Constitutive expression of heat shock proteins hsp90, hsc70, hsp70 and hsp60 in the rat during postnatal development

by

Sandra Maria D'Souza

A thesis in conformity with the requirements
for the degree of Master of Science
Graduate Department of Zoology
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This thesis examines the expression of heat shock proteins hsp90, hsc70, hsp70 and hsp60 in neural and non-neural rat tissues during postnatal development. Western blot analyses showed abundant levels of hsp90 and hsc70 in the brain throughout postnatal development. Levels of hsp60 (which is localized primarily to mitochondria), and cytochrome oxidase, subunit IV (a mitochondrial marker), increased during development, reflecting a developmental elevation in mitochondrial content. In addition, low basal levels of hsp70 were detected throughout postnatal development in certain brain regions. In the developing kidney, hsp90 and hsc70 levels decreased while hsp70, hsp60 and cytochrome oxidase, subunit IV levels increased. Tissue-specific differences in the relative levels of these heat shock proteins were also evident. Hsp90, hsc70, hsp60 and cytochrome oxidase, subunit IV were all localized to neurons in the developing and adult brain by immunocytochemistry.

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ABBREVIATIONS

Ad	adult
ATP	adenosine triphosphate
BS	brain stem
BSA	bovine serum albumin
c	cytoplasm
°C	degrees Celsius
Cb	cerebellum
CH	cerebral hemispheres
C.OX IV	cytochrome oxidase, subunit IV
DAB	3,3'-diaminobenzidine
DCN	deep cerebellar nuclei
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
grp	glucose regulated protein
HBD	hormone binding domain
H_2O_2	hydrogen peroxide
hr	hour
hsc	heat shock cognate
HSE	heat shock element
HSF	heat shock factor
hsp	heat shock protein
IgG	immunoglobulin G
Kid, K	kidney
L	liver
µg	microgram
µm	micrometer
M	molar
mM	millimolar
P	Purkinje neuron
p	neuronal process
PBS	phosphate buffered saline
PBT	phosphate buffered saline (0.02% Trition X-100, 0.01% BSA)
mRNA	messenger ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TBST	Tris buffered saline Tween-20

Figures 1, 2, 4, 5, 7, 9 and 11 of this thesis have been submitted for publication. This is to certify that I, Sandra M. D'Souza, carried out the research documented in the following publication.

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1. INTRODUCTION

1.1 Overview

Heat shock proteins (hsps) are a set of proteins which are up-regulated in cells following elevated temperatures and other forms of cellular stress, and are thought to play a repair and protective role (Parsell and Lindquist, 1993; Welch, 1995). In addition to being required during stressful conditions, most hsps are also needed in cells under normal physiological conditions. In the unstressed cell, hsps participate in protein folding, degradation and transport (Gething and Sambrook, 1992; Hartl *et al.,* 1994). They have also been shown to be essential for normal cellular growth and maintenance (Lindquist and Craig, 1988) and function in cell division and differentiation during development (Heikkila, 1993; Walsh *et al.,* 1993).

Previous studies from our laboratory have shown that following physiologically relevant hyperthermia, hsp70 mRNA and protein are induced primarily in glial cells of the mammalian brain (Manzerra *et al.,* 1993; Foster and Brown, 1997). Induction of hsp70 protein is not detected in certain populations of large neurons, such as Purkinje cells of the cerebellum. These same neurons exhibit high constitutive levels of other heat shock proteins, such as hsp90 and hsc70, which may dampen the

induction of hsp70 following hyperthermia (Quraishi and Brown, 1995; Manzerra *et al.,* 1997). In addition, basal levels of hsp70 mRNA are detected in hippocampal and cortical neurons (Foster and Brown, 1996a). This thesis examines the developmental expression of the heat shock proteins hsp90, hsc70, hsp70 and hsp60 during postnatal development of the rat, using Western blotting and immunocytochemistry.

1.2 The heat shock response

In 1962, F.M. Ritossa showed that elevated temperature induced a specific puffing pattern in the polytene chromosomes of *Drosophila* salivary glands. Tissières (1974), later demonstrated that these chromosomal puffs corresponded to sites of induction of mRNAs encoding a group of proteins, now known as heat shock proteins. Since these early studies, this 'heat shock response' has been shown to occur in all organisms following other metabolic insults, such as exposure to amino acid analogs (Kelly and Schlesinger, 1978; Hightower, 1980; Welch and Suhan, 1986), and glucose deprivation (Lee *et al.,* 1983; Lee, 1987).

A main trigger of the heat shock response seems to be the presence of denatured and aggregated proteins. Injection of denatured, but not native proteins into *Xenopus laevis* oocytes resulted in the increased expression of a reporter gene driven by a heat shock promoter (Ananthan *et al.,* 1986).

Mifflen and Cohen (1994), extended this study and showed that aggregation of denatured proteins is a key factor in this heat shock response. Hsp70 is the major heat shock protein induced following a physiological insult, consequently, hsp70 expression has been used as an indicator of cellular stress (Nowak *et al.*, 1990).

The heat shock response is considered a universal defense mechanism. Heat shock proteins are thought to protect cells from the damaging effects of cellular trauma. Riabowol *et al.*, (1988), investigated the protective effects of hsp70 and demonstrated in cultured fibroblasts that microinjection of antibodies against hsp70 resulted in cell death following an otherwise non-lethal heat shock. Heat shock proteins such as hsp70 are thought to protect compromised cells by (i) binding to and preventing aggregation of damaged or unfolded proteins (Kampinga, 1993), (ii) participating in the renaturation/repair of denatured proteins (Ohtsuka and Laszlo, 1992; Brown *et al.*, 1996), and (iii) promoting degradation of abnormal proteins under both normal and stress conditions by presenting them to proteases within lysosomes (Chiang *et al.*, 1989; Hayes and Dice, 1996).

1.3 The heat shock proteins

Heat shock proteins can be classified into major families based on

their molecular weight (Welch, 1992). These include hsp100s, hsp90s, hsp70s, hsp60s and hsp20s. This thesis focuses on hsp90, hsp60 and two members of the hsp70 family of proteins, the stress-inducible hsp70 and the constitutively expressed hsc70.

1.3.1 Hsp90

Hsp90 is an abundant, cytosolic protein which is found in association with a variety of other intracellular proteins including calmodulin (Minami *et al.,* 1993), actin (Nishida *et al.,* 1986), tubulin (Sanchez *et al.,* 1988), several kinases (Oppermann *et al.,* 1981; Rose *et al.,* 1987) and steroid receptors (Kang *et al.,* 1994). Hsp90 exhibits general chaperone functions, that include protein folding (Wiech *et al.,* 1992), intracellular trafficking (Pratt, 1993), and stabilizing target proteins in an inactive or unassembled state (Jakob and Buchner, 1994).

One major function of hsp90 is in modulating the activities of steroid hormone receptors and protein kinases (Ziemiecki *et al.,* 1986; Xu and Lindquist, 1993). Hsp90 forms hetero-oligomeric complexes with other proteins such as hsp70 (Sanchez *et al.,* 1990a) and hsp56 (Sanchez *et al.,* 1990b). These heterocomplexes function in maintaining steroid receptors, such as the glucocorticoid receptor, in an inactive, unliganded state (for review, see Pratt and Welsh, 1994). Hsp90 binds to the hormone binding

domain (HBD) of steroid receptors. When the appropriate hormone binds to the HBD of the steroid receptor, hsp90 dissociates from the receptor and the receptor moves into the nucleus and activates transcription. Hsp90 binding to the glucocorticoid receptor has been shown to be necessary for efficient hormone binding and subsequent transcriptional activation by the receptor (Bresnick *et al.,* 1989).

Certain protein kinases, such as the oncogenic Rous sarcoma virus transforming protein pp60 $^{v\text{-}src}$, also exist in heterocomplexes containing hsp90, hsp70 and p50, a 50kDa protein of unknown function (Brugge, 1986; Whitelaw *et al.,* 1991). This heterocomplex is involved in the maturation of pp60 $^{v\text{-}src}$ and in the translocation of the protein to the plasma membrane. As mentioned above, hsp90 binds to actin (Koyasu *et al.,* 1986) and tubulin (Sanchez *et al.,* 1988) and it is through this interaction with these cytoskeletal proteins that hsp90 is thought to function in intracellular trafficking of various target proteins.

Hsp90 exists as a dimer, exhibits ATPase activity and undergoes autophosphorylation (Nadeau *et al.,* 1993). Mammalian hsp90 consists of two isoforms, hsp90α and hsp90β, which are encoded by separate genes (Moore *et al.,* 1987; Moore *et al.,* 1989). Hsp90 exists as either homodimers of α-α, or β-β in the mouse (Minami *et al.,* 1991). Dimerization occurs through the carboxy-terminal region (Minami *et al.,* 1994). Hsp90 comprises 1-2% of total cellular protein (Lai *et al.,* 1984) and is found in

abundant levels within the adult mammalian brain (Itoh *et al.,* 1993), where it is localized to neurons (Izumoto and Herbert, 1993; Quraishi and Brown, 1995).

1.3.2 Hsp70/hsc70

The hsp70 multigene family of proteins includes (i) the stress-inducible hsp70 (Wu *et al.,* 1985), found in the cytoplasm and nucleus, (ii) the cytoplasmic and constitutively expressed hsc70 (Chappell *et al.,* 1986), (iii) grp75 (or mitochondrial hsp70), localized to the mitochondria (Mizzen *et al.,* 1989), and (iv) grp78 (BiP), localized to the endoplasmic reticulum (Munro and Pelham, 1986). Other hsp70-related proteins exist, such as the testis-specific hsp70.2 or hst70 in mouse and rat, respectively (Zakeri *et al.,* 1988; Wisniewski *et al.,* 1990).

Both hsp70 and hsc70 bind to and stabilize nascent polypeptides before assembly and aid in the translocation of proteins into organelles, such as the endoplasmic reticulum and the mitochondria (Beckmann *et al.,* 1990; Brown *et al.,* 1993). The structure of all hsp70-related proteins is conserved. A typical hsp70 protein contains an ATP-binding site located at its N-terminal domain and a peptide binding domain at the C-terminal (Flaherty *et al.,* 1990). Hsc70 self-associates into dimers and trimers (Benaroudj *et al.,* 1995) via its C-terminal peptide binding domain

(Benaroudj *et al.*, 1997). Unfolded proteins tend to promote the dissociation of hsc70 oligomers into monomers (Benaroudj *et al.*, 1996). Monomers of hsc70 bind to unfolded proteins in an ATP-dependent manner (Palleros *et al.*, 1991). Polymerization of hsc70 may serve to store it in an inactive state (Gao *et al.*, 1996).

Hsc70 mRNA and protein are highly expressed within neurons of the adult mammalian brain (Aquino *et al.*, 1993; Manzerra *et al.*, 1993; Foster *et al.*, 1995). In addition, basal levels of hsp70 mRNA are localized to neurons in the unstressed adult brain (Foster and Brown, 1996a, 1996b). Hsc70 was first identified as the ATP-dependent enzyme that is involved in removing clathrin coats from endocytic vesicles (Schlossman *et al.*, 1984; Chappell *et al.*, 1986). In neurons, it may function in this manner in the synaptic vesicle recycling pathway (Maycox *et al.*, 1992). Hsc70 has also been implicated in axonal transport (de Waegh and Brady, 1989; Black *et al.*, 1991) as well as neuronal signaling (Thekkuveettil and Lakhotia, 1996).

1.3.3 Hsp60

Hsp60, also known as chaperonin 60, belongs to a class of proteins termed 'chaperonins', which includes hsp10, as well as a distant homologue of hsp60, the Tcp-1 proteins (Gupta, 1995). These proteins serve as molecular chaperones which promote protein folding and assembly (Martin,

1997). Hsp60 is a highly conserved protein, which is localized to mitochondria and plant chloroplasts (for review, see Parsell and Lindquist, 1993). Mammalian hsp60 exhibits a high degree of homology (40-50%) to the GroEL protein of *E.coli*, the Rubisco subunit binding protein of chloroplasts, and to the 65kDa 'common antigen' of mycobacteria (Jindal *et al.*, 1989). Hsp60 was first identified in *Tetrahymena thermophila* as a heat-inducible mitochondrial protein (McMullin and Hallberg, 1987).

In bacteria, fungi and plants, hsp60 homologues exist as double-ring complexes consisting of fourteen 60kDa subunits. Mammalian hsp60 is made up of seven 60kDa subunits, which form a single toroidal structure (Viitanen *et al.*, 1992). Within the mitochondria, hsp60 aids in the folding and assembly of proteins into oligomeric structures (Cheng *et al.*, 1989; Hallberg *et al.*, 1993). This process has been shown to be ATP-dependent (Ostermann *et al.*, 1989). A co-chaperonin, hsp10, regulates the ATP hydrolytic activity of hsp60 (Dickson *et al.*, 1994).

1.3.3.1 Mitochondrial protein import

Import of nuclear-encoded proteins into the mitochondria requires the cooperation of a number of cytosolic as well as mitochondrial molecular chaperones (Ryan *et al.*, 1997). Following translation of most nuclear-encoded mitochondrial proteins within the cytosol, an amino-terminal leader

sequence targets the precursor protein to the mitochondria (Hartl *et al.*, 1989). Mitochondrial targeting signals are typically 15 - 40 amino acid residues long and are rich in hydrophobic and basic residues (von Heijne, 1986; Hartl *et al.*, 1989). Cytosolic hsc/hsp70 is thought to bind to the preprotein and maintain it in a translocation-competent conformation (Murakami *et al.*, 1988). Proteins are transported into mitochondria through protein channels at contact sites between inner and outer membranes (Pfanner *et al.*, 1990). Import of proteins into the mitochondria, requires the presence of the mitochondrial membrane potential, $\Delta\Psi$, across the inner membrane (Schleyer *et al.*, 1982), as well as ATP hydrolysis in the matrix (Ungermann *et al.*, 1994). Within the matrix, mitochondrial hsp70, also called grp75, binds to the precursor protein and initiates the translocation process (Kang *et al.*, 1990; Ungermann *et al.*, 1994; Horst *et al.*, 1997). The presequence is proteolytically cleaved within the mitochondrial matrix and the protein is usually transferred to hsp60 where its folding and assembly begins. It should be noted that not all mitochondrial proteins require hsp60 in order to fold. Following completion of folding, proteins are sorted to the appropriate mitochondrial subcompartment (Glick *et al.*, 1992).

