Independent functions of Hsp90 in neurotransmitter release and in the continuous synaptic cycling of AMPA receptors

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The delivery of neurotransmitter receptors into synapses is essential for synaptic function and plasticity. In particular, AMPA-type glutamate receptors (AMPA receptors) reach excitatory synapses according to two distinct routes: a regulated pathway, which operates transiently during synaptic plasticity, and a constitutive pathway, which maintains synaptic function under conditions of basal transmission. However, the specific mechanisms that distinguish these two trafficking pathways are largely unknown. Here, we evaluate the role of the molecular chaperone hsp90 in excitatory synaptic transmission in the hippocampus. On one hand, we found that hsp90 is necessary for the efficient neurotransmitter release at the presynaptic terminal. In addition, we identified hsp90 as a critical component of the cellular machinery that delivers AMPA receptors into the postsynaptic membrane. Using the hsp90-specific inhibitors radicicol and geldanamycin, we show that hsp90 is required for the constitutive trafficking of AMPA receptors into synapses during their continuous cycling between synaptic and non-synaptic sites. In contrast, hsp90 function is not required either for the surface delivery of AMPA receptors into the non-synaptic plasma membrane, or for the acute, regulated delivery of AMPA receptors into synapses upon plasticity induction (long-term potentiation). The synaptic cycling of AMPA receptors was also blocked by an hsp90-binding tetratricopeptide repeat (TPR) domain, suggesting that the role of hsp90 in AMPA receptor trafficking is mediated by a TPR domain-containing protein. These results demonstrate new roles for hsp90 in synaptic function by controlling neurotransmitter release, and independently, by mediating the continuous cycling of synaptic AMPA receptors.

Keywords: AMPA receptor trafficking/hippocampus/LTP/radicicol/TPR domain

Most excitatory synaptic transmission in the brain is mediated by AMPA-type glutamate receptors (AMPA receptors), and it is being increasingly appreciated that the targeting and delivery of AMPA receptors into synapses is critical for controlling synaptic function, maturation and remodeling (Sheng and Lee, 2001; Barry and Ziff, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002). AMPA receptors are hetero-oligomeric molecules composed of different combinations of GluR1 to GluR4 subunits (Hollmann and Heinemann, 1994). In hippocampus, most AMPA receptors are composed of a combination of either GluR1/GluR2 subunits or GluR2/GluR3 subunits (Wenthold et al., 1996). Targeting of AMPA receptors into synapses depends mainly on their subunit composition, and two main pathways have been proposed (Malinow et al., 2000). GluR2/GluR3 oligomers are continuously cycling in and out of synapses in a manner largely independent from synaptic activity (constitutive pathway). This synaptic cycling depends on the direct interaction between GluR2 and the hexameric ATPase N-ethylmaleimide-sensitive fusion protein (NSF) (Nishimune et al., 1998; Song et al., 1998; Lüscher et al., 1999). In contrast, GluR1/GluR2 receptors are added into synapses in an activity-dependent manner upon NMDA receptor activation (regulated pathway) (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 2001; Esteban et al., 2003). For this regulated delivery, protein-protein interactions mediated by the GluR1 subunit are critical (Hayashi et al., 2000; Shi et al., 2001). According to this scenario, the constitutive pathway would serve to maintain synaptic strength despite protein turnover, and it would act in a relatively fast manner (half-time of minutes). The regulated pathway would act transiently upon plasticity induction, leading to long-lasting changes in synaptic strength

(Malinow et al., 2000). However, the molecular mechanisms mediating these two trafficking pathways are still unclear.

Heat-shock protein 90 (hsp90) is expressed constitutively in brain from early development into adulthood (D'Souza and Brown, 1998) and it is especially abundant in limbic system-related structures, such as the hippocampus (Izumoto and Herbert, 1993). Hsp90 was proposed to be a mediator of protein trafficking over a decade ago (Pratt, 1992; Pratt, 1993). Since then, ample evidence has accumulated indicating that hsp90 is required for the subcellular targeting of a variety of proteins, including the glucocorticoid receptor (Czar et al., 1997; Galigniana et al., 2001; Owens-Grillo et al., 1996; Silverstein et al., 1999), the dioxin receptor (Kazlauskas et al., 2001), the receptor tyrosine kinase ErbB2 (Xu et al., 2002), the epidermal growth factor receptor (Supino-Rosin et al., 2000), the CFTR chloride channel (Loo et al., 1998) and the G protein $G_{\alpha 12}$ (Waheed and Jones, 2002). Hsp90 is also required for protein translocation into mitochondria (Young et al., 2003) and peroxisomes (Crookes and Olsen, 1998). The trafficking functions of hsp90 depend on cytoskeletal elements (Galigniana et al., 2002; Galigniana et al., 1998; Pratt et al., 1999), and involve specific interactions between C-terminal sequences of hsp90 and tetratricopeptide repeat (TPR) domains in several effector molecules (Chen et al., 1996; Russell et al., 1999; Scheufler et al., 2000; Ward et al., 2002; Young et al., 1998). TPR domains are involved in a variety of cellular functions, including protein transport and targeting (reviewed in Blatch and Lassle, 1999). Therefore, we considered that hsp90 was an interesting candidate to mediate AMPA receptor trafficking.

Here we examine the potential role of hsp90 in the transport and targeting of AMPA receptors into synapses. Using a combination of biochemical and

electrophysiological techniques on organotypical hippocampal slice cultures, we have found that hsp90 is a critical component of the cellular machinery that delivers AMPA receptors into synapses during their continuous cycling. In addition, we also describe an independent role for hsp90 in the control of neurotransmitter release at the presynaptic terminal.

MATERIALS AND METHODS

Expression of recombinant proteins in hippocampal neurons from organotypic slice cultures. The TPR domain (Chen et al., 1996) and the GluR2 (R586Q) (Shi et al., 2001) have been described before. The TPR domains were co-expressed with GFP by means of an IRES construct. All constructs were expressed in hippocampal CA1 pyramidal neurons from organotypic slice cultures using the Sindbis virus expression system (Malinow et al. 1999), except for the coexpression of GluR1 with the t-CaMKII, where we used the biolistic delivery method with plasmids bearing the CMV promoter (Lo et al., 1994). Briefly, hippocampal slices are prepared from young rats (postnatal day 5 to 7) and placed in culture on semiporous membranes (Gahwiler et al., 1997). After 4-7 days in culture, the recombinant gene is delivered into the slices with the Sindbis virus expression system (Schlesinger, 1993). This is a replication-deficient, low-toxicity, neurotropic virus that allows us to express recombinant proteins exclusively in neurons by injecting the viral solution extracellularly in the desired area of a hippocampal slice. Expression of the TPR domain was for 15 hours and that of GluR2 (R586Q) and GluR1 was for 36 hrs. Neurons remain morphologically and electrophysiologically intact during these expression times. All biosafety procedures and animal care protocols were approved by the University of Michigan.

