

**Characterization of osmoregulatory control centers in the
brain of *Rana pipens* with *c-fos* immunocytochemistry**

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ABSTRACT

In order to maintain homeostasis, the body has many self-correcting controls that function to return conditions to levels necessary for physiological processes. Previous studies in rats used *c-fos* immunocytochemistry to identify regions that were activated upon desiccation, indicating possible osmoregulatory control centers. *C-fos*, a transcription factor, was used because its excess expression signifies cell activity. To study osmoregulatory control centers in frogs, *Rana pipens* were used. *C-fos* immunocytochemistry is presently being utilized to study differences in brain activity between desiccated *R. pipens* and *R. pipens* in environments with normal water levels.

Osmoregulatory Control Centers Indicated with *c-fos*

INTRODUCTION

C-fos, an oncogene product, may cooperate with *jun*, another oncogene product, to foster gene transcription (Marx 1988). Oncogenes respond to several growth factors and nerve impulses (Marx 1988). *c-fos* has been a widely accepted marker for neural activity (Chan et al. 1993).

Upon increased blood osmolarity, arginine vasopressin (AVP) is released to mediate body fluid balance (Ding et al. 1994). AVP is mainly synthesized in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) (Ding et al. 1994). Axons from these cells pass down the pituitary stalk and terminate in the posterior lobe of the pituitary gland from which the hormone is secreted (Ding et al. 1994). After being transported through the circulatory system, AVP acts on (1) the collecting ducts in the kidneys to regulate water reabsorption and (2) arterioles in many organs to produce vasoconstriction (Ding et al. 1994).

In an earlier study, rats were intracellularly dehydrated. After the animals were anesthetized, the brains were removed, sectioned, and fixed onto slides. Immunocytochemistry for the distribution of *c-fos* was performed. *c-fos* mRNA was positively identified in the hypothalamic magnocellular neurons in the SON, nucleus circularis, and adjacent accessory cell groups clustered along blood vessels in the dehydrated rats, while only background levels of *c-fos* mRNA was detected in the control group. This demonstrates that the increase of *fos* mRNA after acute dehydration is greater in the SON than in the PVN (Ding et al. 1994). Similar studies in birds demonstrate increased *fos* activity in the SON and PVN (Sharp et. al. 1995).

For this study, 20 *Rana pipens* were dehydrated to determine the brain's osmoregulatory center. Afterward, the brains will be remove, section, and fixed onto slides. Immunocytochemistry will be utilized to determine changes in *fos* activity, in turn, indicating brain activity.

MATERIALS AND METHODS

Rana pipens were used to evaluate the *c-fos* activity within the brain after desiccation. *c-fos* activity can then be correlated with the portion of the brain involved in osmoregulatory.

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20 frogs were gathered. 10 control frogs were kept in 1 cm of tap water and 10 frogs were towel dried and deprived of water for 48 hours. Histologies were performed on the frogs. The distribution of *c-fos* will be determined with immunochemistry.

Protocols:

Brain Removal Procedure for General Histology Protocol

1. Frogs were deeply anesthetized with benzocaine (0.02% solution: 10 g benzocaine in 100 ml 95% ethanol, diluted with H₂O right before use).
2. Fixative was prepared. Decapitated frog using scissors.
3. Removed lower jaw with large scissors.
4. Trimmed head at sides, remove eyes and cut off tip of nose (until you see into cranial cavity) using scissors.
5. Oriented head so that the ventral surface is up and caudal end to the left.
6. Stabilized the head with long, curved forceps.
8. Gently lifted the parasphenoid bone by sliding scissors from the anterior to posterior. Remove the ventral piece of cranium.
9. Used a fine scissors to cut all nerves, beginning with the olfactory and optic and continuing back.
10. Use a blunt probe to "push" the brain gently all around and make use that it is free of the skull.
11. Use watchmaker's forceps to grasp the olfactory nerve. Pull the brain out by sliding it forward and slightly up.
12. Drop the brain into a glass scintillation vial containing fresh fixative and fix for at least another 6-24 hours, at 4°C.

Formaldehyde Fixative

1. Dissolve 4 g paraformaldehyde in 100 ml of dH₂O by heating the mixture to 65°C with stirring.
2. Add a few drops of 40% NaOH until the solution becomes clear. Allow the solution to cool.
3. Prepare a 0.2 solution of dibasic sodium phosphate (1.7805 g in 59 ml dH₂O water).
4. Prepare a 0.2 M solution of monobasic sodium phosphate (1.38 g in 50 ml dH₂O).