1.4 The heat shock response in the mammalian brain

Heat shock proteins are induced in the mammalian brain in response to various stresses, such as hyperthermia, ischemia and tissue injury (for review, see Brown, 1994; Nowak, 1994). Following a physiologically relevant increase in temperature, hsp70 mRNA and protein are induced primarily in glial cells within the mammalian brain (Marini *et al.*, 1990; McCabe and Simon, 1993; Manzerra *et al.*, 1993; Foster *et al.*, 1995). Induced hsp70 mRNA is localized to oligodendrocytes and microglia in the hyperthermic rabbit forebrain (Foster and Brown, 1997). Induction of hsp70 protein is not detected in certain populations of large neurons, such as the Purkinje cells of the cerebellum. High endogenous levels of other heat shock proteins such as hsc70 and hsp90 may dampen the heat shock response in neurons following hyperthermia (Manzerra *et al.*, 1993; Quraishi and Brown, 1995; Manzerra and Brown, 1996). In contrast to the glial induction of hsp70 that is observed following hyperthermia, a neuronal induction of hsp70 mRNA and protein as well as hsp90 mRNA is observed in the mammalian brain following ischemia (Vass *et al.*, 1988; Aoki *et al.*, 1993; Kawagoe *et al.*, 1993). In addition, tissue injury of the rat brain results in the induction of hsp70 mRNA in both neurons and glial cells (Brown *et al.*, 1989; Gower *et al.*, 1989). These results imply that different types of neural stress induce characteristic responses in specific brain cell

types. Endogenous levels of stress proteins may affect the extent to which a particular cell responds to stress (Manzerra *et al.*, 1993).

1.5 Regulation of heat shock gene expression

Heat shock gene expression is mediated via a family of heat shock transcription factors, known as HSFs (for review see Morimoto *et al.*, 1994; Scharf *et al.*, 1994). HSF1, which exists as two isoforms (HSF1α and HSF1β), is activated to a DNA-binding form in cells exposed to stressful conditions, such as heat shock (Sarge *et al.*, 1993), or oxidative stress (Benjamin *et al.*, 1990), and thus is considered a mediator of the heat shock response. Under normal physiological conditions, HSF1 exists as a monomer and is maintained in a non-DNA binding state via hydrophobic interactions between its amino- and carboxy-terminal domains (Rabindran *et al.*, 1993; Zuo *et al.*, 1994). In response to a stressful stimulus, HSF1 trimerizes (Sarge *et al.*, 1993) and binds to consensus sequences known as heat shock elements (HSEs) within the promoter regions of heat shock genes. This triggers transcription of heat shock proteins, such as hsp70 (Fernandes *et al.*, 1994). An HSE consists of multiple arrays of the sequence AGAAn (Amin *et al.*, 1988). As hsp70 accumulates, it is thought to negatively regulate HSF1 by binding to it (Abravaya *et al.*, 1992; Baler *et al.*, 1996). HSF1 then dissociates from the DNA and returns to its original monomeric

state. In addition to hsp70, other heat shock proteins, such as hsp90 (Nadeau *et al.,* 1993) and hsc70 (Lis and Wu, 1993; Morimoto, 1993) are also thought to interact with HSF1.

HSF2 may activate heat shock gene expression in the absence of stress, such as during differentiation or developmental processes. Activation of HSF2 was demonstrated during hemin-induced differentiation of human K562 erythroleukemia cells (Sistonen *et al.,* 1992). This activation correlated with the induction of hsp70, hsp90 and grp78. HSF2 exists in the non-DNA binding state as a dimer (Sistonen *et al.,* 1994). Two isoforms of HSF2 exist, HSF2α and HSF2β, formed by alternative splicing. Results from Leppa *et al.,* (1997) suggested that the HSF2β isoform negatively regulates the activation of HSF2α during hemin-mediated erythroid differentiation. When activated, HSF1 and HSF2 have been shown to interact differently with HSEs (Kroeger *et al.,* 1993). Simultaneous activation of both HSF1 and HSF2 resulted in the synergistic induction of hsp70 gene expression in human erythroleukemia cells, suggesting a complex regulatory mechanism (Sistonen *et al.,* 1994).

1.6 Developmental expression and regulation of heat shock proteins

Expression of heat shock genes as well as their transcriptional activators has been shown to be developmentally regulated in a number of

processes including mammalian embryogenesis and spermatogenesis. These systems are frequently used to study differentiation and developmental phenomena (Bensaude *et al.*, 1991).

1.6.1 Mammalian embryogenesis

Heat shock proteins are present very early in mammalian embryogenesis. Members of the hsp70 family of proteins are among the first products of zygotic genome activation at the 2-cell stage in the preimplantation mouse embryo (Bensaude *et al.*, 1983). HSF1 transcripts have been reported to be present as early as the 1-cell stage in the embryonic mouse (Christians *et al.*, 1997). HSF1 at this stage is considered a maternal factor, and is thought to be involved in the constitutive expression of the hsp70 gene at the onset of zygotic genome activation. HSF2 is present and exhibits constitutive DNA-binding activity at the blastocyst stage of mouse development (Mezger *et al.*, 1994). Interestingly, examination of HSF2 levels, HSF2 activity and heat shock protein expression during the postimplantation phase of mouse embryogenesis revealed no apparent correlation between these phenomena (Rallu *et al.*, 1997).

1.6.2 Spermatogenesis

Expression of heat shock proteins including hsp70 and hsp90 family members has been localized to immature germ cells in the adult rat and mouse testes (Zakeri and Wolgemuth, 1987; Lee, 1990). Hsp70 protein was concentrated in spermatocytes and spermatids of the adult rat testes (Raab *et al.*, 1995). In addition, mitochondrial hsp60 was observed in spermatogonia and primary spermatocytes (Meinhardt *et al.*, 1995). Concurrent with these results, Sarge *et al.*, (1994) reported on the nuclear localization of HSF2 mRNA and protein in spermatocytes and round spermatids in the mouse testis. In addition, HSF2 exhibited constitutive DNA binding activity, suggesting a role for this transcription factor as a regulator of heat shock protein expression during mammalian spermatogenesis. Goodson *et al.*, (1995) reported that HSF2α is the predominant isoform in the mature mouse testis, as well as a more potent transcriptional activator than HSF2β. These studies demonstrate that heat shock proteins function during developmental events, such as spermatogenesis.

1.7 Thesis objectives

1. Our previous studies have shown that hsp90 and hsc70 mRNA and protein are present at abundant levels in certain large neurons in the adult mammalian brain. In addition, basal levels of hsp70 mRNA are found in forebrain neurons. In order to investigate when this constitutive expression of heat shock proteins is attained during postnatal neuronal development, the developmental expression of heat shock proteins hsp90 and hsc70 was examined using immunocytochemistry. Additionally, the localization of hsp60 in the developing brain was studied to observe whether this heat shock protein exhibited a similar neuronal pattern of expression as that seen for hsp90 and hsc70.

2. Western blot analysis was carried out on rat tissues using antibodies specific to hsp90, hsc70, hsp70 and hsp60 to determine the developmental profiles of these heat shock proteins through postnatal development. In addition, the developmental profile of cytochrome oxidase subunit IV was compared with that of hsp60, another mitochondrial protein.

3. The relative levels of heat shock proteins between various neural and non-neural rat tissues was investigated at postnatal day 1 and in the adult in order to observe any tissue-specific differences in the expression

of these heat shock proteins at these two developmental stages.

4. Examination of the developing kidney by both Western blotting and immunocytochemistry using antibodies to hsp90, hsc70, hsp70 and hsp60 was carried out so that a comparison could be made between the brain regions and a non-neural tissue.

2. MATERIALS AND METHODS

2.1 Western blot analysis

2.1.1 Isolation of protein homogenates

Wistar rats (Charles River) were sacrificed by decapitation at postnatal days 1, 5, 10, 15, 20 and 90 days (adult). Tissue was dissected from brain regions, kidney and liver and homogenized in 0.32M sucrose. For each stage of development, samples from two or three rats were pooled for a given brain region or tissue type. Protein concentrations were determined using the BioRad protein assay. Tissue samples were stored at -20°C.

2.1.2 One-dimensional gel electrophoresis

Protein samples were solubilized by boiling for 5 min with an equal volume of dissociation buffer (8M urea, 2% SDS, 2% β-mercaptoethanol, 20% glycerol). Polyacrylamide gel electrophoresis was carried out in the presence of SDS on either 10% (for hsp90, hsc70 and hsp60) or 12% (for hsp70 and C.OX IV) gels with 5% stacking gels using the discontinuous buffer system of Laemmli (1970). Either 15μg of protein (for hsp90 and

hsp60), 30µg of protein (for hsc70) or 100µg of protein (for hsp70) was loaded per lane. Equal loading of protein in the gel lanes was tested by staining parallel gels with Coomassie Blue stain. The proteins were electrophoretically transferred onto nitrocellulose membranes for 16 - 18 hr in a solution of 50mM boric acid, 4mM β-mercaptoethanol and 2mM EDTA, at 400 mA. The blots were briefly stained with Ponceau S in order to verify equal loading and efficient transfer of protein, and then processed for Western blot analysis.

2.1.3 Western blot procedure

For Western blotting, the blots were washed 4 x 5 min in TBST buffer (10mM Tris, 0.25M NaCl, 0.5% Tween-20, pH 7.5), blocked for 1 hr at room temperature in 5% Carnation milk powder in TBST buffer, and then incubated overnight in primary antibody (see 2.1.4 for dilutions of antibodies) in 1% purified BSA in TBST. Following incubation with primary antibody, blots were washed 4 x 10 min in 1% BSA (Sigma grade) in TBST, incubated for 2 hr at room temperature with horseradish peroxidase-coupled secondary antibodies (Sigma), anti-mouse IgG diluted 1:5000 in 1% BSA in TBST (for monoclonal antibodies), or with anti-rabbit IgG diluted 1:10,000 in 1% BSA in TBST (for polyclonal antibody), and then washed 6 x 5 min in TBST. Immunoreactive bands were visualized by use

of enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, RPN 2106). All antibodies used produced a signal which was specific for the respective protein being analyzed. Non-specific bands were not detected with any of the antibodies.

Some of the Western blots that were originally incubated with the anti-hsp90 antibody (Figure 1A) were stripped with 0.1M sodium citrate, pH 3.5, and processed again using the anti-hsp60 antibody (Figure 7A). Similarly, some of the immunoblots probed with the anti-hsp70 antibody (Figure 11A) were stripped and reprobed with the anti-C.OX IV antibody (Figure 9A). Exposed X-ray film was scanned using a BioRad GS-700 imaging densitometer. Bar graphs represent data from two to four sets of animals. Values at postnatal day 1 (P1) were standardized to a value of 1.0. Error bars indicate the SEM.

2.1.4 Antibodies used for Western blot analysis

The primary antibodies which were diluted in 1% purified BSA in TBST and used for Western blot analysis were as follows:

1) '29A' mouse monoclonal anti-rat hsp90 (gift from A.C. Wikström, Karolinska Inst., Huddinge, Sweden) diluted 1:5000.
2) '1477' rabbit polyclonal anti-human hsc70 (gift from R. Tanguay, Laboratory Inst. of Cellular and Molecular Genetics, Quebec) diluted

1:50,000.

3) mouse monoclonal anti-hsp60 (gift from R. Gupta, McMaster University, Ontario) diluted 1:16,000.
4) '20E8' mouse monoclonal anti-bovine cytochrome oxidase subunit IV (Molecular Probes, A-6431) diluted 1:10,000.
5) 'C92' mouse monoclonal anti-human hsp70 (StressGen, SPA810) diluted 1:5000.

The specificity of antibodies #1, 4, and 5 has been demonstrated (Welch and Suhan, 1986; Akner *et al.*, 1992; Taanman *et al.*, 1993).

2.2 Immunocytochemical analysis

2.2.1 Preparation of tissue

Rats were perfused intracardially with 4% paraformaldehyde in 0.1M PBS, pH 7.4. Brain and kidney tissue were removed and placed in 4% paraformaldehyde overnight at 4°C and then allowed to equilibriate in 25% sucrose in PBS. The tissue was then mounted in OCT embedding compound (Miles Inc.), and kept at -70°C until use. 20µm cryostat sections were floated on water and collected on gelatin-coated glass microscope slides (1% gelatin, 0.05% chromium potassium sulfate) and air-dried overnight.

2.2.2 Immunocytochemistry protocol

Brain and kidney tissue sections were rehydrated for 30 min at room temperature in PBT buffer (0.1M PBS, pH 7.4, 0.2% Triton-X 100, 0.1% BSA), and then blocked for 1 hr in PBT buffer with 1.5% normal horse serum (for monoclonal antibodies) or 2.0% goat serum (for polyclonal antibody). Sections were incubated overnight at room temperature in primary antiserum diluted 1:100 for hsp90, 1:500 for hsc70, 1:200 for hsp60, and 1:200 for C.OX IV. After washing for 2 x 5 min in PBT buffer, sections were incubated in biotinylated rat-adsorbed anti-mouse IgG (for monoclonal antibodies) diluted 1:200, or biotinylated anti-rabbit IgG (for polyclonal antibody) diluted 1:400 for 1.5 hr at room temperature. Following another 2 x 5 min wash in buffer, sections were incubated in 0.3% H_2O_2 in methanol for 30 min to block any endogenous peroxidase activity. After a 20 min wash in buffer, sections were processed with the Vectastain Elite ABC kit (Vector Labs, Burlingame,CA). Diaminobenzidine (DAB) was used as the chromagen. As negative controls, the primary or secondary antibody were individually omitted from the protocol. Under these conditions, immunostaining was not detected. Data shown are representative of independent experiments carried out on three sets of animals. A Zeiss Axiophot microscope was used with either 100X or 40X objective lenses to photograph cells in Figures 3, 4, 6, 8, and 10.