Electrophysiology. Simultaneous double whole-cell recordings were obtained from nearby pairs of infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. The recording chamber was

perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin and 2 μM 2-chloroadenosine, at pH 7.4, gassed with 5% CO_2/95% O_2. Patch recording pipettes (3-6 MΩ) were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine and 0.6 mM EGTA at pH 7.25. In the rectification experiments, i.e. Fig 5, 0.1 mM spermine was added. Voltage-clamp whole-cell recordings were carried out with multiclamp 700A amplifiers (Axon Instruments, Union City, California, USA). Synaptic responses were evoked with two bipolar electrodes with single voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 μm and 500 μm from the recorded cells. Synaptic AMPA receptor-mediated responses were measured at –60 mV and NMDA receptor-mediated responses at +40 mV, at a latency when AMPA receptor responses have fully decayed (60 ms). Synaptic responses were averaged over 50-100 trials. In the rectification experiment (Fig 5), NMDA receptor-mediated responses were blocked pharmacologically using 0.1 mM DL-APV. Synaptic AMPA receptor-mediated responses were measured at –60 mV and +40 mV and their ratio was used as an index of rectification. Electrophysiological experiments in the presence of the pep2m/G10 peptide were carried out by including 1 mM peptide in the internal solution, together with the following protease inhibitors: 100 μM pepstatin A, 10 μM leupeptin and 100 μM bestatin. LTP experiments were carried out as previously described (Hayashi et al., 2000), by pairing 0 mV postsynaptic depolarization with 3 Hz presynaptic stimulation (300 pulses). All electrophysiological experiments were carried out in organotypic hippocampal slices.

Surface cross-linking assay. Hippocampal slices were treated either with 20 μM radicicol or with the vehicle (0.1% DMSO) for 30 min. Slices were then immersed for 5 min in perfusion solution containing 2 mM BS^3 (Pierce, cat. # 21580), a membrane impermeant cross-linker. This technique has been used previously to determine the fraction of AMPA receptors exposed on the cell surface of hippocampal neurons (Hall and Soderling, 1997) and cerebellar granule cells (Archibald et al., 1998). Extracts from treated hippocampal slice were prepared in homogenization buffer containing protease inhibitors (10 mM HEPES, 500 mM NaCl, 10 mM EDTA, 4 mM EGTA, 0.1 mM PMSF, 2 μg/ml Chymostatin, 2 μg/ml Leupeptin, 2 μg/ml Antipain, 2 μg/ml Pepstatin and 1% triton X-100). AMPA receptors were then analyzed by western blot with anti-GluR1 antibody (Chemicon). The fraction of intracellular receptors was calculated as the ratio of the intensity of the intracellular (non-crosslinked) band over the intensity of the sum of intracellular and surface (crosslinked) bands. All quantifications were carried out by densitometric scanning of autoradiographic films under linear exposure conditions.

RESULTS

Distinct presynaptic and postsynaptic roles of hsp90 in synaptic transmission

As a first step to evaluate whether hsp90 function is important for synaptic transmission, we monitored evoked AMPA and NMDA receptor-mediated responses in CA1 cells from hippocampal slices upon addition of radicicol, a cell-permeable, highly-specific inhibitor of hsp90 (Schulte et al., 1999). As shown in Fig. 1A, radicicol added to the perfusion system produced a rapid decrease in AMPA receptor-mediated currents. Separate recordings carried out at +40 mV also showed a significant, although smaller, depression of NMDA receptor-mediated responses. As control, addition of an equivalent amount of the radicicol vehicle (0.1 % DMSO) did not alter AMPA transmission. Fig. 1 B indicates that the changes in response amplitude shown in Fig. 1A are not due to systematic variations in access resistance during the recordings.

The effect observed upon bath-application of radicicol may be due to the interference with presynaptic and/or postsynaptic functions of hsp90. To evaluate more directly a potential presynaptic function of hsp90, we measured paired-pulse facilitation of synaptically evoked excitatory responses in hippocampal slices. Paired-pulse facilitation is a well-established paradigm of short-term plasticity that is very sensitive to changes in the probability of neurotransmitter release (Dobrunz and Stevens, 1997). We compared paired-pulse facilitation between control hippocampal slices and slices that had been under perfusion in the presence of radicicol for at least 30 min before recordings (radicicol was also present during the recordings). As shown in Fig. 2, paired-pulse facilitation was significantly enhanced in slices pre-treated with radicicol, indicating that

the probability of neurotransmitter release was reduced upon blockade of hsp90 function. To our knowledge, this is the first evidence of a presynaptic role of hsp90 during synaptic transmission.

A reduction in neurotransmitter release should affect, to a large extent, similarly AMPA and NMDA receptor-mediated responses. However, our results shown in Fig. 1A suggest that the depression induced upon blockade of hsp90 function is more pronounced for AMPA receptors than for NMDA receptors. To address this point more rigorously, we kept hippocampal slices under perfusion in the presence of radicicol for at least 30 minutes and then, measured the ratio between AMPA and NMDA receptor-mediated responses from individual cells by recording evoked synaptic currents at -60 mV (AMPA) and +40 mV (NMDA). As shown in Fig. 3, slices pre-incubated in radicicol showed a significant decrease in AMPA/NMDA ratio, as compared with naïve slices or with slices treated with vehicle (DMSO). To further control for the specificity of this effect, we used a chemically different hsp90 inhibitor, geldanamycin. Both radicicol and geldanamycin block hsp90 with high specificity by occupying its atypical nucleotide binding pocket (Roe et al., 1999). Indeed, slices preincubated with geldanamycin also showed significantly reduced AMPA/NMDA ratios (Fig. 3). These results, taken together, strongly suggest that hsp90, in addition to its presynaptic function, plays a direct postsynaptic role in AMPA receptor-mediated transmission. The following experiments are then oriented to test this interpretation and elucidate the postsynaptic function of hsp90.

Inhibition of hsp90 does not alter the fraction of AMPA receptors on the cell surface nor the total number of AMPA receptors

The fraction of AMPA receptors present at synapses is a small fraction of the total population present on the neuronal cell surface (synaptic plus extrasynaptic; Shi et al., 1999). Therefore, in order to investigate whether hsp90 is required specifically for the synaptic delivery of AMPA receptors or whether it is involved in their global delivery to the cell membrane, we used a surface cross-linking assay (see Methods). Hippocampal slices were pretreated with radicicol or DMSO for 30 minutes and then exposed to BS^3, a membrane impermeant bifunctional cross-linker that reacts with primary amine groups in proteins. In the case of AMPA receptors, this reagent crosslinks the different subunits of the receptor, leading to a marked increase in its apparent molecular weight in polyacrylamide gel electrophoresis. As shown in Fig. 4, radicicol treatment did not increase the percentage of non-crosslinked, intracellular AMPA receptors, as compared to the slices treated with the vehicle only. Importantly, radicicol did not change the total amount of receptors (intracellular plus surface), suggesting that blocking hsp90 function for 30 minutes did not cause degradation of AMPA receptors. In conclusion, these data indicate that hsp90 is not needed for the global surface delivery of the receptor. Thus, hsp90 may be important for the local delivery of receptors into synapses, which constitute only a small fraction of the total number of surface AMPA receptors (Shi et al., 1999).