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5. Prepare the 0.1 M solution phosphate buffer by mixing 30.5 ml of 0.2 M dibasic solution with 19.5 ml of 0.2 M monobasic solution and dilute to 100 ml with dH₂O.
6. Add 100 ml buffer to 100 ml paraformaldehyde solution.
Note: Must be made fresh, before use, as it oxidizes quickly.
The amount of dibasic and monobasic sodium phosphate used must be recalculated, depending upon whether the powder is hydrated.
Wear gloves and safety glasses throughout.

0.2 M Phosphate buffer

1. Prepare 0.2 M stock of monobasic sodium phosphate (NaH₂PO₄-H₂O; MW=138). Dissolve 13.8 g in 500 ml of dH₂O.
2. Prepare 0.2 M stock of dibasic sodium phosphate (Na₂HPO₄-7H₂O; MW=268.1). Dissolve 53.6 g in 1 L of dH₂O.
3. To prepare 0.2 M phosphate buffer at physiological pH, mix 100 ml dibasic stock with 37.5 ml monobasic stock.
4. Double-check pH around 7.4. Store refrigerated.
5. 0.1 M buffer can be prepared by diluting the 0.2 M with an equal volume of dH₂O.

ICC Protocol

1. In 1 L volumetric flask, add 5 g gelatin (knox), 0.5 g chrome alum (chrominum potassium sulfate), and fill up to 1 L with hot dd H₂O. Double sub slides with 30 min. in between. Let dry overnight. Solution only good 48 hrs.
2. Transfer brains to new cryoprotectant with 30% sucrose in 0.2 PBS at 4°C for up to one week.
3. Cut 50 µm thick sections on cryostat. Thaw-mount onto subbed slides. Re-freeze. Store slides at -80°C until use.
4. Remove slides from -80°C, allowing them to dry for one hour. Wash in 0.05 M PBS--2 times 5 min each.
5. Incubate in 1% H₂O₂ in PBS for 30 min (1.36 ml of 30% H₂O₂ in 40 ml 0.05 M PBS; add H₂O₂ just before use).

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6. Wash in twice with 0.05 M PBS, 10 min each. Wipe slide edges and draw ring around sections with PAP pen (on dry slide area).
7. Block with 5% NGS in PBS (100 μ l vector NGS in 1.9 ml 0.05 M PBS). Fill PAP ring with solution 1 drop at a time. Block for 30 min at room temperature with slide flat on counter.
8. Remove NGS by blotting the slides with a kimwipe, dry edges of slide. Place slides flat in incubation chamber, on white slide holder, with damp kimwipes in bottom of chamber to provide humidity.
9. Incubate sections overnight (24 hr), covered, at 4°C with solution containing with a 1: 4000 dilution of *c-fos* primary antibody:
 - 20 ml 0.05 M PBS
 - 40 μ l of Triton X-100
 - 5 μ l *c-fos*, rabbit polyclonal antisera (Oncogene Research Products, Cat# PC38)
10. Wash 2 times for 5 min with 0.05 M PBS.
11. Incubate sections for 90 min (flat) with a 1:50 dilution of the secondary at room temperature:
 - 30 μ l vector NGS (1.5% NGS)
 - 40 μ l vector biotinylated secondary antibody (goat, anti-rabbit)
 - 1930 μ l 0.05 M PBS
12. Wash 2 times for 10 min each in 0.05 M PBS.
13. Incubate sections with ABC complex for 90 min at room temperature.
14. Wash 2 times for 10 min with 0.05 M PBS.
15. Develop in DAB solution for 30 min.
16. Wash 2 times, 10 min each in 0.05 M PBS.
17. Dry, coverslip with Permount and mount.

PBS

Prepare 0.05 M phosphate buffer with 0.9% saline, pH 7.3.

A. In 4 liter volumetric, add <4 L dH₂O + 27.6 g NaH₂PO₄-H₂O + 32.4 g NaCl.

B. Adjust pH to 7.3 with about 35 ml 5 N NaOH. Bring volume up to 4 L line on volumetric with dH₂O.

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C. Double-check pH; store refrigerated (indefinitely) in plastic roller bottle.

ABC reagent

- A. 1 drop reagent A
- 1 drop reagent B
- 2.5 ml 0.05 M PBS

B. Stir and let sit for 30 min at room temperature before use.

DAB/Nickel Solution

A. To 9 ml 0.05 M PBS, add:

5 mg of Diaminobenzidine (use 1 ml of aliquot of frozen stock)

4 μ l of 30% H₂O₂ (immediately before use)

4 drops of Ni solution from Vector DAB kit

B. Mix in plastic conical test tube.

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