3. RESULTS

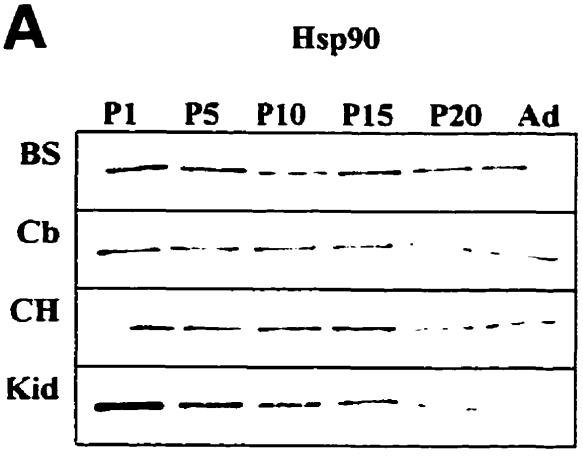
3.1 Developmental analysis of hsp90 protein levels

A Western blot analysis of hsp90 protein levels during postnatal development of the rat was carried out. As shown in Figure 1A, hsp90 was present at abundant levels throughout postnatal development from postnatal day 1 (P1) to the adult (Ad) in the brain stem (BS), cerebellum (Cb) and cerebral hemispheres (CH). In the kidney (Kid), a dramatic developmental decrease in hsp90 was observed. Exposed X-ray film was scanned with an imaging densitometer and histograms were constructed (Figure 1B). These results confirmed the marked developmental decrease in hsp90 in the kidney. In the brain regions, little developmental change in hsp90 levels was observed.

A comparison of hsp90 levels between various adult (Ad) rat tissues (Figure 2A) showed hsp90 protein levels to be greater in neural regions such as the brain stem, cerebral hemispheres, and cerebellum, than in non-neural tissues, such as the kidney (K) and liver (L). However, at postnatal day 1 (P1), hsp90 levels in the kidney were comparable to the neural levels.

Figure 1: Western blot analysis of hsp90 protein levels during postnatal development of the rat.

Hsp90 levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1-20), and in the adult (Ad). **A:**15µg of protein were loaded per lane for brain stem (BS), cerebellum (Cb), and cerebral hemispheres (CH). 30µg of protein were loaded per lane for kidney (Kid). For each tissue shown, a parallel gel was stained with Coomassie blue to ensure equal loading. **B:**Exposed X-ray film was scanned using an imaging densitometer. Protein levels at postnatal day 1 (P1) were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on two to four sets of animals. Error bars indicate the SEM.


A
Hsp90
P1
P5
P10
P15
P20
Ad
BS
Cb
CH
Kid

B

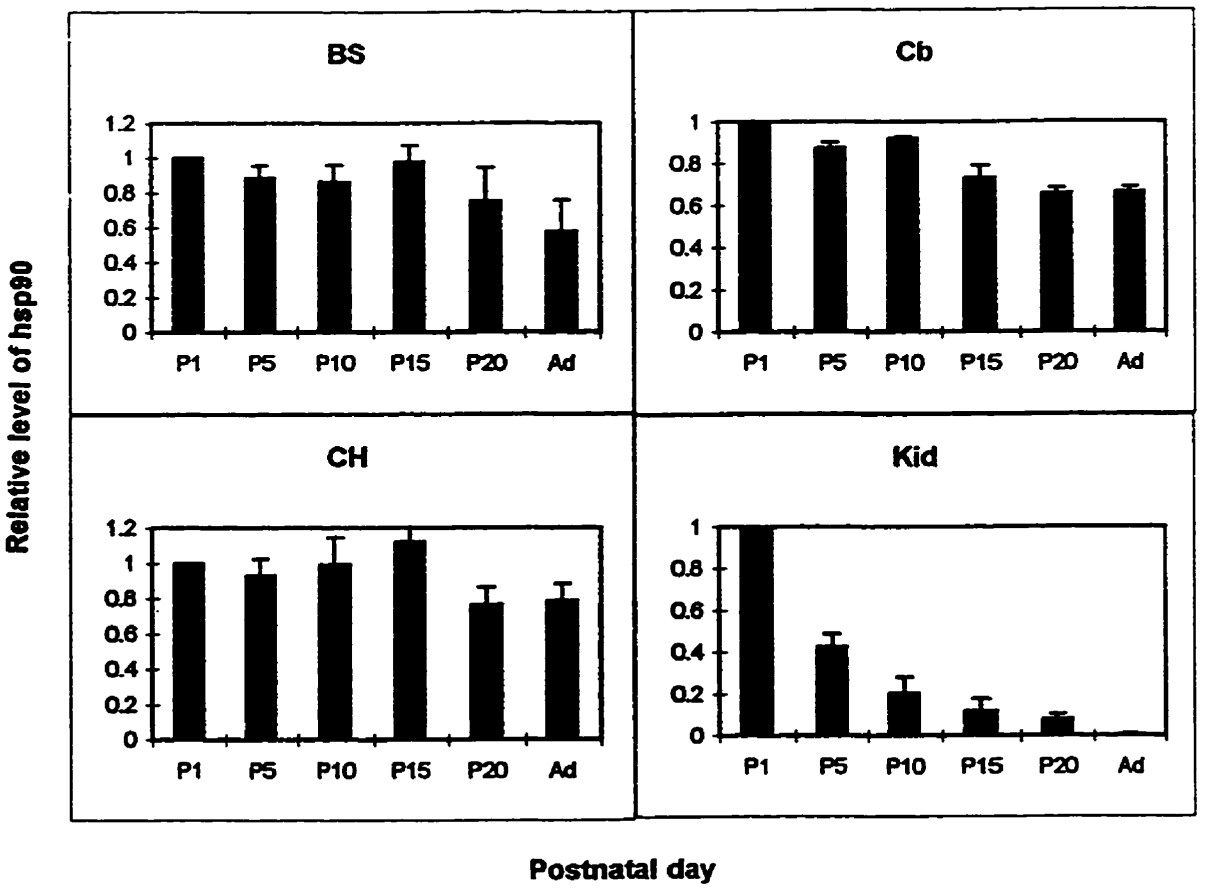
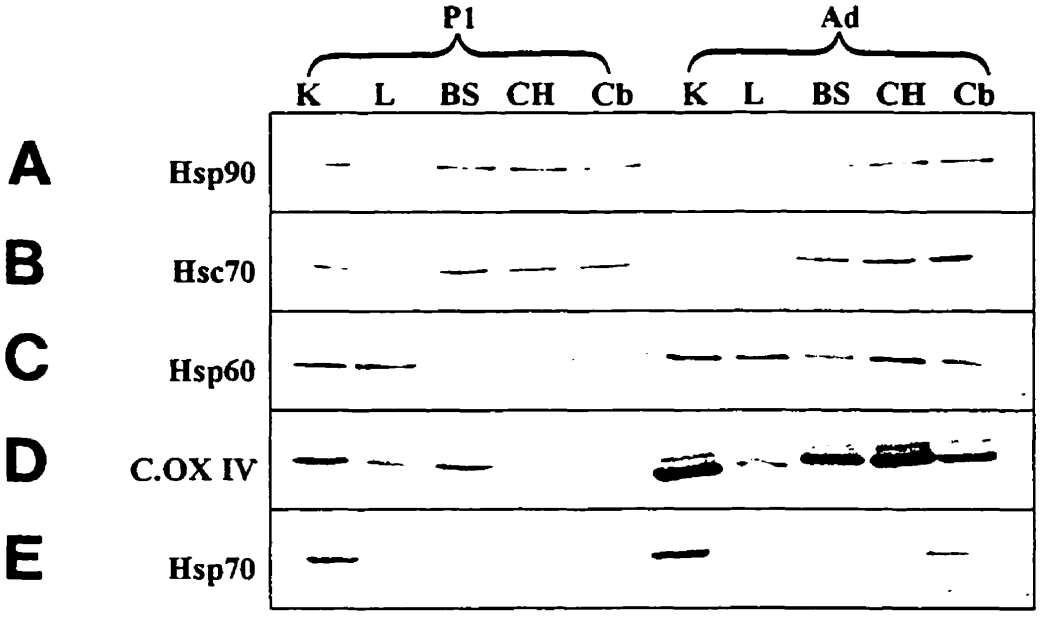

Hsp90
BS
Cb
CH
Kid
Relative level of hsp90
Postnatal day
P1
P5
P10
P15
P20
Ad

Figure 2: Tissue comparisons of hsp90, hsc70, hsp60, C.OX IV and hsp70 protein levels at postnatal day 1 and the adult rat.

Levels of hsp90 (**A**), hsc70 (**B**), hsp60 (**C**), C.OX IV (**D**) and hsp70 (**E**) were analyzed in kidney (K), liver (L), brain stem (BS), cerebral hemispheres (CH), and cerebellum (Cb) at postnatal day 1 (P1) and in the adult. For (**A**) and (**C**), 15µg of protein was loaded per lane. For (**B**), 30µg of protein was loaded per lane. For (**D**) and (**E**), 100µg of protein was loaded per lane.


P1
Ad
K L BS CH Cb K L BS CH Cb
A
Hsp90
B
Hsc70
C
Hsp60
D
C.OX IV
E
Hsp70

3.2 Cellular localization of hsp90 protein in brain and kidney

Immunocytochemical analysis of hsp90 in the developing rat brain revealed that hsp90 protein was expressed in neuronal-enriched regions of the cerebellum and brain stem. Glial-enriched areas of the brain, such as the deep white matter of the cerebellum, were examined and were found to be immunonegative for hsp90 (data not shown). Figure 3 shows that hsp90 was expressed in neurons of the brain stem, deep cerebellar nuclei, as well as Purkinje neurons of the cerebellum, at postnatal days 1, 15 and in the adult. Neurons were identified by their relatively large size and morphology. High magnification allowed the intracellular localization of hsp90 within these cells to be determined. As seen in Figure 3, immunoreactivity was present in the cytoplasm as well as in neuronal processes. Immunostaining within dendritic extensions was not always evident, depending on the plane of section. The hsp90-positive neurons shown for each brain region are representative of the staining pattern of neurons in that region.

In the kidney, hsp90 was localized to the convoluted tubules of the renal cortex as well as to straight tubules of the renal medulla. Figure 4 (hsp90 panel), shows the renal cortex. Within the cortex, hsp90 was not detected in the glomeruli of the renal corpuscles. In agreement with the Western data shown in Figure 1, hsp90 levels decreased

Figure 3: Immunocytochemical analysis of hsp90 in the rat brain during postnatal development.

Hsp90 was detected in brain stem neurons (BS), deep cerebellar neurons (DCN) and cerebellar Purkinje neurons (P) at postnatal days 1, 15, and in the adult (Ad). Both cytoplasm (c) as well as neuronal processes (p) immunostained. The immunostained neurons shown for each brain region are representative of the staining pattern of neurons in that region.
Bar = 10.9μm