Hsp90 is necessary for the continuous synaptic delivery of AMPA receptors

In order to test directly the role of hsp90 in the delivery of AMPA receptors into synapses, we expressed a recombinant AMPA receptor subunit (GluR2 R586Q) in

organotypic hippocampal slices using a viral delivery system (see Methods). Recombinant GluR2 receptors behave as endogenous GluR2/GluR3 heteroligomers, and therefore, can be used to monitor the constitutive pathway of AMPA receptor synaptic delivery (Shi et al., 2001). In addition, the mutation R586Q, at the channel pore, prevents the receptor from conducting outward currents at positive membrane potentials (inward rectification). Therefore, the synaptic delivery of the recombinant receptor can be detected as an increase in the ratio of the AMPA receptor-mediated response at -60 mV versus the response at +40 mV (rectification index; Hayashi et al., 2000; Zhu et al., 2000; Shi et al., 2001; Esteban et al., 2003). Importantly, this rectification index is independent from changes in presynaptic function, which would alter similarly responses at -60 mV and at +40 mV. In the absence of radicicol, expression of the recombinant receptor produced inward rectification (increase in the rectification index) (Fig. 5A, compare control and GluR2(RQ)), as previously described (Shi et al., 2001), indicating the delivery of the homomeric receptor. Thirty minute incubation with radicicol on slices expressing GluR2 (R586Q) blocked this rectification (Fig. 5A, GluR2(RQ)+Rad). Since GluR2 receptors are constitutively cycling in and out of synapses (Shi et al., 2001; Passafaro et al., 2001), these results suggest that hsp90 is necessary either for the synaptic reinsertion of AMPA receptors during their continuous cycling, or for their stability once inserted at synapses.

Hsp90 inhibition does not affect the non-cycling population of AMPA receptors

In order to test whether hsp90 inhibition affects receptor stability at synapses, we evaluated the effect of blocking hsp90 function on the population of AMPA receptors

that is not continuously cycling. To this end, we co-expressed a constitutively active form of αCaMKII (tCaM; Hayashi et al., 2000; Poncer et al., 2002) with the recombinant GluR1 subunit on organotypic hippocampal slices. Recombinant GluR1 receptors behave as endogenous GluR1/GluR2 heteroligomers, and therefore, can be used to monitor the non-cycling receptor population once their synaptic delivery is triggered by active CaMKII (Hayashi et al., 2000). Recombinant GluR1 was detected at synapses when co-expressed with constitutively active CaMKII, as indicated by the increase in rectification index (compare control in Fig. 5A and GluR1-tCaM in Fig. 5B; see also Hayashi et al., 2000). Importantly, thirty-minute incubation with radicicol on slices expressing GluR1-tCaM did not block this rectification (Fig. 5B, GluR1-tCaM+Rad), indicating that hsp90 is not required for the stability of AMPA receptors at synapses. Furthermore, the rectification index was significantly higher in the presence of radicicol than in its absence. This result is consistent with radicicol preventing reinsertion of the endogenous (non-rectifying) recycling pool of receptors, without affecting the stability of the recombinant (rectifying) receptors. As control, radicicol did not change the rectification index when recombinant GluR2 (R586Q) or GluR1 was not expressed (Fig. 5B, Rad). In conclusion, these results indicate that hsp90 is required for receptor cycling, but not for the synaptic stability of the non-cycling population of receptors.

Hsp90 and NSF act on the same pool of cycling AMPA receptors

The efficient constitutive cycling of AMPA receptors into synapses requires a direct interaction between GluR2 and NSF. Hence, when this interaction is prevented by intracellular infusion of a peptide containing the NSF-binding sequence of GluR2

(pep2m/G10), AMPA receptor-mediated responses rapidly decline (Nishimune et al., 1998; Song et al., 1998; Lüscher et al., 1999). This is interpreted as the depletion of synapses from the constitutively cycling pool of receptors. In order to test whether hsp90 and NSF act on the same population of AMPA receptors, we incubated hippocampal slices with radicicol for thirty minutes and then recorded AMPA receptor-mediated responses while loading the recorded cell with the GluR2/NSF interfering peptide. As shown in Fig. 6A, pre-incubation with radicicol virtually abolished the depression of AMPA responses induced by the peptide. As control, recordings from non-treated slices showed the expected run-down of AMPA transmission upon infusion of the peptide. Fig. 6B indicates that the changes in response amplitude shown in Fig. 6A are not due to variations in access resistance during the recordings.

This result indicates that blocking hsp90 function with radicicol depletes synapses from the same pool of cycling receptors on which NSF acts. This interpretation, based on monitoring endogenous AMPA receptors, is consistent with the results shown in Fig. 5A, where radicicol prevented the constitutive synaptic delivery of recombinant GluR2 receptors.

Hsp90-TPR domain interactions are involved in AMPA receptor trafficking

As described in the introduction, all known cases of protein transport mediated by hsp90 are dependent on specific interactions between hsp90 and TPR-containing proteins. To test whether this is the case for AMPA receptors, we overexpressed in CA1 hippocampal neurons the TPR domain of protein phosphatase 5, which binds specifically to hsp90 and acts as a dominant negative on hsp90 function (Chen et al., 1996). As

control, we also used a TPR domain with a point mutation that abolishes binding to hsp90 (Arg101 to Ala, (Russell et al., 1999)). It is also important to note that the expression of the TPR constructs is targeted exclusively to the CA1 neurons (by means of local virus injection; see Methods). Since we monitor synaptic responses from CA3 to CA1 neurons, this experimental configuration implies that the TPR domain is only expressed in the postsynaptic cell. As shown in Fig. 7A, overexpression of the TPR domain significantly decreased AMPA receptor-mediated transmission, without altering NMDA receptor-mediated responses (data not shown). Importantly, overexpression of the TPR mutant that does not bind hsp90, TPR (R101A), did not have any effect on AMPA (Fig. 7B) or NMDA (not shown) transmission. These results support the hypothesis that hsp90 is necessary for the trafficking of AMPA receptor into synapses, and that this role may be mediated by a TPR domain-containing protein. Additionally, since the depression of AMPA responses shown in Fig. 7A was obtained under conditions of basal transmission, these results suggest that hsp90 is necessary for the constitutive (activity-independent) delivery of AMPA receptors into synapses, supporting the results obtained with radicicol treatment. We can also conclude from these experiments that the depression of NMDA responses upon bath-application of radicicol (Fig. 1A) was solely due to a reduction in presynaptic function, since postsynaptic expression of the TPR domain did not affect NMDA responses (Fig. 7A).

