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Molecular Chaperones and Their Roles in Neural Cell Differentiation

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Abstract
During the development of the nervous system, a large number of neurons are eliminated through naturally occurring neuronal death. Many morphological and biochemical properties of such dying neurons are reminiscent not only of apoptosis, a type of death involving the action of genetically programmed events, but also of epigenetic phenomena such as oxidative stress. Increasing evidence demonstrates that oxidative stress alters the expression of antioxidant enzymes and enhances expression and/or DNA binding of numerous transcription factors, including heat shock factor. The latter is a transcription factor for specific promoter elements located upstream of the heat shock genes. Heat shock proteins (Hsps) are essential, highly conserved proteins that are needed for normal cell growth and maintenance, and expression of Hsps has been detected during embryogenesis in various organisms. Developmental profiles of Hsps indicate that they are differentially regulated during neural maturation, suggesting a role for Hsps in neural cell differentiation. Their putative function in cell remodeling during migration and differentiation, as well as during postnatal development, a time of extensive cell differentiation, is considered in the present review. Moreover, the function of Hsps in cell signaling, protein transport and the effect of heat shock on neural plate induction and brain development are discussed.

Introduction

Free radicals are an integral part of metabolism and are formed continuously in the body. Many sources of stress, such as heat, irradiation, hyperoxia, inflammation and any increases in metabolism, including exercise, injury, and even repair processes, lead to increased production of free radicals and associated reactive oxygen or nitrogen species. Evidence is accumulating that free radicals have important functions in the signal network of cells, including induction of growth and apoptosis.

On the one hand, dying neurons often display signs of oxidative stress, including an elevation of their intracellular concentration of free radicals. Antioxidants may reduce the extent of neuronal death, suggesting a causal

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implication of free radicals in the death process. Endogenous and nutritional antioxidant systems have to be adjusted to ensure adequate removal of radicals during stress to prevent damage to membranes, proteins, or nucleic acids. Excessive stress will induce DNA damage in the form of oxidized nucleosides, strand breaks, or DNA-protein crosslinks. Possible events associated with DNA damage are repair, apoptosis/necrosis, or defective repair leading to DNA sequence alterations and possibly to the developmental alterations or, in case of mitochondrial DNA, to metabolic dysfunction. The good message is that moderate stress may have protective effects against oxidant-induced DNA damage. Upregulation of endogenous antioxidant defense systems and complex regulation of repair systems such as heat shock proteins (Hsps; Hsp70, Hsp27, HO-1) are seen in response to moderate stress. Up-regulation of antioxidants and modulation of the repair response may be mechanisms by which mild oxidative stress can beneficially influence our health. Massive intervention into the redox state by pharmaceutical doses of exogenous antioxidants should be regarded with caution due to the ambiguous role of free radicals in regulation of growth, apoptosis, and cytotoxicity by immunocompetent cells, thereby constituting a potential threat for proliferative processes of regionalization which, endowed with positional information, are fundamental for production of neuronal patterns.

Although an alteration in Hsp expression in pathological or stress brain compared to normal adult brain is now well documented [1, 2], far less is known about the expression of these proteins during brain development and differentiation. The expression of heat shock genes during embryonic development has been investigated in different vertebrate and invertebrate models. Indeed, many heat shock genes display complex patterns of constitutive and inducible expression and, although significant progress has been made in understanding the chaperone machinery, relatively little is known about the specific role of the heat shock system during brain development.

Neurons and glial cells of the central nervous system (CNS) originate from a specialized region of ectoderm, the neural plate. Following neural induction, the neuroectodermal cells lining the neural tube proliferate, migrate and differentiate into various cell types, such as neurons and glial cells of adult brain. At the lateral margin of the neural plate a significant number of cells migrate into mesoderm forming the neural crest, from which some cells originate giving rise to neurons and glial cells of the peripheral nervous system [3]. Cell proliferation and cell fate specification are under strict spatiotemporal control in the developing neural plate and early brain formation. During mammalian development of the brain, precursor cells undergo characteristic patterns of cell division before commitment to specific fates. Such invariant division may result from dependence of cell fate on cell lineage and also from a precise pattern of divisions, which are essential to achieve the required distribution of cytoplasmic determinants. Cell division generally is under tight control, even in conditions whereby cells adopt fates that are independent on their lineage program. At the molecular level a number of regulatory genes are expressed to ensure regional distribution of restricted patterns in the developing brain [4]. This process of regionalization provides positional information that is fundamental for production of neuronal pattern, and also for migration and axon elongation [5]. Neuronal differentiation begins in the mouse between embryonal stage E8.5 (time of neural tube closure) and E11 [6, 7]. Glial cells generally appear later, at E11 for radial glia, E16 for astrocytes, while generation of myelin forming cells, oligodendrocytes, starts postnatally [8]. Neuronal cell death is an important phenomenon in the series of steps involved in development. In fact, during the development of mammalian CNS 50% or more of many types of neurons die soon after they fail to form synaptic connections with their target cells [9]. The molecules involved in the formation of the neural tube are largely unknown; hence, any molecule displaying a specific pattern of expression and paralleling important morphogenetic events can be a valuable candidate to play an active part in brain development. Hsp expression appears to be closely linked in early mammalian development to critical differentiation and proliferation stages. Increasing evidence now indicates that Hsps are developmentally activated around blastula stage and constitutively expressed at high levels during neural tube closure [10]. The chaperone function of Hsps, as molecules involved in cell protection against injury and death, control of cytoskeletal structure and intracellular transport, as well as in the activation of transcription factors, has been well established. However, only recently, Hsp47 and Hsp27 have been shown, during gastrulation, to specifically bind and fold to nascent collagen and actin molecules, and this role has been recognized to be essential for the formation of the basement membrane, extracellular matrix and neural crest migration during neural plate development. In this context, the demonstration of a developmental activation of the heat shock element has been crucial for underscoring the role of Hsps in neural cell fate. Indeed, during neuroectoderm differentiation the activation of heat shock factors 1 and 2 (HSF1, HSF2) appears to correlate with

