

## Analysis of Synapsin I and II mRNAs and Proteins During Murine Development

Michael RR\*

Department of Biology, University of Puerto Rico at Cayey, Puerto Rico

### Abstract

The synapsins are a family of neuronal phosphoproteins that have been previously shown to play an important regulatory role in the release of neurotransmitter from the presynaptic terminal and in the process of synaptogenesis. The mechanisms that regulate the formation of synaptic terminals are a central process in determining the specificity of synaptogenesis and the development of the nervous system. Proteins involved in neurotransmitter release and the control of this release process (specifically synaptic vesicle proteins) have been implicated as being important for synaptogenesis. To determine whether synapsin is expressed at times during mouse development when synaptogenic activity is high, we examined the time course of synapsin I and II mRNA and protein expression in embryonic and postnatal mice using Northern blot and Western blot analyses. Quantification of these blot analyses demonstrated that synapsin RNA and protein can be detected as early as 13.5 days of mouse embryogenesis and that expression of five of the six isoforms of synapsin increase throughout embryonic and postnatal development reaching characteristic high levels by adulthood. This early expression pattern suggests an important role for the synapsins in the development of the mammalian nervous system.

**Keywords:** Synapsin; Phosphoprotein; Synaptic vesicle protein; Gene expression; Mouse neuronal development; mRNA; Protein; Northern blot; Western blot

### Introduction

The three mammalian synapsin genes encode a family of neuronal synaptic vesicle phosphoproteins that are important for the control of neurotransmitter release from the presynaptic nerve terminal [1-5]. In the mouse, the synapsin I gene is located on chromosome X, the synapsin II gene on chromosome 6 and the synapsin III gene on chromosome 10. The primary transcript of each murine synapsin gene is alternatively spliced to produce two isoforms. The six isoforms comprising the mouse synapsin family each contain shared and unique domains. These genes encode peripheral membrane proteins that are associated with the cytoplasmic surface of synaptic vesicles in the presynaptic nerve terminal [1].

*In vitro* binding studies have shown that the synapsins bind to cytoskeletal components such as actin as well as purified synaptic vesicles in a phosphorylation state-specific manner [1,6,7]. Thus synapsin I is thought to tether synaptic vesicles to the cytoskeleton. The six isoforms of synapsin are all endogenous substrates for phosphorylation by numerous kinases including cyclic AMP-dependent protein kinase (protein kinase A) and calcium/calmodulin-dependent protein kinase I. The two isoforms of synapsin I are major substrates for calcium/calmodulin-dependent protein kinase II. The phosphorylation state of synapsin I was shown to regulate the release of neurotransmitter in the squid giant axon [8]. This regulation of synaptic transmission is thought to be controlled by the phosphorylation of synapsin I by calcium/calmodulin-dependent protein kinase II that results in a conformational change in synapsin I. The affinity of phosphorylated synapsin I for both actin and synaptic vesicles is decreased and relieves the constraint on the synaptic vesicles. This results in increases in the number of synaptic vesicles in the pools available for fusion with the cytoplasmic membrane and release of neurotransmitter [3].

Synaptogenesis is important both for the development of the complex circuitry of the brain and for plasticity in the functioning of the fully developed adult brain. The process of synaptogenesis is an important area of developmental neurobiology and advances in our understanding of synaptogenesis have implicated proteins involved in neurotransmitter release and the control of this release process

(specifically synaptic vesicle proteins) as important for synaptogenesis [9-13].

It has been shown that the synapsins are also important for the process of synaptogenesis [9,14]. Injection of synapsin I protein into developing *Xenopus* spinal neurons resulted in the functional maturation of these neurons [14]. Transfection of synapsin IIb into NG108-15 neuroblastoma/glioma hybrid cells was shown to induce the formation of presynaptic terminals and upregulate other synaptic vesicle proteins [9]. This work was extended to show that synapsin regulates the ability of NG108-15 cells to form functional synapses with muscle [15]. It has been shown that various other synaptic vesicle proteins may also have important regulatory roles in the process of synaptogenesis [10-13].

