Hsp90 activity is necessary to acquire a proper neuronal polarization

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ABSTRACT

Chaperones are critical for the folding and regulation of a wide array of cellular proteins. Heat Shock Proteins (Hsps) are the most representative group of chaperones. Hsp90 represents up to 1–2% of soluble protein. Although the Hsp90 role is being studied in neurodegenerative diseases, its role in neuronal differentiation remains mostly unknown. Since neuronal polarity mechanisms depend on local stability and degradation, we asked whether Hsp90 could be a regulator of axonal polarity and growth. Thus, we studied the role of Hsp90 activity in a well established model of cultured hippocampal neurons using an Hsp90 specific inhibitor, 17-AAG. Our present data shows that Hsp90 inhibition at different developmental stages disturbs neuronal polarity formation or axonal elongation. Hsp90 inhibition during the first 3 h in culture promotes multiple axon morphology, while this inhibition after 3 h slows down axonal elongation. Hsp90 inhibition was accompanied by decreased Akt and GSK3 expression, as well as, a reduced Akt activity. In parallel, we detected an alteration of kinesin-1 subcellular distribution. Moreover, these effects were seconded by changes in Hsp70/Hsc70 subcellular localization that seem to compensate the lack of Hsp90 activity. In conclusion, our data strongly suggests that Hsp90 activity is necessary to control the expression, activity or location of specific kinases and motor proteins during the axon specification and axon elongation processes. Even more, our data demonstrate the existence of a "time-window" for axon specification in this model of cultured neurons after which the inhibition of Hsp90 only affects axonal elongation mechanisms.

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1. Introduction

Neuronal function depends on the establishment of different morphological and functional differentiated domains: soma, dendrites, axon and axon initial segment. The morphological differentiation starts with the specification of one of the initial neurites as the axon, and its subsequent elongation. This process requires the coordinated reorganization and dynamics of actin and microtubule cytoskeletons [1,2]. Cytoskeleton dynamic behavior is controlled by microfilament and microtubule associated proteins (ABPs or MAPs), as well as, tubulin post-translational modifications [2–4]. Different signaling pathways, such as PI3K pathway are involved in axon specification and growth [5], including proteins such as Akt, GSK3 [6–8], Par3/Par6, GTPases Rho, Rac and cdc42. Axonal specification appears as a consequence of the local accumulation of some determined proteins, that implies a fine regulation of its synthesis and local degradation mechanisms. In cultured rat

hippocampal neurons, several proteins, such as Par3 and Par6, Akt, and ζPKC, are concentrated in tip of the neurite specified as the axon and absent from other neurites. Two possible mechanisms have been proposed to concentrate these proteins at the future axon tip. First, proteins can be specifically targeted to the axon tip by kinesins. In fact, KIF5 is expressed in several neurites before axon specification but appears selectively accumulated in the emerging axon and absent from the dendrites when axon is specified [9]. Second, axon selective accumulation of these proteins can be the consequence of protein degradation in other neurites. In this sense, E3-ubiquitin ligase Smurf2 initiates the degradation of the Rap1b GTPase in all neurites of unpolarized neuron stage 2, except in the future axon [10] controlling proper neuronal polarization. Moreover, the E3-ubiquitin ligase Smurf1 when is phosphorylated by PKA changes substrate affinity contributing to RhoA degradation and accumulation of the Par6 protein at the axon tip, contributing to axon initiation and proper polarity formation [11].

While transport and degradation mechanisms contribute to neuronal polarization and axon initiation, mechanisms involved in protecting proteins from degradation have not been studied during neuronal polarization and axon growth. Chaperones are general cellular regulators critical for the folding and regulation of a wide array of cellular proteins. Their functions include folding and/or stability, intracellular protein trafficking, translocation, assembly into multi-protein complexes and

facilitate degradation through the ubiquitin–proteosome or lysosomal pathways [12–16]. Hsps are the most representative group of chaperones. Among them, Hsp90 is a very abundant protein and may possibly represent up to 1–2% of soluble protein. Many proteins involved in the development and maturation of CNS, such as Akt and GSK3, has been described as Hsp90 clients. In mammals, there is up to four different Hsp90 homologs: two cytosolic Hsp90 (constitutive and inducible isoforms), mitochondrial TRAP1 (tumor necrosis factor receptor-associated protein 1), and the endoplasmic reticulum (ER) associated GRP94 (94 kDa glucose-regulated protein). Most eukaryotic species contain at least two genes encoding highly homologous isoforms of cytoplasmic Hsp90 [12,13].

In central nervous system, Hsp90 is widely expressed in all regions at the latest stages of gestation and postnatal development. In the adult mammalian nervous system and at all stages of postnatal development, the Hsp90 constitutively expressed is preferentially localized on neurons [12,13,17–19]. Most studies regarding the role of Hsp90 in nervous system have focused on its involvement on neurodegenerative diseases [20], however its importance in neuron differentiation remains mostly unknown. Since Hsp90 is expressed preferentially in neurons in the nervous system from late embryonic differentiation stages [18], and local stability and degradation mechanisms are responsible for neuronal polarity establishment and axon outgrowth, we asked whether Hsp90 could be a regulator of axonal polarity and growth. Our study shows that Hsp90 activity inhibition at different developmental stages disturbs neuronal polarity formation or axonal elongation in cultured hippocampal neurons. In parallel to these morphological changes, we have observed decreased Akt activity, concomitant with increased GSK3 activity and alteration of subcellular distribution of motor protein KIF 5. Moreover, these effects are seconded by Hsp70/Hsc70 localization changes that seem to compensate the lack of Hsp90 activity.

2. Materials and methods

2.1. Antibodies

Commercial antibodies against the following proteins were used: Hsp90α/β, βIII-tubulin from Santa Cruz Biotechnology; Hsp70/Hsc70 from Stressgen; tau1 from Millipore; α-tyrosinated-tubulin from Sigma; kinesin 5c and MAP2 from Abcam; GSK3α/β from Invitrogen; pGSK3α/β$^{S9/21}$, Akt and pAkt^{S473} from Cell Signaling Technology.