Hsp90 is not required for long-term potentiation. Long term potentiation (LTP) in the CA1 hippocampus is one of the most thoroughly studied forms of synaptic plasticity, and it is accompanied by the synaptic delivery of GluR1-containing AMPA receptors

(Hayashi et al., 2000). To test whether hsp90 is necessary for the movement of AMPA receptors during synaptic plasticity, we examined pairing-induced LTP in CA1 neurons expressing the TPR domain or its inactive mutant (TPR R101A). As shown in Fig. 8, neurons expressing either TPR or TPR (R101A) exhibit LTP levels similar to those of control, uninfected neurons. Therefore, hsp90 is not required for the acute, activity-dependent delivery of AMPA receptors during synaptic plasticity.

DISCUSSION

Hsp90 is an abundant, constitutively expressed protein in neurons (Gass et al., 1994), and different members of the hsp90 co-chaperone machinery have been found in presynaptic and postsynaptic terminals. In particular, a co-chaperone complex composed of hsc70 and CSP is present in the presynaptic neurotransmitter vesicles (Tobaben et al., 2001). This complex, together with hsp90, has been proposed to control neurotransmitter release (Sakisaka et al., 2002). Complementary, two members of the hsp90 co-chaperone machinery, hsp70 and hsp40, have been found at the postsynaptic density (Moon et al., 2001; Suzuki et al., 1999; Walikonis et al., 2000), although the function of this postsynaptic complex was unknown. Here, using electrophysiological techniques, we show that hsp90 plays important roles in synaptic transmission at both the presynaptic and postsynaptic compartments through independent mechanisms. In particular, we have found that hsp90 is necessary for efficient neurotransmitter release at the presynaptic terminal. Although this function had been proposed previously from biochemical studies (Sakisaka et al., 2002), it had never been shown to operate during synaptic transmission. In addition, the most unexpected result of this study is that hsp90 is an essential component of the molecular machinery required for the continuous cycling of AMPA receptors at at the postsynaptic membrane.

We have provided three lines of evidence that suggest that hsp90 is required for AMPA receptor synaptic delivery: (1) radicicol decreased AMPA receptor-mediated responses as compared to those mediated by NMDA receptors (AMPA/NMDA ratio); (2) radicicol prevented the constitutive synaptic delivery of AMPA receptors, as assayed with recombinant GluR2 receptors and by monitoring endogenous receptor cycling with

the GluR2/NSF interfering peptide; (3) expression of an hsp90-binding TPR domain decreased AMPA receptor-mediated synaptic responses, but did not affect NMDA receptor-mediated transmission. In contrast, expression of the TPR domain did not alter LTP induction or expression, which suggests that hsp90 is not involved in the activity-dependent delivery of AMPA receptors. Additionally, surface crosslinking experiments indicated that hsp90 function is not required for the delivery of AMPA receptors into the non-synaptic cell membrane. Overall, these results strongly suggest that hsp90 has a necessary role in the synaptic delivery of AMPA receptors specifically during their continuous cycling. It is worth mentioning that this specific role in the constitutive pathway is consistent with the small, but significant, reduction of AMPA/NMDA ratio (Fig. 3, 7A) or absolute AMPA receptor-mediated responses (Fig. 7A) observed when blocking hsp90 function. These results suggest that the fraction of cycling AMPA receptors at synapses may range around 30% to 50%, in good agreement with previous estimations using GluR2/NSF interfering peptides (Nishimune et al. 1998; Lüscher et al., 1999; Lee et al., 2002), dominant negative GluR2 C-terminus constructs (Shi et al., 2001) and rectifying receptors (Shi et al., 2001).

While the involvement of hsp90 in the synaptic cycling of AMPA receptors is a novel observation, it is worth mentioning that this is the second chaperone described as an important component of this process. As mentioned above, the hexameric chaperone NSF interacts directly with AMPA receptors and disruption of this interaction cause a rapid loss of synaptic AMPA receptors (Kim et al., 2001; Luscher et al., 1999; Luthi et al., 1999; Osten et al., 1998; Song et al., 1998; Nishimune et al., 1998; Noel et al., 1999;

Shi et al., 2001). Therefore, our results indicate that both hsp90 and NSF are required for the rapid synaptic cycling of these receptors.

How does hsp90 mediate AMPA receptor synaptic delivery? Hsp90 may be acting as a classical molecular chaperon, catalyzing the assembly and disassembly of transient protein complexes required for receptor cycling. This would actually be similar to the role that has been proposed for NSF, which has been shown to catalyze the ATP-dependent dissociation of the complex formed between PICK1 and GluR2 (Hanley et al., 2002). The formation and dissociation of this complex, together with the one formed between GluR2 and GRIP, is thought to regulate receptor delivery into synapses (Chung et al., 2000; Daw et al., 2000; Perez et al., 2001; Braithwaite et al., 2002; Seidenman et al., 2003). We have indeed tested whether complex assembly between GluR2, NSF, PICK1 and GRIP was dependent on hsp90 activity, but the results were negative (not shown).

On the other hand, as mentioned above, hsp90 is known to control intracellular protein transport and targeting. Through its TPR acceptor site, hsp90 interacts with a variety of proteins that bind motor proteins, therefore linking hsp90 with movement along the cytoskeletal tracts (reviewed in Pratt et al., 1999; Pratt and Toft, 2003). For instance, the TPR acceptor site of hsp90 interacts with the TPR domain of unc45, and unc45 binds to the actin-dependent motor protein myosin through its carboxyl terminal region (Barral et al., 2002). Interestingly, AMPA receptor presence at synapses is dependent on an intact actin cytoskeleton (Kim and Lisman, 1999; Kim and Lisman, 2001; Shen et al., 2000; Zhou et al., 2001). Our results with the dominant negative TPR

domain are consistent with a model in which hsp90, via a TPR-dependent interaction, mediates the actin-dependent insertion of AMPA receptors into synapses.

In summary, the present findings advance our understanding of the distinct molecular machinery that catalyzes the continuous versus the regulated exocytosis of AMPA receptors. In addition, these results uncover a new role for hsp90 in synaptic function as a mediator of the constitutive delivery of AMPA receptors into synapses.

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FIGURE LEGENDS

Figure 1. Radicicol, an inhibitor of hsp90, diminishes AMPA and NMDA receptor-mediated responses. (A) AMPA and NMDA receptor-mediated evoked synaptic responses were recorded in CA1 neurons of the hippocampus upon stimulation of the Schaffer Collateral pathway. Radicicol (20 μM Rad) or the equivalent amount of the radicicol vehicle (0.1 % DMSO) was added to the perfusion system at the time indicated with an arrow. AMPA and NMDA receptor-mediated currents were recorded in separate experiments, at -60 mV and +40 mV, respectively. Values are presented as mean ± sem. Insets, sample trace of evoked AMPA or NMDA receptor-mediated synaptic responses (as indicated) before (thin line) and 15 min after (thick line) the addition of radicicol or DMSO. Scale bars, 20 pA and 20 ms. (B) Average series resistance for the recordings shown in (A). In addition, we confirmed that radicicol application does not alter membrane input resistance (control: 182 ± 13 MΩ; plus radicicol: 194 ± 15 MΩ).