To determine whether synapsin is expressed at times during mouse development when synaptogenic activity is high, we examined the time course of synapsin I and II mRNA and protein expression in embryonic and postnatal mice using Northern blot and Western blot analysis. Quantification of these blot analyses demonstrated that synapsin RNA and protein can be detected as early as 13.5 days of mouse embryogenesis and that expression of the five isoforms of synapsin increases throughout embryonic and postnatal development reaching characteristic high levels by adulthood.

### Materials and Methods

#### Isolation of brain tissue from developmentally staged mice

RNA and protein were isolated from outbred CRL ICR Br (Charles River, Kingston) staged (timed) pregnant females and mice of a precise age.

\*Corresponding author: Michael RR, Department of Biology, University of Puerto Rico at Cayey, Puerto Rico, Tel: (787)-738-7473; E-mail: [michael.rubin@upr.edu](mailto:michael.rubin@upr.edu)

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**Embryos:** Pregnant females, at the appropriate time post-conception, were sacrificed by cervical dislocation and embryos were removed by dissection. Embryos were briefly placed on ice and then dissected into heads and bodies. The tissues were flash frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until use.

**Postnatal mice:** Litters of mice at various precise postnatal ages were sacrificed, and heads or brains were dissected and flash frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until use.

### **Purification of RNA from developmentally staged mouse head or brain**

RNA extracts were prepared from frozen head or brain tissue and quantified [16]. Approximately 0.5 g of frozen head or brain tissue from developmentally staged mouse head or brain was pulverized in liquid nitrogen using a mortar and pestle and 5 ml of denaturing solution (4 M guanidine thiocyanate, 20 mM sodium acetate, 0.5% Sarkosyl and 0.1 mM DTT) was added to the pulverized frozen tissue. The samples were vortexed, the solubilized tissue was passed through an 18 gauge needle 10 times to shear the DNA, 0.5 ml of 2 M sodium acetate (pH 4) was added, and the resulting solution was gently vortexed. 5 ml of phenol (pH 5.2) was added followed by gentle vortexing. One (1) ml of chloroform/isoamylalcohol (49:1) was added followed by gentle vortexing. The resulting solution was incubated on ice for 15 min followed by centrifugation at  $10 \text{ K} \times \text{g}$  for 20 min at  $4^{\circ}\text{C}$ . The upper aqueous phase containing the total RNA was precipitated by adding an equal volume of 100% isopropanol (5-6 ml), freezing at  $-20^{\circ}\text{C}$  for 30 min followed by centrifugation at  $10 \text{ K} \times \text{g}$  for 20 min at  $4^{\circ}\text{C}$ . The RNA pellet was dissolved in 0.3 ml denaturing solution by gentle vortexing and precipitated by adding 0.3 ml of 100% isopropanol and freezing at  $-20^{\circ}\text{C}$  for 30 min followed by centrifugation at  $10 \text{ K} \times \text{g}$  for 10 min at  $4^{\circ}\text{C}$ . The RNA pellet was resuspended in 75% ethanol, vortexed, and incubated for 15 min at room temperature. After centrifugation for 5 min at  $10 \text{ K} \times \text{g}$ , the pellet was air dried for 10 min and the RNA was resuspended in 50  $\mu\text{l}$  of RNase free DEPC treated water. The purified RNA was quantified by spectrophotometry at a wavelength of 260 nm.

### **Purification of protein from developmentally staged mouse head or brain**

Protein extracts were prepared from frozen head or brain tissue and quantified. Approximately 0.8 g of frozen head or brain tissue from developmentally staged mouse head or brain was placed in a screw capped microcentrifuge tube and 1.5 ml of boiling 1% SDS was added. The samples were sonicated for 15 s and then boiled for 30 s. The samples were sonicated again for 30 s and then boiled for 5 min. The samples were then centrifuged at  $15^{\circ}\text{C}$  for 15 min at  $2 \text{ K} \times \text{g}$  and the protein containing supernatant was transferred to a clean microcentrifuge tube. The protein samples were quantified using the BCA protein assay reagent system (Pierce, Rockford, IL).