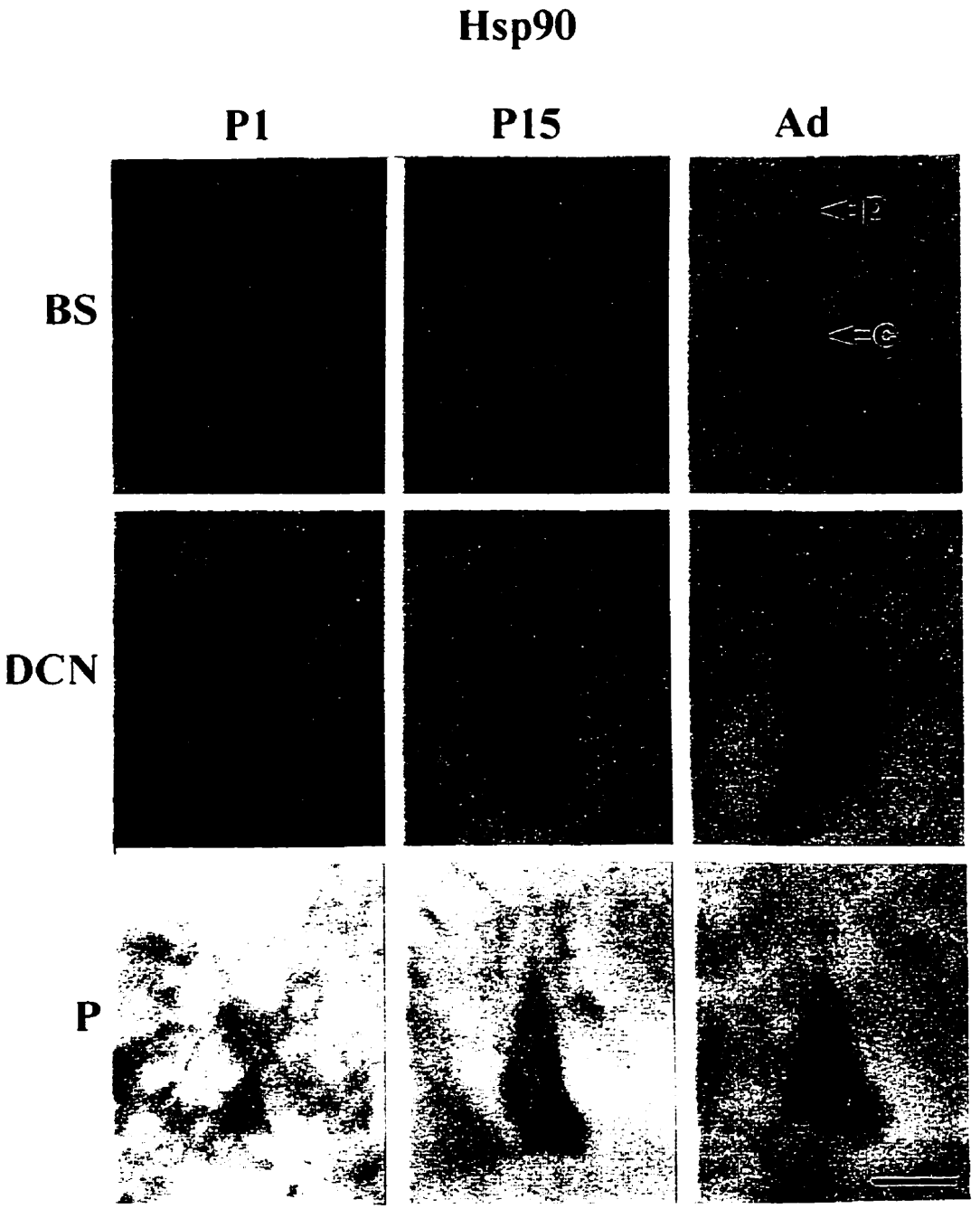
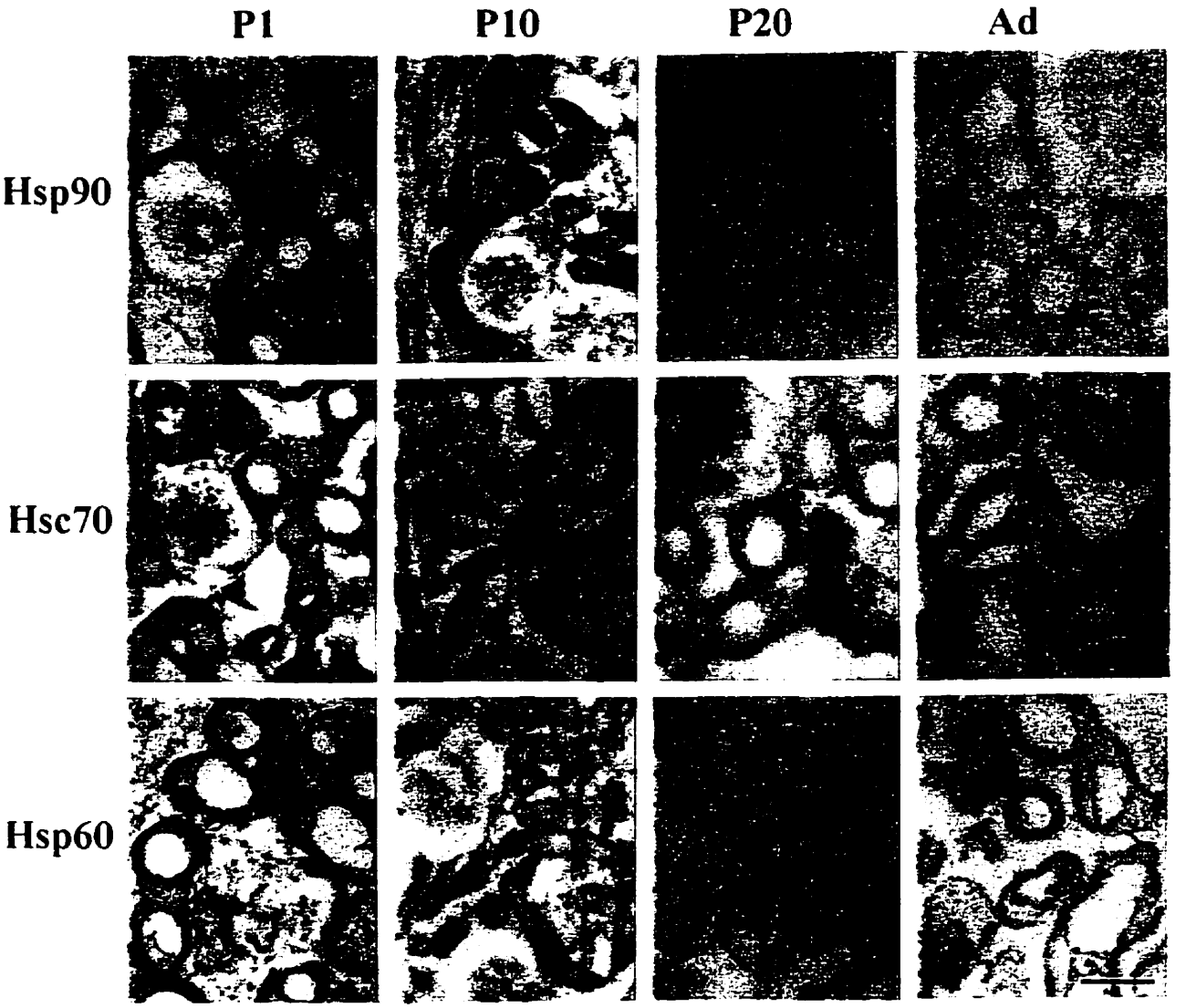

Hsp90
P1
P15
Ad
BS
DCN
P

Figure 4: Immunocytochemical analysis of hsp90, hsc70 and hsp60 in the developing rat kidney.

Rat kidney sections from postnatal days 1, 10, 20, and the adult were immunostained with antibodies to hsp90, hsc70 and hsp60. The cortex region of the kidney is shown. Bar = 26.4μm


P1
P10
P20
Ad
Hsp90
Hsc70
Hsp60

in the kidney through postnatal development.

3.3 Developmental analysis of hsc70 protein levels

Western blot analysis revealed abundant levels of hsc70 protein in the three brain regions with little change during postnatal development (Figure 5A). In the kidney, a developmental decrease in hsc70 protein levels was observed. Densitometric scanning confirmed the developmental decrease in hsc70 protein levels in the kidney which was observed by Western blotting.

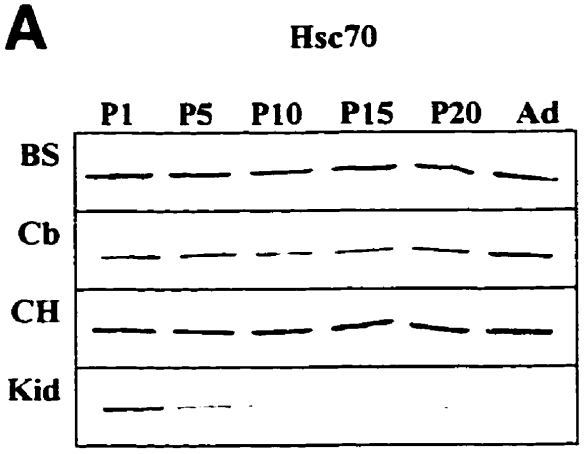
The levels of hsc70 protein in different rat tissues was investigated at postnatal day 1 and in the adult (Figure 2B). In concurrence with the tissue comparison of hsp90 levels (Figure 2A), Western blot analysis revealed that hsc70 protein levels were greater in the three neural regions than in kidney and liver, particularly in the adult. In summary, abundant levels of hsc70 protein appear to be present early in postnatal development of the nervous system and are maintained through to the adult.

3.4 Cellular localization of hsc70 protein in brain and kidney

The cellular localization of hsc70 protein in the developing rat brain was determined by immunocytochemistry (Figure 6). Hsc70 protein was

Figure 5: Western blot analysis of hsc70 protein levels during postnatal development of the rat.

Hsc70 levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1-20), and in the adult (Ad). **A:**30µg of protein were loaded per lane for brain stem (BS), cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). **B:**Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on three to four sets of animals. Error bars indicate the SEM.


A
Hsc70
P1
P5
P10
P15
P20
Ad
BS
Cb
CH
Kid

B

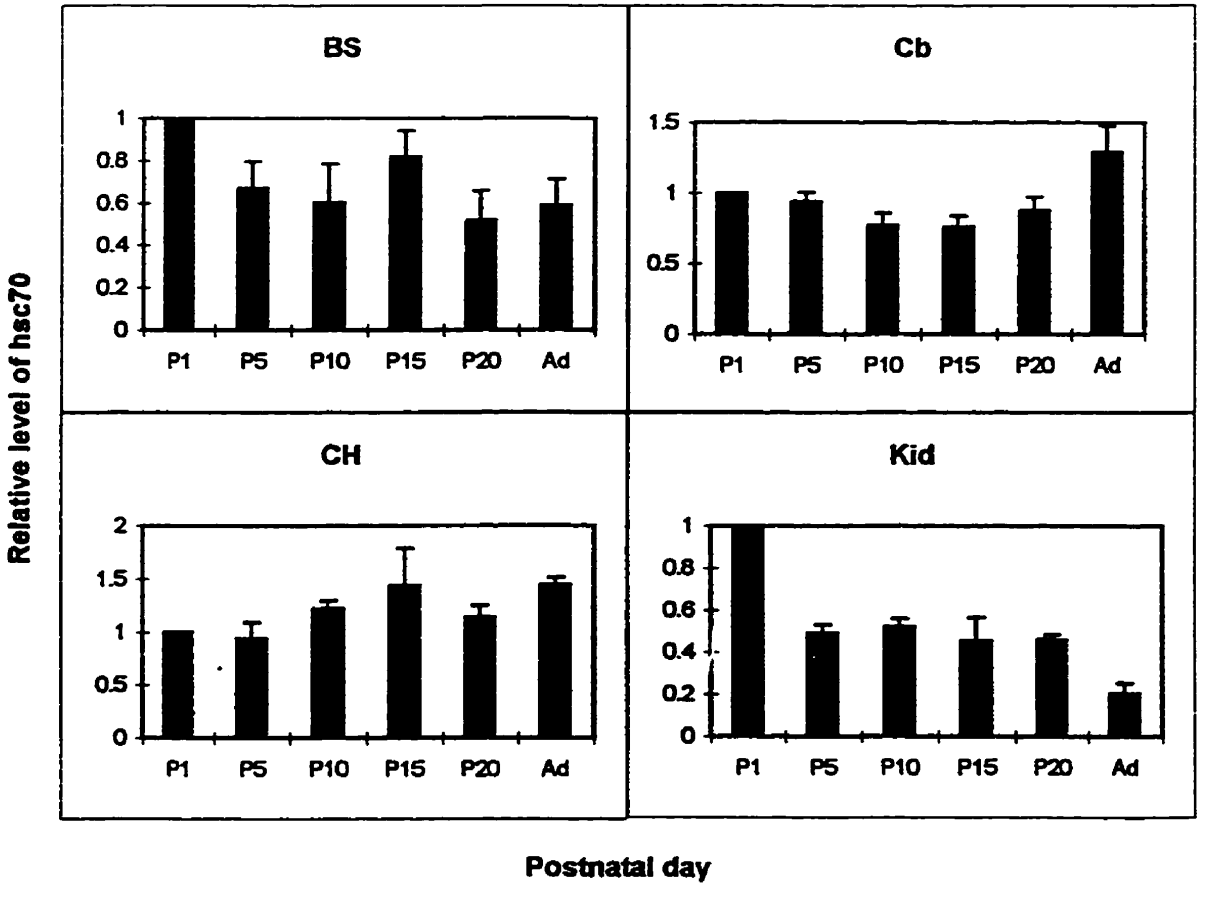
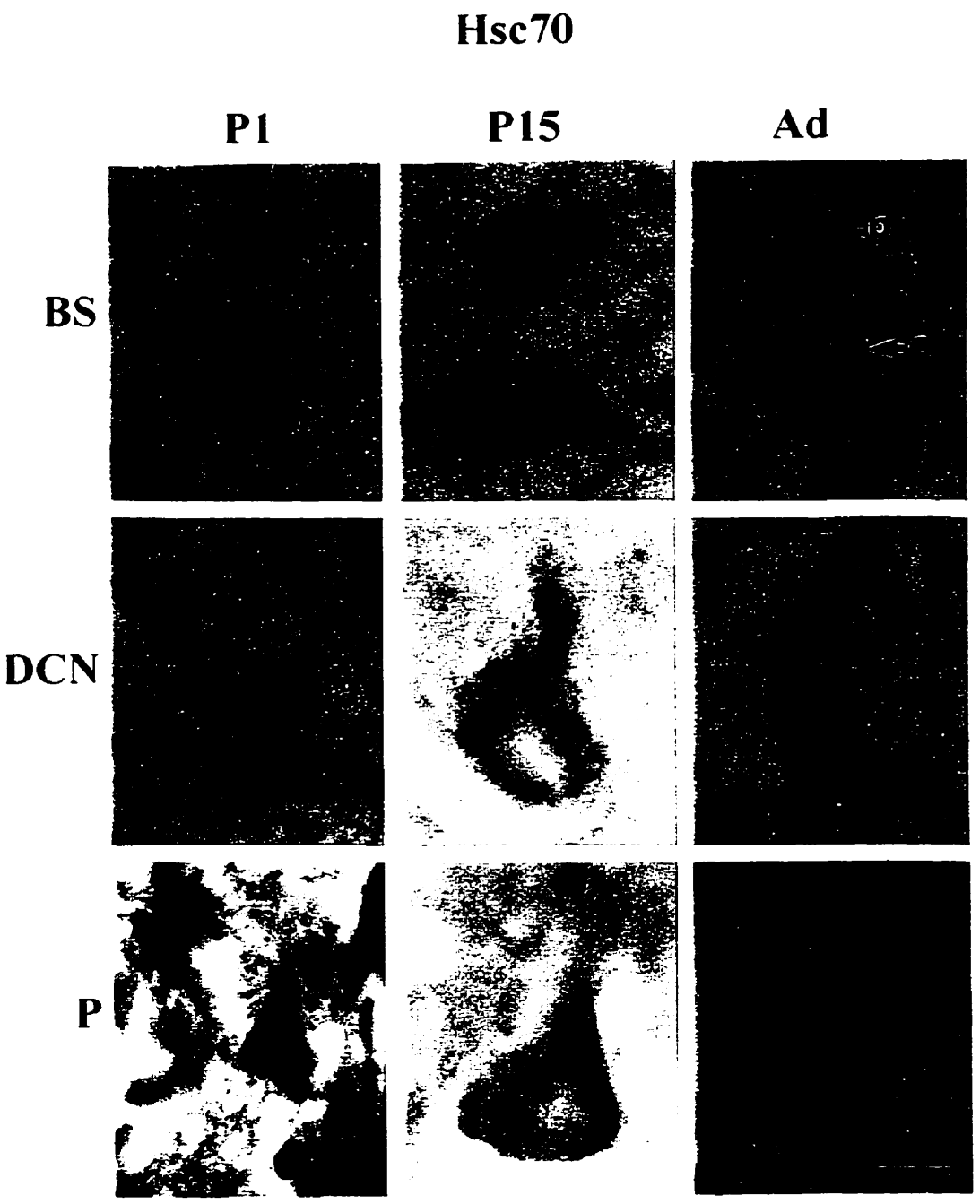

Hsc70
BS
Cb
CH
Kid
Relative level of hsc70
Postnatal day
P1
P5
P10
P15
P20
Ad

Figure 6: Immunocytochemical analysis of hsc70 in the rat brain during postnatal development.