2.2. Neuronal cultures and treatment

Neurons were obtained from E17 mouse embryos as described previously [21]. Briefly, hippocampi and cortex were removed and after dissection and washing three times in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (HBSS), the tissues were digested for 15 min in the same solution containing 0.25% trypsin, in the presence of 0.1 mg/ml DNase I in the case of the cortex. The tissue was then washed three times in Ca^{2+} and Mg^{2+}-free HBSS and dissociated with fire-polished Pasteur pipettes. The cells were counted, resuspended in plating medium (MEM, 10% horse serum, 0.6% glucose) and plated at a density of 5000/cm^2 on polylysine-coated coverslips (1 mg/ml) for immunochemistry experiments. Cortical neurons were plated at a density of 200,000/cm^2 on 60 mm plates coated with polylysine for biochemical experiments. Neurons were incubated at 37 °C for 2 h before switching them to neuronal culture medium (Neurobasal, B-27, glutamax-I). For the cultures beyond 6 DIV, neurons were plated and transferred to dishes containing astrocytes, without contact between them.

To analyze the effect of Hsp90 inhibition, the inhibitor 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG, from Sigma A8476), diluted in DMSO, was added at the times and concentrations indicated. Addition times are counted considering time t0 as the moment of switching cells to neuronal culture medium. Vehicle (0.1% DMSO) was added to control cells.

2.3. Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde for 20 min. Coverslips were treated with 50 mM NH$_4$Cl and incubated with 0.22% gelatin and 0.1% Triton X-100 in PBS for blocking non-specific binding. Cells were then incubated with primary antibodies for 1 h at room temperature. The primary antibodies (and dilutions) were Hsp90 α/β (1/50), Hsp70/Hsc70 (1/100), MAP2 (1/10.000), tau 1 (1/1000), KIF5C (1/400), and α-tubulin (1/10,000). After washing, they were incubated with Alexa-Fluor-conjugated secondary antibodies (1/1000) or Phalloidin-Alexa594 (1/500). The coverslips were mounted using Fluoromount G (Southern Biotech) and images acquired on a LSM510 confocal microscope coupled to an Axiovert 200 M (Zeiss) microscope. Axon length was quantified with the NeuronJ program. Images were processed and presented using Adobe Photoshop and Illustrator CS4.

2.4. Western-blot

Cell extracts were prepared from high density cortical neurons in lysis buffer containing 20 mM Hepes [pH 7.4], 100 mM NaCl, 100 mM NaF, 1 mM Na$_3$VO$_4$, 1 μM okadaic acid, 5 mM EDTA, 1% Triton X-100 and a protease inhibitor cocktail (Complete™, Roche). Briefly, the cells were left for 30 min on ice in lysis buffer and, after adding loading buffer, the samples were boiled for 10 min and resolved by SDS-PAGE gels and transferred to nitrocellulose membranes in the presence of 20% methanol and 0.1% SDS, and non-specific signals were blocked incubating with 5% BSA in PBS and 0.1% Tween-20 (PBS-T) and then probed with primary antibodies overnight at 4 °C. The primary antibodies were Hsp90 α/β, βIII-tubulin, Hsp70, GSK3α/β, pGSK3α/β$^{S9/21}$, Akt and pAkt^{S473}. All of them were used at 1/1000 dilution. After washing, the membranes were exposed to the corresponding peroxidase-conjugated secondary antibody (1/5000, Santa Cruz Biotechnology) for 1 h at room temperature. Antibody binding was visualized by ECL Amersham) and the results were quantified with the Image J software. The quantified values were normalized to the values obtained for a control antibody to correct for variation in the amount of total protein loaded.

2.5. Statistical analysis

All experiments were repeated at least three times and the results are presented as the mean ± standard error of the mean. Statistical differences between experimental conditions were analyzed by unpaired *t*-test tool of the SigmaPlot software.