### **Northern blots**

Northern blots were prepared using equal amounts (based on optical density measurements) of developmentally staged mouse brain total RNA and probed with rat synapsin I or II cDNA probes at high stringency. Twenty micrograms (20  $\mu\text{g}$ ) of developmentally staged mouse brain total RNA were electrophoresed on a 1.0% agarose gel containing formaldehyde and blotted onto nitrocellulose filters by capillary action from a reservoir of  $20 \times \text{SSC}$  [18]. The 18S and 28S ribosomal RNA served as molecular weight markers. Blots were prehybridized and subsequently hybridized to radioisotopically labeled probes (prepared by random primed labeling of purified rat synapsin Ib

[PS4A] and synapsin IIb [pSKB] cDNA fragments) in 50% deionized formamide in hybridization buffer and  $^{32}\text{P}$  labeled probe at  $42^{\circ}\text{C}$  for 16-20 h. Blots were washed to  $0.1 \times \text{SSC}$ , 0.1% SDS at  $65^{\circ}\text{C}$  and exposed in a Molecular Dynamics Phosphoimager and/or to XAR-5 X-ray film (Kodak) followed by film development. The integrity and amount of RNA on the blots were confirmed by the intensity of the 18S and 28S rRNA bands after ethidium-bromide staining and by rehybridization with labeled human beta-actin and/or human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) probes (Clontech, CA). Reacted bands were quantified using a Molecular Dynamics Phosphoimager and Imagequant software (Molecular Dynamics). Total RNA levels were normalized for each sample using RNA band intensities measured and quantified for the beta-actin and/or G3PDH hybridizations.

### **Western blots**

Western blots were prepared using equal amounts of developmentally staged mouse brain protein and probed with antibody recognizing synapsin isoforms (G357/G358). Fifty or 250  $\mu\text{g}$  of developmentally staged mouse brain proteins were separated by electrophoresis on a 7.5% polyacrylamide gel (with a 3.75% stacking gel) and transferred by electro blotting onto nitrocellulose membranes. After air-drying, the blots were washed in TBST (containing 0.05% Tween 20), and blocked using TBST with 5% dried milk. The blots were reacted (1:100 in TBST (with 0.05% Tween 20)) at room temperature with an antibody (G357/G358) that recognized synapsin isoforms (Ia, Ib, IIa, IIb and IIIa). After the antibody reaction, the blots were washed 3 times in TBST (with 0.05% Tween 20). The blots were then blocked in TBST with 5% milk and then incubated with a 1:500 dilution of  $^{125}\text{I}$  in TBST (with 0.05% Tween 20). The blots were then washed in TBST (with 0.05% Tween 20), air dried, wrapped in plastic wrap and exposed in a Molecular Dynamics Phosphoimager and/or to XAR-5 X-ray film (Kodak) followed by film development. Reacted bands were quantified using a Molecular Dynamics Phosphoimager and Imagequant software (Molecular Dynamics). Quantitative Western blots were prepared with known amounts of purified bovine synapsin I and varying diluted amounts of total rat or mouse brain homogenates and reacted with antibody recognizing synapsin isoforms (G357/G358).