Hsc70 was detected in brain stem neurons (BS), deep cerebellar neurons (DCN) and Purkinje neurons (P) at postnatal days 1, 15, and in the adult (Ad). Both cytoplasm (c) as well as neuronal processes (p) immunostained. The immunostained neurons shown for each brain region are representative of the staining pattern of neurons in that region. Bar = 10.9μm


Hsc70
P1
P15
Ad
BS
DCN
P

expressed in brain stem neurons, deep cerebellar and Purkinje neurons of the cerebellum at postnatal day 1, 15 and in the adult. Immunopositive staining was concentrated in the cytoplasm as well as in neuronal processes.

In the developing rat kidney, hsc70 immunoreactivity was detected in the convoluted tubules of the renal cortex (Figure 4, hsc70 panel). In addition, signal was detected in glomeruli of renal corpuscles as well as in medullary straight tubules (data not shown).

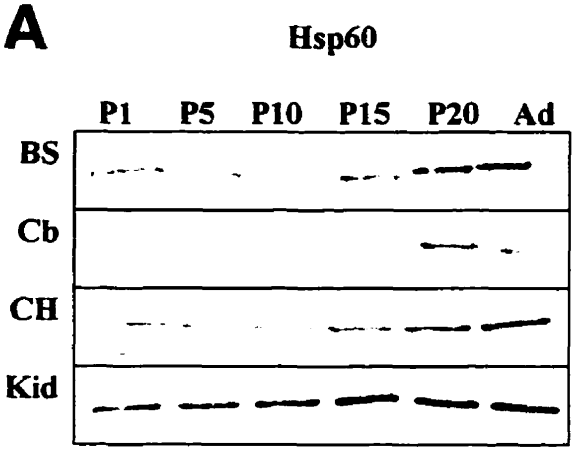
3.5 Developmental analysis of hsp60 protein levels

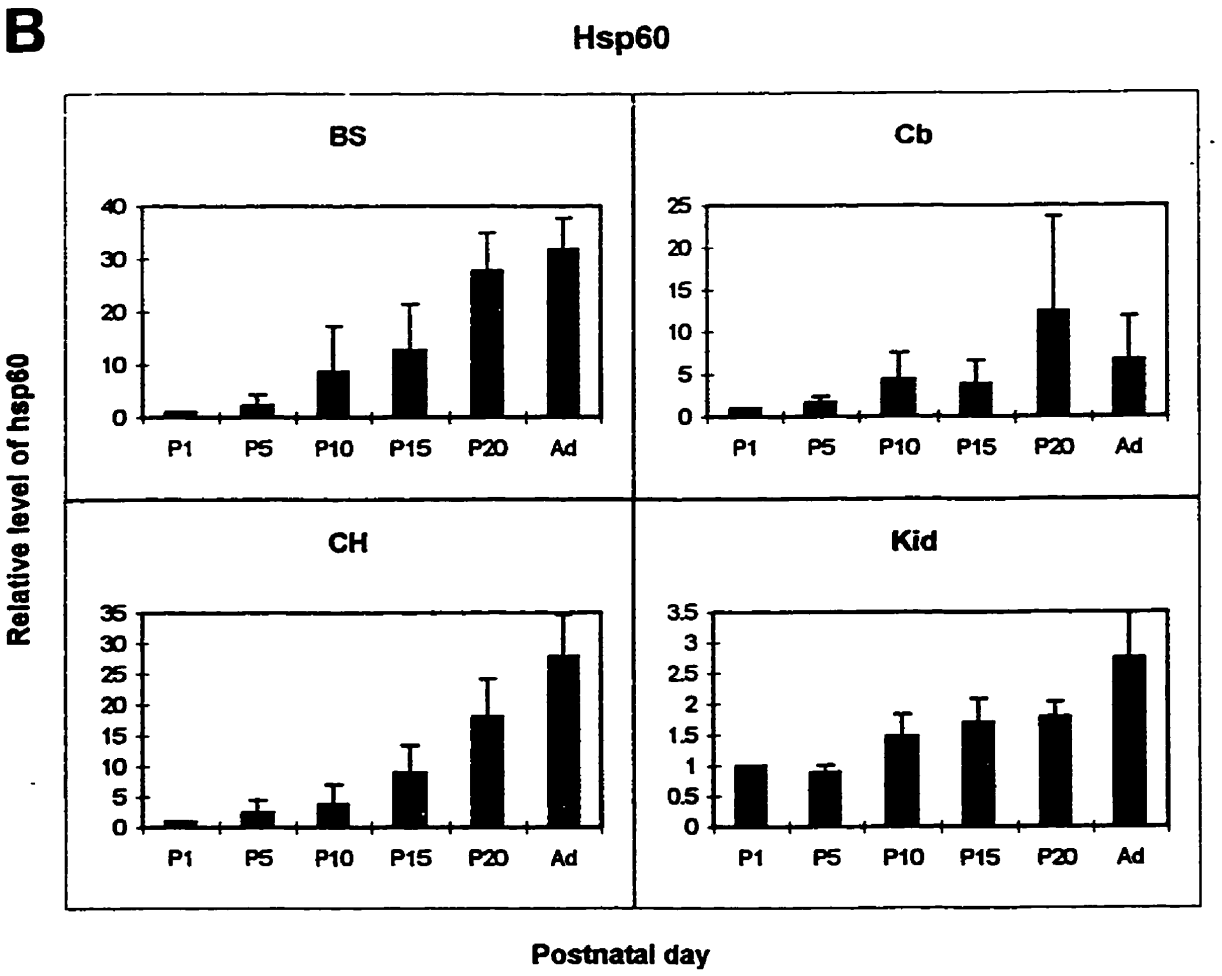
A major increase in hsp60 protein levels was observed during postnatal development of all three brain regions examined. A developmental increase of lesser magnitude was observed in the kidney (Figure 7A). Densitometric scanning of exposed X-ray film revealed a 25-30-fold increase in hsp60 protein levels during postnatal development in the brain stem, and the cerebral hemispheres (Figure 7B). In the kidney, a less dramatic increase of 3-fold was evident.

A comparative analysis of hsp60 protein levels in various rat tissues was undertaken by Western blotting (Figure 2C). In contrast to hsp90 and hsc70 protein levels, hsp60 levels were greater in non-neural tissues, such as the kidney and liver, than in neural regions, such as the brain stem, cerebral hemispheres, and cerebellum, at postnatal day 1. In the adult,

Figure 7: Western blot analysis of hsp60 protein levels during postnatal development of the rat.

Hsp60 levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1-20), and in the adult (Ad). **A:**15µg of protein were loaded per lane for brain stem (BS), cerebellum (Cb) and cerebral hemispheres (CH). 30µg of protein were loaded per lane for kidney (Kid). **B:**Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on two to three sets of animals. Error bars indicate the SEM.


A
Hsp60
P1
P5
P10
P15
P20
Ad
BS
Cb
CH
Kid


B
Hsp60
BS
Cb
CH
Kid
Relative level of hsp60
Postnatal day
P1
P5
P10
P15
P20
Ad

hsp60 protein was present in abundant levels in both brain and non-brain regions.

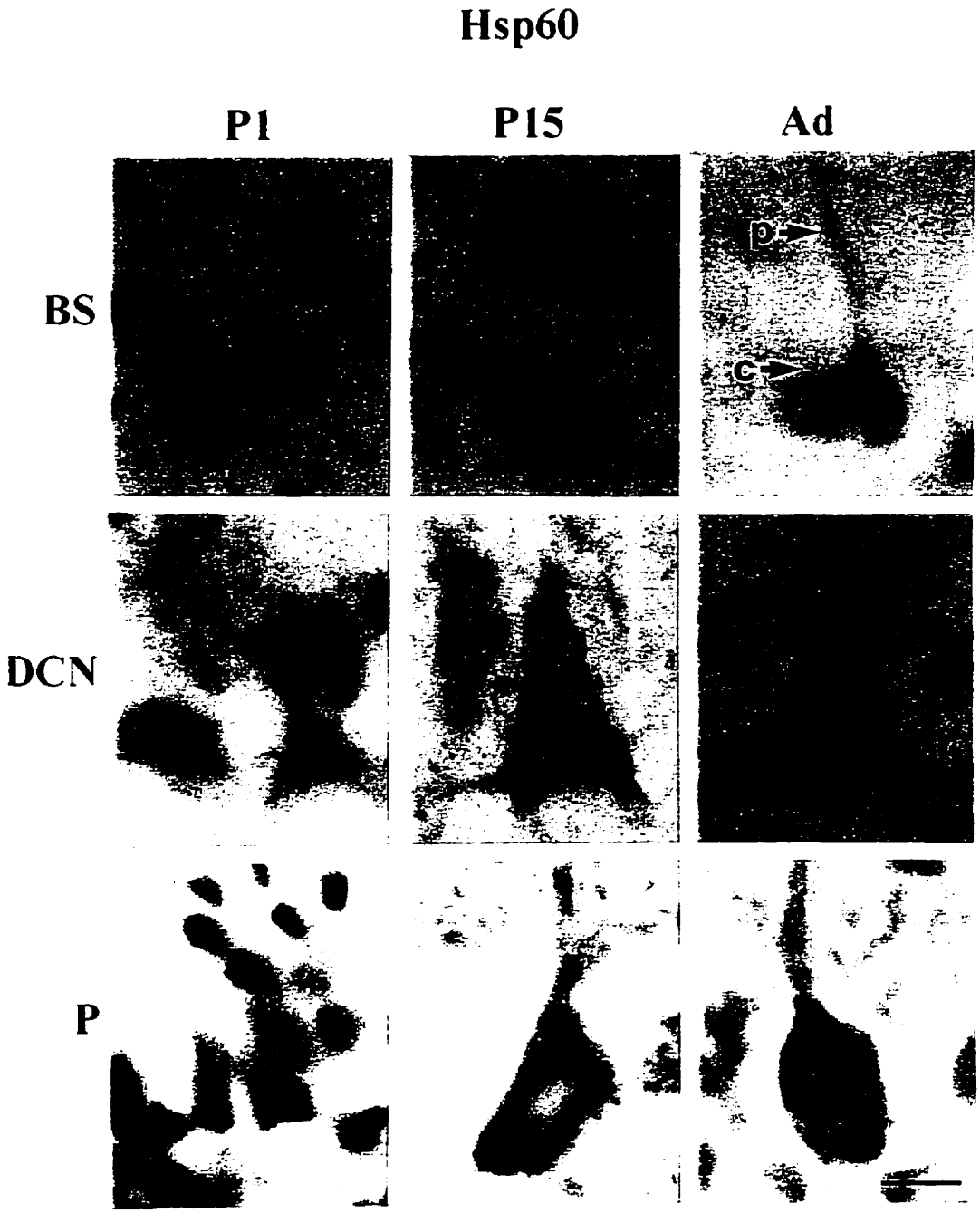
3.6 Cellular localization of hsp60 protein in brain and kidney

Similar to the immunocytochemical results shown for hsp90 and hsc70 protein (Figure 3, Figure 6, respectively), hsp60 protein was localized to neurons in the brain stem, deep cerebellar nuclei, and Purkinje cell layer in the developing rat brain (Figure 8). Hsp60 immunoreactivity was present in the cytoplasm as well as in dendritic processes. In contrast to the Western blot analysis (Figure 7A), a developmental increase in hsp60 was not detected by immunocytochemistry. Western blotting, which detects proteins in a denatured state, may be used to analyze changes in protein levels. However, this is not possible with immunocytochemistry, which detects native proteins within the cell, and immunoreactive epitopes may be blocked due to interactions with other cellular proteins. Immunocytochemical analysis is generally used to show cellular localization of proteins, and may not accurately represent total levels of a particular protein within a cell.

In the developing rat kidney, hsp60 was localized to convoluted tubules of the renal cortex (Figure 4, hsp60 panel). In addition, immunopositive signal was observed in the straight tubules of the medulla

Figure 8: Immunocytochemical analysis of hsp60 in the rat brain during postnatal development.

Hsp60 was detected in brain stem neurons (BS), deep cerebellar neurons (DCN) and cerebellar Purkinje neurons (P) at postnatal days 1, 15, and in the adult (Ad). Both cytoplasm (c) as well as neuronal processes (p) immunostained. The immunostained neurons shown for each brain region are representative of the staining pattern of neurons in that region.
Bar = 10.9μm


Hsp60
P1
P15
Ad
BS
DCN
P
p
c

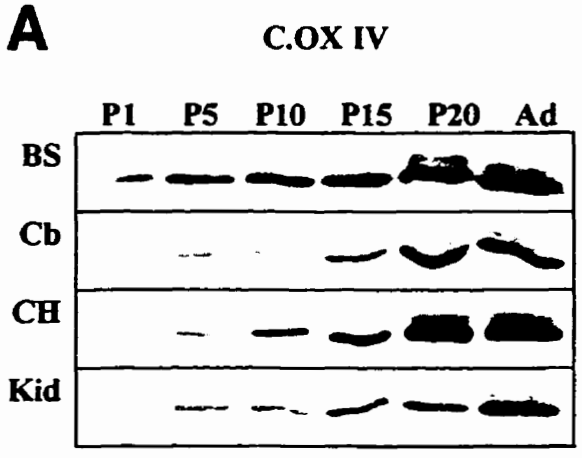
(data not shown). Hsp60 was not detected in glomerular cells, similar to what was observed for hsp90. The increase in hsp60 levels seen in the developing kidney by Western blot analysis (Figure 7A), was not evident by immunocytochemistry.