3. Results

3.1. Early expression of Hsp90 in growth cones during neuronal development

As mentioned in Section 1, Hsp90 is highly expressed in brain from late embryonic development to adulthood. However, its function during neuronal development, as well as, that of other chaperones remains mostly unknown. We asked if Hsp90 may have a role as a chaperone that protects proteins during neuronal polarization. Thus, we first studied Hsp90 subcellular distribution in cultured hippocampal neurons in different developmental stages, including neurites' growth, axon elongation and dendrites' development and maturation. Hippocampal neurons were fixed at different developmental stages (1, 2, 3, 6, 21 days in vitro, DIV) and stained with antibodies against Hsp90 and MAP2 or the F-actin marker, Phalloidin (Fig. 1A,B). In all stages, Hsp90 was mostly localized in neuronal soma, showing higher expression around the nucleus. Hsp90 was also expressed in all the neurites at 1 DIV, showing a higher Hsp90 expression in their growth cones identified by F-actin staining (Fig. 1A). Once the axon is established and starts its elongation (2 DIV), Hsp90 is localized in the axonal growth
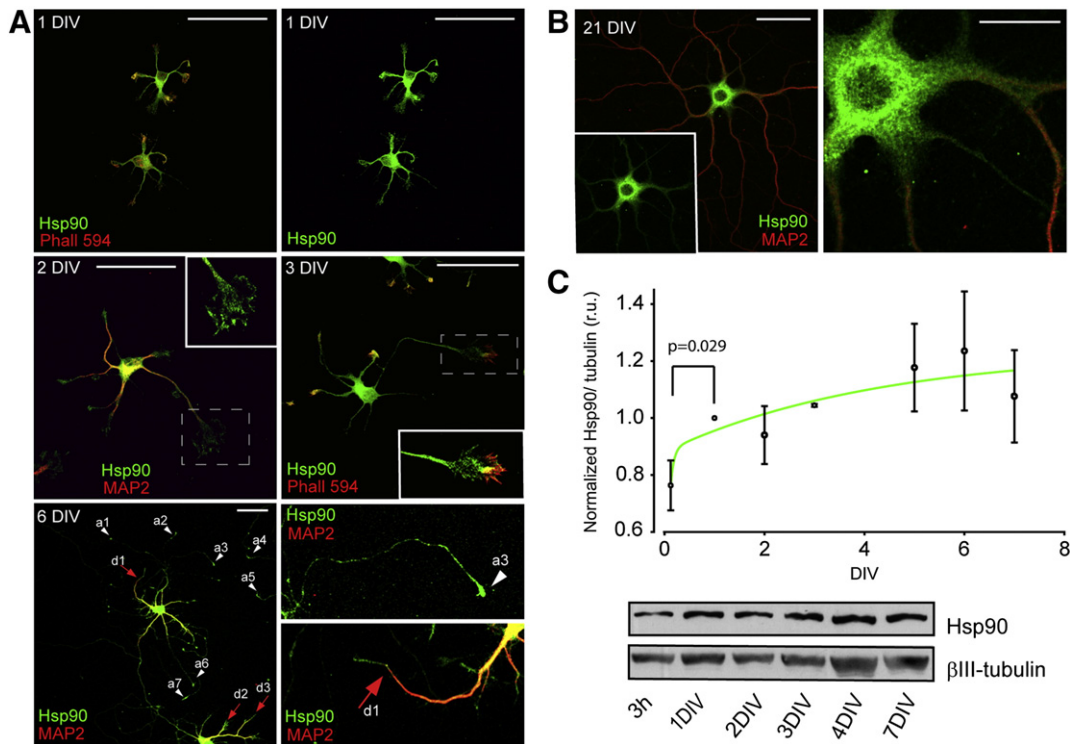
Fig. 1. Hsp90 subcellular localization during neuronal polarization and axonal growth. (A) Hippocampal neurons cultured for 1, 2, 3 and 6 days in vitro (DIV). Neurons were fixed and stained with antibodies against Hsp90 (green) and MAP2 (red, somatodendritic domain) or Phalloidin-Alexa 594 (F-actin marker) to define the neuronal morphology and structures (scale bars = 50 μm). Distal regions of axons in 2 and 3 DIV neurons are indicated into gray dashed lines and 2× magnifications of Hsp90 expression (green) in axonal distal regions are included in the image. Hsp90 expression in axonal tips is indicated as a1–a7 and arrow heads in 6 DIV neurons image. Dendrites are indicated as d1–d3 and red arrows. 4× magnifications of a1 and d3 are shown. (B) Image shows Hsp90 expression in a 21 DIV hippocampal neuron. Dendrites are identified by MAP2 staining. Scale bar = 20 μm. (C) The graph represents the Hsp90 expression level increment normalized to βIII-tubulin along neuronal development. Data are normalized to 1 day in vitro normalized expression levels. Data are expressed as the mean ± s.e.m. of three independent experiments. A representative western blot of total cell extracts obtained from cortical neurons cultured from 1 to 7 DIV is shown. Samples were incubated with antibodies against Hsp90 (upper blot) and βIII-tubulin as a loading control (lower blot).

cone associated with microtubules and F-actin regions (see magnification). Hsp90 is still associated to other growth cones of other neurites (future dendrites). After 3 DIV, Hsp90 axonal localization is characterized by an increasing proximal to distal gradient, and continues to be associated to microtubules and F-actin in the growth cone. At later stages (6 DIV), axonal localization of Hsp90 is mainly observed in axonal growth cones (white arrow heads), keeping a higher concentration in the somatodendritic domain. In 21 DIV mature neurons Hsp90 expression was concentrated in soma showing a higher perinuclear density (Fig. 1B). Interestingly, a faint Hsp90 staining was observed in the axon initial segment of these neurons, that was absent along the axon. In view of this Hsp90 expression pattern along neuronal development, we analyzed Hsp90 expression levels in neurons from the beginning of their development to 7 DIV (Fig. 1C). We obtained protein extracts from 3 h and 1, 2, 3, 4 and 7 DIV cultured cortical neurons. Interestingly, βIII-tubulin normalized Hsp90 expression levels were significantly augmented between 3 h and 1 DIV (p = 0.029). Hsp90 expression levels in later stages were increased, around 20%, without any significant change compared to 1 DIV neurons (Fig. 1C).

The earlier Hsp90 expression and increment during neuronal development, as well as, its presence in the growth cones of all neurites at 1 DIV, when neuronal polarization mechanisms are in place, suggest that Hsp90 may have a relevant role in the decision of neuronal polarization, as well as in axonal elongation due to microtubules and F-actin colocalization with Hsp90 in axon growth cone.

3.2. Hsp90 activity is necessary for proper neuronal polarization and axonal elongation

Then, we analyzed whether Hsp90 activity inhibition may interfere with axon specification and/or elongation. A previous study

demonstrated that axon specification and neuronal polarization occurs during the first 24 h [7], when our study shows a significant change in Hsp90 expression levels. Hence, we studied the morphology and polarization of cultured hippocampal neurons at 3 DIV with or without the previous addition of an Hsp90 specific inhibitor at different times to analyze the consequences of Hsp90 lack of function (Fig. 2C). Neurons were treated with 17-AAG (10 μM) when medium was changed to neuronal medium from plating medium (0 h) or 3, 6 or 24 h later (Fig. 2A,B). Neurons were then kept till 3 DIV and stained with axonal (tau-1) and somatodendritic (MAP2) markers. First, we quantified the number of tau-1 positive axons per neuron (Fig. 2D). As expected, most control hippocampal neurons in culture (80.02 ± 1.31%) are polarized showing a single tau-1-positive process and only 15.91 ± 1.42% of neurons have more than one axon. Interestingly, Hsp90 inhibition at the time of switching to neuronal medium (0 h) resulted in a significant percentage of neurons with multiple tau-1 positive processes or multiple axon morphology (57.01 ± 1.95%). Similar results were obtained when Hsp90 was inhibited 3 h later (51.81 ± 2.77%). However, when Hsp90 was inhibited at 6 h of culture in neuronal medium only 26.74 ± 2.47% of neurons showed a multiple axon pattern. Similar results were obtained when inhibition was carried out after 24 h (22.62 ± 4.23%). In all conditions, the percentage of non-polarized neurons without detectable axon was limited and did not differ from that observed in control neurons (4.06 ± 0.91%). Interestingly, the percentage of neurons showing only one axon augmented from 35.34 ± 2.50 when hsp90 was inhibited at 0 h to 71.67 ± 3.35% or 73.76 ± 3.01% after inhibition at 6 or 24 h (Fig. 2D). Thus, our results suggest that Hsp90 activity is necessary for proper neuronal polarization at least during the first 3 h in culture.