high constitutive expression of many of the Hsps. Specifically, Hsps90, 73, 71, 47 and 27 are tightly regulated by the cell cycle at neurulation. Similarly, only recently, Western blot and immunocytochemical studies have demonstrated a neuronal localization of Hsp90, Hsc70 and Hsp60 at all stages of postnatal development, exhibiting a developmental profile which indicates that they are differentially regulated at different stages of neural maturation. In the present review, we discuss the role of Hsps in cell remodeling during migration and differentiation, as well as during postnatal development, a time of extensive cell differentiation. We then review the evidence for Hsp function in cell signaling, protein transport and the effect of heat shock on neural plate induction and brain development.

Roles of Molecular Chaperones in the Nervous System

It is well known that living cells, when challenged by conditions which cause acute or chronic stress, can activate networks of responses which detect and control diverse forms of stress. One of these responses, known as the heat shock response, represents a fundamental mechanism necessary for cell survival under a wide variety of toxic conditions. In mammalian cells Hsp synthesis is induced not only after hyperthermia, but also following alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs or cytotoxic drugs. It is now ascertained that transient exposure to elevated temperatures has a cross-protective effect against sustained, normally lethal exposures to diverse pathogenic stimuli. Hence, the heat shock response contributes to establish a cytoprotective state in a variety of metabolic disturbances and injuries [11]. This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies [12]. In mammalian cells the induction of the heat shock response is regulated by trans-acting heat shock factors and a cis-acting heat shock element present at the promoter region of heat shock genes [13, 14]. In mammalian cells, three heat shock factors have been identified: HSF1, HSF2 and HSF4 [15]. HSF1 mediates the induction of heat shock genes in response to temperature elevation and other stress [16]. In response to stress conditions, HSF1 undergoes oligomerization from a non-DNA-binding monomeric form to a trimeric, DNA-binding form which interacts with hsp gene promoter sequences to increase transcription of these genes [17]. Two isoforms of HSF1 protein exist in murine cells, which arise from alternative splicing of HSF1 pre-mRNA [16]. HSF1 does not show constitutive DNA-binding activity in postimplantation mouse embryos and, also, constitutive Hsp expression in cultured embryonic murine cells remains unaffected by the disruption of the HSF1 gene [18]. In addition, *hsf1* (–/–) knockout mice have an increased chance of prenatal death and females are infertile, present chorioallantoic placenta, growth retardation and increased production of tumor necrosis factor-α which results in increased mortality after endotoxin challenge [19]. Because basal Hsp expression is not altered by disruption of the HSF1 gene, it seems conceivable that HSF1 might be involved in regulating other important genes or signaling pathways.

HSF2 is not activated in response to heat shock and most other forms of stress [20] and appears to function as a regulator of *hsp* gene expression under nonstress conditions, particularly in cells involved in processes of differentiation and development [16, 21]. HSF2 mRNA is subjected to developmental cell-type-dependent regulation and, as with HSF1, two isoforms HSF2α and HSF2β exist in murine cells, arising from alternative splicing of HSF2 pre-mRNA. HSF2α, a larger isoform, is predominantly expressed in testis, whereas HSF2β is predominantly expressed in brain. HSF2β is a less potent transcriptional activator than the larger HSF2α isoform [16]. HSF2β appears to act as a negative regulator of HSF2 DNA-binding activity and transcriptional induction of heat shock genes during hemin-mediated erythroid differentiation of K562 cells [22]. Also, HSF2 exists in a DNA-binding form in mouse embryos during postimplantation development and it may have a role in neural proliferation [23]. HSF2 is activated when the ubiquitin-proteasome pathway is inhibited, thus providing a heat shock gene-regulatory mechanism to respond to changes in the protein-degradative machinery [24]. It also interacts with the PR65 (A) subunit of protein phosphatase 2A (PP2A) thereby blocking its interaction with the catalytic subunit for the same binding site on PR65 [25]. PP2A is involved in the regulation of various important cellular processes, including intermediary metabolism, signal transduction and cell cycle progression [26].

It is now widely accepted that most Hsps have a molecular chaperone activity involved in various aspects of protein biogenesis [27]. A molecular chaperone is a protein that binds to and stabilizes not only an otherwise unstable conformer but also apparently stable proteins, and by controlled binding and release of protein substrate, facilitates its correct fate in vivo: folding, oligomeric assembly,

transport to a particular subcellular compartment or controlled switching between active/inactive conformations.

Hsps can be classified into major families, based on their molecular weight [28]. These include Hsp100, Hsp90, Hsp70, Hsp60, Hsp47, Hsp32 and Hsp20. It is well documented that in adult nervous system some Hsp genes are constitutively expressed. For example Hsp90 accounts for 1–2% of total cellular proteins in rabbit CNS [29] and Hsc70 for 2–3% of the total protein content of the rat spinal cord [30]. This constitutive expression is preferentially localized to the neurons [31].

