Cellular/Molecular

Tyrosine Phosphorylation of the 2B Subunit of the NMDA Receptor Is Necessary for Taste Memory Formation

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We aimed to test whether tyrosine phosphorylation of the NMDA receptor (NMDAR) in the insular cortex is necessary for novel taste learning. We found that in rats, novel taste learning leads to elevated phosphorylation of tyrosine 1472 of the NR2B subunit of the NMDAR and increases the interaction of phosphorylated NR2B with the major postsynaptic scaffold protein PSD-95. Injection of the tyrosine kinase inhibitor genistein directly into the insular cortex of rats before novel taste exposure prevented the increase in NR2B tyrosine phosphorylation and behaviorally attenuated taste-memory formation. Functionally, tyrosine phosphorylation of NR2B after learning was found to determine the synaptic distribution of the NMDAR, since microinjection of genistein to the insular cortex altered the distribution pattern of NMDAR caused by novel taste learning.

Introduction

The postsynaptic NMDA receptor (NMDAR) is a major regulator of synaptic plasticity. NMDARs are ligand-gated ion channels that when activated, cause neuronal depolarization by generating fast EPSPs. Opening of the channel causes a transient increase in intracellular calcium, which initiates intracellular signaling. The channel is composed of NR1, which is fundamental for channel function, and NR2A-D subunits, which modulate channel gating as well as pharmacological and functional properties of the channel (Monyer et al., 1994; Cull-Candy et al., 2001).

One of the key mechanisms for regulating NMDAR function involves tyrosine phosphorylation of its NR2 subunits by the cytosolic tyrosine kinases Src and Fyn (Wang and Salter, 1994; Wang et al., 1996; Smart, 1997). Although both NR2A and NR2B undergo tyrosine phosphorylation, the Fyn kinase target tyrosine 1472 NR2B was identified as the major substrate at the postsynaptic density (Moon et al., 1994; Nakazawa et al., 2001). *In vitro* studies confirmed that Y1472 phosphorylation is significantly increased under various conditions, including nerve injury (Abe et al., 2005) and ethanol administration (Yagi, 1999).

Whereas NMDARs in mature neurons were initially thought to display stable expression on the plasma membrane (Lüscher et al., 1999; Ehlers, 2000), recent findings suggest that this process is dynamic. Several findings suggest that Y1472 NR2B phosphorylation plays a significant role in the trafficking of NMDARs: (1) NMDA trafficking after long-term potentiation (LTP) induction in the hippocampus of adult rats requires Src activity (Grosshans et al., 2002); (2) internalization of NR2B requires a consensus motif that includes Y1472 (Roche et al., 2001); (3) mutations in

Y1472 NR2B disrupt binding of the clathrin-associated adaptor protein AP-2 to the channel and inhibit NMDAR internalization (Lavezzari et al., 2003; Prybylowski et al., 2005); and (4) *in vivo*, knock-out of Y1472 results in impaired synaptic plasticity, accompanied by skewed localization of NR1 and NR2B toward the peripheral and perisynaptic regions of the synapse (Nakazawa et al., 2006).

The potential importance of tyrosine phosphorylation of the NMDAR in learning processes was initially proposed *in vitro* when selective tyrosine kinase inhibitors were shown to block LTP (O'Dell et al., 1991) in a Src/Fyn-dependent manner (Grant et al., 1992; Lu et al., 1998). These findings were later corroborated *in vivo* in the dentate gyrus of rats (Rosenblum et al., 1996; Rostas et al., 1996). Since then, tyrosine phosphorylated content of NR2B was shown to increase in response to several learning paradigms, including learning of fear (Kojima et al., 2005; Nakazawa et al., 2006) and of taste aversion (Rosenblum et al., 1995; Miyakawa et al., 1997). However, despite the strong correlation between the increase in tyrosine phosphorylation of NR2B and novel taste learning, it has not been determined whether it is necessary for learning a new taste. Here, we hypothesize that introduction of animals to a novel taste results in changes in tyrosine phosphorylation of NR2B, and that prevention of this phosphorylation impairs novel taste memory formation. We further argue that the mechanism underlying taste memory formation involves adequate cellular localization of NMDARs, which also depends on tyrosine phosphorylation of NR2B.

Materials and Methods

Animal handling
Adult male Wistar Hola or Sprague Dawley rats (Harlan), weighing 200–250 g, were used in all experiments. Standard commercial rat chow and water were available *ad libitum*, and animals were kept on a 12 h light/dark cycle. Animals were handled according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee of the University of Haifa. Protocols were also in accordance with the guidelines laid down by the US National Institutes of Health.

Behavioral paradigms

Incidental taste learning. At the beginning of each biochemical or behavioral experiment, animals were separated into individual housing cages and underwent a 3 d water-restriction training session, in which they received a daily ration of 20 ml of water in two 10 ml pipets, once a day for 20 min. Training ensured that on the day of the experiment the rats drank a sufficient amount of the new taste within the 20 min time frame. On the fourth day, the control group received water, and the experimental group was exposed to an unfamiliar taste (0.1% saccharin or 0.3% NaCl) (Rosenblum et al., 1993).

