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Regulation of HSP70 in excitatory neurons: Possible implications for neuronal functioning

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Abstract. Neurons maintain an intricate organization of cytoplasmic and membrane proteins for their integrity, quick communication across synapses and for other complex activities. Molecular chaperones such as members of the 70 kDa heat shock protein (HSP70) family may play very important roles in these functions. However, in spite of a recent report suggesting the presence of HSP70 related proteins in the synaptic vesicle docking complex at presynaptic sites and the known significant roles for HSP70 in excitotoxicity, there are remarkably few studies that have explored the potential role of HSP70 family proteins in physiological functions of neurons. Here we bring together direct and indirect evidences which suggest that several different pathways involved in long-term potentiation can influence the HSP70 levels at the synapse and hypothesize on possible physiological significance of this family of proteins in neuronal functions.

Keywords. Long-term potentiation; glutamate receptor; HSP70 gene promoter; protein kinase C; tyrosine kinase.

1. Introduction

The 70 kDa group of heat shock proteins or HSP70 is a highly conserved family of proteins, being present from bacteria to man. In most species, there are multiple genes for HSP70 (Günther and Walter 1994). Members of this protein family include constitutive or cognate (HSC70) and the stress inducible forms (HSP70). The term HSP70 is used here, unless otherwise specified, to refer to inducible as well as the cognate forms. These proteins are believed to function mainly as molecular chaperones helping in protein transport and translocation (for reviews see Craig *et al* 1994; Hartl *et al* 1994). Among the very diverse and wide-ranging roles of the HSP70 family proteins, an emerging field of considerable significance concerns their expression in neurons. Several isoforms of HSP70, including the constitutively present cognate forms (HSC70), are known to exist in neurons (Green and Liem 1989; Pardue *et al* 1992). In fact, one of the first glimpses of the function of HSP70 came from studies on bovine brain (Schlossman *et al* 1984) showing an interaction of HSP70 family protein with folded proteins like clathrin. Clathrin uncoating of the synaptic vesicles (SV) is an important step in SV recycling pathway (Zhang *et al* 1994; Ungewickell *et al* 1995). Before the vesicle is recycled and can fuse to early endosomes, its clathrin cover has to be removed. β-internexin, a cytoskeletal-associated protein, with clathrin-uncoating ATPase activity in rat brain (Green and Liem 1989) is a member of the HSP70 family. This HSP70 family protein interacts with conformationally flexible regions of clathrin light

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chains (DeLuca-Flaherty *et al* 1990) and allows the release of bound clathrin while ATP is hydrolyzed.

Additionally, stress or heat shock response is elicited in glutamate related excitotoxic neuropathologies like hypoxiaischemia, cerebral artery occlusion, transient ischemia, limbic seizures, status epilepticus, trauma and following certain drug treatments (reviewed by Koroshetz and Bonventre 1994; Nowak and Abe 1994). Furthermore, the reduction in HSP70 synthesis in neurons during aging has been implicated in old age neuropathologies like Parkinson's and Alzheimer's diseases (Heydari *et al* 1994). However, despite the evidence for involvement of HSP70 in excitatory neuropathologies, the physiological functions of these molecules in neurons have yet to be fully defined. In this article we consider some possible roles of the HSP70 family proteins in neuronal functions and based on the available information in literature, we hypothesize that HSP70 has a physiological role in neuronal integrity and that neurotransmitter release and depolarization may be regulated through control of relative amount of HSP70 at the synaptic terminal.

