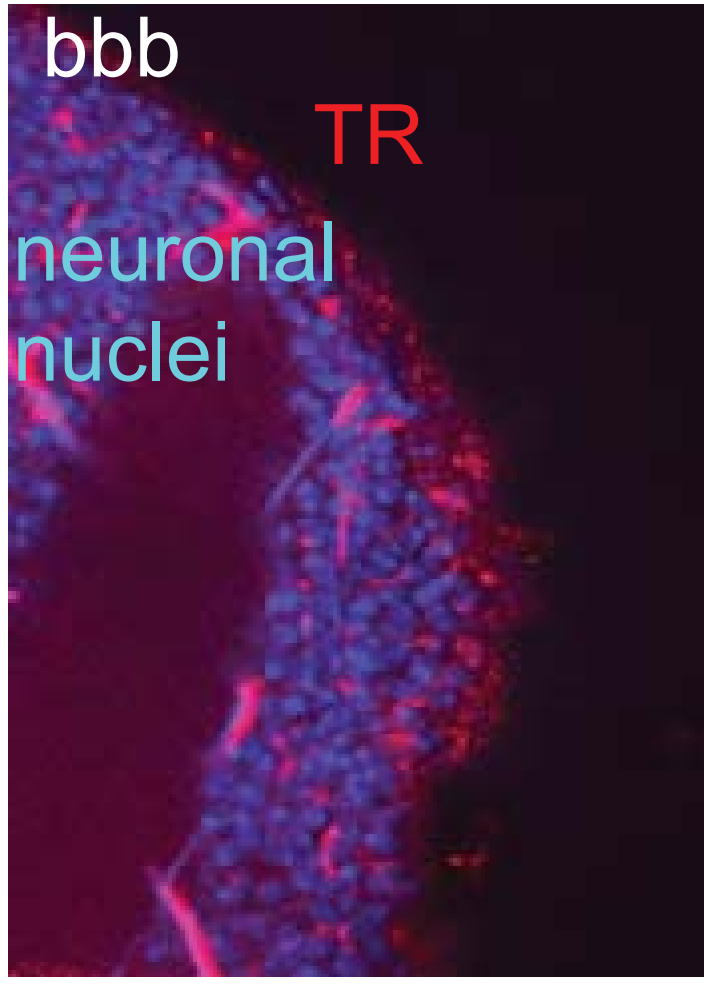

moody mutant with leaky bbb

Isolated brains before Collagenase treatment

Brain after Collagenase treatment

Isolated SPG cells