3.7 Developmental analysis of cytochrome oxidase (subunit IV) protein levels

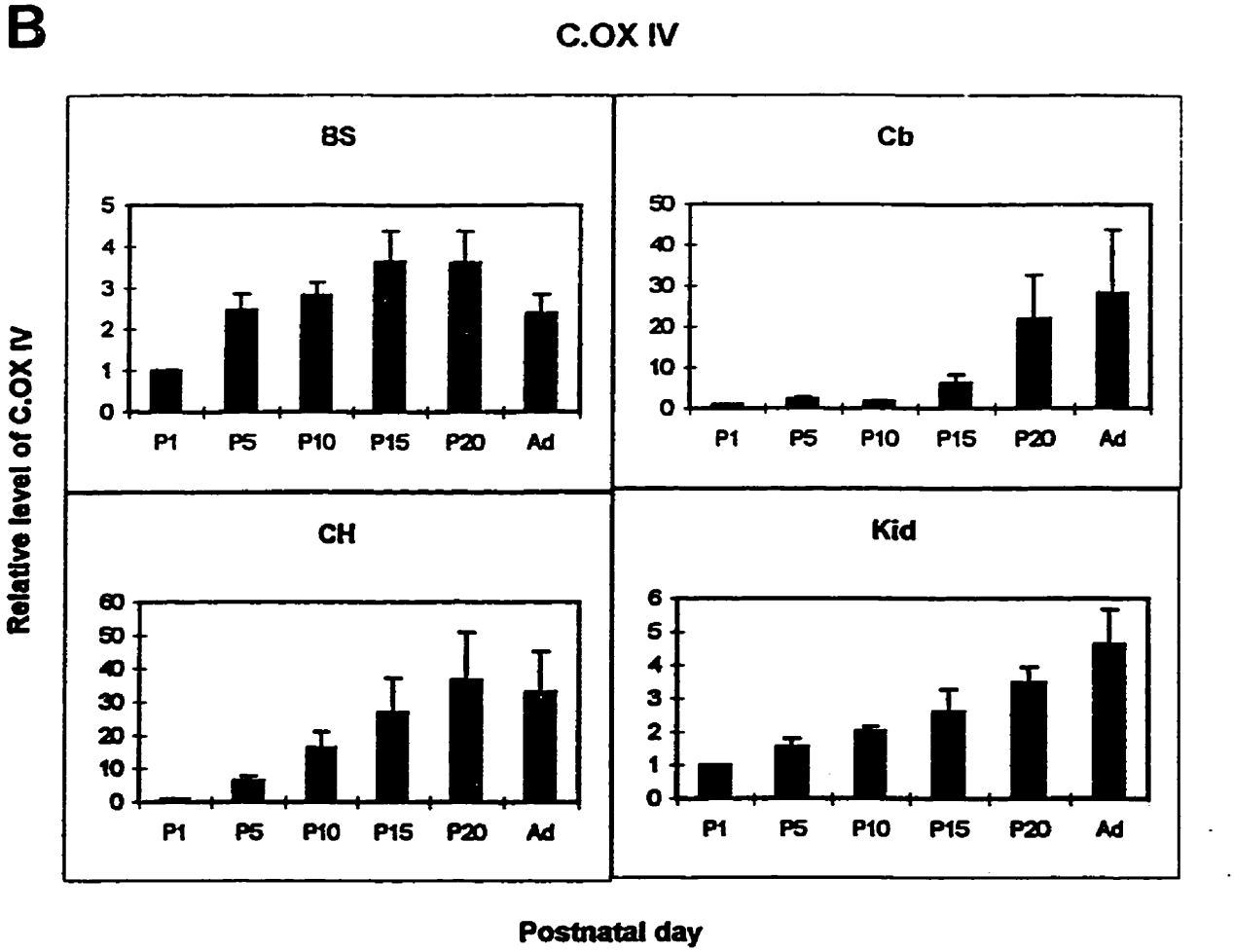
To investigate whether the observed developmental increase in hsp60 was due to an increase in mitochondrial content, the developmental pattern of another mitochondrial protein, cytochrome oxidase, subunit IV (C.OX IV) was studied. As shown in Figure 9A, a developmental increase in C.OX IV was observed in the brain regions as well as in the kidney. Scanning of exposed X-ray film (Figure 9B) revealed that the cerebellum and cerebral hemispheres showed increased levels of C.OX IV of more than 30-fold, while the brain stem and kidney showed developmental increases in C.OX IV levels of lesser magnitude. A comparative analysis of C.OX IV protein levels in various neural and non-neural rat tissues was investigated (Figure 2D). Levels of C.OX IV at P1 appeared higher in non-neural tissues such as the kidney and liver, compared to neural regions such as the cerebral hemispheres and cerebellum. C.OX IV levels in the brain stem were similar to that found in the kidney and liver. In the adult, levels of

Figure 9: Western blot analysis of C.OX IV protein levels during postnatal development of the rat.

C.OX IV levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1-20), and in the adult (Ad). **A:**100µg of protein were loaded per lane for brain stem (BS), cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). **B:**Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on two to three sets of animals. Error bars indicate the SEM.


A
C.OX IV
P1
P5
P10
P15
P20
Ad
BS
Cb
CH
Kid

B


C.OX IV
BS
Cb
CH
Kid
Relative level of C.OX IV
Postnatal day
P1
P5
P10
P15
P20
Ad

C.OX IV in neural and non-neural regions were comparable. With the exception of the brain stem at P1, this pattern of expression was similar to that found for hsp60 (Figure 2C).

3.8 Cellular localization of cytochrome oxidase (subunit IV) protein in brain

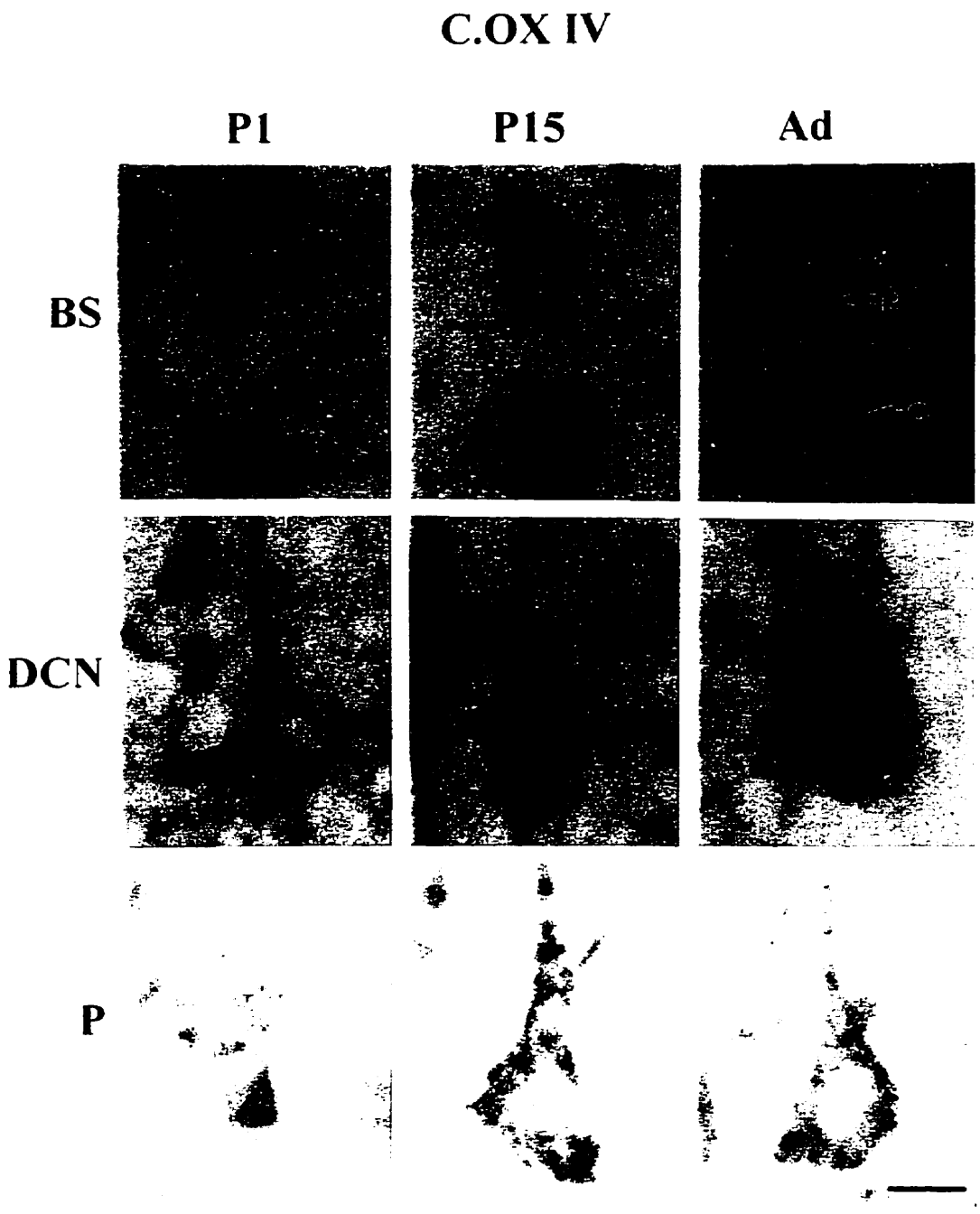
To determine if the observed cellular localization of hsp60 correlated with that of C.OX IV, immunocytochemistry was performed on the rat brain with the C.OX IV antibody. As shown in Figure 10, a neuronal pattern of expression of C.OX IV was observed in the Purkinje cells of the cerebellum, brain stem and deep cerebellar nuclei at P1, P15 and in the adult. Immunoreactivity was present in the cytoplasm as well as in the neuronal processes. Glial-enriched areas of the brain were immunonegative for C.OX IV. Similar to the results observed for hsp60 (Figure 8), a developmental increase in C.OX IV levels was not observed by immunocytochemistry.

3.9 Developmental analysis of hsp70 protein levels

Previous studies from our laboratory using Western blotting have found basal levels of hsp70 protein in regions of the unstressed rabbit brain (Manzerra *et al.*, 1997); moreover, *in situ* hybridization techniques have revealed a neuronal localization of hsp70 mRNA (Foster and Brown, 1996a).

Figure 10: Immunocytochemical analysis of C.OX IV in the brain during postnatal development.

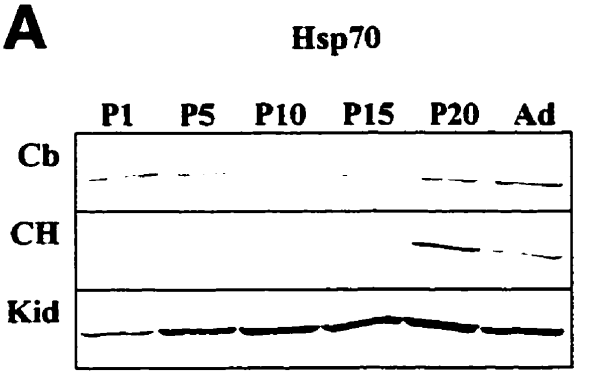
C.OX IV was detected in brain stem neurons (BS), deep cerebellar neurons (DCN) and Purkinje neurons (P) at postnatal days 1, 15, and in the adult (Ad). Both cytoplasm (c) as well as neuronal processes (p) immunostained. The immunostained neurons shown for each brain region are representative of the staining pattern of neurons in that region.
Bar = 10.9μm

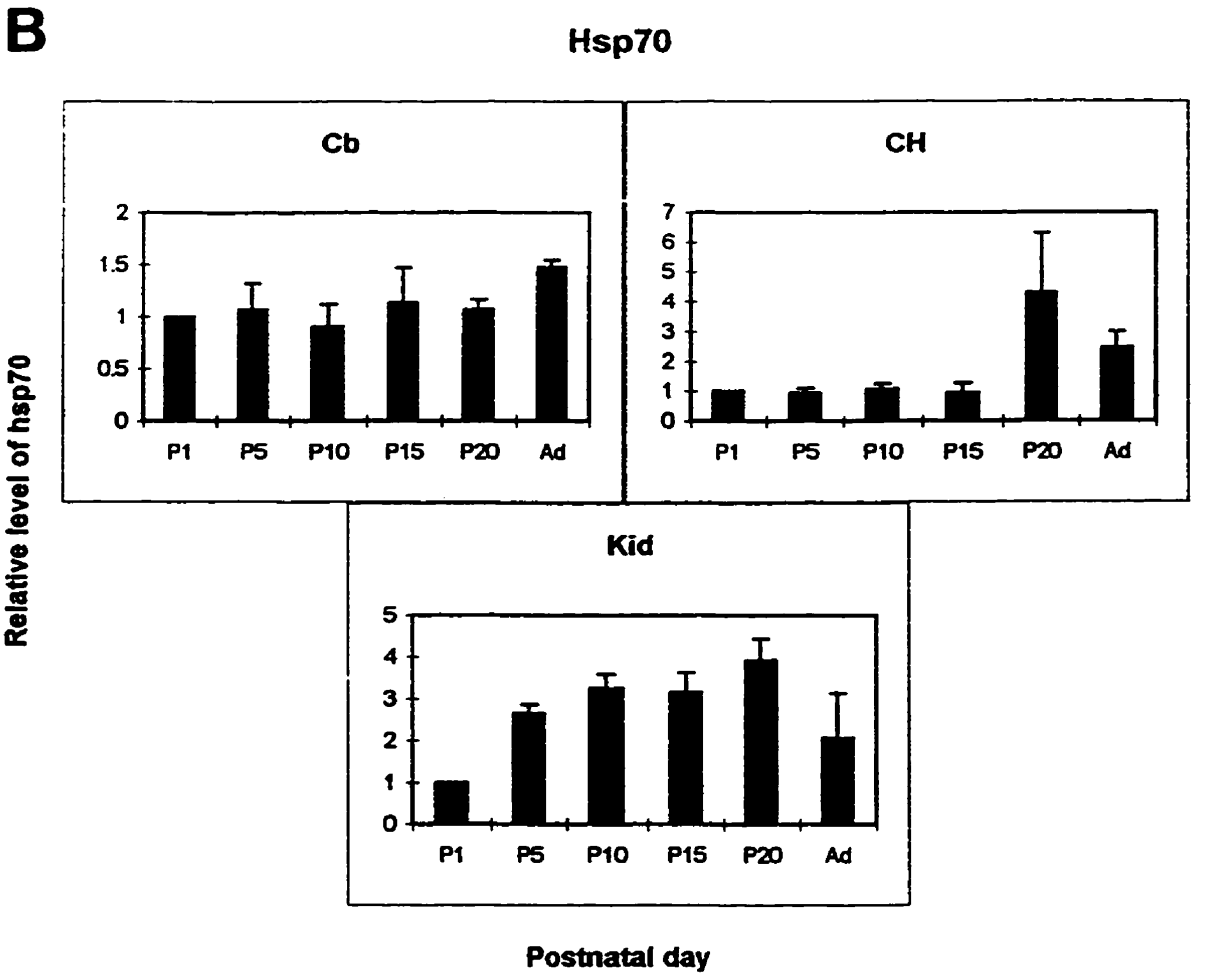

C.OX IV
P1
P15
Ad
BS
DCN
P

In view of this, the developmental profile of hsp70 protein was analyzed in the postnatal rat. As shown in Figure 11A, basal levels of hsp70 were present in the cerebellum, cerebral hemispheres and the kidney from the earliest postnatal days examined. Densitometric scanning of exposed X-ray film (Figure 11B), revealed an increase in hsp70 levels in the kidney, which peaked at postnatal day 20 (P20). In the cerebellum and cerebral hemispheres, comparatively little change in hsp70 levels was observed during postnatal development. Levels of hsp70 were higher in kidney compared to brain regions at P1 and in the adult (Figure 2E). Cellular localization of hsp70 in the developing brain was not possible due to the very low levels of the protein. For the developmental Westerns, the amount of protein loaded per lane had to be increased to 100µg in order to detect a signal.