The mammalian brain is highly sensitive to different stresses, such as fever, ischemia, seizure and drugs, such as amphetamines and LSD. These stressful conditions induce Hsp70 and Hsp90 synthesis generally in glial cells. Depending on the stress type, Hsp70 is also expressed in neurons [32]. Hsp27 has been reported to be induced in a large number of astrocytes of ischemic or kainic acid-stressed rats [33], and also in neurons in pathological conditions, such as Alzheimer's disease and Creutzfeldt-Jakob disease [34]. The molecular chaperone activity of various members of Hsp70 is regulated by the Hsp40 family, as they work as a chaperone complex which is ubiquitous from bacteria to mammals. Cytosolic Hsp70 and Hsp40 are known to bind to nascent polypeptide chain emerging from translating ribosomes and to facilitate their correct folding [35]. As demonstrated in vitro, the binding of Hsp70 to a substrate prone to aggregation is dependent on Hsp40-enhanced ATP hydrolysis. Thus, Hsp70 can protect protein from heat-induced aggregation and promote the renaturation of thermally denatured proteins [14]. Other Hsps, such as Hsp90, Hsp60 and Hsp27, have molecular chaperone activity and participate in various aspects of protein biogenesis and in protecting cells from deleterious environmental stresses. The protective role of different classes of Hsps in pathological or stressed adult brain has been widely recognized, and emerging evidence is now indicating that they may offer strategies for tissue protection in developing nervous system.

Hsp90

The Hsp90 family is present constitutively in relatively large amounts in the cytosol of normal unstressed cells. After heat stress, it is only moderately induced. It has transient interaction with steroid receptors, actin tubulin and is associated with microtubules of interphase cells. GRP94, a glucose-regulated protein, is a member of this family. In differentiating neuroectoderm Hsp90 was highly expressed in the G0 phase or resting phase of the cell cycle. Following severe heat stress a reduction in Hsp90 gene expression has been observed [10] allowing cells to progress through to G1+S phase, thus suggesting that Hsp90 may be important to maintain cell G0 phase [10]. The ansamycin antibiotics herbimycin and geldanamycin bind to a conserved pocket of Hsp90. Occupancy of this pocket prevents ATP-dependent release of protein undergoing refolding. The stabilized complex is then ubiquinated and degraded. This leads to degradation rather than maturation of target key signaling proteins, such as Raf serine kinase, met tyrosine kinase, steroid receptors, and members of the HER kinase family, with resulting growth arrest and possibly cell death. Hsp90 are expressed early during neural crest cell migration, follow the differentiation of the first tracts, and are present in many differentiating fields, where neurons out of the proliferating zone start to undergo their morphogenesis [5]. During brain development, Hsp90α and Hsp90β are widely expressed after E15.5. The coincident localization with neuronal marker suggests that this class of stress proteins is necessary for specific neuronal function [36]. In addition, Hsp90 has been assigned chaperone functions in the mechanisms of cellular signaling through its interaction with protein kinases and transcription factors. Hence, a disruption of the Hsp90 pathway has strong repercussions for development, as demonstrated in *Drosophila* [37]. Growth and/or neurothropic factors play important biological roles in the development and maturation of CNS. They act as instructive signals for lineage commitment in developmental brain, promoting graded stages of cell differentiation, axonal guidance, survival of subsets of neurons, and also maintaining neural phenotype. A wide variety of neurotrophic factors have been described, any of which may require Hsp90 to function properly.

Potential targets for Hsp90 include steroid receptors [38], basic helix-loop-helix (bHLH) factors such as MyoD [39] or single-minded proteins [40], HSF1 and a mutated form of the suppressor p53. Recent studies indicate that bHLH genes play basic functions in CNS morphogenesis. In neural development the transition from the initial phase of growth in dividing precursor cells to the subsequent differentiation phase of postmitotic cells is controlled antagonistically by multiple bHLH genes. Indeed, cascades of neuronal bHLH genes promote determination and differentiation, whereas antineuronal bHLH genes repress them under the control of *Notch*, thus maintaining cells at the precursor stage [41].

Western blot analysis of Hsp expression during embryonic brain development in mouse embryo [5] revealed that at stage E9.5, when the neural tube has just closed and is undergoing an active proliferation phase, inducible

Hsp90α are not detectable, whereas Hsp90β are ubiquitously expressed in all embryonic tissues. At embryonic stage E12.5 brain development has progressed with the formation of numerous nuclei, the ontogeny of tracts and the first signs of neurodifferentiation, as revealed by positive immunostaining with anti-βIII-tubulin. At this stage Hsp90β are widely expressed throughout the CNS, with higher levels of expression in the derivatives of the subventricular layer of the neural tube. Conversely, Hsp90α are not detectable under normal conditions. Embryonic stage E15.5 is characterized by the formation of a stratified primary cortex in the telencephalon, the neopallium, and the first steps in the formation of the cerebellum. In addition, olfactory bulbs and choroid plexi appear fully differentiated. Hsp90β is ubiquitously expressed, but with a lower level in the ventricular layer of the neuroepithelium. From the anterior to posterior part of the brain, Hsp90β is most abundant in the preoptic areas, hypothalamus, pons and medulla. At this stage, however, Hsp90α exhibits a distribution similar to that of Hsp90β, but the expression is comparatively lower. Conversely, Hsp90α, and not Hsp90β, is expressed at a high level in the choroidal plexi in the lateral and fourth ventricles in proximity of the epithelial layer bordering the cavity of the ventricle, where secretion of cerebrospinal fluid occurs. At embryonic stage 17.5 many Hsps are expressed. Hsp90α is strongly expressed in the bed nucleus of the stria terminalis, which is a part of the hypothalamus, and also in the medial part of the hypothalamus, but not in the hypophysis or in the trigeminal ganglia. Hsp90α is specifically expressed in the ventricular epithelium of the mes-metencephalic border. Consistently, glucocorticoid receptor is detected in the rat at E14 in hippocampal formation and at E16 in hypothalamic paraventricular nucleus [42], and the hypothalamus is in mammalian systems an important regulation center for physiological and endocrine functions. As demonstrated by Olazabal et al. [43], estradiol induces Hsp90 and progestin receptor in adult rat ventromedial hypothalamus. All this lends support to the hypothesis that the specific labeling observed in the hypothalamic nuclei might be related to the Hsp90α steroid-receptor-binding activity. However, this activity seems assigned only to Hsp90α and not to Hsp90β, suggesting that the distinct localization observed for the two Hsp90 forms underlies the possibility that Hsp90α and -β perform different early developmental functions.