Conditioned taste aversion. Conditioned taste aversion (CTA) experiments were performed as described previously (Rosenblum et al., 1993). Briefly, the rats underwent a 3 d training session as described above, and on the fourth day they received either water or an unfamiliar taste, i.e., the conditioned stimulus (CS), comprising 0.1% saccharin or 0.3% NaCl. One hour after termination of drinking, both groups were administered the unconditioned stimulus (UCS) in the form of an intraperitoneal injection of the malaise-inducing agent LiCl (0.15 M, 2% body weight). The rats then received water for two successive days after which a taste-preference test was performed: all the animals were exposed to a multiple choice of either water or the CS for 20 min. Aversion to the novel taste in both the conditioned and the unconditioned animals was measured by calculation of an aversion index, defined as: [(volume of water/(volume of water + volume of saccharin)] consumed during each test. Accordingly, an aversion index of 0.5 was designated as the chance level, and an increasing index value indicated increasing preference for water over the novel taste.

Latent inhibition. The latent inhibition paradigm was combined with CTA to isolate the effect of taste learning from the potentially confounding effects of the USC and the CS–UCS association. In this procedure, pre-exposure of the animals to a sensory stimulus diminishes its efficacy to serve as an associative stimulus in subsequent learning. Under such conditions, the degree of aversion after CTA training is a measure of memory of the novel taste acquired incidentally in the pre-CTA trial (Rosenblum et al., 1993). Rats in these experiments were pre-exposed to the novel taste 2 d before the CTA experiment, which significantly reduced their acquired aversion. Testing was performed as described above.

Surgery

Surgery was performed as previously described (Meiri and Rosenblum, 1998). Male Wistar Hola rats were anesthetized with equithesin (0.45 ml/100 g) (2.12% w/v $MgSO_4$, 10% v/v ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotactic apparatus (Stoelting), and implanted bilaterally with a stainless-steel guide cannula (23 gauge) in the gustatory cortex (coordinates, with reference to bregma: anteroposterior = +1.2 mm, lateral = ±5.5 mm, ventral = 5.5 mm). The position of the cannula was secured with acrylic dental cement and two skull screws. A stylus was placed in the guide cannula to prevent clogging. After surgery, animals were allowed 1 week of recovery time before the experimental manipulations.

For micro-injection, the stylus was removed from the guide cannula and a 28-gauge injection cannula was carefully inserted, to extend 0.5 mm from the tip of the guide cannula. The injection cannula was connected via PE20 tubing to a Hamilton micro-syringe, driven by a CMA/100 micro-injection pump (Carnegie Medicin), for an injection rate of 1 μl/min. After injection, the injection cannula was left for an additional 30 s before withdrawal, to minimize dragging of injected liquid along the injection track.

Sample preparation and immunoprecipitation

Experiments were terminated by decapitation 60 min after termination of drinking, and only animals that ingested a sufficient amount of the novel taste (>10 ml) were included in the study (Merhav et al., 2006). After decapitation, the insular cortex was bilaterally dissected and was homogenized in a glass Teflon tissue homogenizer in 1 ml of homogenizing buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1% v/v NP-40, 0.5%, 0.5% w/v deoxycholic acid, 10 mM NaF, 10 mM pyrophosphate, 100 μM sodium orthovanadate, leupeptin at 10 μM/ml, aprotinin

at 10 μM/ml, 0.1 mM PMSF, 0.1% w/v SDS). Aliquots of 100 μl were removed for total fraction assays. Ten minutes after homogenization, samples were centrifuged at 1000 × g and 4°C for 2 min, and the supernatants were collected. Protein concentration was determined with the Bradford Assay (Bio-Rad Laboratories), and samples of supernatant, each containing the same amount of protein, were precleared with 2 μg of preimmune serum and 20 μl of protein A/G at 4°C for 60 min. The samples were then centrifuged at 20,000 × g for 10 min, and the supernatants were collected and subjected to immunoprecipitation with either mouse anti-phosphotyrosine Sepharose 4B (Zymed Laboratories) or rabbit anti-NR2B (Upstate Cell Signaling Solutions) overnight at 4°C. On the next day, samples were washed three times with 0.75 ml of homogenizing buffer, diluted in 30 μl of 2 × SDS sample buffer, and stored pending analysis.

Synaptoneurosomal fractionation

Synaptoneurosomal fractions of insular cortex were obtained according to Quinlan et al. (1999). Briefly, the tissues were homogenized in 4 ml of homogenization buffer (10 mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 10 mM NaF, 10 mM pyrophosphate, 100 μM sodium orthovanadate, leupeptin at 10 μM/ml, aprotinin at 10 μM/ml, 0.1 mM PMSF), and 70 μl samples of the homogenized tissue were removed for analysis of total protein fractions. The remaining homogenate was then filtered through a 100 μm filter followed by a 5 μm filter (Millipore) and centrifuged at 1000 × g for 10 min at 4°C. The pellet containing the synaptoneurosomal fraction was resuspended in homogenizing buffer and subjected to SDS-PAGE after protein determination.