Next, we analyzed whether Hsp90 activity may be involved in the regulation of axonal elongation once the axon is specified. Thus, we
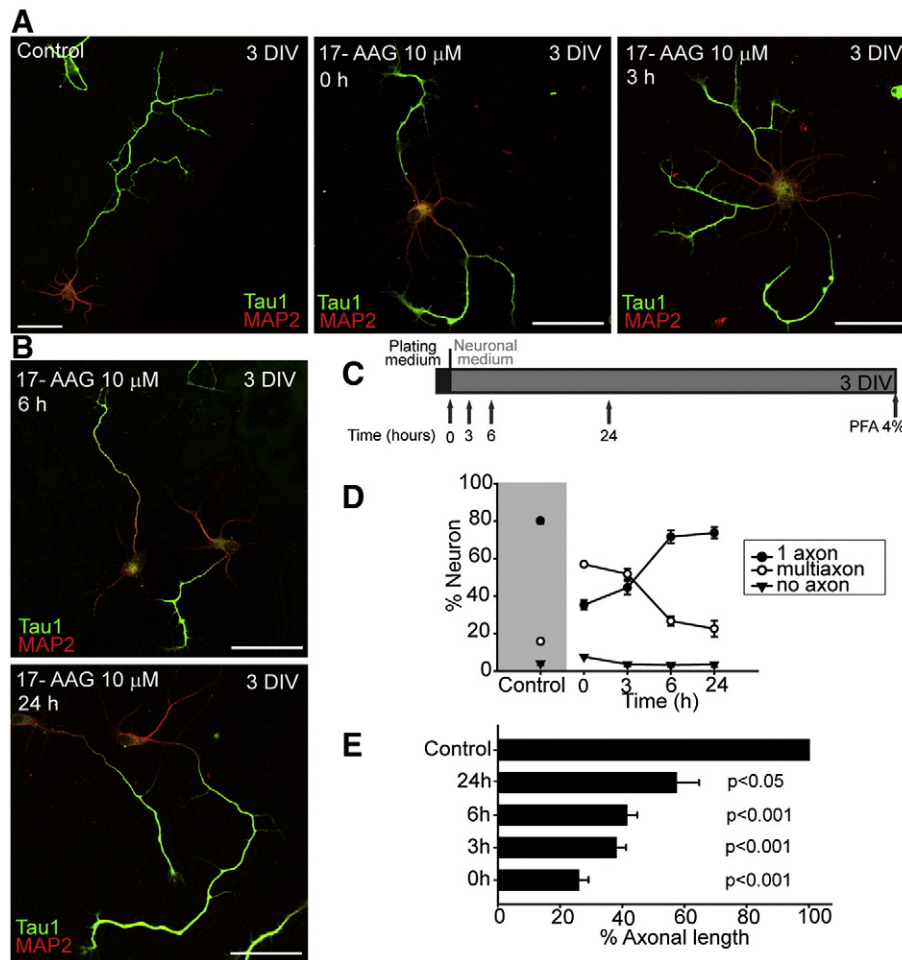
Fig. 2. Hsp90 inhibition disrupts neuronal polarization and slows down axonal elongation in different stages of hippocampal neuron development. (A,B) Hippocampal neurons cultured in the presence or absence of 17-AAG 10 μM, added at different times after switching to neuronal medium and kept in culture for 3 days in vitro. Neurons were fixed and stained with antibodies against tau-1 (green, axon) and MAP2 (red, somatodendritic domain) to define the neuronal morphology and quantify axon length and number. Scale bars = 50 μm. (C) Schema represents a 3 day time bar and the time of Hsp90 inhibition for each condition. After switching to neuronal medium, the Hsp90 inhibitor, 17-AAG, was added at time 0, 3, 6 or 24 h. Then neurons were kept till 3 DIV before fixation (PFA 4%). (D) Graph represents the percentage of neurons, shown in A and B, extending one, multiple or without axons. The percentage distribution in cultured control neurons are shown in the gray column. Data are the means ± s.e.m. from three independent experiments and a minimum of 50 neurons analyzed in each experiment and condition. (E) Graph represents the normalized axonal length of the neurons extending only one axon and treated with 17-AAG (10 μM) at different times. Data are the means ± s.e.m. from three independent experiments and a minimum of 50 neurons analyzed in each experiment and condition.

examined all neurons with one axon and we confirmed that neurons treated with 17-AAG at different times extended significantly shorter axons than control neurons (Fig. 2E). Moreover, axonal length reduction was correlated to the number of hours that neurons were exposed to Hsp90 inhibitor. Axons of neurons treated at 0 h had only 25.84 ± 3.28% of the length of control neurons. Axonal length increased progressively when Hsp90 was inhibited at 3, 6 or 24 h (37.90 ± 3.28%; 41.35 ± 3.48% or 57.28 ± 7.45%, respectively), compared to 100% in control neurons. These data demonstrate also a role of Hsp90 in the regulation of axonal elongation.