Hsp90 protein expression during postnatal development has recently been described [44]. As demonstrated, Hsp90 protein levels decreased slightly during postnatal development in brain regions such as cerebellum and cerebral hemispheres. Immunocytochemical analysis of Hsp90 expression during this developmental period reveals that Hsp90 was expressed in the Purkinje neurons at all stages of postnatal development. Immunoreactivity was localized to the cytoplasm as well as to apical dendrites in these neurons at P1, P15 as well as in the adult. Moreover, neurons in the deep cerebellar nuclei and in the brain stem were found immunopositive for Hsp90, whereas glial enriched areas of the brain, such as the white matter of the cerebellum, was immunonegative for Hsp90. Additionally, a comparison of Hsp90 levels between various adult rat tissues showed Hsp90 protein levels to be greater in neural regions such as the brain stem, cerebral hemispheres and cerebellum, compared to non-neural tissues [44].

Hsp70

The 70-kDa family of stress proteins is one of the most extensively studied. Included in this family are Hsc70 (heat shock cognate, the constitutive form), Hsp70 (the inducible form, also referred to as hsp72), Grp75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum). After a variety of CNS insults, Hsp70 is synthesized at high levels and is present in the cytosol, nucleus and endoplasmic reticulum. Denaturated proteins are thought to serve as stimulus for Hsp70 induction. After heat shock, for instance, the synthesis of hsp70 increases to a point where it becomes the most abundant single protein in a cell. Once synthesized, Hsp70 binds to denaturated proteins in an ATP-dependent manner. The N-terminal end contains an ATP-binding domain, whereas the C-terminal region contains a substrate-binding domain.

In the nervous system Hsp70 are induced in a variety of pathological conditions, including cerebral ischemia, neurodegenerative disorders, epilepsy and trauma. Expression of the gene encoding Hsp70 has been found in various cell populations within the nervous system, including neurons, glia and endothelial cells [45]. Whether Hsp70 are neuroprotective has been the subject of much debate, as it has been speculated that these proteins might be merely an epiphenomenon unrelated to cell survival. Only recently, however, with the availability of transgenic animals and gene transfer, has it become possible to overexpress the gene encoding Hsp70 to test directly the hypothesis that stress proteins protect cells from injury, and it has been demonstrated that overproduction of Hsp70 leads to protection in several different models of nervous system injury [46]. A recent study reported that Hsp70 protects the brain against injury produced by isch-

emia and seizures [47]. In this study, after a defective herpes simplex virus vector, expressing the gene encoding Hsp70 was injected directly into the rat brain, the neuronal survival was significantly increased, from 62 to 95%, in the striatum following middle cerebral artery occlusion [47], and from 22 to 62% in the hippocampus following kainic acid-induced administration [47]. Following focal cerebral ischemia, mRNA encoding Hsp70 is synthesized in most ischemic cells except in areas of very low blood flow, because of limited ATP levels. Hsp70 protein is produced mainly in endothelial cells, in the core of infarcts in the cells that are most resistant to ischemia, in glial cells at the edges of infarcts and in neurons outside the areas of infarction. It has been suggested that this neuronal expression of Hsp70 outside an infarct can be used to define the ischemic penumbras, which means the zone of protein denaturation in the ischemic areas [48]. A number of in vitro studies show that both heat shock and Hsp overproduction protect CNS cells against both necrosis and apoptosis. Mild heat shock protects neurons against glutamate-mediated toxicity and astrocytes against injury produced by lethal acidosis [49]. Transfection of cultured astrocytes with Hsp70 protects them from ischemia or glucose deprivation [50]. Hsp70 has been demonstrated to inhibit caspase-3 activation caused by ceramide, and also affect JUN kinase and p38-kinase activation [51]. In addition, Hsp70 binds to and modulates the function of BAG-1, the bcl-2 binding protein [52]. Hence, Hsp70 appears to act upstream in some apoptotic cascade thereby modulating some but not all types of apoptosis-related cell death [1]. A large body of evidence now suggests a correlation between mechanisms of oxidative and/or nitrosative stress and Hsp induction. Current opinion holds also the possibility that the heat shock response can exert its protective effects through inhibition of NFkB signaling pathway [12]. We have recently demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of Hsp70 stress proteins. Increase in Hsp70 protein expression was also found after treatment of cells with the NO generating compound sodium nitroprusside, thus suggesting a role for NO in inducing Hsp70 proteins. The molecular mechanisms regulating the NO-induced activation of the heat shock signal seems to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and antioxidant enzymes [53, 54].