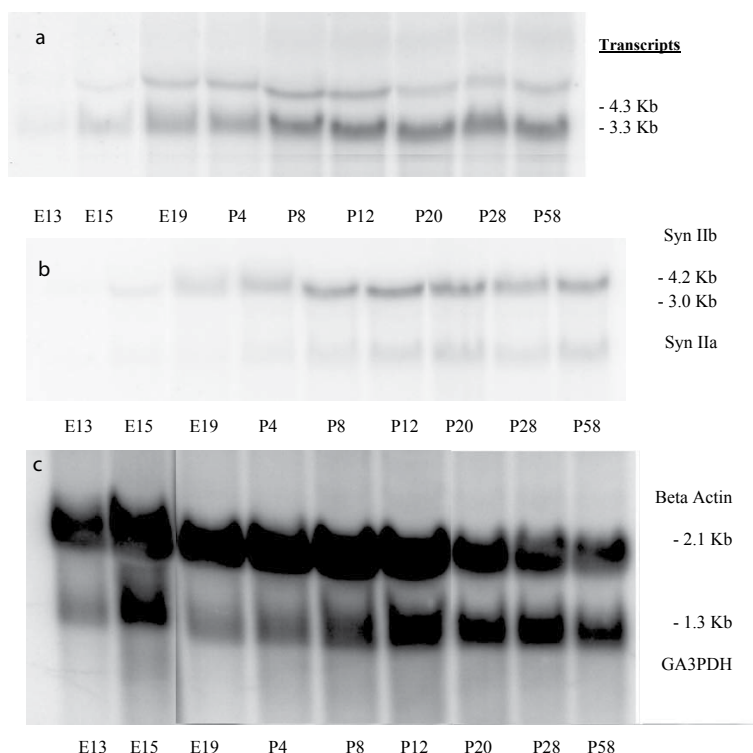
### **Quantification and analysis of results**

Northern and Western blots were exposed in a Molecular Dynamics Phosphoimager and reacted bands on Northern and Western blots quantified. Raw data was exported into Microsoft Excel. For the Northern blots, radioactivity (CPM) was normalized to actin levels and expressed as a percentage of the synapsin I 3.3 Kb transcript or the synapsin IIb transcript. The results were plotted according to the developmental stage. For the Western blot, standard curves were constructed to determine nanograms (ng) synapsin/micrograms ( $\mu\text{g}$ ) total protein. A previously measured ratio of 2:4:1:2 of synapsin Ia:Ib:IIa:IIb in adult brain was used to calculate ng synapsin isoforms/ $\mu\text{g}$  total protein for other synapsin isoforms in the different developmentally staged protein samples.

## **Results**

### **Analysis of synapsin mRNA levels during murine development**

Rat synapsin I and II cDNA probes were used in Northern blot analyses to detect and quantify synapsin I and II mRNA transcripts during prenatal and postnatal mouse development. The Northern blot shows the result obtained using the synapsin I cDNA probe (Figure 1A). An approximately 3.3 Kb hybridizing transcript (synapsins Ia and Ib) can be detected at embryonic day E13.5, the earliest stage examined. An



**Figure 1:** Expression of synapsin I (Figure 1A) and synapsin II (Figure 1B) mRNA during mouse development. Northern blot containing 20 µg of total RNA isolated from outbred CRL ICR Br (Charles River, Kingston) mouse embryonic brains at days (p.c.) E13.5 (lane 1), E15.5 (lane 2), E17.5 (lane 3), E19.5 (lane 4) and postnatal mouse brains at days P4 (lane 5), P8 (lane 6), P12 (lane 7), P16 (lane 8), P20 (lane 9), P28 (lane 10) and P58 (lane 11). After isolation and quantification, RNA was electrophoresed on 1.0% agarose gels containing formaldehyde, blotted onto nitrocellulose filters, and fixed by UV cross linking. The 18S and 28S rRNAs served as molecular weight markers. Blots were hybridized to probes prepared by random priming of rat synapsin I cDNA insert (Figure 1A) or rat synapsin II cDNA insert (Figure 1B). Blots were hybridized at 42°C for 12 to 16 hours. Blots were washed with 0.1X SSC - 0.1% sodium dodecyl sulfate at 68°C and exposed for 3 days at -70°C with an intensifying screen and also exposed and quantified using a Phosphoimager. The approximate sizes of the transcripts are shown in kilobases. The panel below the autoradiogram confirms that equal amounts of RNA were present in the lanes as determined by rehybridization to Actin and G3PDH probes (Figure 1C).

approximately 4.3 Kb (synapsins Ia and Ib) hybridizing transcript can first be detected at embryonic day E15.5. The Northern blot hybridized to the human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) and beta actin cDNA control probes used to normalize the synapsin RNA expression levels. Quantification of the synapsin I Northern blot. The 4.3 Kb transcripts reach characteristic levels found in the adult by embryonic day E19.5 (shortly before birth). In contrast, the 3.3 Kb transcript increases dramatically after birth and is approximately five times more abundant than the 4.3 Kb transcript at 58 days postnatal [P58] in the adult.