3.3. Hsp90 modulates Akt and GSK3 expression and activity in neurons

Some Hsp90 clients have been related to neuronal polarity and axonal elongation. Among them, proteins of the PI3-kinase pathway, such as Akt or GSK3, are involved in neuronal polarity and axon elongation mechanisms. Thus, we analyzed whether Akt, GSK3 or their phosphorylated forms could be modified by the lack of Hsp90 activity during axon development. High density cultured cortical neurons were treated with 17-AAG 10 μM after neuronal medium switching or 24 h later. Then, protein extracts were obtained and analyzed using antibodies against Akt, pAkt, GSK3 or pGSK3. Our data show that Akt protein levels were notably reduced after 17-AAG treatment at 0 h (32.5 ± 9.9%) or

24 h (21.6 ± 5.2%) (Fig. 3A). In parallel, the Akt regulatory phosphorylation on serine 473 was also reduced at 0 h (38.3. ± 5.0%) or 24 h (24.8 ± 3.1%). When we analyzed the pAkt/Akt ratio obtained from neurons treated with 17-AAG at 0 h, a 22.2 ± 6.6% reduction was detected, reflecting a lower Akt activity compared to non-treated neurons. However, only a 6.7 ± 4.8% reduction was detected in neurons treated at 24 h, which was not significantly different compared to 0 hour treatment. These changes in Akt phosphorylation were corroborated by changes in the phosphorylation of its substrate, GSK3 (Fig. 3B). GSK3 expression was reduced a 31.5 ± 8.1% after treatment at 0 h, and, in a lesser extent when treatment was applied at 24 h (12.6 ± 9.5%). In both cases, GSK3α/β phosphorylation on serine 21/9 was severely reduced to 44.6 ± 7.5% or 36.1 ± 5.3% after 0 and 24 hour treatment, respectively. The analysis of pGSK3$^{S9/21}$/GSK3 ratio showed a reduction to values of 47.2 ± 10.6% and 31.8 ± 5.6%, respectively; reflecting a relevant activation of GSK3 at both times. Despite the tendency to a higher GSK3 activity at 24 h compared to 0 hour treatment, no statistical difference was found.

3.4. Kinesin-1 distribution is altered by hsp90 inhibition in neurons

The precedent data demonstrate changes in these kinases, but they are not enough to explain the different neuronal morphology and
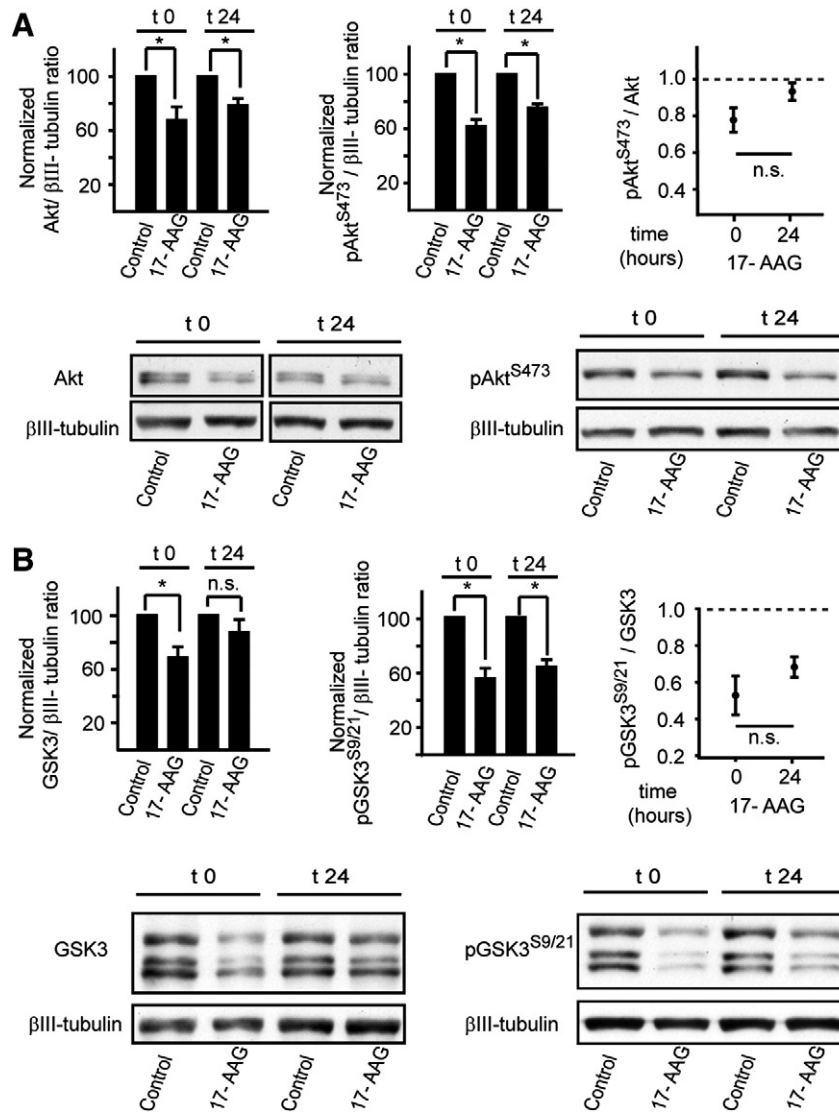
Fig. 3. 17-AAG modifies Akt and GSK3 expression and phosphorylation levels in neurons. (A,B) Quantification of Akt and GSK3 expression levels in extracts of cortical neurons cultured in the absence or presence of 17-AAG (10 μM) from 0 or 24 h till 3 days in vitro. Samples were analyzed by western blot and incubated with antibodies raised against total Akt, pAkt^{S473} (panel A) and GSK3α/β or pGSK3α/β$^{S9/21}$ (panel B). βIII-tubulin was used as a loading control. Data were normalized to protein/βIII-tubulin control levels. Data are expressed as the mean ± s.e.m. of three independent experiments. (*p < 0.05; n.s., not significant). Representative western-blots are shown below the graphs. The graph on the right on each panel represents the pAkt^{S473}/Akt (panel A) or pGSK3α/β$^{S9/21}$/GSK3α/β (panel B) ratios of 17-AAG treated neurons at t0 or t24, normalized to control cells (gray dotted line). Note that decreased phosphorylation levels represent an Akt activity reduction or a GSK3α/β increased activity. Although there are differences between t0 and t24 treatments, any statistical difference is observed between them.