Figure 2. Inhibition of hsp90 enhances paired-pulse facilitation (PPF). Hippocampal slices were perfused with 20 μM radicicol (Rad) for at least 30 min, and then PPF was monitored at different interstimulus intervals (50 ms, 100 ms, 200 ms and 400 ms). PPF is expressed as the ration between the amplitude of the second response *versus* the amplitude of the first response. Note that PPF was significantly enhanced in slices perfused with radicicol at interstimulus intervals of 50 ms, 100 ms and 200 ms. At 400 ms there was no significant facilitation either with or without radicicol. Insets, sample

trace of evoked AMPA receptor-mediated synaptic responses with an interstimulus interval of 100ms. Scale bars, 20 pA and 50 ms.

Figure 3. Inhibition of hsp90 decreases AMPA/NMDA ratio. Hippocampal slices were perfused with 20 μM radicicol (Rad), 20 μM geldanamycin (Geld) or its vehicle (0.1% DMSO) for at least 30 min and then AMPA and NMDA responses were recorded from individual cells. Control slices were maintained in regular perfusion solution. Both radicicol and geldanamycin significantly decreased AMPA/NMDA ratio (p is the probability measured by student t-test comparing AMPA/NMDA ratio in the presence of the vehicle and in the presence of the drug). Values are presented as mean ± sem.

Figure 4. Surface crosslinking of AMPA receptors in hippocampal slices exposed to radicicol. Top. Western blot analysis of the fraction of AMPA receptor GluR1 subunit crosslinked on the cell surface with the membrane-impermeant crosslinker BS^3. Slices were treated with radicicol or DMSO, as indicated, for 30 min. $-BS^3$ indicates control slices not crosslinked. Each lane in the western blot is the result of pooling together extracts from four slices treated in parallel. **Bottom.** Quantification by densitometric scanning of six independent experiments as the one shown on top. Quantification of the intracellular fraction was calculated as described in Methods.

Figure 5. Hsp90 is necessary for the constitutive cycling of GluR2 receptors but not for the stability of GluR1 receptors at synapses. Average rectification values (AMPA-mediated response at –60 mV / AMPA-mediated response at +40 mV) for CA1 neurons

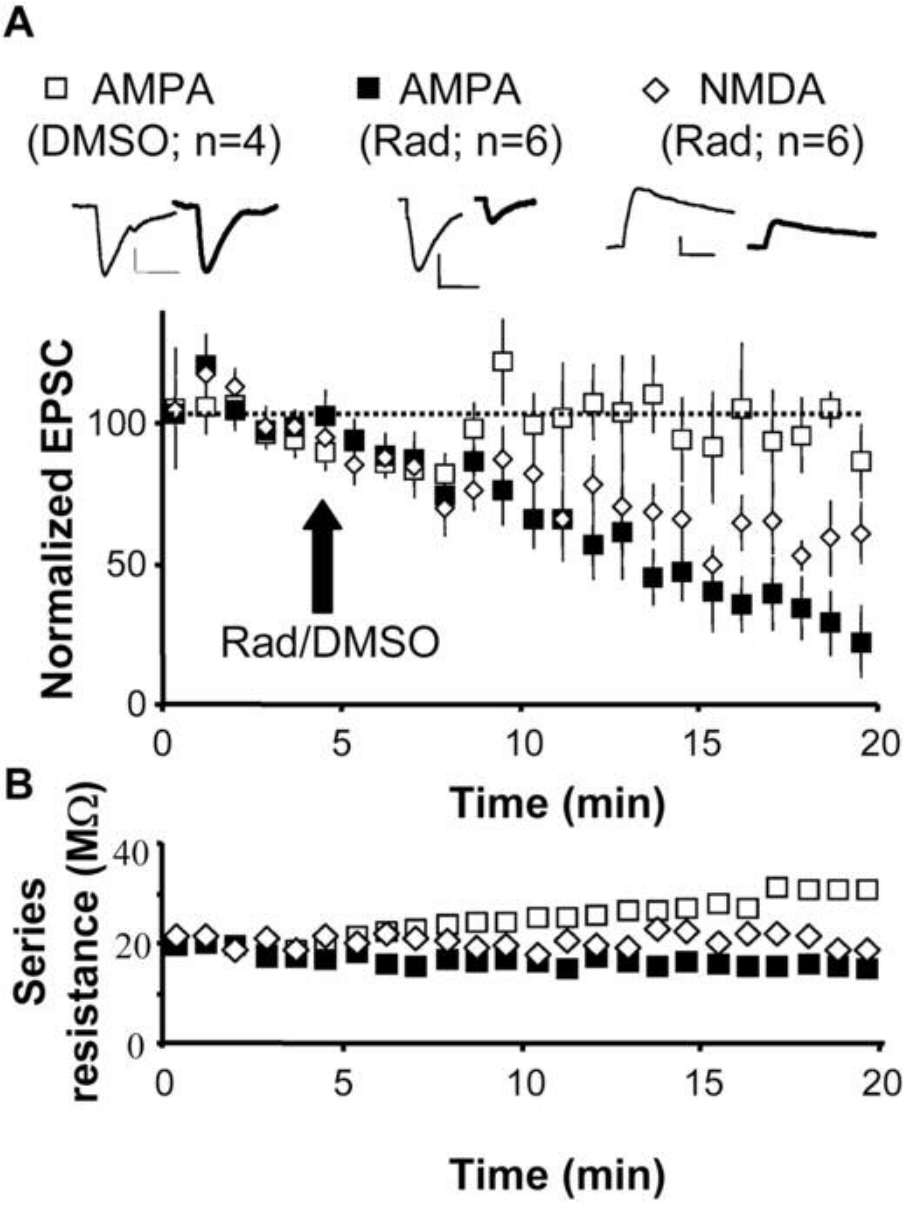
in absence (control) and presence of radicicol (Rad), as indicated, with or without expression of the rectifying GluR2 (R586Q) (A) or GluR1 plus constitutively active αCaMKII (B). Insets, sample trace of evoked AMPA receptor mediated synaptic responses recorded at –60 mV and +40 mV from control, GluR2 (R586Q) infected cells or GluR1-tCaM transfected cells in absence or presence of radicicol, as indicated. Scale bars, 20 pA and 20 ms.

Figure 6. Hsp90 and NSF act on the same pool of cycling AMPA receptors. (A) Whole-cell recordings of AMPA receptor-mediated responses in the presence of the pep2M/G10 peptide in the internal solution. Recordings were carried out on naïve slices or on slices pre-treated with radicicol for at least 30 min, as indicated. (B) Average series resistance from the recordings shown in (A).