2. Neuronal signaling

Signaling pathways inside neurons are formed by a series of cross-talking proteins. One of the well documented messenger pathways in neurons relates to long term potentiation (LTP, for reviews see Bliss and Collingridge 1993; Malinow 1994; Nicoll and Malenka 1995). LTP is an activity dependent synaptic plasticity that has been considered as a model for studying the molecular basis of memory. During LTP the neurotransmitter, L-glutamate, bind to specific receptors at the post synaptic terminal. These glutamate receptors are ligand gated ion channels that are widely distributed in mammalian brain and are classified, based on pharmacological and electrophysiological data, as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, kainate receptors, N-methyl-D-aspartate (NMDA) receptors and G-protein coupled metabotropic receptors (Hollmann and Heinemann 1994). Binding of glutamate to receptors elevates the internal calcium concentration in neurons. This signals activetion and expression of different proteins, both in pre- and post-synaptic terminals, to regulate neuronal excitability and plasticity. The mechanism of expression of increased synaptic strength during LTP is controversial. These are three possible ways through which the neurons can produce this effect: (i) by increasing the quanta release of neurotransmitters, (ii) by enhancing the efficiency of existing post-synaptic receptors, and (iii) by expressing a greater number of active post-synaptic receptors. Despite circumstantial evidence for the existence of all three possibilities, the molecules involved in these pathways have yet to be fully defined (Bliss and Collingridge 1993; Malinow 1994). Here we propose a model (see figure 1) suggesting HSP70 as one of the candidates that helps in upregulation of neurotransmitter release and post-synaptic glutamate receptor density, thus satisfying many of the requirements for the maintenance of LTP. As considered below several pathways can directly or indirectly regulate *hsp70* genes in neurons which in turn can modulate activity at the synapse.

3. Multiple regulation of transcription of *hsp 70* genes

The swift induction of heat shock genes during stress is under control of the heat shock factor (HSF) which binds after trimerization and phosphorylation with the upstream