polarity pattern obtained after Hsp90 inhibition. However, previous studies demonstrate that GSK3 activity is involved in the regulation of cargo release from kinesins together with Hsc70 [22], which raise the possibility that local changes in GSK3 activity may modulate axonal transport. Hence, we established the hypothesis that Hsp90 inhibition mediated changes in polarity and axonal elongation may be due to altered kinesin distribution and changes in protein transport and cargo release. In this sense, kinesin-1 is distributed in all neurites before polarization to the nascent axon, and is polarized to the neurite that is specified as the axon [9]. Thus, we analyzed the distribution of KIF5C in 3 DIV control neurons or treated with 17-AAG at 3, 6 or 24 h (Fig. 4). KIF5 was localized in soma, distal axon and axonal growth cones in control neurons (Fig. 4A). However, KIF5 distribution was modified in 3 DIV hippocampal neurons when Hsp90 was inhibited after 3 h in culture. In this context, KIF5 was detected along the several axons observed in these neurons, without a distal gradient distribution. Moreover, KIF5 expression was mostly concentrated in the soma (Fig. 4B). Hsp90 inhibition after 6 h in culture resulted in KIF5 concentration at the soma, and its absence from axons (Fig. 4C). Finally,

neurons treated with 17-AAG after 24 h in culture showed the same pattern in soma, but KIF5 staining was detected in axonal growth cone in a lesser extent than in control neurons (Fig. 4D). Hence, changes in polarity and axonal elongation induced by early Hsp90 inhibition are confirmed by the modifications observed in kinesin-1 neuronal distribution.

3.5. Hsp90 inhibition alters Hsc70/Hsp70 expression levels and location

As mentioned in Section 3.4, another member of the chaperone protein family, Hsc70, is also involved in cargo release from kinesins. Thus, we analyzed whether Hsp90 inhibition may affect in some extent Hsc70 expression or localization. Moreover, in some cell systems the blocking of Hsp90 activity causes the up-regulation of other chaperones, such as Hsp70 (for review see [14,16]). First, using an antibody that recognizes both Hsc70 and Hsp70, we analyzed Hsc70/Hsp70 expression levels in 3 DIV neurons treated with the Hsp90 inhibitor at 0 h, which modify neuronal polarization, or 24 h, which affects axonal elongation. Hsc70/Hsp70 expression levels were significantly increased
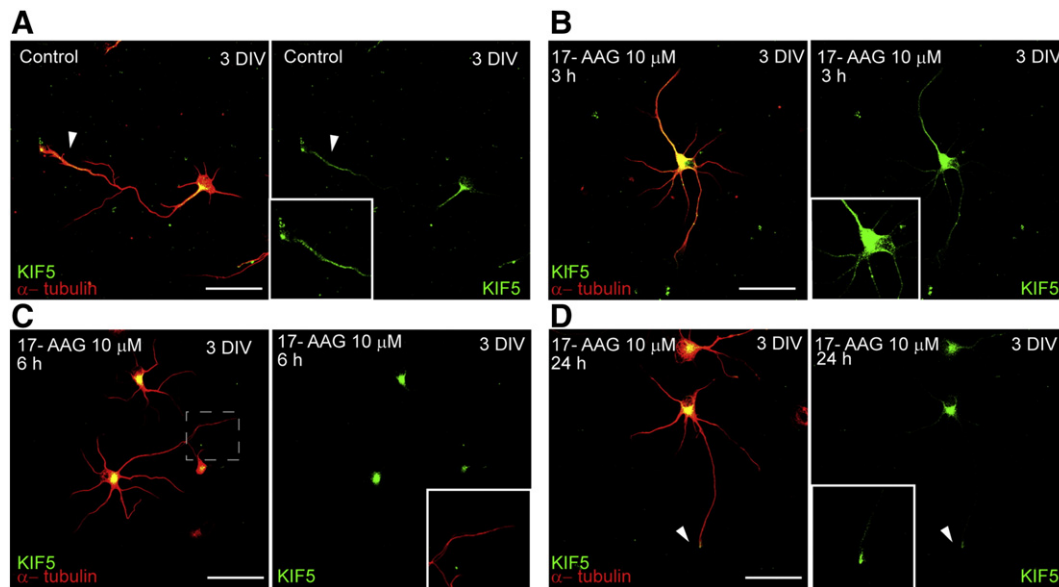
Fig. 4. The kinesin-1 KIF5C localization is sensitive to Hsp90 inhibition in hippocampal neurons. 3 DIV hippocampal neurons cultured in the absence (A) or presence of 17-AAG (10 μM), added at 3 (B), 6 (C) or 24 (D) hours after switching to neuronal medium. Neurons were fixed and stained with antibodies against KIF5C (green) and α-tubulin (red) to define the neuronal morphology. Scale bar = 50 μm. Head arrows indicate the KIF5C expression in the distal region of axon and growth cones. Boxes show 2× image magnification. Note the expression of KIF5C in the several axons generated after Hsp90 inhibition at 3 h, the absence of KIF5C expression in axon growth cones of neurons treated at 6 h, and the partial recovery of KIF5C expression in axon growth cones when neurons were treated at 24 h.

after Hsp90 inhibition at 0 or 24 h (148.40 ± 4.34% and 160.80 ± 11.40%, respectively) (Fig. 5C). Then, we analyzed Hsp90 and Hsc70/Hsp70 localization in control and treated neurons. Hsp90 localization was unaltered by 17-AAG treatment in all conditions compared to control neurons, and most staining was observed in soma and distal region of axons (Fig. 5A). In contrast, to the neuronal soma expression of Hsp90, Hsc70/Hsp70 was not detected in neuronal somas and was mainly expressed in neuronal processes in control neurons (Fig. 5B). Same Hsc70/Hsp70 expression pattern was observed when Hsp90 was inhibited at 24 h. Interestingly, Hsp90 inhibition at 0 h did alter Hsc70/Hsp70 distribution in neurons, which was concentrated in neuronal somas and the several axonal processes.