The Northern blot shows the result obtained using the synapsin II cDNA probe. The synapsin IIa transcript (approximately 3.0 Kb) can first be detected at embryonic day E15.5 (Figures 1B and 1C). The synapsin IIb transcript (approximately 4.2 Kb) can also first be detected at embryonic day E15.5. The Northern blot hybridized to the human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) and beta actin cDNA control probes used to normalize the synapsin RNA expression levels. Quantification of the synapsin II Northern blots (Figures 1C, 2A and 2B). Synapsin IIa and IIb transcripts reach characteristically high levels found in the adult by twenty (20) days after birth (P20). The synapsin IIb transcript is approximately two and a half times more abundant than the synapsin IIa transcript.

Synapsins I and II exhibit different time courses during development. Both genes are expressed at levels detectable by Northern blot analysis during mid-gestational mouse development. The mRNA

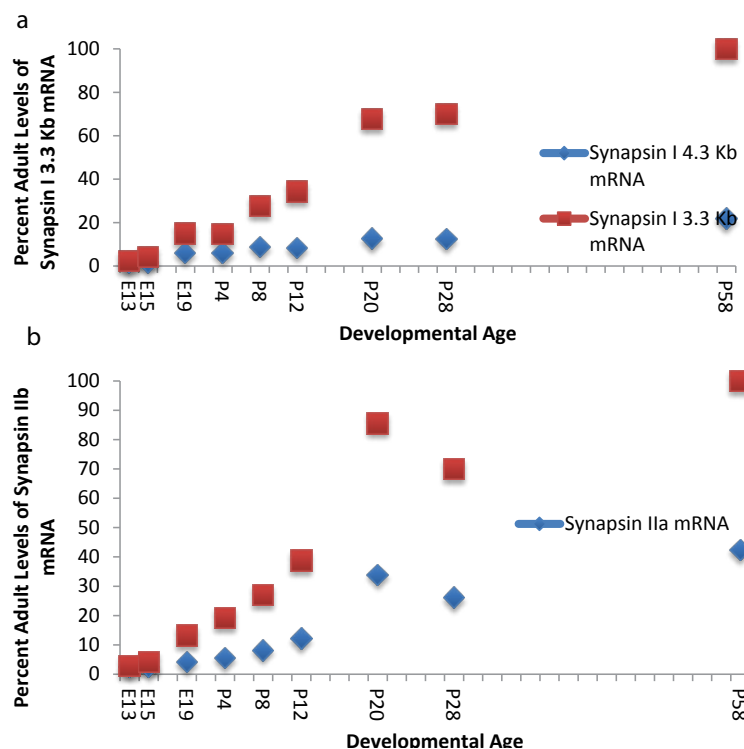
levels transcribed from these genes increase steadily until from mid gestation until adult.

### Analysis of synapsin protein levels during murine development

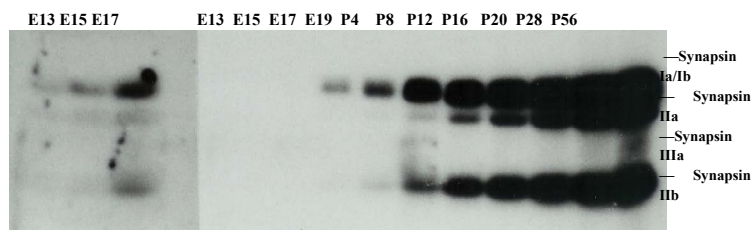
Monoclonal and polyclonal antibodies were used in Western immunoblot analyses to detect and quantify the isoforms of synapsin during mouse development. The Western blot shown shows the result obtained using an antibody that recognizes several isoforms of synapsin (synapsins Ia, Ib, IIa, IIb and III) (Figure 3). This antibody seems to have a lesser affinity for synapsin IIIa that is only detected in the adult. Reacting protein bands from synapsins I and II were detected by embryonic day E13.5, the earliest day examined. Although the synapsin proteins reach characteristically high levels found in the adult by approximately eight (8) days after birth (P8), levels continue to rise until adulthood (P58). The quantification of the synapsin I and II protein levels as detected by Western blot analyses (Figure 4). The synapsin isoforms are detectable during mid-gestational mouse development. Sharp increases are found from postnatal day four until postnatal day 28. Increasing levels are detected throughout embryonic and postnatal development until adult levels are reached at postnatal day 58 (P58).

