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Protocol: Brain Dissection, RNA extraction, DNAse Treatment, and cDNA synthesis for using RT-qPCR to Measure Differential Gene Expression in the Poison Dart Frog *Oophaga sylvatica*

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JUNE 16 – AUGUST 22, 2014

NSF/EDEN grant number IOS # 0955517

Rationale

This protocol describes the methodology used to dissect whole brains of the poison dart frog *Oophaga sylvatica* into five specific brain regions. RNA can then be extracted from these brain regions using the Trizol method described here. DNAse treatment can then be used to eliminate unwanted genomic DNA contamination of the RNA extracted from the brain regions of interest. Finally, qScript reverse transcription can be used to generate cDNA from the RNA extracted from the brain regions providing the means to test for differential gene expression by RT-qPCR. Methods of capturing and euthanizing individual poison dart frogs are not herein described.

Materials Required

- Whole brains frozen at -20°C stored in RNAlater.

For Dissection:

- Dissection kit
- BEAD BUG Microtube Homogenizer, Prefilled Tube with 1ml Trizol. 5 per brain labelled by brain region.
- Dissection microscope
- RNAlater
- Ethanol

For Trizol RNA Extraction:

- BEAD BUG Microtube Homogenizer
- 1.5ml Microfuge tubes
- Chloroform

- Isopropanol
- 75% Ethanol
- Microcentrifuge
- Heat Block
- Nuclease free H_20

For TURBO DNAse-free Treatment:

- 10x TURBO DNAse buffer
- TURBO DNAse reagent
- RNAse OUT reagent
- DNAse inactivation reagent
- PCR tubes

For qScript Reverse Transcription:

- qScript cDNA Supermix
- PCR tubes
- Thermal cycler

Brain Layout and Dissection

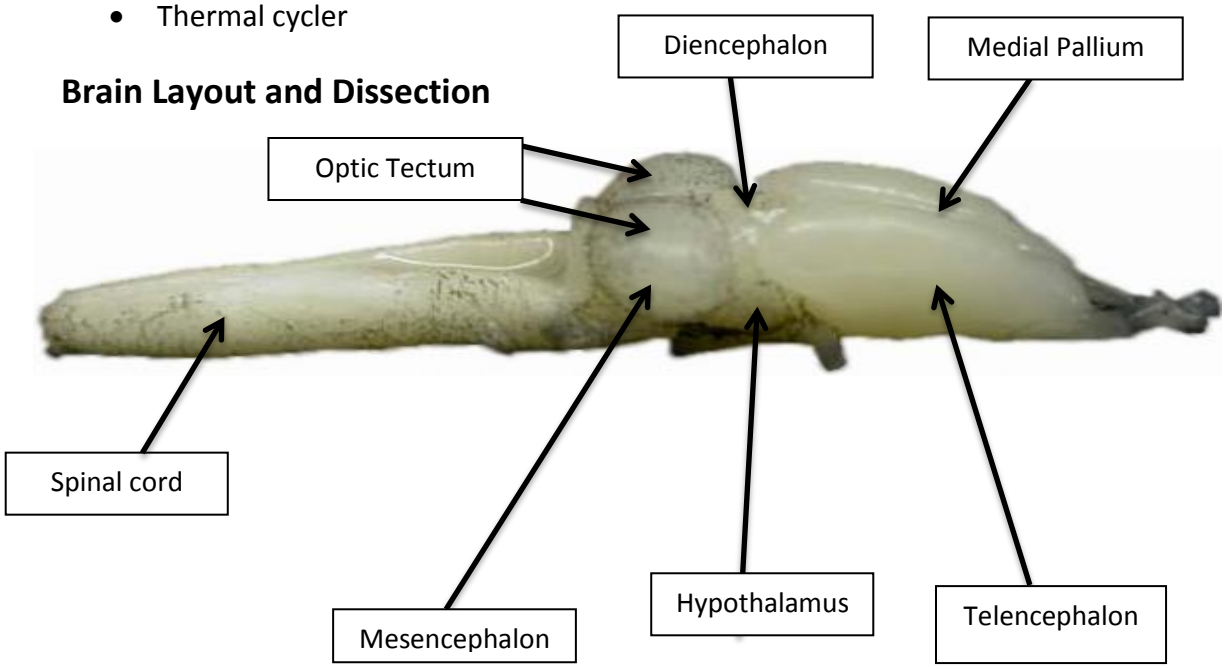


Figure 1. Anatomy of the frog brain.

