Winter 2000

Goldfish Brain GAP-43: Analysis by One and Two-Dimensional Gel Electrophoresis

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Available at: http://scholarworks.gvsu.edu/mcnair/vol4/iss1/7
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ABSTRACT
The concept of learning and memory is a neurological process believed to reside in the nervous system of living organisms. In particular, the growth-associated protein, GAP-43, has been shown to alter its state of phosphorylation via a protein kinase C mediated reaction following long term potentiation, a paradigm of learning and memory. Most recent studies of GAP-43 in goldfish (Carassius auratus L.) have focused on its presence in regenerating or developing optic nerves. The present study seeks to determine the existence of GAP-43 in the goldfish brain and secondly, quantify phosphorylated isoforms of GAP-43 before and after active avoidance training.

Introduction
Biochemical changes occurring in the brain following a learning task have best been studied using the artificially induced process of long term potentiation (LTP), which is an animal model that has been proposed to mimic in vivo changes (Bliss and Collingridge, 1993; Derrick and Martinez, 1994). LTP is defined as a prolonged enhancement of synaptic activity following high voltage stimulation (Bliss and Collingridge, 1993; Soderling and Derkach, 2000). Phosphorylation of GAP-43 by protein kinase C is critical for the induction of LTP (Benowitz and Routtenburg, 1997; Perrone-Bizzozero et al., 1998).

The focus of this research is to study the changes in the phosphorylation of GAP-43 following training tasks that rely on formation of memories. GAP-43 (growth associated protein) is a synaptic protein with a molecular weight of 43 kDa (Oestreicher et al., 1997). This protein is made of 211 amino acids. The first four amino acids provide membrane attachment for the protein. Between the thirty-first amino acid and the forty-sixth amino acid in the sequence of GAP-43, there is 100% homology across all species. There is a calmodulin-binding domain in this protein between the forty-third and the fifty-third amino acid. GAP-43 is associated with neuron outgrowth and regeneration, spontaneously occurring action potentials, and increased neurotransmitter release (Benowitz and Routtenburg, 1997; Meiri et al., 1991; and Oestreicher et al., 1997).

GAP-43 plays an important role in LTP. Neurotransmitter release into the synapse is dependent on presynaptic levels of calcium. As protein kinase C (PKC) phosphorylates GAP-43 at ser41 (Benowitz and Routtenburg, 1997), the calmodulin bound to the protein is released (Oestreicher et al., 1997). The free calmodulin in the presynaptic cell bonds to calcium, causing an increase in intracellular
levels. As a result there is an increase in the amount of neurotransmitter (glutamate) released into the synapse, leading to LTP. In goldfish, the learning process has been blocked with the administration of drugs that interrupt various stages of LTP (Xu et al., 1998).

In order to investigate the hypothesis that the phosphorylated isoforms of GAP-43 are increased in the goldfish brain after learning and memory exercises, techniques to detect GAP-43 by protein extraction, one and two-dimensional gel electrophoresis, western blotting and immunodetection need to be established. These techniques will be used to analyze GAP-43 in a way that will enable its phosphorylation states to be visualized and compared. Immunocytochemical detection will be used to localize GAP-43 in specific regions of the goldfish brain. During this project, any other proteins that change during shock-avoidance training will be analyzed and identified.

This research is unique because, while GAP-43 has been identified in the optic nerve of the goldfish, it has never been detected in the goldfish brain. If detected, the goldfish can serve as an additional animal model to study the biochemistry underlying learning and memory.