### Discussion

The time course during mouse embryogenesis of synapsins I and II mRNA and protein expression was analyzed in embryonic



**Figure 2:** Synapsin I (Figure 2A) and synapsin II (Figure 2B) mRNA transcripts were imaged using a molecular dynamics phosphorimager (molecular dynamics) and quantified using Imagequant software. Raw data was exported into Excel (Microsoft). Radioactivity (CPM) was normalized to actin levels and expressed as a percentage of the synapsin I 3.3 Kb transcript or the synapsin IIb transcript. The results were plotted according to the developmental stage.



**Figure 3:** Expression of synapsin proteins during mouse development. Western blot containing 50  $\mu$ g (or 250  $\mu$ g) of protein isolated from out bred CRL ICR Br mouse embryonic brains at days (p.c.) E13.5 (lane 1), E15.5 (lane 2), E17.5 (lane 3), E19.5 (lane 4) and postnatal mouse brains at days P4 (lane 5), P8 (lane 6), P12 (lane 7), P16 (lane 8), P20 (lane 9), P28 (lane 10) and P58 (lane 11). After isolation and quantification, 50 or 250 micrograms of protein was electrophoresed on 7.5% SDS polyacrylamide gels (with a 2 cm 3.75% stacking gel) at 30 V for 18 h. Gels were electroblotted onto nitrocellulose filters at 30 V for 18 h. Blots were reacted to antibodies that recognize four isoforms of synapsin at 37°C for 2 h. Blots were washed with TBST and exposed for 16 h in a phosphorimager cassette, scanned using a phosphorimager and quantified using Imagequant software (Molecular Dynamics).

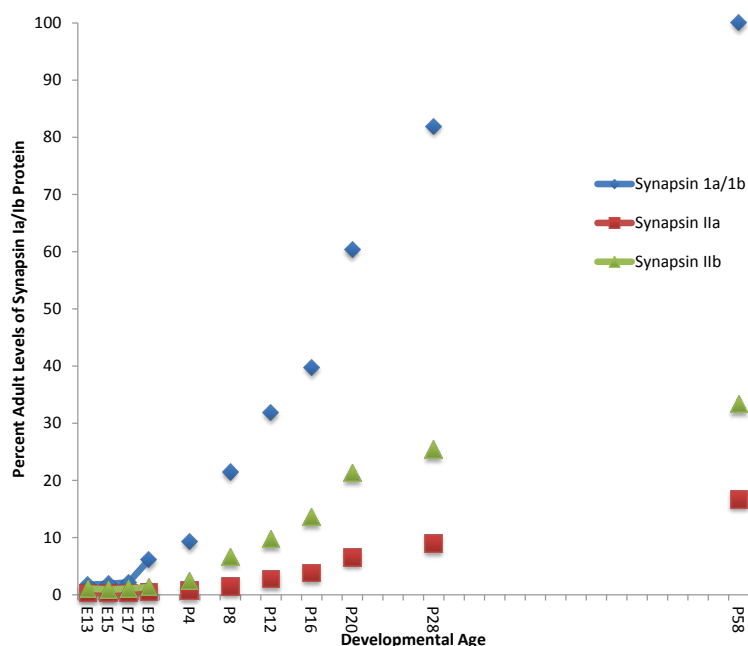
and postnatal mice using Northern blot and Western blot analyses. Quantification of these blot analyses demonstrated that synapsins I and II RNAs and proteins can be detected as early as 13.5 days of mouse embryogenesis and that expression of four of the synapsin isoforms increase throughout embryonic and postnatal development reaching characteristic high levels found shortly after birth and in adulthood.