Whole brains should be stored in RNAlater at -20°C until dissection.

1. Remove brains for dissection from freezer and allow to thaw on ice.
2. Label 5 BEAD BUG Microtube Homogenizer, Prefilled Tubes with 1ml Trizol according to brain region:
    - Mp = hippocampus
    - TEL = basal ganglia
    - HYP = hypothalamus

- DIE = diencephalon
- SC = spinal cord

3. Transfer whole brain sample to a petri dish containing 1ml chilled RNAlater to preserve RNA in sample whilst dissecting.
4. Sterilise dissection equipment using ethanol.
5. Using dissection microscope (necessary as brain size ~4-5mm), remove the Telencephalon.

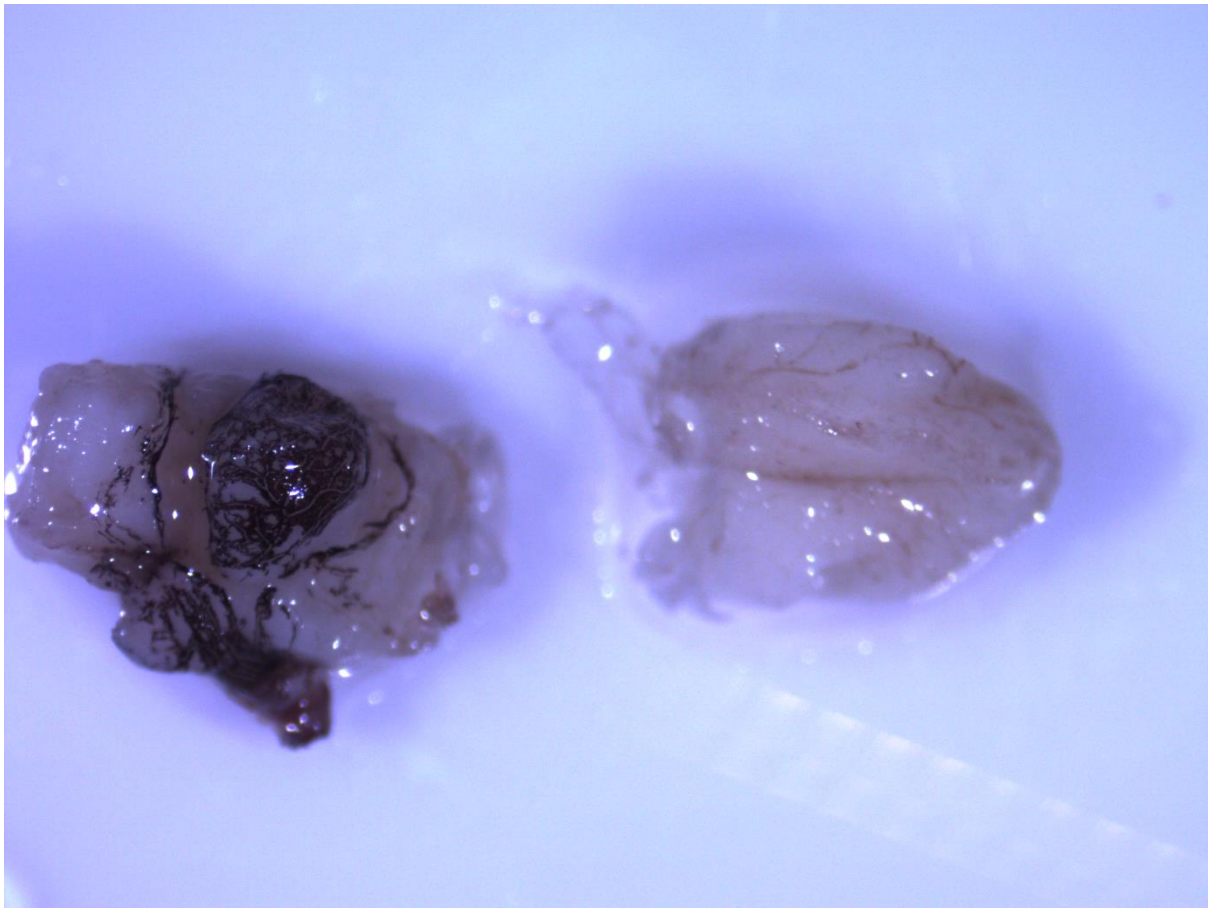