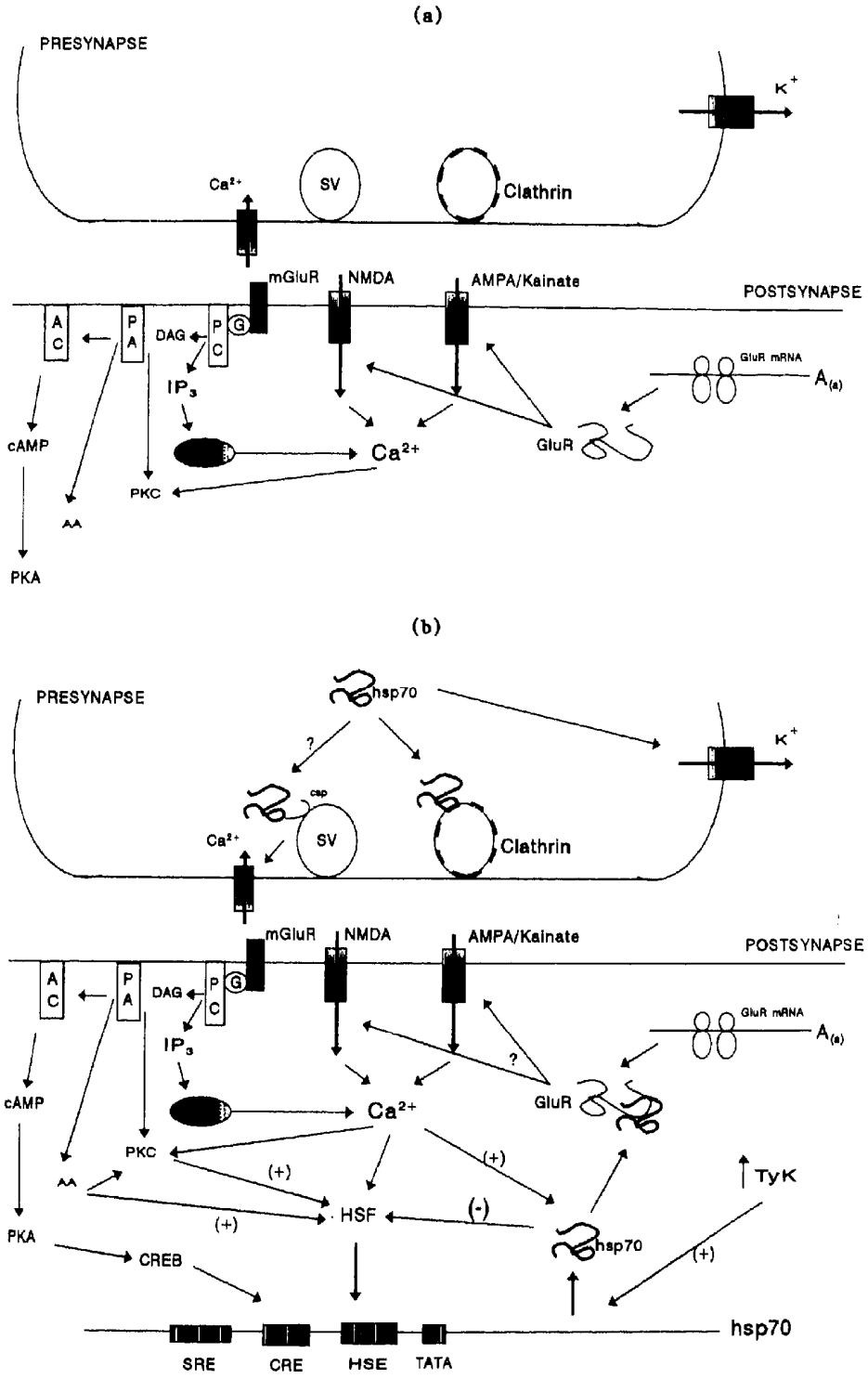

Figure 1. For caption, see p. 634.

heat shock element (HSE) sequences of heat inducible genes (for a recent review see Morimoto *et al* 1994). In addition, the 5′ flanking sequence of the human *hsp70* gene has a cyclic adenosine monophosphate (cAMP) response element (CRE, Choi *et al* 1991). This CRE functions as a major basal level regulatory element and is also required to maintain high promoter activity under stress induced conditions (Alexandre *et al* 1991). The human *hsp70* gene is also transiently activated by neuronal growth factor (NGF) through a serum response element (SRE) sequence present in the *hsp70* promoter region (Visvader *et al* 1988). The presence of such multiple enhancer elements close to the promoter region of *hsp70* (see figure 2) suggests that regulation of this gene is complex. Since the *hsp70* gene promoter remains in an open conformation (see Morimoto *et al* 1994), it is likely that these multiple upstream regulatory elements remain accessible to transcriptional activation.

4. Possible pathways that may regulate *hsp70* in neurons

Possible pathways that may regulate expression of *hsp70* in neurons during LTP are considered below.

It is known that during LTP, the metabotropic glutamate receptor controls activity of adenyl cyclase, the enzyme that converts ATP to cAMP (Cooper *et al* 1995). cAMP in turn activates the cAMP dependent protein kinase A (PKA) to phosphorylate the CRE binding protein (CREB). This is a well established pathway for consolidation of the late phase of LTP (Bourtchuladze *et al* 1994; Yin *et al* 1994). Since the 5′ flanking sequence of the human *hsp70* gene has a CRE sequence (Choi *et al* 1991), it is possible that transcription of the *hsp70* gene can be activated by cAMP during LTP.

Persistence of cAMP induced LTP requires transcription during a very critical period (within 1–2 h); a failure in transcription within this time period results only in short term potentiation which does not require protein synthesis while a blockage in RNA synthesis after 2 h has no effect on LTP maintenance (Nguyen *et al* 1994). This critical time window of transcriptional requirement for LTP, approximately tallies with the observed timing of increase in neuronal *hsp70* transcription (1–4 h) during glutamate related excitotoxicity (Walker and Carlock 1993). Since cAMP-inducible

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Figure 1. (**a**) Pathways involved in LTP: The neurotransmitter glutamate release from presynaptic terminal result in postsynaptic Ca^{2+} transient through the three pharmacologically distinct glutamate receptors (G1uR), known as NMDA, AMPA and kainate receptors, becomes amplified by the release of intracellular stores by Ca^{2+} and inositol triphosphate (IP_3 Parallely, a G-protein coupled metabotropic glutamate receptor (mGluR) can produce diacylglycerol (DAG), arachidonic acid (AA) and regulate the cAMP level through phos–phoinositide-specific phospholipase C (PC), phospholipase A_2 (PA) and adenylate cyclase (AC), respectively. (**b**) A model showing various ways in which HSP70 may affect the processes involved in LTP: Ca^{2+} in association with various kinases, viz, cAMP dependent kinase (PKA), PKC and tyrosine kinase (TyK), then leads to activation of *hsp70* transcription either through HSF which binds to the HSE of the gene, or through cAMP response element (CRE) binding protein (CREB), or by a still independent pathway as in the case of TyK. Additionally, *hsp70* gene promoter can also be regulated by NGF through the SRE. TATA stands for the TATA box. At the presynaptic terminal a SV associated protein, the CSP, can regulate the Ca^{2+} entry and thus control the neurotransmitter release. HSP70 can also regulate outwardly rectifying K^+ channel activity. Various assumptions, which seem most likely based upon current knowledge are highlighted with a question mark. For details see text.