These data may suggest that the lack of Hsp90 activity before axon specification is in somehow compensated by changes in Hsc70/Hsp70 expression and location, taking Hsc70/Hsp70 the position of Hsp90. However, Hsc70/Hsp70 up-regulation or modified distribution did not compensate for the morphological changes generated by Hsp90 inhibition. Thus, while different chaperones can compensate each other functions in some cell types [23,24], in neurons Hsc70/Hsp70 is unable to compensate Hsp90 functions in axonal polarization.

4. Discussion

Our results show a role for Hsp90 during axonal polarization process and subsequent axonal elongation. Hsp90 activity inhibition before axon specification generates a multiple axon morphology in cultured hippocampal neurons. Once the axon is specified and neuronal polarity achieved, Hsp90 inhibition diminish axonal elongation rate. In both cases, Akt and GSK3 expression and activity are modified and the distribution of motor proteins, such as kinesin-1, is altered. Finally, Hsp90 inhibition increases the expression of Hsc70/Hsp70 chaperones. Interestingly, a change in Hsc70/Hsp70 distribution is correlated with multiple axon morphology.

Hsp90 function in neuronal development has been poorly studied. Besides the known expression of heat shock proteins (Hsps) in the neural tube from early mouse embryonic tissue (E9.5), the role of Hsps during neuronal differentiation is unknown. Hsp90β and Hsp90α are widely distributed in βIII-tubulin positive cells after E15.5, suggesting that Hsp90s may be required for specific functions in

neurons [18]. In fact, Hsp90 mediates neurites' outgrowth in PC12 cells [25]. Our results demonstrate that Hsp90 function is necessary to acquire a proper neuronal polarization with a unique axon. Moreover, Hsp90 activity is necessary in later stages to promote axonal elongation. In this sense, differentiation of P19 cells to neurons is accompanied by a six time increment of Hsp90 expression that is localized in soma and neurites [26]. We also observed an increment of Hsp90 expression in hippocampal neurons during the early stage of neuronal polarization that was also previously described during the first 3 h of N2A neuroblastoma differentiation in response to FK506 treatment [27]. Concomitantly, FK506 treatment of hippocampal neurons increases the expression in axons of the Hsp90 associated protein FKBP52. Regarding the Hsp90 location in neurites' tips in our experiments, same location was previously observed in differentiating N2A neuroblastoma cells [27,28]. These data suggest a role of Hsp90 in the modulation of neurites' growth, and raise the question whether Hsp90 function in one of the neurites or absence of function in the other neurites' controls axonal polarization? In fact, our experiments show that Hsp90 inhibition generates multiple axons, demonstrating that Hsp90 activity is necessary for axonal polarization. This morphological pattern in neurons has been previously described after GSK3 inhibition [29,30] or Akt activation [6]. However, opposite results demonstrating that GSK3 inhibition does not generate multiple axon morphology have been obtained by other groups [7,8,31,32]. Nevertheless, these data indicate an important role of these kinases in axon specification and development. In fact, our data show that Hsp90 inhibition decreases GSK3 and Akt expression levels, and affect their activities. The Akt activity was reduced and concomitantly GSK3 activity was enhanced, as inferred from the pGSK3/GSK3 ratio. Hsp90 inhibition at time 0 increases GSK3 activity around 50%, and it is tempting to propose that this increment may be responsible for the multiple axon morphology. However, GSK3 inhibition did not reverse the multiple axon morphology generated by Hsp90 inhibition, but impaired axon outgrowth (data not shown). Several studies have shown that active Akt (pAkt) is accumulated at the axon growth cone during axon specification [33]. Moreover, Akt inhibition increases its ubiquitination and degradation [6]. This process is controlled in neurons, where neuronal polarity requires dendritic Akt degradation by the ubiquitin–proteosome system. According to these data, expression of constitutively active Akt generates multiple axons [6]. Previous
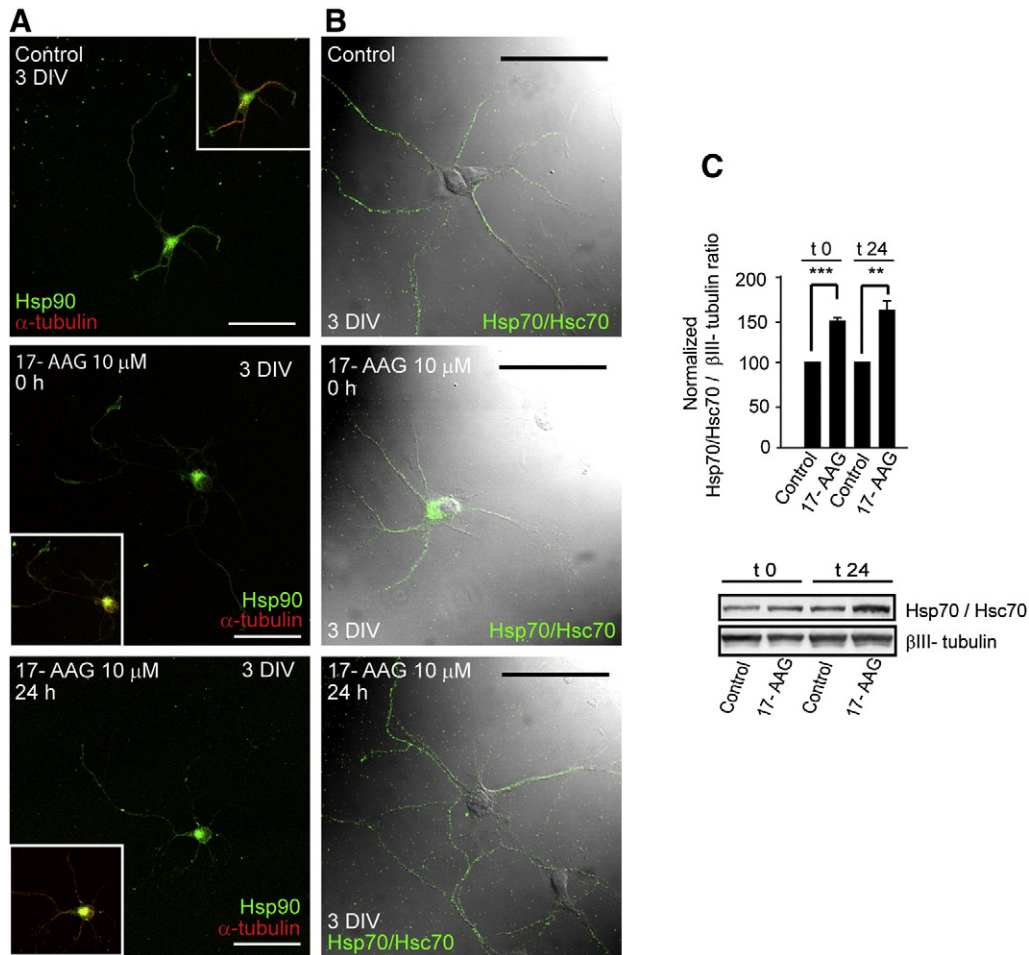
Fig. 5. Hsp70/Hsc70 expression and localization is altered by Hsp90 inhibition. 3 DIV hippocampal neurons were cultured in the absence or presence of the Hsp90 inhibitor 17-AAG (10 μM), added at the moment of neuronal medium switching (0 h) or after 24 h. (A) Neurons were fixed and stained with antibodies against Hsp90 (green) and α-tubulin (red, in white boxes). Scale bar = 50 μm. Note the absence of changes in Hsp90 localization. (B) Neurons in the same experiments were stained with antibodies against Hsp70/Hsc70 to analyze their localization. Neuronal morphology and integrity were confirmed in images obtained by differential interference contrast, DIC. Scale bars = 50 μm. (C) Cell extracts were obtained from 3 DIV cultured cortical neurons previously exposed to the Hsp90 inhibitor, 17-AAG (10 μM), at 0 or 24 h. Bottom panel shows a representative western-blot of Hsp70/Hsc70 expression in different experimental conditions. βIII-tubulin was used as a loading control. Graph (upper panel) represents the mean ± s.e.m. Hsp70/Hsc70 of three independent experiments normalized to βIII-tubulin levels. ***p < 0.001, **p < 0.01.