Figure 7. Overexpression of an hsp90-binding TPR domain decreases AMPA receptor-mediated transmnission. Left, average AMPA receptor-mediated current amplitude from infected neurons co-expressing TPR domain and GFP (A) or TPR (R101A) mutant and GFP (B) and control neighboring cells not expressing the recombinant protein (uninf); n represents the number of pathways from cell pairs. Right, average AMPA/NMDA ratios for uninfected and infected cells (n represents the number of pathways).

Figure 8. Blocking hsp90 postsynaptically does not impair long term potentiation (LTP). Organotypic slice cultures were infected with virus expressing either TPR or TPR

(R101A). Whole cell recordings were established from neurons expressing the desired proteins or uninfected cells, and LTP was induced by pairing, as previously described (Hayashi et al., 2000). Experiments were done blind with respect to which construct was expressed. Pairing significantly increased AMPA receptor-mediated responses in control, TPR- and TPR (R101A)-expressing neurons. No significant difference in the amount of potentiation was observed among the three groups at any time point ($p=0.95$). Inset, sample trace of evoked AMPA receptor-mediated synaptic responses before pairing (thin line) and 30 min after pairing (thick line). Scale bars, 20 pA and 40 ms.



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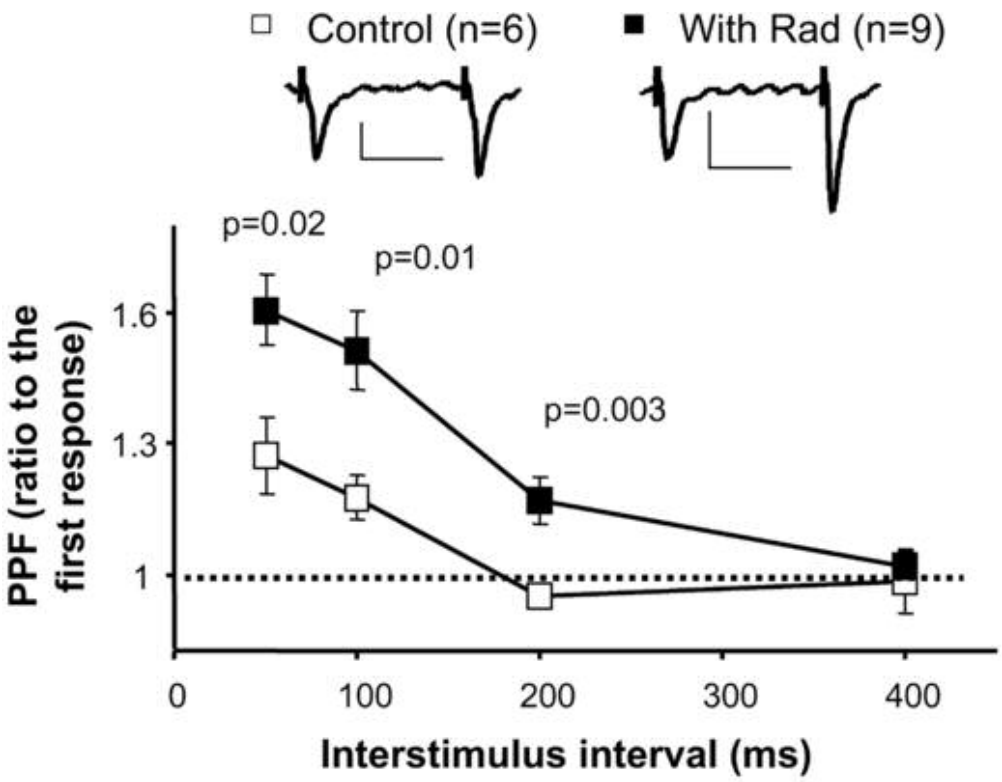

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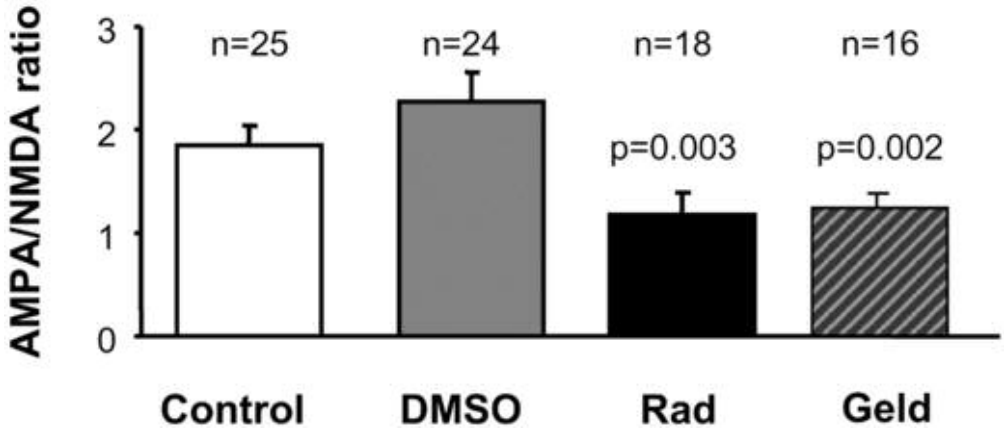