Western blot analysis

All samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories) for immunoblotting. Blots were visualized by enhanced chemiluminescence with ECL Plus (Amersham Biosciences) and quantified with an XRS charge-coupled device camera (Bio-Rad Laboratories) and Quantity One software. Phosphorylated protein levels were normalized against total levels of unphosphorylated protein.

Reagents

Rabbit polyclonal anti-phosphotyrosine was obtained from Zymed Laboratories [polyclonal anti-NR2A, polyclonal anti-NR1, and monoclonal PSD 95 (clone K28/43) were purchased from Upstate Biotechnology]. Goat NR2B and β actin were obtained from Santa Cruz Biotechnologies. Polyclonal anti-NMDA NR2B (pTyr1472) and protein G plus/protein A-agarose were obtained from Calbiochem. Mouse anti-syntaxin (clone SP8) was obtained from Millipore Bioscience Research Reagents. Mouse anti-synapsin (clone 8) was obtained from Transduction Laboratories (Millipore). All primary antibodies were used at a dilution of 1:1000. Horseradish peroxidase-conjugated donkey anti-goat IgG, goat anti-rabbit IgG, and goat anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories and were used at a dilution of 1:10,000. Genistein and ifenprodil were from Sigma. All other reagents were standard laboratory grade.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by means of one-way ANOVA. *Post hoc* analysis was applied with a Tukey–Kramer multicomparison test when appropriate. A value of $p < 0.05$ was considered significant.

Results

To study the role of tyrosine phosphorylation of NR2B in novel taste learning, tyrosine phosphorylated proteins were immunoprecipitated from the insular cortices of rats 1 h after exposure to either familiar taste water or to the novel taste saccharin. Probing of these samples with a general anti-phosphotyrosine antibody revealed that exposure to saccharin elicits a significant increase in the phosphorylation of a 180 kDa band corresponding to NR2B (Fig. 1 *A*, left). Subsequent probing of the same blots for their NR2B content revealed similar increases in NR2B levels (Fig. 1 *A*,
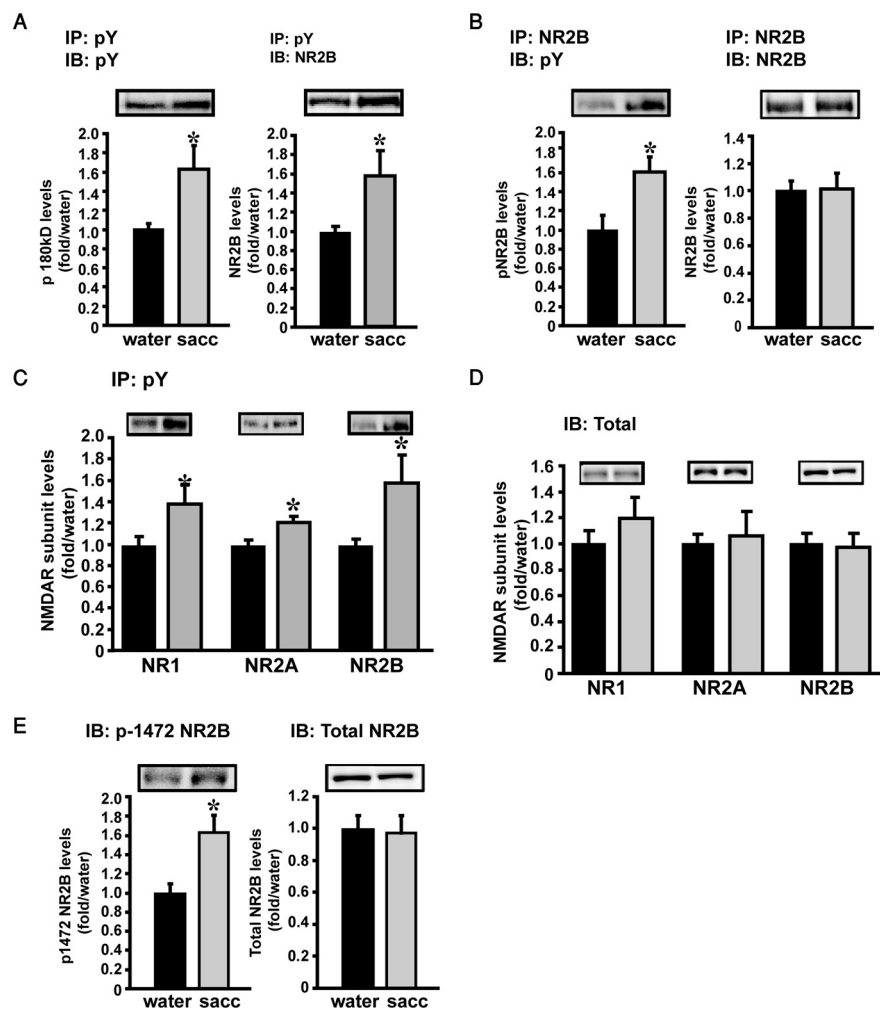
Figure 1. Novel taste learning induces phosphorylation of Y1472 NR2B and increases levels of NMDAR in the phosphotyrosine fractions of the insular cortex. ***A***, Immunoprecipitation (IP) of tyrosine-phosphorylated fractions from the insular cortex after novel taste learning. Insular cortices of rats were removed bilaterally 1 h after ingestion of a familiar taste (water, black bars) or a novel taste [0.1% saccharin (sacc), gray bars]. Tyrosine-phosphorylated proteins were immunoprecipitated with phosphor-Tyr-specific antibodies (pY) as described in Materials and Methods. The phosphotyrosine content of a band corresponding to 180 kDa was determined from these experiments by immunoblot (IB) (left). The identity of this band was confirmed as NR2B by probing the same immunoprecipitates with anti-NR2B-specific antibody (right). ***B***, Immunoprecipitation of total NR2B content from the insular cortex after novel taste learning. Total NR2B protein was immunoprecipitated from brains of water- and saccharin-receiving rats by means of anti-mouse NR2B antibodies, and was probed for phosphotyrosine content with rabbit polyclonal anti-phosphotyrosine (left) and rabbit anti-NR2B (right). ***C***, NR1 and NR2 subunits of NMDA are elevated in the phosphotyrosine fractions of the insular cortex. Immunoprecipitates of tyrosine-phosphorylated proteins from animals treated with water or saccharin were probed with specific anti-NR1, anti-NR2A, or anti-NR2B antibodies. ***D***, No change in total levels of NMDAR after novel taste learning. The different NMDAR subunits in total tissue lysates of insular cortex were probed 1 h after treatment. ***E***, Novel taste exposure elevates tyrosine phosphorylation of the Fyn kinase target Y1472 NR2B. Phospho-Y1472-specific anti-NR2B antibody (left) and total anti-NR2B antibody were used to probe total tissue lysates of insular cortex of rats 1 h after exposure to water or saccharin. In each panel, a representative immunoblot is shown above a graph depicting mean $\pm$ SE for three or four independent experiments. *Saccharin versus water, $p < 0.05$.