Figure 11: Western blot analysis of hsp70 protein levels during postnatal development of the rat.

Hsp70 levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1-20), and in the adult (Ad). **A:**100µg of protein were loaded per lane for cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). **B:**Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on two to three sets of animals. Error bars indicate the SEM.


A
Hsp70
P1
P5
P10
P15
P20
Ad
Cb
CH
Kid


B
Hsp70
Cb
CH
Kid
Relative level of hsp70
Postnatal day
P1
P5
P10
P15
P20
Ad

4. DISCUSSION

Studies on the developmental expression of heat shock proteins have often focused on the time frame of embryogenesis in various organisms, such as mouse (Kothary *et al.*, 1987), zebrafish (Krone *et al.*, 1997), amphibian (Ali *et al.*, 1996; Coumailleau *et al.*, 1997; Heikkila *et al.*, 1997), sea urchin (Bédard and Brandhorst, 1986) and fruit fly (Zimmerman *et al.*, 1983; Ding *et al.*, 1993). These studies have shown that heat shock proteins are essential proteins that are needed for normal cellular division and growth. Comparatively few studies have examined the expression of heat shock proteins in the mammalian brain during postnatal development. In the present study, the constitutive expression of a number of heat shock proteins was investigated during postnatal development of the rat using Western blotting and immunocytochemistry. The rat is an appropriate animal model to use when studying postnatal brain development since its brain is structurally and functionally immature at birth, and matures postnatally. A number of significant processes occur during postnatal development of the rat brain, such as cell growth, cell migration, neurite outgrowth, and synaptogenesis. Other important developmental events also occur, such as the emergence of motor coordination, eye opening and a distinctive electroencephalogram. Since heat shock proteins have been associated with growth and differentiation processes, it is interesting to

examine these proteins during postnatal development, a crucial window in the development of the rat brain.

4.1 Developmental expression of hsp90 protein

Western blot analysis revealed a large developmental decrease in hsp90 levels in the kidney during postnatal development. Tanguay *et al.*, (1993), also found a decrease in hsp90 protein levels in the adult kidney compared to that of 11-day old mice, although the magnitude of the decrease was not as great as that observed in this study. Hsp90 has been shown to interact with several transcription factors, such as myoD (Shaknovich *et al.*, 1992), and has thus been implicated in the process of myogenesis (Sass *et al.*, 1996). The expression of hsp90 mRNA was down-regulated in fully differentiated muscle cells, along with myoD, implying a role in muscle differentiation, but not in the maintenance of the mature muscle. Although no direct evidence is available, hsp90 may play a similar role in the transcriptional regulation of kidney cell growth during postnatal development. For example, hsp90 has been shown to interact with steroid receptors, such as the glucocorticoid receptor (Sanchez *et al.*, 1985), and is thought to keep these receptors in an inactive, unliganded conformation. It has been suggested that glucocorticoids play a role in regulating the transcription of several mitochondrial enzymes during postnatal

development of the rat kidney (Djouadi *et al.*, 1996). Therefore, hsp90, through its association with the glucocorticoid receptor, may function during maturation of the kidney.

The localization of hsp90, by immunocytochemistry, to the convoluted tubules of the kidney is similar to that observed in the adult rat by Matsubara *et al.*, (1990), who suggested that the presence of this heat shock protein reflected a stress response to toxic agents within the kidney. In the present study, hsp90 immunostaining decreased in the adult kidney, consistent with results obtained from Western blot analysis. Therefore, hsp90 appears to function in the developing kidney, but the requirement is decreased in the mature organ.

In vitro studies have implicated a role for hsp90 in developmental and differentiation processes. Hemin-induced differentiation of K562 erythroleukemia cells resulted in elevated levels of hsp90 (Sistonen *et al.*, 1992). In addition, hsp90 levels increased during differentiation of the monoblastoid cell line U937 to a macrophage-like phenotype (Twomey *et al.*, 1993; Galea-Lauri *et al.*, 1996). In murine embryonal carcinoma F9 cells, however, discrepancies in hsp90 levels have been found. While Kohda *et al.*, (1991), demonstrated that hsp90 mRNA levels increased after differentiation of F9 cells, other researchers have observed a decrease in hsp90 mRNA levels following differentiation of the same cell line (Levine *et al.*, 1984; Barnier *et al.*, 1987).

A tissue comparison of hsp90 levels at postnatal day 1 and in the adult showed higher amounts of the protein in neural regions compared to non-neural tissues. Immunocytochemical studies demonstrated a neuronal localization of hsp90 in the rat brain at all stages of postnatal development examined. Previous studies in the adult mammalian brain have demonstrated high levels of hsp90 mRNA and protein expression in neurons of the forebrain, cerebellum, brain stem and spinal cord (Itoh *et al.*, 1990; Izumoto and Herbert, 1993; Gass *et al.*, 1994; Quraishi and Brown, 1995). In addition, hsp90 has been found to exist at higher levels in brain regions compared to non-brain regions in the adult rabbit (Quraishi and Brown, 1995) as well as in the rat (Itoh *et al.*, 1993) by Western blot analysis. The present Western blot analysis revealed no marked changes in hsp90 levels during postnatal development of the brain. By immunocytochemistry, hsp90 protein was detected in neurons of the postnatal brain as well as in neurons of the adult brain. This suggests a role for hsp90 in both the developing and the fully differentiated neuron.

What role might hsp90 play in the brain? The presence of hsp90 in the brain may reflect the sensitivity of this neural tissue to glucocorticoids (Vamvakopoulos, 1993). Glucocorticoid receptor mRNA was localized to cerebellar and forebrain neurons in the adult rat brain (Aronsson *et al.*, 1988). It is not known if hsp90 associates with glucocorticoid receptors in the developing brain. In addition to interacting with glucocorticoid

receptors, hsp90 also interacts with various other cellular proteins, such as actin (Koyasu *et al.*, 1986), and tubulin (Sanchez *et al.*, 1988). These cytoskeletal proteins play important roles in the maintenance of cell shape, cell migration, and in the intracellular transport of proteins. In the postnatal brain, hsp90 was detected in neuronal processes of both cerebellar and brain stem neurons, by immunocytochemistry. Hsp90 may therefore be involved in neurite outgrowth, which requires cytoskeletal elements, such as actin and tubulin (Schmitt *et al.*, 1977; Riederer, 1990). Hsp90 may play other molecular chaperone-like roles at postnatal day 1, when neuronal processes have not yet formed, and in the adult, when synaptogenesis is complete.

4.2 Developmental expression of hsc70 protein

Abundant levels of hsc70 were attained early in postnatal development of the nervous system and were maintained in the adult. A developmental decrease in hsc70 protein levels was evident in the kidney, similar to that observed for hsp90. Pak *et al.*, (1996), showed that the collagen-binding heat shock protein, gp46, decreased through postnatal development of the rat kidney. Gp46 is thought to play a role in the metabolism of collagen types I and IV, which are extracellular matrix components. The high levels of gp46 early in postnatal development

were postulated to reflect the increase in extracellular matrix synthesis and deposition that is observed at this time of rapid tissue growth and differentiation. In the present study, hsc70 may be playing molecular chaperone-like roles that are intrinsic to kidney development. Immunocytochemical analysis demonstrated that hsc70 was present in all regions of the kidney, consistent with results obtained by other researchers (Komatsuda *et al.*, 1992; Muller *et al.*, 1996).

Similar to the hsp90 data, higher amounts of hsc70 were detected in neural regions than in non-neural tissues at postnatal day 1 and in the adult. Analysis of hsc70 distribution in the developing rat brain by immunocytochemistry revealed a neuronal pattern of expression. High amounts of hsc70 mRNA and protein have been observed in neurons, such as Purkinje cells of the cerebellum, and spinal cord neurons, in the adult mammalian central nervous system (Aquino *et al.,* 1993; Manzerra *et al.,* 1993; Foster *et al.,* 1995). In addition, the adult mammalian brain appears to express higher levels of hsc70 mRNA and protein compared to non-neural tissues (Giebel *et al.,* 1988; Manzerra *et al.,* 1997). The present results suggest that hsc70 plays an important role in the developing as well as adult mammalian brain, particularly within neurons. Hsc70 has been shown to act as an ATPase in the removal of endocytic clathrin coats (Chappell *et al.,* 1986). In neurons, hsc70 may function in this manner in the synaptic vesicle recycling pathway. Maycox *et al.,* (1992),

demonstrated that coated vesicles from brain are primarily involved in the recycling of synaptic membranes. Western blot data from Giebel *et al.*, (1988), demonstrated that mouse tissues such as brain, exhibited high levels of hsc70 as well as clathrin protein, while tissues such as liver, expressed both hsc70 and clathrin at lower levels. The importance of hsc70 as an uncoating ATPase within the brain was confirmed by Buxbaum and Woodman, (1995), who showed that the extent of uncoating by hsc70 was greater in vesicles from brain than from non-brain tissues, such as placenta. This difference in uncoating efficiency in brain and placenta vesicles was thought to reflect differences in coat structure. Research on hsc70 levels during brain development has revealed that the molecular chaperone is present during human embryogenesis (Aquino *et al.*, 1996). Hsc70 protein levels remained constant in the human fetal brain during the second trimester. In addition, the high levels of hsc70 observed in the developing brain were shown to approximate those in the adult brain. These findings along with results obtained from the present study suggest a role for hsc70 in both the developing and adult brain.

4.3 Developmental expression of hsp60 protein

Western blot analysis of hsp60 protein levels during postnatal development showed large increases in all brain regions as well as a smaller

increase in the developing kidney. Since hsp60 is a mitochondrial protein, an increase in levels of expression of hsp60 during postnatal development may reflect an increase in mitochondrial content. Indeed, mitochondrial biogenesis has been reported to occur postnatally in the rat brain (Giuffrida *et al.,* 1979; Renis *et al.,* 1989). Increased amounts of hsp60 during postnatal development might be required to promote the folding of newly synthesized mitochondrial proteins during mitochondrial growth. Additionally, chronic stimulation of skeletal muscle, which induces mitochondrial biogenesis has been shown to lead to an increase in hsp60 protein levels in the adult rat (Ornatsky *et al.,* 1995). Levels of hsp60 were also shown to increase in porcine cardiac and skeletal muscle during postnatal development (McComb and Spurlock, 1997).

In order to strengthen the correlation between hsp60 and mitochondrial content, the developmental profile of another mitochondrial protein, cytochrome oxidase, subunit IV (C.OX IV), was investigated. In mammals, cytochrome oxidase exists as a 13-subunit complex (Kadenbach *et al.,* 1983) which is embedded into the inner mitochondrial membrane. It acts as the terminal component of the electron-transport chain and therefore plays a crucial role in oxidative metabolism. Similar to the hsp60 results, levels of C.OX IV increased greatly during postnatal development of the brain and kidney. However, the magnitude of increase in C.OX IV for a given region did not always parallel that of hsp60. For example, hsp60

levels increased approximately 30-fold in the brain stem (Figure 7B), whereas C.OX IV levels (Figure 9B) increased less than 4-fold in the same brain region during postnatal development. Kim *et al.,* (1995), observed an increase in the mRNA levels of several cytochrome oxidase subunits (including subunit IV), in various mouse tissues during postnatal development. Furthermore, the activity of cytochrome oxidase was demonstrated to increase in skeletal muscle under conditions of mitochondrial biogenesis (Hood *et al.,* 1989). These results suggest that the increase in hsp60 and C.OX IV protein levels observed in this study during postnatal development of the brain and kidney may reflect an increase in mitochondrial content, possibly due to increased energy demands as development proceeds.

The localization of hsp60, by immunocytochemistry, to the convoluted tubules of the kidney is consistent with observations from Muller *et al.,* (1996). The kidneys play an important role in regulating the salt and water balance of the blood, as well as in excreting harmful waste products (Hill and Wyse, 1989). The convoluted tubules of the kidney are involved in the active reabsorption of sodium. This energy-requiring process is mediated through the Na^+/K^+ ATPase. The activity of this enzyme was shown to increase within the convoluted tubules of the mammalian kidney during postnatal development (Schmidt and Horster, 1977; Horster, 1978). Additionally, the transcription of several mitochondrial enzymes increased

in the postnatal rat kidney, and this increase correlated with the time frame of mitochondrial biogenesis (Djouadi *et al.*, 1996). These results imply an increased energy demand during postnatal development of the mammalian kidney. The developmental increase in hsp60 observed in the present study by Western blot analysis may reflect an increase in mitochondria that is required to meet the increased energy needs of the developing and adult kidney.