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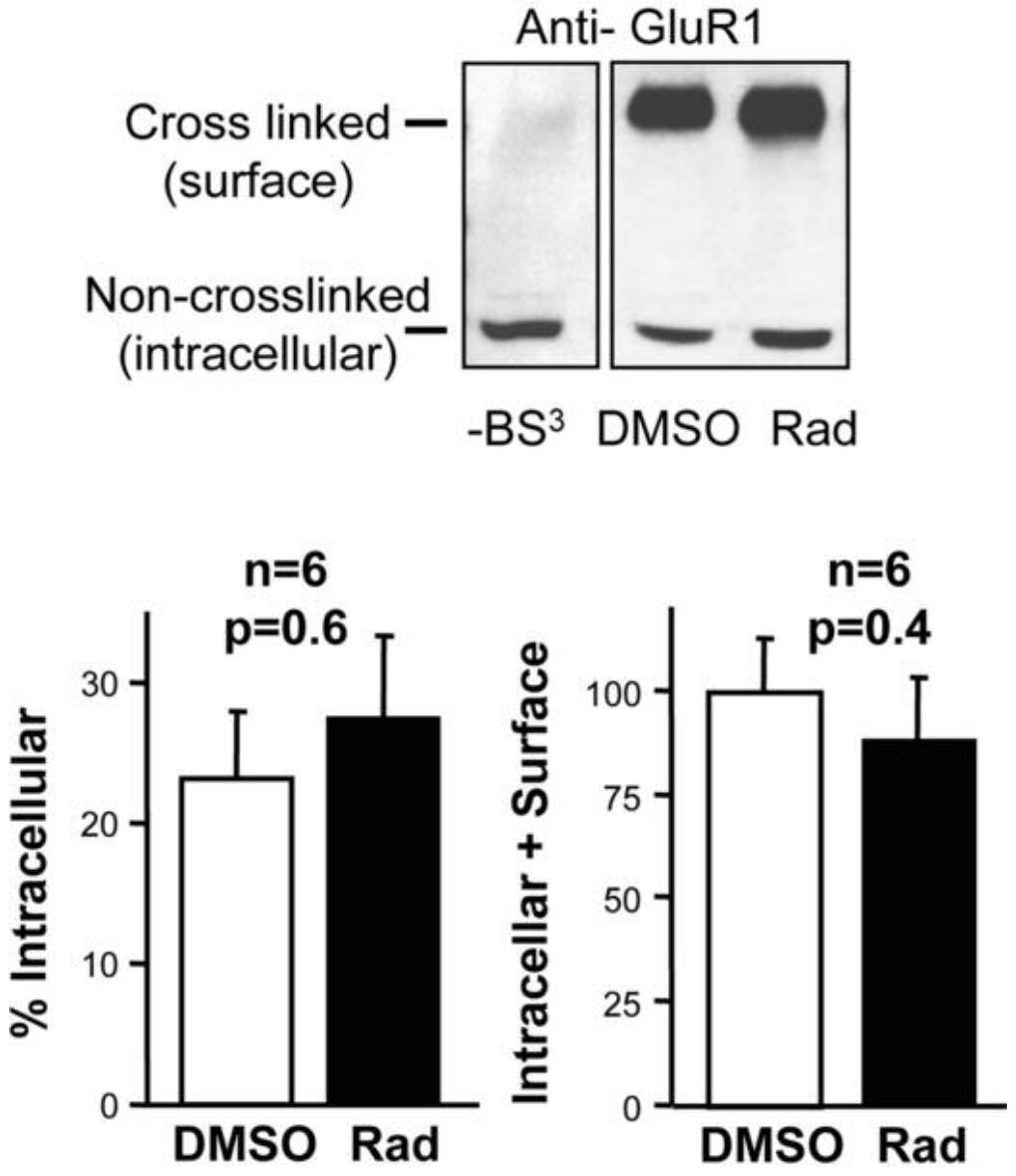

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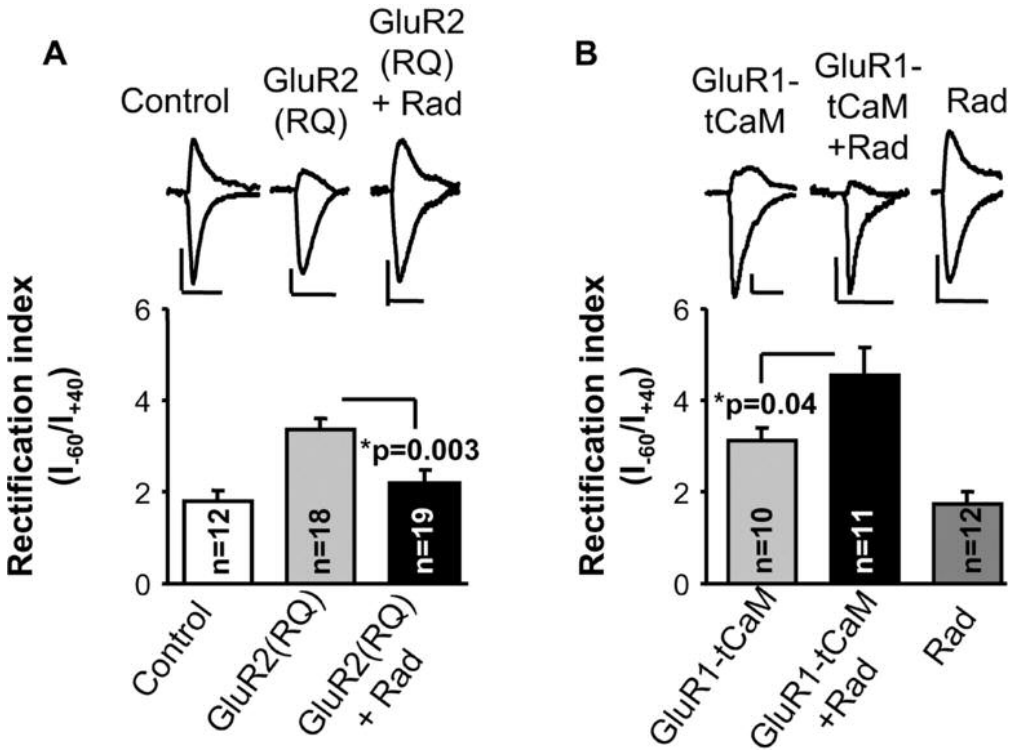

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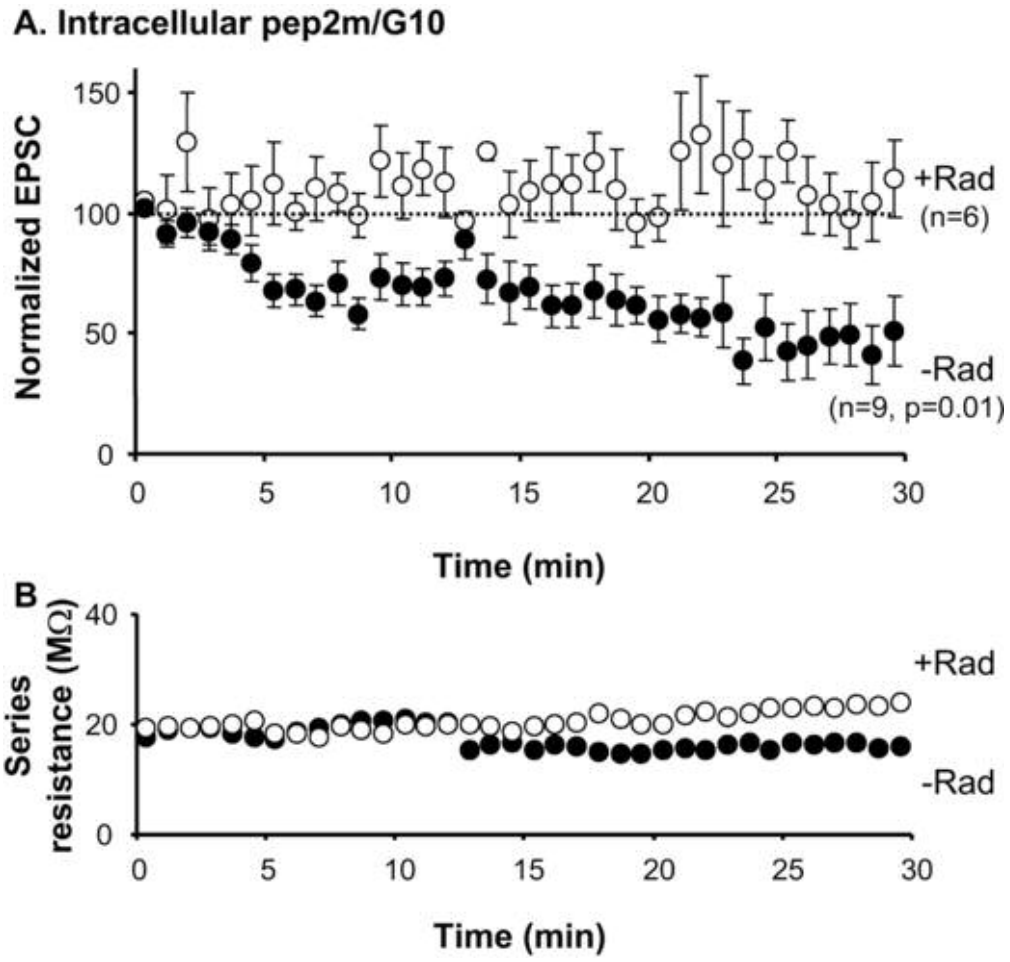