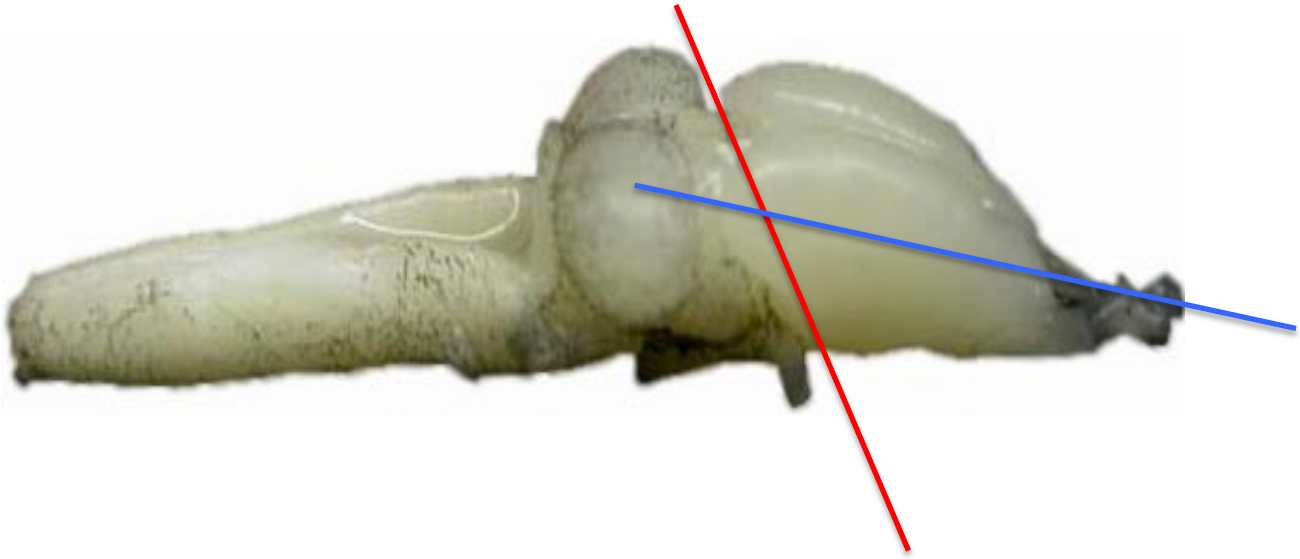
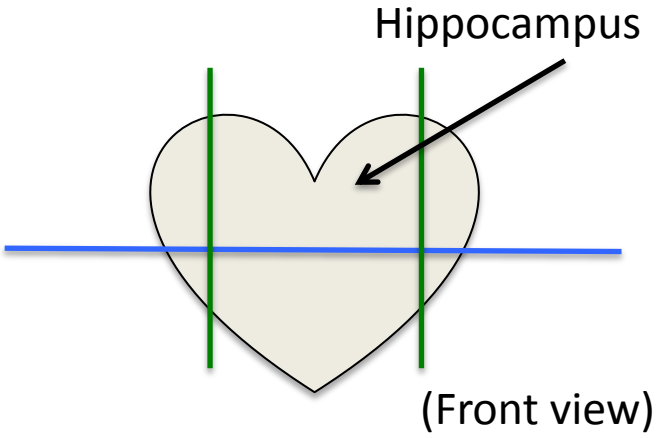


Figure 2. *Oophaga sylvatica* brain with the Telencenphalon (right) removed. Hypothalamus, Diencephalon and Spinal Cord on the left.



6. Cut the Telencephalon laterally, the bottom part is the Basal Ganglia, put this in the tube labelled TEL with Trizol
7. Remove the medial edges of the upper Telencephalon. This is the Hippocampus. Put this in the tube labelled Mp with Trizol.



8. Cut away the Diencephalon, the region in between the Diencephalon and the already removed Telencephalon is the Hypothalamus; put this in the tube labelled HYP with Trizol.