Comparison of Hsp levels between various brain regions shows comparable levels for Hsp90β and Hsc70 during CNS development. Hsp70 is greater in mesencephalon than in diencephalon, hindbrain or telencephalon. At embryonic stage E9.5 inducible Hsp70 is not detectable, whereas Hsc70 is expressed at a significant level in the cells of the diencephalon and in the basal plate of the rhomboencephalic plate. Embryonic stage E12.5 is characterized by a Hsc70 localization in strongly βIII-tubulin immunoreactive (neuronal) cells of the stria medullaris, as well as in the marginal layer of the spinal cord. At this stage inducible Hsp70 are not expressed [5]. At embryonic stage E15.5 Hsc70 is present in the neocortex, basal ganglia, preoptic areas of the anterior brain as well as in the posterior commissure. Hsp70 is not detectable in the CNS before E15.5. At this stage, cortical localization of Hsp70 is highly specific, compared to the other Hsps. Hsp70 is expressed in the external plexiform layer in the olfactory bulb, ventrally in the septum and dorsally in the neocortex. Also, Hsp70 immunoreactivity is found in the diencephalon, in the preoptic area, in the mesencephalon, metencephalon and in the cerebellar vermix. Peripherally, in the medulla Hsp70 is expressed at the level of the ventral pyramidal tracts. Interestingly, glial fibrillary acidic protein (GFAP) colocalizes perfectly with Hsp70. At E17.5 the specific pattern of Hsp70 expression reflects GFAP distribution, whereas Hsc70 is more highly expressed in βIII-tubulin-positive cells (neurons) in the subventricular zone. It is known that neurons of hippocampus and of dentate gyrus appear at E11 and their formation stops at E16. At E17.5 in the hippocampus all Hsps are expressed, with Hsp70 distributed in GFAP immunoreactive cells and Hsc70 distributed in neuronal cells (immunoreactive to the neurospecific βIII isoform of tubulin).

The levels of Hsc70 during postnatal brain development are significant in many brain regions and are maintained through to the adult. At the cellular level, they are primarily concentrated in the neuronal cytoplasm as well as to dendritic processes. The same pattern has been observed in neurons of the deep cerebellar nuclei and brain stem [44]. Developmental analysis of Hsp70 in postnatal rat brain reveals that basal levels increased in the cerebral hemispheres until P20 and then decreased in the adult, whereas in the cerebellum comparatively little changes were observed during postnatal development, as compared to the adult. Developmental expression of Hsp73, the constitutive form of Hsp70 in human brain, begins as early as 6 weeks of gestation, increasing in intensity and extending rostrally during fetal life. In the postnatal period Hsp73 immunoreactivity was always positive in neurons and glial cells [55], but with regional differences. It is noteworthy that the regions with low levels of Hsp73 at 30 weeks of gestation, such as pontine nuclei in

the brain stem, or CA1 and subiculum pyramidal cells in the hippocampus, are vulnerable at this stage of perinatal hypoxic-ischemic insults, which often cause a distinct lesion known as pontosubicular necrosis. This coincidence rises the possibility that Hsp73 protects neurons from hypoxic-ischemic insults. The time course of developmental Hsp73 immunoreactivity in human brain is consistent with the time courses of overall neuronal and glial maturation, suggesting an increasing role of Hsp73 during neural cell differentiation [55].

Hsp60, Glucose-Regulated Protein 75 (GRP75) and Hsp10 Chaperone Proteins within Mitochondria

GRP75 and GRP78, also called oxygen-regulated proteins, are produced by low levels of oxygen and glucose. These protect brain cells against ischemia and seizures in vivo, after viral-induced overexpression [56]. Developmental profile of Hsp60 suggests that it increases during postnatal development in the rat brain stem and the cerebral hemispheres, while cerebellum shows an increase of lesser magnitude. In these regions Hsp60 levels are significantly higher at P20 and in the adult compared to P1. Similar to expression of Hsp90 and Hsc70, in the developing rat brain, Hsp60 protein is localized in the cytoplasm as well as in dendritic processes of neurons. Remarkably, the neuronal developmental patterns of subunit IV of cytochrome oxidase paralleled that of Hsp60, a protein localized predominantly to mitochondria. Hsp60 is indeed associated with mitochondria, where it participates to import, folding and assembly of transported proteins into the mitochondrion [57].

Hsp32

Hsp32, also known as heme oxygenase 1 (HO-1), is synthesized mainly in microglia and is one of several related heme oxygenase proteins that metabolizes heme to carbon monoxide, iron and biliverdin [58]. The gene encoding heme oxygenase 2 (HO-2) is constitutively expressed and found in neurons and endothelial cells. HO-1 and HO-2 regulate heme protein turnover, iron metabolism and oxidative stress [59]. The mechanism of HO-1 induction and Hsp response is dependent on the discovered heat shock factors [60]. The role of HO in cell protection is evident in some forms of nitrosative stress and oxidative stress, such as exposure to light, free radicals, irradiation and other forms of degenerative diseases and ageing process in which enhanced HO activity seems to have an antioxidant effect. Induction of HO occurs together with the induction of other Hsps in the brain during various experimental conditions, including ischemia [61]. In the cortex and hippocampus of patients with Alzheimer's disease, where oxidative stress is extensive [62], HO-1 has been shown to be overexpressed and also colocalizes to senile plaques and neurofibrillary tangles [63]. Up-regulation of HO-1 in the substantia nigra of subjects with Parkinson's disease has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximum HO-1 immunoreactivity [64]. Injection of blood or hemoglobin results in increased expression of the gene encoding HO-1, which has been shown to occur almost exclusively in microglia throughout the brain [65]. This suggests that microglia take up extracellular heme protein following cell lysis or hemorrhage. Once in the microglia, heme induces the transcription of HO-1. HO-1 then metabolizes heme to biliverdin, CO and iron. The iron released by HO-1 is bound by ferritin, perhaps via a HO-1 chaperone function [66].