Northern and Western blots were used to detect and analyze levels of mRNA and protein, respectively. These techniques permit the sizes of the expressed products to be directly compared to determine if there are any changes during this period of development. Differences in synapsin I transcript sizes, although it is not clear whether this is an artifact due to anomalies during the gel electrophoresis (Figure 1A). For synapsin IIb, a size difference is observed in the mRNA with slightly larger transcript sizes around the time of birth after E15 and before P8, including days E19 and

P4. Since Northern blot analysis was used to determine mRNA levels, we can not distinguish between increased mRNA stability and increased *de novo* expression of the synapsin I and II genes. Additional transcription experiments are necessary to elucidate the molecular mechanisms involved in the increase in synapsin transcripts found during mouse development.

To determine the synapsin protein levels during murine development, it was assumed that the various isoforms are expressed, at the earliest times detected and throughout embryogenesis and postnatal development, in the 2:4:1:2 (SynIa:SynIb:SynIIa:SynIIb) proportion characteristic of adult animals. This protein ratio was used as a basis for the calculations carried out to determine synapsin quantification during development. These experiments were normalized using Western blots containing a series of predetermined concentrations of synapsin proteins (data not shown).





**Figure 4:** Quantification of synapsin protein levels during murine development Synapsin I and II proteins were imaged using a molecular dynamics phosphoimager and quantified using Imagequant software. Raw data was exported into Microsoft Excel. Radioactivity (CPM) was plotted according to developmental age.

Differences in post translational modifications, specifically phosphorylation states of the synapsin isoforms, would not be detected in the Western blot experiment as the modified phosphorylation states of the synapsin proteins would be expected to comigrate during electrophoretic separation (Figure 3). This study does not address the relative abundance of the multiple phosphorylation states and combinations of states of the various synapsin isoforms during development. Synapsin protein sizes seem to be unchanged throughout the period of development examined in these studies.

From a comparison of synapsin mRNA and protein levels quantified in, it can be observed that the amount of synapsin IIa and IIb mRNA levels decreases after postnatal day 20 (P20) to lower levels at P28 and thereafter increases to higher adult quantities by P58 (Figure 2B). Synapsin IIa and IIb protein levels continue to increase during this same period to reach their adult levels at P58, not demonstrating a reduction in amounts of protein at P20 (Figure 4). Enhanced protein translation or stability could account for these differences. The Synapsin Ia and Ib mRNA and synapsin I protein levels are not observed to exhibit reduction in amounts between P20 and P58 (Figure 2A).

The expression of synapsin mRNA and protein reported here is supported by previous work using RT-PCR, Western blots, and immunohistochemical staining of brain sections [17,19]. Both synapsins I and II were detected using RT-PCR in total RNA purified from mouse lenses isolated at postnatal days 21 and 42 as well as detected using Western blot analyses of adult mouse brains and lenses [17]. Synapsin I was detected using immune histochemical staining of brain sections at embryonic day eighteen [19]. Direct sequence analyses of synapsin mRNAs during embryonic and postnatal development could be used to quantitatively determine the relative abundance of the synapsin gene products during these developmental stages.

The expression of the synapsin gene family at early times during embryogenesis is coincidental to a major period of synaptogenesis during

development. This could indicate a role for the synapsins in synaptogenesis by promoting axonal outgrowth or targeting. The function of the synapsins in synaptogenesis could result from the involvement of synapsin in the regulation of neurotransmitter release and the importance of such release in the formation of functional synapses.

Synapsin is an important molecule with a role in the regulation of neurotransmitter release and in the formation of functional synapses. Further studies are needed to elucidate the functions of the synapsins during mouse embryogenesis and the molecular mechanisms whereby this family of proteins contributes to the development and maintenance of the functional nervous system.

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