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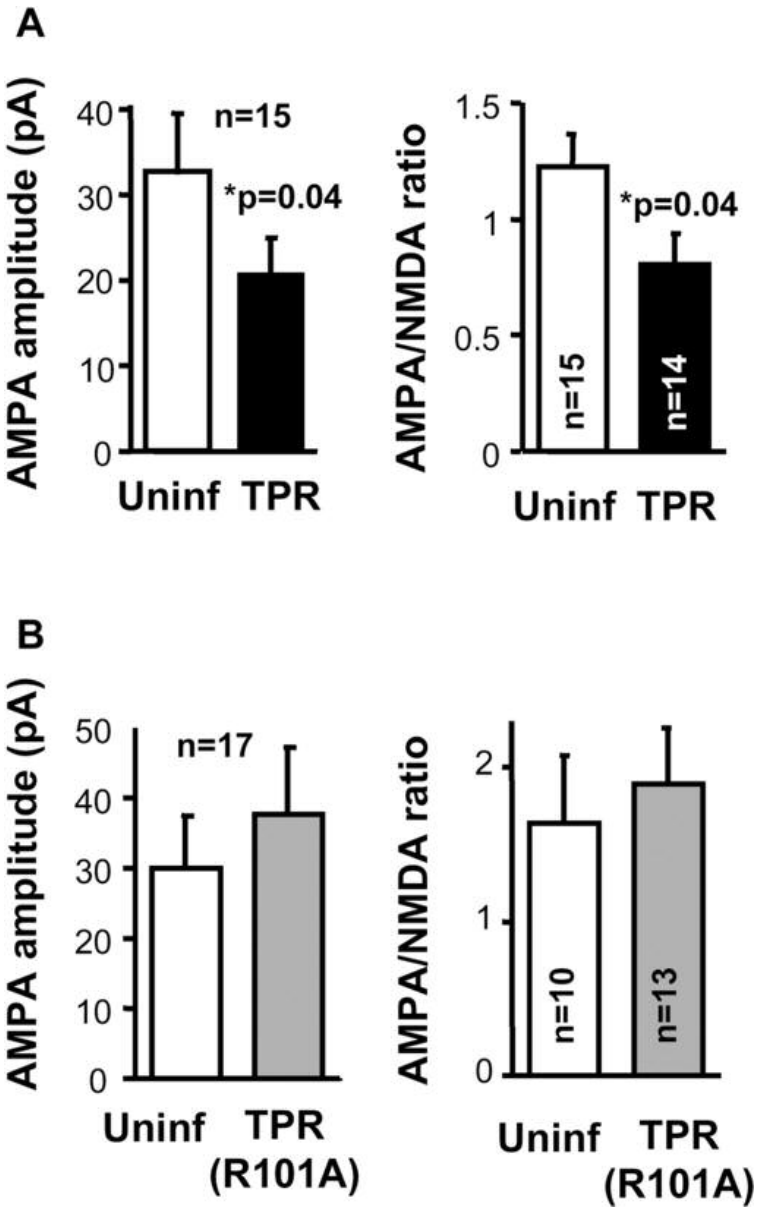

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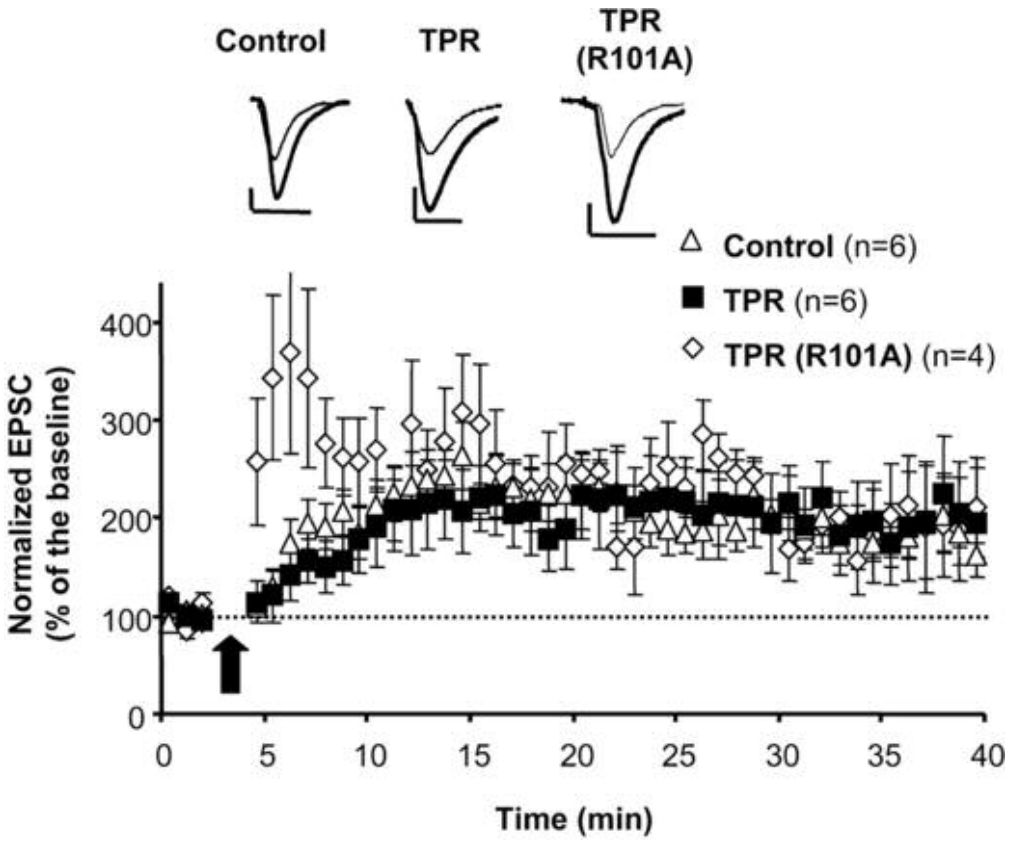

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