Hsp27

Small heat shock proteins (sHsps) are a family of Hsps categorized by their molecular mass ranging from 15 to 30 kDa. Although sHsps in mammalian cells are initially identified as a component of a single protein (Hsp27, also known as Hsp25 or Hsp28), recent studies have revealed that αB-crystallin, a component of the vertebrate eye lens protein, is also a member of the Hsp family [67]. Hsp27 and αB-crystallin form oligomeric structures that are modified by phosphorylation, reducing the multimeric size [68]. Phosphorylation of Hsp27 is increased in response to various stimuli such as serum, ionophore, and a set of growth factors or cytokines [69].

In the CNS sHsps are predominantly localized in glial cells. Marked induction of both Hsp27 and αB-crystallin is found mainly in astrocytes in response to stress stimuli, and also in astrocytes and oligodendrocytes associated with various neurological diseases such as Alzheimer's disease, Alexander's disease, Creutzfeldt-Jakob disease and multiple sclerosis [70]. The function of sHsps is still unclear. sHsps are thought to act as molecular chaperones in the maintenance of the native conformation of cytosolic proteins, allowing cells to survive under stress conditions [1]. Furthermore, recent studies have shown that sHsps may all play a physiological role. Expression of sHsps is developmentally regulated in several organisms [71]. In mammalian cells an increase in sHsps has been demonstrated during differentiation [72] and the constitutive expression of sHsps results in inhibition of Fas/APO-1-mediated apoptosis and cell proliferation [73] as well as in protection against staurosporine, tumor necrosis

factor and etoposide-induced apoptotic cell death or H_2O_2-induced necrosis [74]. These results raise the possibility that sHsps could regulate cell differentiation and proliferation under both physiological and pathological conditions. Moreover, in response to brain injury, astrocytes extend numerous processes to form scar tissues, a process called reactive gliosis. The transformation of 'resting' astrocytes to their 'reactive' form is characterized by hypertrophy, stellate shape, and an increase in GFAP expression. Although little is known about the precise mechanism underlying this transformation in response to physiological insults it is intriguing that emerging evidence suggests β-adrenergic receptor together with sHsps might play an important role in cytoskeletal reorganization associated with developing reactive gliosis [70]. This view is corroborated by the finding that sHsps can modulate not only actin microfilament dynamics, but also GFAP assembly by the phosphorylation of their non-α-helical head domains [70, 75]. At embryonic stage E9.5 Hsp25 is present at very low level. It is localized in the first differentiating motoneurons of the truncal spinal cord [5]. At embryonic stage E12.5 Hsp25 is expressed throughout the brain, where it is very selectively expressed in isolated or grouped neurons of the tracts limiting the different prosomeres, in the ventral regions of the tegmentum, pons and medulla. At embryonic stage E15.5, besides its ubiquitous synthesis, Hsp25 exhibits a specific pattern of expression in the epithelium of the olfactory bulbs, in the rhinencephalon, the septum and the striatum in the basal ganglia and the neocortex in the anterior brain. Hsps are ubiquitously expressed at embryonic stage E17.5. During development, in the cerebellum, several distinct phases in Hsp25 expression can be distinguished and, as it has been demonstrated, this seems to reflect the topographical complexity of cerebellar compartmental organization [76]. Hsp25 immunopositive Purkinje cells are, in fact, seen in the mouse at birth, and by postnatal days 6–9 a phase of widespread expression begins. Also, Hsp25 is heavily expressed in the earlier stages of brain vasculature development, when the first endothelial cells, originating from the neural crest cells, invade the periphery of the neural tube and migrate inside, hence proliferating and differentiating into brain vessels. These cells are strongly immunoreactive to anti-Hsp25 antibody. Indeed, Hsp25 are strongly expressed in the adult meninges, primarily in the dura mater.