right). To ascertain that these changes were not a result of modifications in protein levels, we performed reciprocal experiments in which total NR2B in the insular cortex was immunoprecipitated and probed for tyrosine phosphorylation. These data show that exposure to novel taste does not change the total NR2B levels in the insular cortex, but instead affects only the levels of phosphorylated NR2B (Fig. 1*B*).

To test the effect of novel taste learning on the other subunits of the NMDAR, the levels of NR2A and NR1 in the tyrosine phospho complexes of both groups were analyzed. As depicted in Figure 1*C*, all subunits of the NMDAR were found to be elevated

in the phospho complex in response to saccharin. Since no changes were observed in the total amounts of the NMDAR 1 h after exposure to saccharin (Fig. 1*D*), these increases may either be attributed to increases in the tyrosine phosphorylation contents of NR2A and NR1, in addition to that of NR2B, or simply be attributable to their association with hyperphosphorylated NR2B. Whereas there is no evidence of the occurrence of major tyrosine phosphorylation of NR1 (Lau and Huganir, 1995), NR2A contains several putative tyrosine phosphorylation sites. Probing two of these sites with the phospho-specific antibodies against Y1353 NR2A and Y1387 NR2A has not revealed any significant increase in phosphorylation after novel taste learning (data not shown). Together, these data suggest that that the increased presence of NR2A, as well as that of NR1, was likely attributable to their association with hyperphosphorylated NR2B.

Residue Y1472 was previously identified as the main tyrosine site of NR2B to undergo phosphorylation *in vitro* (Nakazawa et al., 2001). This Fyn kinase target was shown to play an important role in the formation of LTP and in fear learning (Nakazawa et al., 2001, 2006). To test whether phosphorylation of Y1472 NR2B increases in response to a novel taste, samples from both the familiar and the novel taste groups were probed with a specific anti-Y1472NR2B antibody. As shown in Figure 1*E*, saccharin caused an increment of ~50% in phosphorylation of Y1472, which suggests that most of the detected increase in tyrosine phosphorylation of NR2B in response to saccharin exposure likely occurs on this site.