Methods and Materials

Enrichment

Thirty goldfish brains were obtained from Dr. Xandra Xu. The brains were stored at -70°C. The brains were placed in 1.0 ml of homogenization buffer (.32 M sucrose, 10mM Tris-HCl, 2mM EDTA at pH 7.4) and diced with a scalpel. The tissue was homogenized inside of a pestle using Kontes Pellet Pestle Motor. The procedure used in the enrichment of GAP-43 was described by McMaster et al. (1998). Following homogenization, the tissue in the eppendorff tubes was combined into two Beckman Ultracentrifuge tubes. The tissue was centrifuged at low speed (1000 x g for 10 minutes) at 4°C using a LE 80 Beckman Ultracentrifuge SW40 Ti Rotor. The supernatant (S1) was combined into one Beckman Ultracentrifuge tube and centrifuged at 13000 x g at 4°C for 20 minutes. The pellet (P2) was resuspended in 5.0 ml of lysing buffer (10mM EGTA, 2mM EDTA, 10mM Tris-HCl at pH 7.5). This solution was stirred in an ice bath for 45 minutes (NUOVA II Thermolyne Stirplate). The solution was then centrifuged at 175000 x g at 40°C for 20 minutes. To perform the alkaline extraction, 2.0 ml of 1mM Magnesium Acetate buffer was used to resuspend the pellet (P3). The pH was brought to 11.5 using 1M NaOH. This solution was centrifuged at 75000 x g at 4°C for 20 minutes. To perform the acid precipitation of the proteins, approximately 10 drops of 1mM sodium acetate (pH 5.0) was added to the supernatant (S4) to bring the pH to 5.5. This solution was centrifuged at 75000 x g at 4°C for 20 minutes. The supernatant (S5) was decanted into a graduated cylinder to measure the volume (2.6 ml). The concentration of ammonium sulfate in this solution was brought to 40% ammonium sulfate. This solution was allowed to stand on ice for 20 minutes. The S5 was centrifuged at 100000 x g at 4°C for 20 minutes. This solution was decanted into a graduated cylinder for the purpose of measuring its volume (2.8ml). The concentration of ammonium sulfate in the solution was then brought to 80%. This solution was incubated on ice for 20 minutes and the centrifuged at 100000 x g at 4°C for 20 minutes. The pellet (P7) was collected and stored at 4°C for future use. This same procedure was performed under the same conditions using three rat brains, the P7 sample was collected and stored at -20°C.

One-Dimensional Gel Electrophoresis

The 80% ammonium sulfate fraction was analyzed using one-dimensional gel electrophoresis. In this procedure the proteins are separated by molecular weight in a gel-matrix using electric current. Approximately 50ul of sample buffer (containing 10% sodium dodecyl sulfate (SDS), 1% bromophenol blue, 5% 2-mercaptoethanol and 7% glycerol) was added to 20ul of the P7. Molecular weight standards and sample proteins were separated, on a 13% SDS-acrylamide gel and then placed in 300ml running buffer (60ml stock solution and 240ml distilled water). The proteins were electrophoresed at 200v for 50 minutes.

Two-Dimensional Gel Electrophoresis

In two-dimensional gel electrophoresis the proteins in the 80% ammonium sulfate fraction were separated first by isoelectric point and secondly by molecular weight. The 80% ammonium sulfate fraction was analyzed using the BIO-RAD Mini-PROTEAN II 2-D Cell, Mini-PROTEAN II Tube Cell, and Mini-PROTEAN II Tube Module system for two-dimensional electrophoresis. A sample of 15ul was added to the tube gels and focused at 200v, 1mA for approximately 20 hours. The tube gels were the electrophoresed on 13% acrylamide separating gels at 200v, 80mA for 50 minutes. The gels were then stained using the silver staining procedure (Bio-Rad silver staining kit).

Coomassie Blue Stain

After electrophoresis, gels were fixed for 30 minutes in 50% methanol and 7% acetic acid. The fixed gels were placed in the Coomassie blue stain for a period of 30 minutes and then destained using a solution of 5% acetic acid and 5% methanol.

Silver Staining Procedure

Immediately after electrophoresis, the gel was placed in a fixative enhancer solution (50% reagent grade methanol, 10% reagent grade acetic acid, 10% fixative enhancer concentrate, and 30% deionized distilled water) for 20
minutes, with gentle agitation. The gels were washed three times in 500ml glass-distilled water over a period of 60 minutes. The gels were placed in a staining solution for approximately 20 minutes, a 5% acetic acid solution was used to stop the reaction. Gels were rinsed in distilled water and scanned.

**Immunodetection Procedure**

The gels were transferred to a PVDF (Bio-Rad) membrane using 100v, 300mA of electricity for approximately 1.5 hours using the Mini-trans-Blot electrophoretic transfer cell (Bio Rad). The membrane containing proteins was incubated in the blocking solution TTBS(100mM Tris-Cl, pH 7.5, and 0.9% NaCl) for 30–60 minutes or until the entire membrane was hydrated. This blocks any unoccupied sites on the PVDF membrane to prevent any non-specific binding of the primary antibody. The primary antibody (Goat polyclonal IgG N-19 GAP-43) in a dilution of 40 ul in 10 ml TTBS was applied to the membrane, this solution incubated for approximately 30 minutes. TTBS was used to wash the membrane three times over a 15 minute time span. The membrane was allowed to incubate in the secondary antibody (biotinylated donkey anti-goat IgG - Chemicon) which was diluted to 10 ul in 20 ml TTBS for 30 minutes. Again the membrane was washed in TTBS three times over a period of 15 minutes. The avidin-biotin-enzyme solution was applied to the membrane and left to incubate for 30 minutes (2 drops Vectastain reagent A and 2 drops Vectastain reagent B, diluted in 50ml of TTBS). After washing the membrane three times in TBS over a 30 minute span, the membrane was developed using a DAB Substrate Kit for Peroxidase (Vector Laboratories). The color reaction was stopped by rinsing the membrane five minutes in glass distilled water.