Discussion and Conclusions

Hsps such as Hsp90, Hsp70, Hsp60, Hsp40 and Hsp28 are constitutively expressed in normal conditions, playing basic and indispensable functions in the life cycle of proteins as molecular chaperones, as well as in protecting against metabolic and oxidant insults [77]. Recently, constitutive Hsp70 (Hsc70) and Hsp40 have been localized to the synapse in the mammalian CNS, indicating a synaptic role for these Hsps [78]. In addition, several lines of evidence indicate that molecular chaperones are related to synaptic plasticity phenomena [79]. As constitutive Hsps are present at certain embryonic stages, it can also be inferred that they play a role in normal development. Hsp expression appears to be closely linked in early mammalian development to critical differentiation and proliferation stages, as they are developmentally activated around blastula stage, constitutively expressed at high levels during neural tube closure, and are heat shock-responsive. These constitutive proteins form a relatively large proportion of the total protein content of the neuroectoderm at day 8 in the mouse or days 9.5–11.5 in the rat, the stages of neural induction and major organogenesis. The role of Hsps in protein folding and degradation has been well documented by in vitro and in vivo studies. However, despite increasing research efforts, understanding the developmental role of Hsps and chaperones is still elusive, owing to the main limits represented by the still scarce information we have on what these specific functions might be. With the known functions of Hsps, and the evidence that the onset and duration of inducibility of Hsps coincides with onset and duration of the most critical stage of organogenesis, it appears that a function of the heat shock response could be to provide protection against embryonic damage by heat and other stresses at vulnerable stages of development. Moreover, cells of developing tissues undergo extensive architectural remodeling during either migration, adhesion, or differentiation. This remodeling leads to extensive reorganization of the cytoskeletal system (microtubules, microfilaments and intermediate filaments). It has been shown that many Hsps bind to cytoskeletal elements [80]. Coprecipitation studies have indicated that Hsp90 and Hsc70 bind to actin and microtubules. Also, it has been suggested that Hsp70 may regulate tubulin polymerization through two mechanisms: directly, by acting as antagonist of microtubule-associated proteins, thereby inhibiting tubulin polymerization required for cell division and differentiation, or indirectly, through association with the microtubule-associated protein tau [81]. It has also been shown that Hsp25,

by interacting with peripherin, vimentin and GFAP, which are three intermediate filament proteins, might be responsible for the maintenance of the different networks of these filaments [82]. In addition, the association of Hsp25 with F-actin in a phosphorylation-dependent manner [68], and its ability to affect cell motility and shape, provide a rational explanation for Hsp25 being selectively expressed in migrating neurons.

Cell differentiation, especially dendrite and axonal process formation, leads to an activation of protein transport. This involves clathrin-coated vesicles shuttling back and forth. It has been demonstrated that Hsc70 functions as an ATP-dependent uncoating enzyme which releases clathrin from coated vesicles. Two components of axonal transport have been described with different rates of transport. Increasing evidence suggests that Hsc70 binds to clathrin during the fast axonal transport [83] and this could be important in shuttling correctly folded proteins to synaptic plasma membrane during cell differentiation. Slow axonal transport is generally utilized by the cell for soluble proteins which are associated to elements of the cytoskeleton. Hsc70 may serve as a cross-linker molecule between transported molecules and neurofilaments, through binding of Hsp70 directly with the cellular targeting sequence [84]. Hence, an increased Hsp synthesis during neuronal development may occur as a consequence of an increased demand for axonal transport.

The intimate relationship linking developmentally regulated phenomena and the heat shock system in the brain is also confirmed by those studies describing teratogenic effects of hyperthermia on neural plate induction and brain development. Hyperthermia refers to a higher than normal body temperature with the induction of a characteristics cellular heat shock response and the induction of Hsps interrupting and altering the physiological homeostatic mechanisms of the cell [85]. Records from many sources indicate that approximately 3% of newborn children have a developmental defect requiring medical attention, and approximately one third of these conditions can be regarded as life-threatening. The emotional and financial cost of birth defects is enormous; approximately one half the children are in hospitals because of a birth defect. In most instances the cause of the defect remains unknown, about 25% is genetic in origin and less than 10% can be ascribed to a teratogenic agent. Human studies have shown a number of defects to be associated with a maternal hyperthermic episode [86]. The *hsp* genes have been highly conserved through evolution in all species investigated. The relatively high and stable body temperatures of mammals confer many advantages in survival compared with poikilothermic animals. It could be inferred that evolutionary pressures could result in even higher body temperatures unless prevented by some biochemical and physiological barriers. It has been suggested that the deleterious effects of heat on spermatogenesis and cellular proliferation in embryos are two of such restrictions. The initiation of early CNS and brain formation begins at neural tube closure at approximately 3 weeks of human pregnancy. This is one of the most sensitive and critical periods of mammalian development. Interruption to neural plate development and induction of the neuroectoderm results in major craniofacial defects and mental retardation. Differentiating neuroectoderm exposed to heat shock causes rapid cell cycle changes resulting in either thermotolerance or cell death. In general, the type of defect caused by heat shock depends largely on the stage of embryonic development at the time of exposure and the severity depends largely on the dose of heat, which is both the product of the temperature elevation and the duration of elevation. Heat shock-induced defects encompass a broad developmental spectrum corresponding to the interruption of cell differentiation during organogenesis at the relevant stages of development. These include, besides the aforementioned neural tube and craniofacial defects, microphthalmia, heart defects, coloboma, kyphosis, scoliosis and skeletal defects when exposed about the time of neural tube closure. Cataract, hypodactyly, microencephaly, renal and dental agenesis, exomphalos, cranial nerve defects, and behavioral abnormalities follow exposure at later stages of embryogenesis. Embryos can tolerate variable doses of heat shock depending mainly on the stage of development. In rat embryos (E9.5) a defined threshold elevation of temperature causing defects in vivo has been calculated and is 2–2.5°C over a total heating and cooling period of 60 min. It has been suggested that this threshold exists because of the presence of constitutive Hsp90, 70, 47 and 27 which can afford protection against denaturation, so that the threshold represents an indirect quantitative measure of the noxa required to titrate out the constitutive Hsps [87]. Furthermore, it has been known that even at normal body temperatures (37–40°C) proteins are denatured, with a loss at 37°C of over 0.2% of cells per hour [88].