Association of the NMDAR with the postsynaptic density protein PSD-95 through its PDZ domains was shown in many cases to regulate its function (Kornau et al., 1995; Lavezzari et al., 2003). To test the nature of the interaction between NR2B and PSD-95 after novel taste learning, the PSD-95 content was probed in both the total and phosphotyrosine fractions of rats exposed to a familiar or a novel taste. Similarly to the NMDAR findings, whereas the total levels of PSD-95 remained the same in both groups (Fig. 2*A*, right), the content of PSD-95 in the phosphotyrosine fractions was significantly elevated (Fig. 2*A*, left). To confirm that the observed increase in PSD-95 levels was not attributable to a possible increase in its own tyrosine phosphorylation levels, we immunoprecipitated PSD-95 in both groups and probed the samples for phosphotyrosine and PSD-95 protein levels. A faint band at 95 kDa appeared in both groups after immunoblotting with the general phosphotyrosine antibody. However, no changes were observed in these bands after treatment (Fig. 2*B*, left). Immunodetection with anti-PSD-95 con-
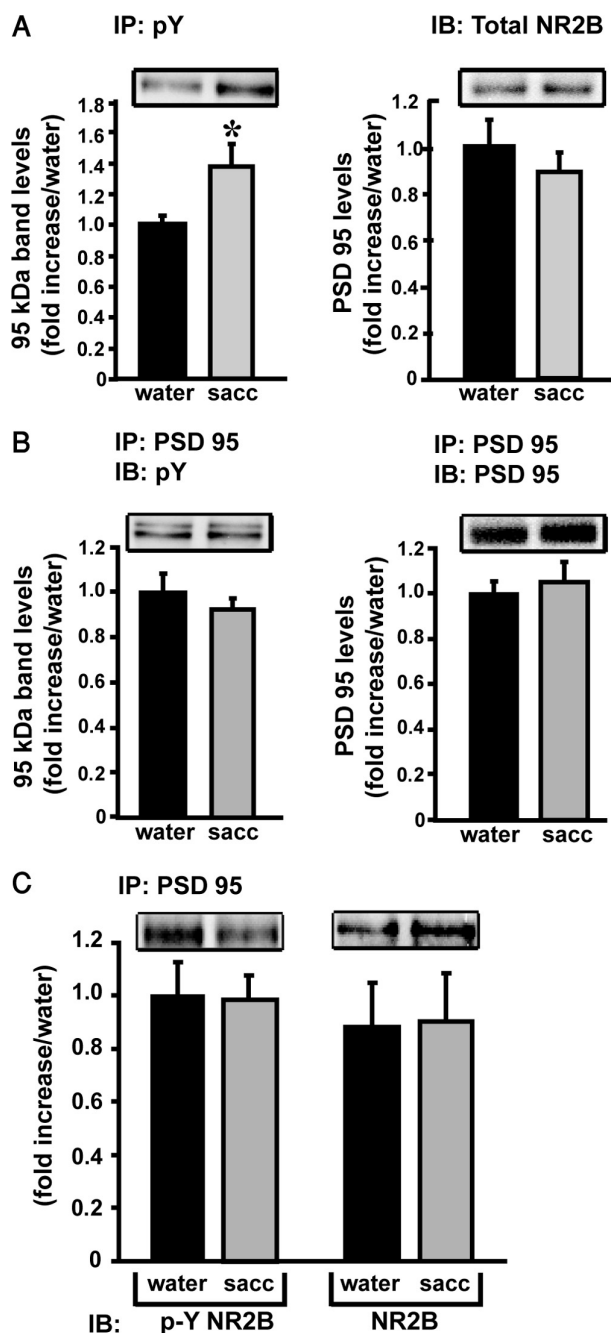
Figure 2. Novel taste learning increases the association of PSD-95 with phosphorylated NR2B. **A**, Novel taste learning increases the levels of p-NR2B-associated PSD-95. Insular cortices of rats were removed bilaterally 1 h after ingestion of a familiar taste (water, black bars) or saccharin (sacc; gray bars). The phosphotyrosine content (p-Y) of a band corresponding to 95 kDa was determined from these experiments (left). The identity of this band was confirmed as PSD-95 by probing the same immunoprecipitates with mouse anti-PSD-95-specific antibody (right). **B**, No change in total PSD-95 levels after exposure to novel taste. PSD-95 protein was immunoprecipitated from insular cortices of rats 1 h after ingestion of water or saccharin and probed with a general phosphotyrosine antibody (left) or total PSD-95 (right). **C**, Total NR2B content and phospho-NR2B levels were probed in the same PSD-95 immunoprecipitates. In each panel a representative immunoblot is shown above a graph depicting mean ± SE for three or four independent experiments. Other abbreviations are as defined in the legend to Figure 1. *Saccharin versus water, $p < 0.05$.

firmed that 1 h after novel taste learning there were no changes in total PSD-95 levels (Fig. 2B, right). To further ascertain that the increase in PSD-95 content in the phosphotyrosine fraction occurs solely because of its association with hyperphosphorylated

NR2B, PSD-95 immunoprecipitates were probed for the presence phosphorylated and total NR2B. As shown in Figure 2C, no changes were observed in the amount of total or phosphorylated NR2B precipitated with PSD-95, suggesting that exposure to a novel taste causes a significant change only in the fraction of PSD-95 that is associated with hyperphosphorylated NR2B and not in the general NR2B-PSD-95 pool.

The observed increases in phosphorylation and association of NR2B with PSD-95 in response to novel taste exposure were merely correlative, and reflect an observation made at a specific time point after exposure. To find out whether tyrosine phosphorylation of NR2B is in fact necessary for novel taste learning, we inhibited its phosphorylation chemically, and examined the effect this had on the ability of the animals to learn a new taste. Initially, to determine whether phosphorylation of NR2B can be effectively reduced, we applied the general tyrosine kinase inhibitor genistein locally to the insular cortex of naive animals. The phosphotyrosine fractions were isolated by immunoprecipitation and were probed for both phosphorylated and total NR2B. As shown in Figure 3A, bilateral application of genistein to the insular cortex caused an ~60% reduction in the levels of phosphorylated NR2B. In accordance with this, there was a marked reduction in the amounts of NR2B in the phosphorylated fraction of animals that received genistein.