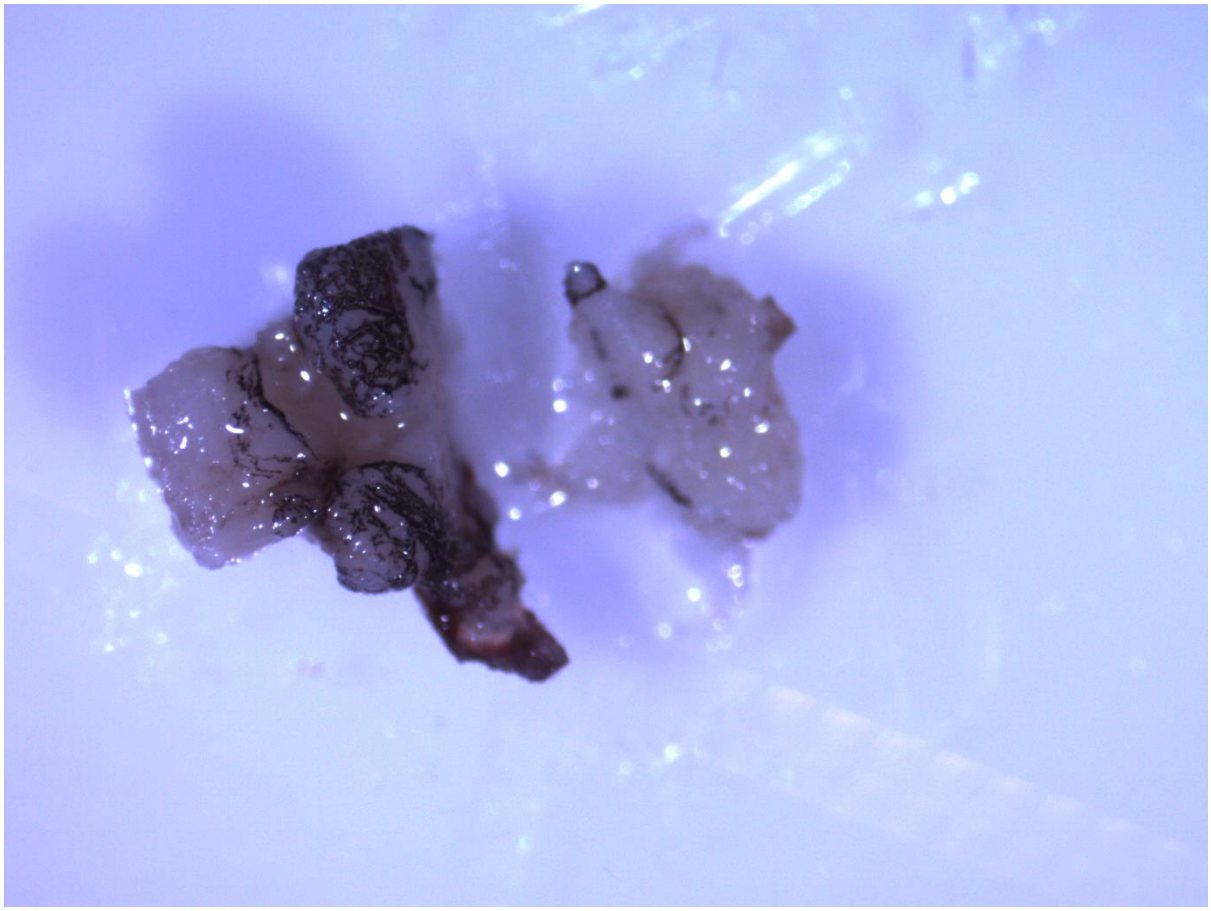
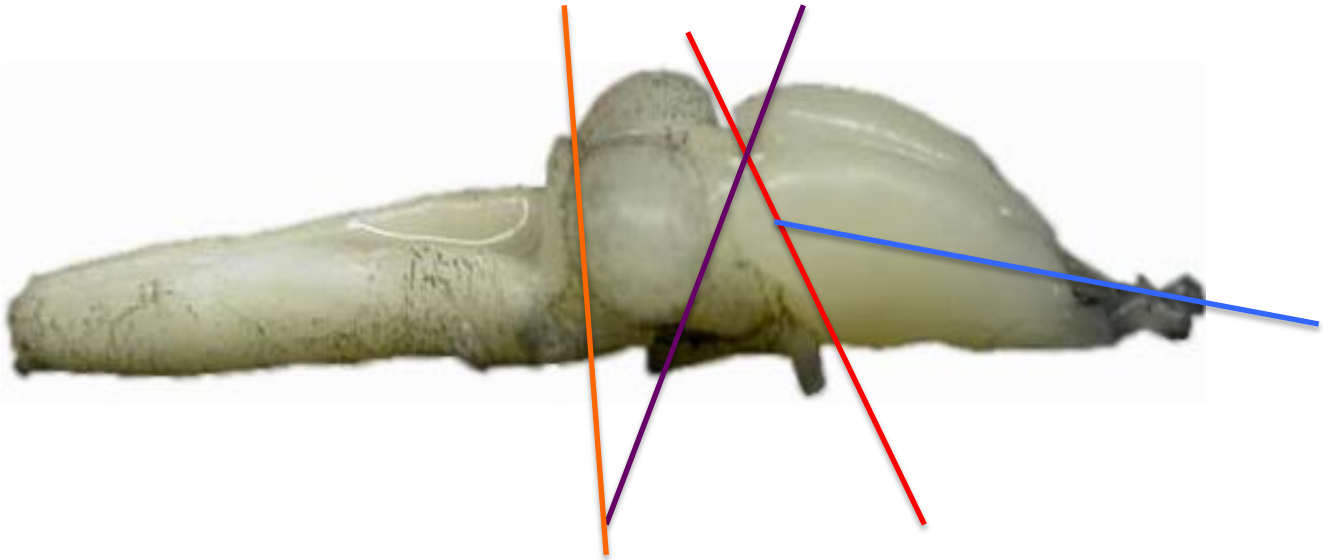