In contrast to the developmental increase in hsp60 and C.OX IV protein levels observed by Western blot analysis (Figure 7 and Figure 9, respectively), levels of hsp60 and C.OX IV did not increase during postnatal development when examined using immunocytochemistry (Figure 8 and Figure 10, respectively). In Western blotting, proteins are solubilized, and therefore the accessibility of an epitope to a particular antibody is increased. In immunocytochemistry, proteins within the tissue are present in their native conformation and possibly bound to other proteins. For example, hsp60 oligomerizes into a heptamer in its natural state (Viitanen *et al.*, 1992). Therefore, although immunocytochemical analysis allows the cellular localization of proteins to be determined, it may not be as useful in detecting changes in protein levels over a given time course.

A comparison of hsp60 levels in neural and non-neural tissues revealed higher amounts of the protein in non-neural tissues at P1, in contrast to results obtained for hsp90 and hsc70. In the adult, levels of

hsp60 were similar in both brain and non-brain tissues. Tissue-specific differences in the relative levels of C.OX IV were also comparable to that of hsp60, with the exception of the brain stem, at postnatal day 1, which showed moderate levels of C.OX IV, and very low levels of hsp60. The high hsp60 and C.OX IV levels in the kidney and liver of the newborn rat may correspond to high metabolic rates in these tissues compared to the brain. In the adult, the similar levels of these mitochondrial proteins in brain and non-brain tissues suggest similar energy requirements. For example, the adult mammalian kidney expends a comparable amount of energy to the adult brain in preserving its ionic balance by the Na^+/K^+ ATPase (Soltoff, 1986).

Immunocytochemical analysis demonstrated a neuronal distribution of hsp60 and C.OX IV protein in the developing and adult rat brain. Glial-enriched areas of the brain, such as the deep white matter of the cerebellum, were immunonegative for these mitochondrial proteins. Even though hsp60 and C.OX IV were not detected in glial cells, it is likely that these cells do express both hsp60 and C.OX IV within their mitochondria, since these proteins are essential for mitochondrial function. Functionally active areas of the brain have elevated levels of glucose utilization. This enhanced activity can be visualized by metabolic mapping, using 2-deoxyglucose autoradiography or cytochrome oxidase histochemistry (Hevner *et al.*, 1995). The neuronal expression of C.OX IV protein detected

in this study is consistent with that found by other researchers (Hevner and Wong-Riley, 1991; Hevner *et al.*, 1995). The high metabolic demands of neuronal activity might explain the localization of hsp60 and C.OX IV to neurons in the brain. In general, neurons exhibit a greater oxidative metabolic activity than glia (Hevner *et al.*, 1995).

The developing and adult brain differ in their energy requirements. In the developing brain, energy is utilized for biosynthetic processes, such as cell division, growth, and differentiation, cell migration, dendritic arborization and synaptogenesis. The adult brain's primary energy requirement is for the maintenance of ion gradients. For example, it is thought that 40-60% of ATP in the adult brain is devoted to ion pumping by Na^+/K^+ ATPase (Erecinska and Silver, 1989). Although energy is utilized for different purposes in the developing and adult brain, the requirement for energy seems to increase during postnatal development. For example, an increase in oxygen consumption was observed in the rat brain during postnatal development, suggesting a developmental increase in the brain's energy requirement (Milstein *et al.*, 1968). These results, along with the mitochondrial biogenesis reported by others (Giuffrida *et al.*, 1979; Renis *et al.*, 1989), in the postnatal rat brain, lend support to the findings in the present study of a developmental increase in mitochondrial proteins hsp60 and C.OX IV.

It is important to keep in mind that although hsp60 has been shown

to exist predominantly within the mitochondrial matrix, recent work has suggested that a small amount of the protein may also be present at extra-mitochondrial sites, such as at the cell surface (Soltys and Gupta, 1996), or in the cytosol (Itoh *et al.*, 1995). However, these finding have been refuted by others (San Martin *et al.*, 1995). The importance of hsp60 in mitochondrial function was made apparent in a case report about a fatal mitochondrial disease in which a decrease in mitochondrial enzyme activities and aberrant mitochondrial ultrastructure, among other abnormalities, were noted (Agsteribbe *et al.*, 1993). A deficiency of hsp60 was also observed, and was suggested to be the cause of the defective synthesis and maintenance of the mitochondria in this patient.

4.4 Developmental expression of hsp70 protein

Analysis of hsp70 protein levels showed low basal amounts in the unstressed brain from postnatal day 1 to the adult. In contrast to the brain, the kidney was found to express hsp70 at high levels, and this expression increased during postnatal development, peaking at postnatal day 20. Basal levels of hsp70 mRNA and protein have been previously reported in the unstressed adult brain and kidney (Longo *et al.*, 1993; Tanguay *et al.*, 1993; Foster and Brown, 1996a, 1996b; Manzerra *et al.*, 1997). Constitutive expression of hsp70 mRNA was localized to

hippocampal and cortical neurons in the unstressed rabbit brain (Foster and Brown, 1996a). In addition, other tissues, such as the retina (Barbe *et al.*, 1988; Manzerra *et al.*, 1997) and muscle (Locke *et al.*, 1991) have also been shown to express basal levels of hsp70 protein. Basal levels of hsp70 protein were observed in unstressed swine heart (Locke *et al.*, 1996), and this expression was shown to be independent of an HSF-HSE interaction.

Hsp70 is generally considered to be an indicator of cellular stress. Following heat shock, or other forms of cellular stress, it is induced in both neural as well as non-neural tissues (for review, see Welch, 1993; Brown, 1994). The presence of hsp70 under normal physiological conditions may reflect a state of constant stress. For example, tissues such as the kidney, which experience high concentrations of toxic metabolites, may require the protective effects of basal hsp70 (Tanguay *et al.*, 1993). In addition, constitutive hsp70 levels that were expressed in the retina (Barbe *et al.*, 1988; Manzerra *et al.*, 1997), may result from the stress of light exposure.

Evidence also exists that hsp70 is developmentally expressed. An increase in basal expression of hsp70 protein through postnatal development has been noted in the sheep heart and lung by Western blot analysis (Strandness and Bernstein, 1997). Tanguay *et al.*, (1993) observed an increase in hsp70 levels from young to adult mice in various tissues, including brain and kidney. In the rat retina, an increase in hsp70 transcripts at postnatal day 7 was thought to represent a stress response to

photostimulation at the time of eye opening (Kojima *et al.*, 1996). In addition, hsp70 mRNA was associated with differentiating cells in the embryonic chicken lens, suggesting a role for hsp70 in the development of this tissue (Dash *et al.*, 1994). In cultured HeLa cells, hsp70 expression was coupled to the synthetic phase of the cell cycle (Milarski and Morimoto, 1986). This suggests a role for hsp70 in cell replication. In the postnatal brain, developing glial cells continue to divide for the first few weeks, while most large neurons are post-mitotic at birth (Rappoport and Fritz, 1972; Bayer and Altman, 1991). In the cerebellum, proliferation of granule cell neurons is extensive postnatally (Rappoport and Fritz, 1972). High rates of cell division in the kidney may account , in part, for the high basal hsp70 levels observed within this tissue. Multiple basal elements in the human hsp70 promoter have been identified (Greene *et al.*, 1987; Morgan, 1989; Williams *et al.*, 1989). These elements interact with TATA factors, Sp1, and CCAAT-box-binding transcription factor. Therefore, basal expression of hsp70 may be regulated by one or more of these promoter elements.

4.5 Summary

1. Western blot analyses revealed that in the brain, hsp90 and hsc70 protein levels were abundant throughout postnatal development. Levels of hsp60 increased in the brain during postnatal development,

similar to the increase observed for cytochrome oxidase, subunit IV (C.OX IV). Low basal levels of hsp70 were detected in the cerebellum and cerebral hemispheres throughout postnatal development. In the kidney, levels of hsp90 and hsc70 decreased during postnatal development, while levels of hsp60, C.OX IV, and hsp70 increased. These results suggest that the various heat shock proteins are differentially regulated during postnatal development of the rat.

2. A tissue-comparison between brain and non-brain tissues of the adult rat showed levels of hsp90 and hsc70 protein were greater in neural regions, such as the brain stem, cerebral hemispheres and cerebellum, than in non-neural tissues, such as the kidney and liver. In contrast, hsp60 and C.OX IV protein levels were greater in non-neural tissues than in neural regions at postnatal day 1. The kidney displayed the highest basal level of hsp70 at postnatal day 1 and in the adult. The comparative analysis of heat shock proteins in neural and non-neural rat tissues implies that tissue-specific differences exist in the expression of these heat shock proteins at postnatal day 1 and in the adult.

3. Immunocytochemical studies demonstrated a neuronal pattern of staining for hsp90, hsc70, hsp60 and C.OX IV in the cerebellum and brain stem, at postnatal days 1, 15 and in the adult. In all cases,

immunopositive signal was concentrated in the cytoplasm and neuronal processes of these cells. Glial-enriched areas of the brain, such as the deep white matter of the cerebellum, were immunonegative. High levels of these heat shock proteins may serve to protect neurons from physiological stress. In addition, heat shock proteins may also function in protein folding and protein trafficking in the growth and differentiation of neurons within the brain. The high metabolic requirements of neurons may necessitate high hsp60 (and C.OX IV) levels within these cells.

4. Examination of the expression of hsp90, hsc70 and hsp60 in the developing and adult kidney by immunocytochemistry showed that these heat shock proteins were localized to convoluted tubules of the renal cortex, as well as to medullary straight tubules. Heat shock proteins within the kidney may function in differentiation and/or protective processes. In addition, abundant levels of mitochondrial proteins, such as hsp60 and C.OX IV, may be required to meet the high energy demands of the developing and adult kidney.

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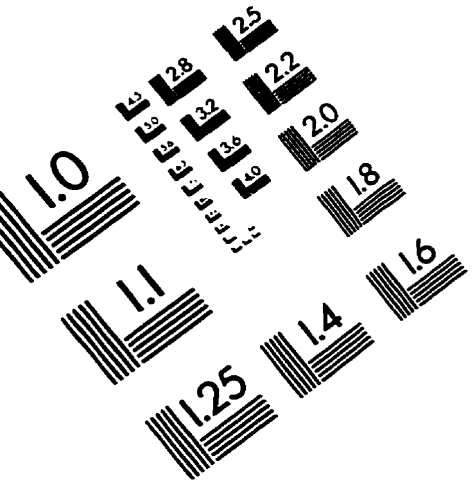
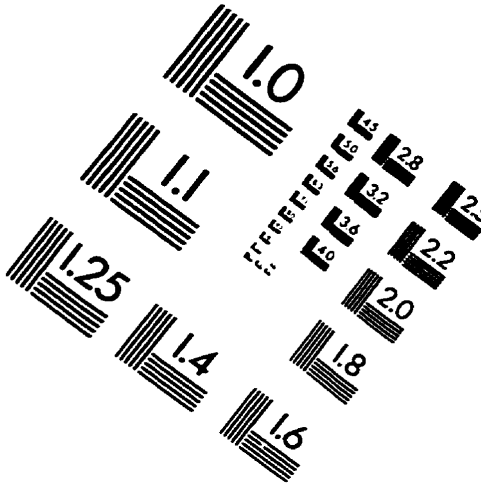
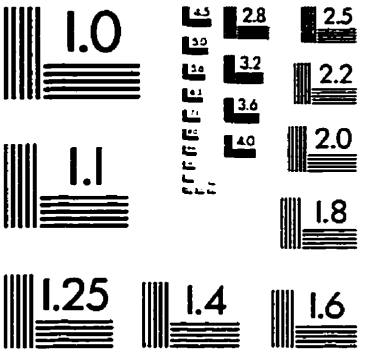
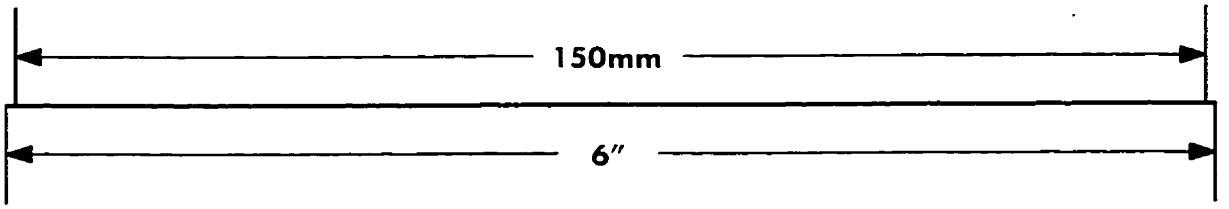
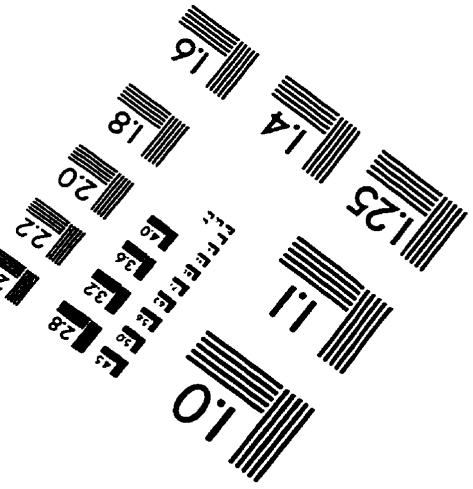
IMAGE EVALUATION TEST TARGET (QA-3)

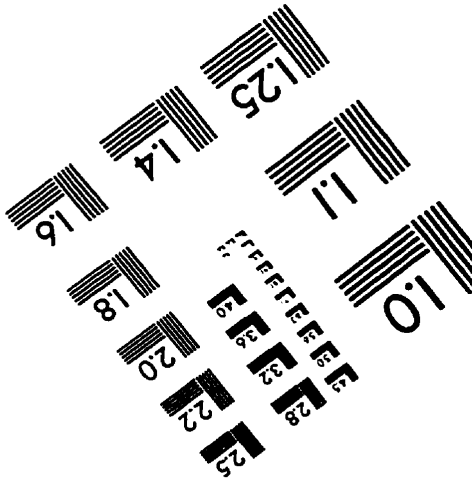












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