We then proceeded to determine whether local application of genistein has a behavioral effect on novel taste learning. For this we used the latent inhibition paradigm, whereby CTA in the animals is attenuated by a pre-exposure to the novel taste, in the absence of coupling to a feeling of malaise. Under such conditions, the degree of aversion after CTA training reflects the memory for the novel taste that was acquired incidentally (Rosenblum et al., 1993). In these experiments animals were cannulated bilaterally, and genistein or vehicle was administered 10 min before the pre-exposure phase. As shown in Figure 3B, animals injected with genistein displayed significantly greater aversion to the novel taste than those in the vehicle-injected group. These levels were similar to those exhibited by animals undergoing CTA without pre-exposure. To show that the effect of genistein was not unique to the saccharin taste, the same type of experiment was repeated with NaCl used as a different new taste. As in the case of saccharin, animals that received genistein before the pre-exposure exhibited increased aversion to the NaCl after training (Fig. 3B, right). Next, to couple the behavioral observations with the biochemical ones, we performed an incidental taste-learning task whereby we exposed four groups of animals to either water or saccharin, having administered injections of genistein or vehicle before their exposure to the novel taste. Samples were collected 1 h after learning, and the tyrosine content of NR2B was analyzed. As depicted in Figure 3C, exposure to saccharin induced a significant increase in NR2B phosphorylation, comparable to those observed previously in the uncannulated animals (see Fig. 1). Application of genistein before saccharin ingestion attenuated this effect, resulting in phosphorylation levels comparable to those displayed by animals receiving the familiar taste. Finally, animals that received a single dose of the NR2B antagonist ifenprodil directly into the insular cortex before CTA training also showed a small but significant decrease in the aversion index (Fig. 3D). Together, these findings indicate that phosphorylation of the NR2B subunit is not only correlated with, but may in fact be necessary for novel taste learning.

Previous studies have indicated that tyrosine phosphorylation of the NMDAR modulates its cellular distribution, and that these changes in receptor localization may be necessary for synaptic
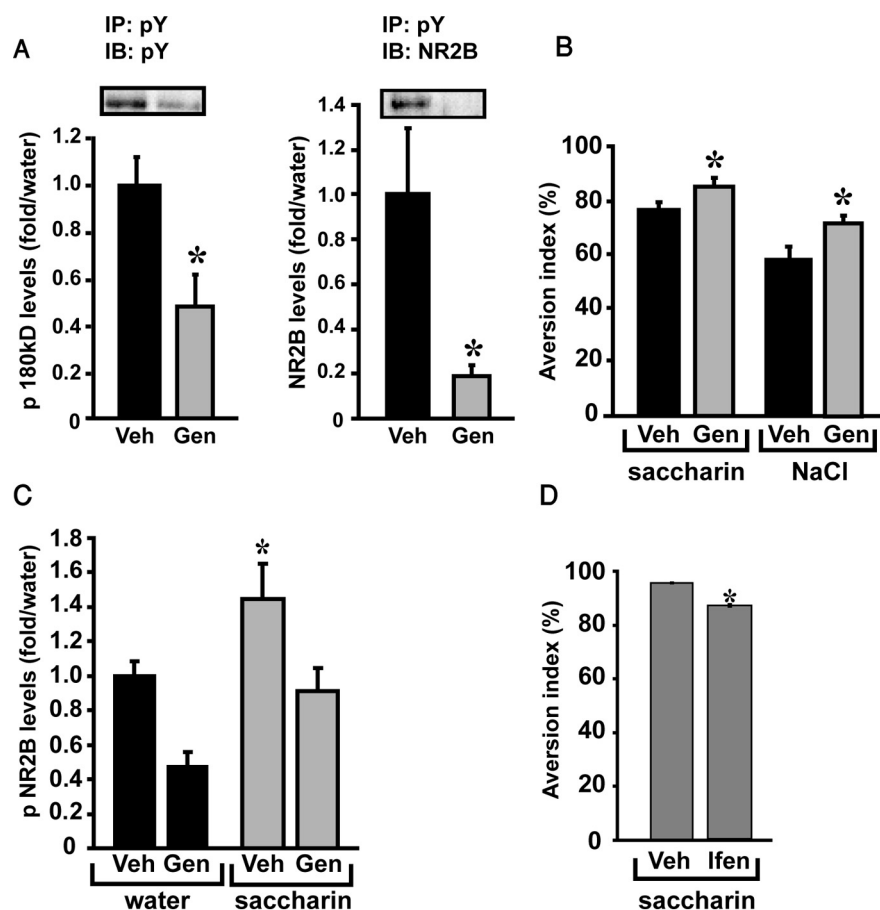
Figure 3. Tyrosine phosphorylation of NR2B is necessary for novel taste learning. ***A***, Genistein inhibits phosphorylation of NR2B in the insular cortex. A 1 μl aliquot of 30 μmol of genistein (Gen) or vehicle (Veh; 0.3% DMSO in saline) was injected bilaterally to the insular cortex of naive rats. The rats were killed 1 h later and tyrosine-phosphorylated proteins were immunoprecipitated with phospho-Tyr-specific antibodies. The tyrosine phosphorylation of the 180 kDa band (left) and total NR2B (right) levels were assayed by using specific antibodies, as described in Materials and Methods. A representative immunoblot is shown above the graph depicting mean ± SE for two independent experiments. *Saccharin versus water, $p < 0.05$. ***B***, Genistein attenuates novel taste learning. Rats received a bilateral injection of 1 μl aliquot of 30 μmol of genistein or vehicle (0.3% DMSO in saline) 10 min before pre-exposure to the novel taste (saccharin or NaCl) or water (CS); 2 d later, both groups were exposed to 0.1% saccharin or 0.3% NaCl followed by intraperitoneal injection of 0.15 M LiCl, as the US. A third group underwent the procedure of CTA without pre-exposure. The animals were tested for their preference for the novel or the familiar taste 2 d later. Aversion to the novel taste was calculated as described in Materials and Methods. Results are mean ± SE for four independent experiments. *Gen or CTA versus Vehicle, $p < 0.05$. ***C***, Genistein prevents the increase in NR2B phosphorylation after exposure to a new taste. Bilaterally cannulated animals were injected with either genistein or vehicle 10 min before a 20 min single exposure to either saccharin or water. The animals were killed 1 h after termination of drinking. Tissue was homogenized and immunoprecipitated with anti-phosphotyrosine antibody, and the levels of phosphorylated NR2B were assessed as before. ***D***, Ifenprodil (Ifen) attenuates novel taste learning. Ifenprodil (5 μM) or vehicle was microinjected into the insular cortex 20 min before CTA training (control $n = 6$, ifenprodil $n = 8$). The animals were tested for their preference for the novel or the familiar taste 2 d later. Aversion to the novel taste was calculated as described in Materials and Methods. Other abbreviations are as defined in the legend to Figure 1. *Ifenprodil CTA versus Vehicle CTA, $p < 0.05$.