Figure 3. Hypothalamus (right) removed from the Diencephalon (left), Diencephalon is also still attached to the Spinal Cord.



9. Cut off the spinal cord separating it from the Diencephalon. Put the Diencephalon in the tube labelled DIE and the spinal cord in the tube labelled SC both with Trizol.

Trizol RNA Extraction

1. Homogenize tissue in Bead Bug tube with 1mL Trizol. Let sit for 5min on bench.
2. Move to 1.5mL microfuge tube with 150µL chloroform. Shake for 15 sec, let sit for 3min on bench.
3. Spin in microcentrifuge at 13000rpm 4°C for 15min (RNA in upper layer).
4. Transfer upper layer to new tube (2x 180µL; as much as you can without the interface).
5. Add 270 µL isopropanol, vortex 1sec. Let sit 10min on bench.
6. Spin in microcentrifuge at 13000 4°C for 10min (RNA will pellet).
7. Remove supernatant.
8. Wash with 600 µL 75% Ethanol.
9. Spin in microcentrifuge at 10000rpm 4°C for 5min
10. Remove Ethanol and repeat 8-9.
11. Quick spin & remove Ethanol. Air dry pellet 1-2m.
12. Add 16µL water to each tube. Incubate in heat block at 60-70C for 10 min.
13. Vortex and quick spin. Proceed to TURBO DNAse-free treatment.

TURBO DNAse-free Treatment

1. Add 4 µL TURBO DNA-free Mix (Consisting of 2µL 10X TURBO DNAse buffer, 1µL TURBO DNAse reagent, and 1µL RNAse OUT reagent). Vortex to mix.
2. Incubate at 37°C for 20 min.
3. Add 2.5 µL Inactivation reagent to each tube.

4. Incubate 5 min at room temperature, ensuring reagents stay mixed by agitating tubes.
5. Spin 9,000rpm for 1.5 min and transfer RNA (~16µL) to PCR tubes.
6. Freeze at -80C or proceed to Reverse Transcription.

qScript Reverse Transcription

1. Add 4µL of qScript cDNA supermix to 16µL RNA.
2. Run Thermal cycling protocol:
    I. 25°C - 5min
    II. 42°C - 120min
    III. 85°C – 5 min
    IV. 4°C - ∞
3. Freeze cDNA at -20°C.

This cDNA can then be used to test differential gene expression via RT-qPCR.

References

Nolan, T., Hands, R. E., & Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. Nature protocols, 1(3), 1559-1582.