**Results**

A characteristic band of proteins near the 45kDa molecular weight marker was observed in gels containing a sample of the 80% ammonium sulfate fraction from the goldfish brain. This band was observed in gels that had been stained in coomassie blue and gels that were stained using the silver staining technique. Gels containing samples of the 80% ammonium sulfate fraction from both the goldfish and rat brain were also observed. In these gels the characteristic band near the 45kDa molecular weight marker was present in both the goldfish and the rat samples. The band of the 80% ammonium sulfate fraction taken from the rat was heavier then the band in the 80% ammonium sulfate fraction taken from the goldfish.

The two-dimensional gel containing a sample of the 80% ammonium sulfate fraction taken from the goldfish brain revealed the separation of one protein into five distinct isoforms.

The immunodetection procedure was performed on a membrane containing samples of the 80% ammonium sulfate fraction from both the rat and the goldfish. A strong positive reaction took place in the lane containing the 80% ammonium sulfate fraction taken from the rat brain. A positive reaction also developed in the lane containing the 80% ammonium sulfate fraction taken from the goldfish brain.

**Discussion**

This is a preliminary study of GAP-43 in the goldfish brain. Proper controls for this project still need to be run. The results of this preliminary study suggest that GAP-43 is indeed present in the goldfish brain. The two-dimensional gel revealed the separation of one protein in to five distinct isoforms. The behavior of this protein is strikingly similar to the way that GAP-43 has been characterized in other animals. GAP-43 is an acidic protein, spots were observed at the very acidic end of the two-dimensional gel.

As GAP-43 is phosphorylated it becomes more acidic, the spots on the gel could represent the various phosphorylation states of this protein. As this study continues, phosphospecific antibodies need to be applied in order to distinguish between these spots and positively identify GAP-43.

During the immunodetection procedure, a positive result was observed in the lanes containing the 80% ammonium sulfate fraction taken from the rat and the 80% ammonium sulfate fraction taken from the goldfish. Although the negative controls have not yet been run, there is reason to suspect that this reaction was specific for GAP-43 in the goldfish brain. On a one-dimensional gel stained in Coomassie blue, a band of protein with a molecular weight of about 45kDa was observed from the 80% ammonium sulfate fraction taken from the goldfish brain, there was also a very heavy band of protein near the 31kDa molecular weight marker. Even though this band stained very heavy in coomassie blue, the band of protein with a molecular weight of about 31kDa did not exhibit any nonspecific binding during the immunodetection procedure. On the other hand the light band of protein with a molecular weight of about 45kDa did show a positive response to the immunodetection procedure.

The positive result for the rat GAP-43 was much stronger than the result of the goldfish GAP-43. A number of factors could account for the difference in the amount of staining in the goldfish and the rat samples. The same amount of both the goldfish brain and the rat brain 80% ammonium sulfate fractions were loaded into the lanes. The 80% ammonium sulfate fraction that is being analyzed is not a pure sample, but an enriched sample. Unfortunately the samples were not quantitated, therefore the amount of GAP-43 contained in each sample is unknown.
There was also a difference in the antibody recognition between the two samples. The primary antibody used was made specifically against the first nineteen amino acids of GAP-43 in the rat. This portion of the protein is not 100% homologous with the goldfish protein. The primary antibody would have less affinity for the goldfish GAP-43 than the rat GAP-43 because of the difference in the amino acid sequence. This could also account for the weaker reaction in the goldfish lane compared to the rat lane.

GAP-43 is known to aid in the regeneration of injured axons and to be involved in neuron growth during developmental periods (Oestreicher et. al., 1997). The interest of this research project is to examine GAP-43 in the goldfish brain and its phosphorylation states before and after training tasks. Using LTP as a model, the chemical changes that GAP-43 undergoes can help in understanding the physiological changes that take place during learning and memory in the human brain.

References