plasticity and learning (Nakazawa et al., 2006). To further elucidate the role of tyrosine phosphorylation of NR2B in the mechanism of novel taste learning, we studied the levels of phosphorylated and total NR2B in a subcellular fraction of the tissue that is enriched for synaptic proteins (Quinlan et al., 1999). Surprisingly, as opposed to the findings in total tissue homogenate, the levels of Y1472-phosphorylated NR2B were greatly reduced in brains of animals that had consumed saccharin (Fig. 4A, left). Probing for total NR2B levels in these fractions revealed a significant reduction in the actual protein levels (middle), but not in the ratio of phosphorylated to total protein (right), suggesting that the observed decrease in phosphorylation was a direct result

of the reduction in the amount of NR2B present in the synaptic area. We next determined the total levels NR1 and NR2A in these fractions, and discovered that 1 h after saccharin exposure their levels too were significantly decreased (Fig. 4B). To better determine whether these changes could be attributed to a global change in synapse size, the same samples were assayed for two presynaptic proteins, synapsin and syntaxin. As shown in Figure 4C, no changes were observed in either protein after novel taste learning, suggesting that the observed changes in NMDA levels were not attributable to an overall change in synapse size. Finally, to establish whether the observed changes in NMDA localization were dependent on tyrosine phosphorylation, levels of NMDA subunits were determined 1 h after the exposure in the synaptoneurosomal fraction. As shown in Figure 4D, genistein did not affect the reduction in NR1 levels, but reversed the relative levels of NR2B and NR2A, yielding synaptic levels similar to those obtained in the animals exposed to the known taste of water.

Discussion

The major finding of our study is that tyrosine phosphorylation of the NR2B subunit of the NMDAR is a necessary step toward novel taste learning. We showed that 1 h after a single exposure of rats to saccharin, there is a marked elevation in phosphorylation of Y1472 NR2B and an increased interaction of phosphorylated NR2B with the major postsynaptic scaffold protein PSD-95. Furthermore, injection of a tyrosine kinase inhibitor directly into the insular cortex of rats before exposure to novel taste prevented the increase in NR2B phosphorylation and resulted in a phenotype of animals that were unable to learn the new taste. Last, we found that although a new taste does not change the overall levels of NMDAR, it does cause a significant reduction in its levels in a subcellular area enriched with synaptic proteins. This reduction is prevented after an *in vivo* injection of the animals with genistein, suggesting a possible role for tyrosine phosphorylation of NR2B in the cellular distribution of the NMDAR after novel taste.

The involvement of tyrosine phosphorylation of the NMDAR in learning processes was indicated by earlier findings that correlated enhanced phosphorylation with various behavioral paradigms. Increased phosphorylation of NR2B was observed in several activities, e.g., hippocampal spatial learning tasks (Mizuno et al., 2003), morphine-seeking behavior in the forebrain (Kato et al., 2006), and in our own study of conditioned taste aversion and novel taste learning in the insular cortex (Rosenblum et al., 1997). The use of transgenic animals overexpressing wild-type or con-