However, there is ample evidence that a strongly protective heat shock response need not be teratogenic, but there is equally strong evidence that a teratogenic dose of heart is associated with highly elevated hsp mRNA and Hsps, and that cell death is a prominent feature of the damage to the nervous system. Perhaps, there is a threshold level for the response, above which the developmental

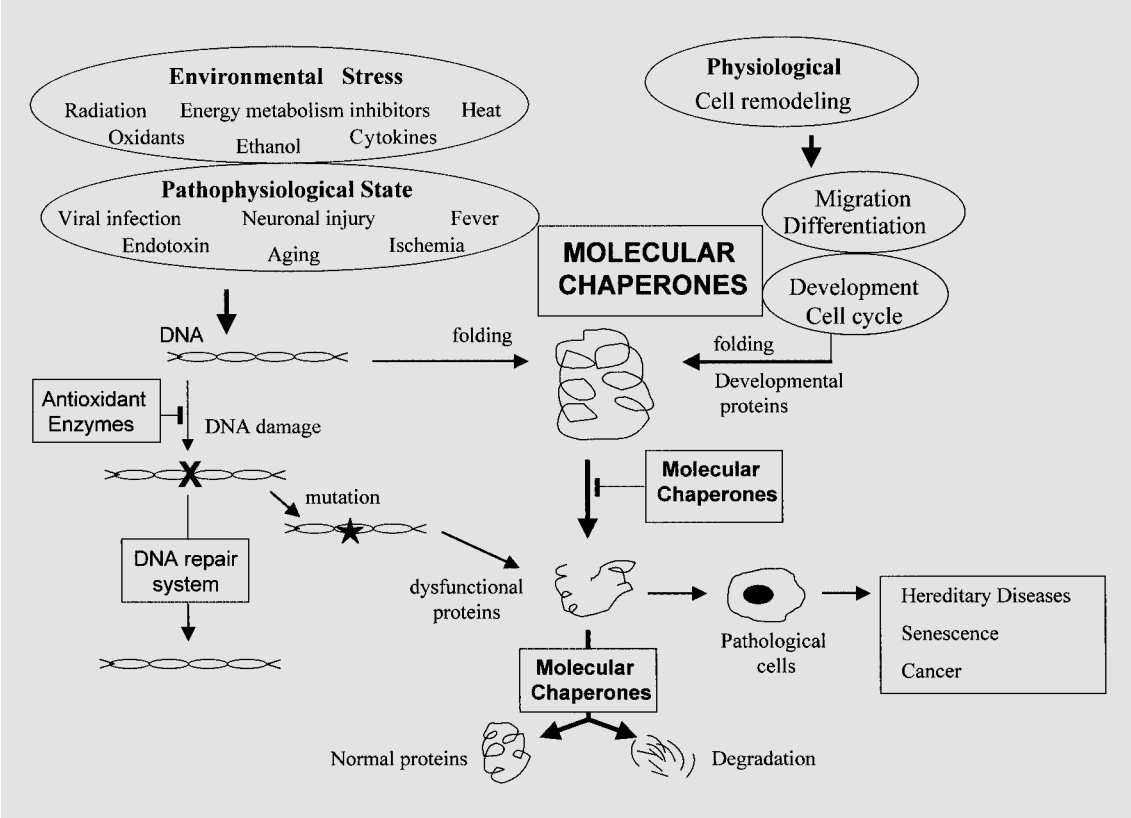

Fig. 1. Moleular chaperones and their roles in brain physiopathology. Accumulation of abnormal, dysfunctional proteins in the cells, elicited by genotoxic and proteotoxic insults, which are dictated by genetic mutations or various environmental or metabolic stresses, is thought to cause senescence and a variety of pathological states. Molecular chaperones, as guardians of proteins, are also critical for cell remodeling, migration, adhesion and differentiation processes during brain development. Thus Hsps are promising for the protection and therapeutic treatment resulting from protein misfolding.

program cannot be rescued, and below which apparently complete recovery occurs. The basic question is whether defects occur because of the activity of the heat shock system or because of its failure to afford protection through replacement and/or regenerating mechanisms.

As Hsp70 is produced in all cell types by a wide variety of stressful stimuli [89–92], the discussion above implies that its modulation might have the greatest chance for protecting the brain against a wide variety of physiopathological processes, including brain developmental defects, seizures, ischemia or neurodegenerative disorders. All these considerations strongly sustain the idea that Hsp signal pathways may represent a basic mechanism of defence against proteotoxic impairment of cell homeostasis (fig. 1). To date, a wide variety of inherited diseases are known to result from the inability of the mutant or abnormal protein to achieve its functional conformation. They are collectively called protein-folding diseases. A moderate overexpression of molecular chaperones by means of mild hyperhermia, nontoxic compounds such as natural polyphenolic antioxidants, caloric restriction or gene transfer, conceivably, may be used for the protection and therapeutic treatment of inherited diseases caused by protein misfolding. However, further efforts must be made in order to clearly address questions such as those on: (a) the mechanism involved and the physiological significance of variations observed in the response of different tissues of an embryo or an adult organism to stress; (b) whether Hsps protect against different kinds of cerebral injury in vivo, or can be administered to protect larger brain regions against various insults.

Finally, the possibility of an intracellular interaction between transcription factors, such as HSF1 and NFkB, HSF1 and Ras, or HSF1 and signal transducer and activator of transcription 1 (STAT-1) [93, 94], as well as an extracellular interaction between Hsps and cell surface receptors (chaperokines) might open up potentially novel therapeutic strategies [95], relying upon the simultaneous activation of cytoprotective genes of the cell life program, which we call vitagenes [77], and down-regulation of those proinflammatory and pro-oxidative genes involved in programmed cell death.

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