Figure 2. Human 70 kDa heat shock gene promoter region (*hsp70*, EMBL Accession no. M19865). The sequences for HSE, SRE, CRE and TATA box are highlighted. The other human *hsp70* sequences in the EMBL data bank also have these regulatory sequences in the promoter region, though their relative locations differ.

genes are believed to be involved in induction of LTP (Nguyen *et al* 1994), a modification in *hsp70* gene expression during LTP may be an important event.

Post LTP modifications in neurons include consolidation of neuronal contacts and structural modifications such as new synaptic connections at activated post-synaptic terminals (Kandel and O'Dell 1992). NGF is essential for neurite outgrowth, increased excitability and change in neurotransmitter synthesis (see Visvader *et al* 1988). Like the *c-fos* gene, an early gene expressed during LTP (Kandel and O'Dell), which is rapidly and transiently expressed in many tissues in response to NGF stimulation through the SRE in its upstream region, the SRE sequence in the promoter region of the human *hsp70* gene promoter is also responsive to NGF (Visvader *et al* 1988).

In addition to the above enhancer sequences for activation of the *hsp70* gene, which may still need HSF binding for transcription, HSF independent pathways are also known for *hsp70* transcription. Well documented among them is the tyrosine kinase pathway. The tyrosine kinase inhibitor geinstein can block transcription of *hsp70* gene without affecting the HSF binding to the gene indicating a HSF independent regulation of *hsp70* gene (Price and Calderwood 1991). The level of tyrosine kinases in hippocampus and cerebellum regions of brain is high. Tyrosine kinase phosphorylates the microtubule associated protein kinase (MAP kinase) during ischemic neuropathology (Campos-González and Kindy 1992) and during LTP (O'Dell *et al* 1991). The tyrosine kinase inhibitor, geinstein, can selectively block the induction of LTP in CA1 pyramidal neurons of hippocampus (O'Dell *et al* 1991). These observations raise the possibility that the high levels of tyrosine kinases in certain regions of brain may activate *hsp70* without the thermal stress.

It is known that the levels of intracellular Ca^{2+}, diacyl glycerol and arachidonate increase during LTP. These in turn activate protein kinase C (PKC), a phospholipids and calcium dependent kinase (Bliss and Collingridge 1993). Calcium ions (Price and Calderwood 1991), PKC (Morimoto *et al* 1994) and arachidonate (Jurivich *et al* 1994) can regulate HSF and/or HSP70 activity. For example, Ca^{2+} is known to autophos-

phorylate HSP70 *in vitro* (O'Brien and McKay 1993), although this has yet to be shown *in vivo* (McKay *et al* 1994).

Arachidonate is known to be a potent activator of HSF1 and heat shock gene transcription (Jurivich *et al* 1994). One of the potential targets of arachidonate could be the ε–isoform of Ca^{2+} /phospholipid dependent kinase (PKC) (Koide *et al* 1992; Morimoto *et al* 1994). PKC is also necessary for maintenance of LTP and for the transition from short term potentiation to long term potentiation (Ben-Art *et al* 1992). Several potential PKC sites are identified on HSF1 which may have a role in its activation; however, it remains to be shown that these sites are indeed phosphorylated by PKC. Alternatively, like palmitic and stearic acids which have been shown to associate with HSC70, archidonate may bind with heat shock cognate proteins and indirectly influence the DNA binding of HSF1 (Morimoto *et al* 1994).

5. Possible functional targets for HSP70

Since HSP70 family proteins have multiple physiological functions, they may be involved in many of the diverging pathways in neurons at the neurotransmitter release site (presynaptic) and/or at the postsynaptic terminal. The following considers some possible functions that HSP70 may perform at the synapse.