studies demonstrate that Hsp90 maintains the native conformation and activity of GSK3β and Akt [34]. Thus, differential regulation of Akt or GSK3 stability through Hsp90 in dendrites and axon maybe control the process of neuronal polarization. However, our data strongly suggest that is not a question of a simple Akt activity reduction, because the inhibition of Akt (data not shown) or the elimination of some Akt isoforms [35] did not generate a loss of axonal polarization. Complementary to this effect we cannot discard that other Hsp90 clients may be additionally affected contributing to the final polarization effect.

On the other hand, our results show that Hsp90 function is necessary for proper axonal elongation once the axon is specified. Hsp90 inhibition after axon specification slows down axonal growth, which may be in agreement with a reduced contribution of Akt activity [35]. Evaluating this data, it is tantalizing to propose that in this hippocampal polarization model exists a time-window for axonal specification. In fact, the inhibition during the initial hours affects polarity.

Besides different kinases involved in neuronal polarization and axonal growth, it has been described that KIF5, kinesin-1, is one of the first proteins to be polarized to the newly specified axon [9]. In fact, axonal growth requires a continued input of proteins from soma to axon that is mediated by motor proteins, such as kinesins [36]. Our results show that KIF5 is expressed in axon tips and shows an increasing distal gradient in the axon in control neurons, but after

Hsp90 inhibition KIF5 is retained in the soma, highly reduced in axon tips and in the case of multiple axons KIF5 staining is higher in the proximal region of the axons. These data prompt to modifications in protein transport mechanisms and/or the supporting structures for kinesin movement and polarization. It has been proposed that cargo liberation from kinesins is mediated by GSK3 phosphorylation of the kinesin light chain, which renders kinesins more accessible to Hsc70 chaperone and eases the delivery of its cargo in the axonal domain [37]. In fact, our data show that Hsp90 inhibition increases GSK3 activity, which may explain the reduced axonal growth through early cargo liberation before reaching its final destination. Finally, our results show a higher Hsc70 expression both time 0 and 24 h, which would support a disrupted transport of proteins to axon. The increased Hsc70 could provoke, combined with the high GSK3 activity, the delivery of kinesin cargos without arriving to its specific destination. Besides the increased Hsc70 expression, Hsc70 location was also modified. Hsc70 remains concentrated in soma when the Hsp90 inhibitor is applied at the first hours after platting, resembling what happens with KIF5 distribution. The apparent Hsp90 compensation by Hsc70/Hsp70 could be a cellular strategy to achieve neuronal polarization in the absence of Hsp90 function. In fact, previous studies have shown that pharmacological Hsp90 inhibition increases the expression of Hsp70 and Hsp40 to compensate

for Hsp90 through the activation of a transcription factor, HSF1 [38]. HSF1 is finely regulated during the neuronal differentiation of NG108-15 neuroblastoma–glioma hybrid cells to change the pattern of Hsps and the constitutive Hscs. This differentiation is co-related to an increased Hsc70 expression [39].

In conclusion, we have described for the first time a critical role for Hsp90 in axonal polarization mechanisms and the subsequent axonal growth. The effects may be due to the reduction of expression of some Hsp90 clients, as well as, to anomalous local distribution of proteins at the first differentiation stages. Further experiments and new experimental tools regarding Hsp90 will be necessary to fully understand the involvement of this chaperone in neuronal polarity and axon growth.

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