5.1 *At presynaptic terminal*

The role of HSP70 in uncoating of clathrin cover of SV during endocytosis has been studied in detail. This process is helped by a co-factor, auxilin, which binds with high affinity to assembled clathrin lattice and recruits a cognate member of HSP70 in the presence of ATP (Ungewickell *et al* 1995). Interestingly auxilin has a conserved Dna-*J* domain (Ungewickell *et al* 1995) which in conjunction with HSP70 can result in a substantial increase in its ATPase activity (Liberek *et al* 1991).

Recently, two members of HSP70 family have been found to be present in a cell-free assay that is claimed to faithfully reconstitute the synaptic vesicle docking and fusion complex (Söllner *et al* 1993), a process involved in exocytosis. Intriguingly, synaptic vesicles also contain a protein called cysteine string protein (CSP), which has a Dna-J domain (Mastrogiacomo *et al* 1994). Studies on the *csp* gene in *Drosophila* have shown that it has an important role in regulation of neurotransmitter release (Zinsmaier *et al* 1994); CSP is believed to regulate a presynaptic calcium channel (Mastrogiacomo *et al* 1994). *csp* mutants show temperature sensitive paralysis and lethality suggesting that CSP may be required in stabilizing the components of neurotransmitter release machinery (Zinsmaier *et al* 1994). Stabilization of proteins and keeping the competency of various components in a functional machinery are well known roles of HSP70 (Scheffield *et al* 1990). If HSP70 members are indeed present in the 20S docking and fusion particle (Söllner *et al* 1993), it is attractive to imagine that the Dna-J domain of CSP may be functioning as a 'receptor' for HSP70 at the synaptic terminal. It is also possible that the CSP and HSP70 complex may interact with a presynaptic calcium channel to convert it from inactive to active form (Mastrogiacomo *et al* 1994).

Additionally, HSP70 may influence intracellular processes through its possible ability to regulate outwardly rectifying K^+ current (Saad and Hahn 1992). Neurons repolarize back or show a neuronal inhibition by opening of the S-type K^+ channels (see Abe *et al* 1995). If the regulation of K^+ channel activity by HSP70, as demonstrated

in fibrosarcoma cells (Saad and Hahn 1992), is operative in neuronal cells too, such a negative feedback loop could control the prolonged depolarization of neurons and help in their repolarization.

5.2 *At postsynaptic terminal*

It has been shown that the polyribosomes that translate the glutamate receptor family proteins are located close to the postsynaptic terminals (Miyashiro *et al* 1994). HSP70 may have an important role in this since binding to the nascent polypeptides and aiding in their translocation are well documented functions of this chaperone family. For example, it is known that HSP70 assists in translocation of the cystic fibrosis transmembrane conductance regulator, a plasma-membrane channel protein (Yang *et al* 1993). Whether HSP70 helps in a rapid transport and translocation of glutamate receptor in a similar fashion is a possibility that needs examination. It is interesting to note in this context that the hippocampal neurons show high levels of constitutive (Pardue *et al* 1992) as well as stress-induced (e.g., ischemia) HSP70 (Nowak and Abe 1994); furthermore, many of these neurons die during excitotoxicity due to high Ca^{2+} inflow (Nowak and Abe 1994), probably by the increased expression of glutamate receptor channels (Choi 1988). As mentioned earlier, glutamates are also known to be inducers of *hsp70* gene (Nowak and Abe 1994). Put together these may, provocatively, suggest an intriguing possibility that some neurons experience excitotoxicity due to a more efficient HSP70 mediated assembly and translocation of the glutamate receptors.

6. A model for HSP70 function in neurons

In view of the above possibilities, we propose (see figure 1) that during normal physiological condition any increase in release of neurotransmitters or calcium ions in neurons would lead to changes in *hsp70* transcription through HSF and/or through HSF independent pathways. One of the effects of HSP70 would be to assist increase in the number of glutamate receptors at the post-synaptic membrane, thereby enhancing the internal calcium concentration resulting in an increase in action potential observed in LTP. Additionally, HSP70 may be promoting the neurotransmitter release by either directly acting on a presynaptic calcium channel or through CSP. Conversely, increase in HSP70 production would be expected to decline the internal Ca^{2+} and PKC activation by promoting outward K^+ channels. Thus excessive neurotransmitter release and depolarization would be checked through the negative feedback loop.

Further studies directed to examine these possibilities are expected to facilitate a better understanding of the role of HSP70 in neuronal activities.

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