stitutively active Fyn kinase further sup-
ported these findings by showing that en-
hanced phosphorylation of NR2B is
associated with behavior modification
such as decreased alcohol withdrawal
(Stork et al., 2002), maintenance of neuro-
pathic pain (Abe et al., 2005), and im-
paired conditioned freezing in fear para-
digms (Kojima et al., 2005). However,
knock-out of Fyn totally eliminated the
phosphorylation of NR2B and prevented
neuropathic pain in mice (Abe et al.,
2005), and acutely increased their sensitiv-
ity to alcohol (Yaka et al., 2003). Further-
more, direct knock-out of Y1472 in mice
resulted in impaired fear-related learning
and reduced LTP in the amygdala (Naka-
zawa et al., 2006). Powerful as these studies
may be, they are limited as to their ability
to evaluate the role of tyrosine phosphor-
ylation of NR2B in the live, adult animals.
Here, we show that phosphorylation of
NR2B is indeed induced in the insular cor-
tex of an adult animal in the course of
novel taste learning, and that attenuation
of this process before exposure prevents
the formation of that memory. In contrast
to other studies in which tyrosine kinase
inhibitors were injected either systemically
or intraventricularly (Mizuno et al., 2003),
we applied the inhibitor locally to the in-
sular cortex, thereby offering a more spe-
cific role for cortical NR2B receptor phos-
phorylation in novel taste learning. The
observed increase in tyrosine phosphory-
lation of NR2B in the insular cortex after
introduction of a novel taste was small,
suggesting that novel taste learning induces a change in only a
specific fraction of the NMDARs of the insular cortex. This hy-
pothesis is supported by observations in cultured neurons, which
showed that among glutamate receptors, NR2 subunits of the
NMDARs were the only ones to undergo tyrosine phosphory-
lation, with an estimated tyrosine phosphorylation of only 3.5% of
NR2B in vivo (Lau and Huganir, 1995).

Both NR2A and NR2B interact directly with PSD-95 at the C
terminus of the NMDAR (Kornau et al., 1995), and this interac-
tion may be modulated by either serine or tyrosine phosphoryla-
tion. Serine phosphorylation of PSD-95 by CaMKII (calcium-
calmodulin-dependent protein kinase II) causes dissociation of
NR2A from PSD-95, without interfering with its interaction with
NR2B (Gardoni et al., 2006). The role of tyrosine phosphoryla-
tion of NR2B in the association with PSD 95 is less clear. Phos-
phorylation of NR2A and NR2B and their associated proteins by
Src or Fyn was found in some cases to enhance their association
with PSD-95 (Rong et al., 2001; Zalewska et al., 2005), whereas
under conditions of hypoxia that lead to impaired long-term
spatial learning and memory there were marked decreases in the
levels of PSD-95-NMDAR complex (Chen et al., 2007). One pos-
sible role for the formation of this complex is to protect NR2
subunits from undergoing cleavage by calcium-dependent pro-
teases, and thereby to provide a mechanism for regulating
NMDAR expression (Dong et al., 2004).

Regulation of NMDAR localization is critical for the forma-
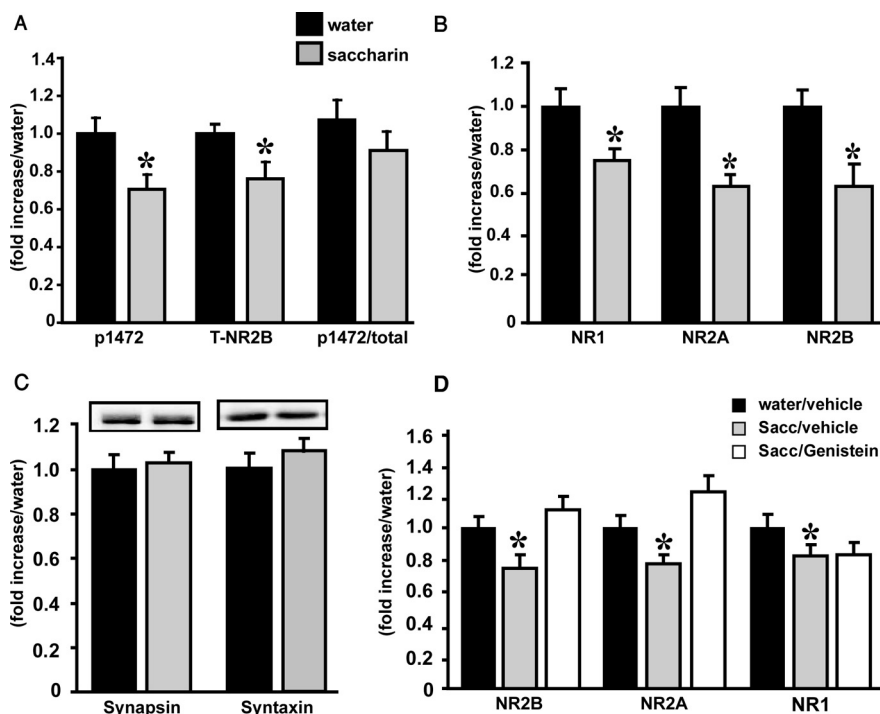
Figure 4. Exposure to novel taste affects the localization of the NMDAR. *A*, Total levels of NR2B are reduced in synaptoneuro-
somes after novel taste exposure. Synaptoneurosomes were prepared from insular cortices of rats exposed to either water or 0.1%
saccharin (sacc) for 1 h. Samples were probed with phospho-Y1472-specific anti-NR2B antibody (left) and total anti-NR2B anti-
body (middle), and the ratios of phosphorylated to total NR2B were calculated (right). *Saccharin versus water, $p < 0.05$. *B*, Total
levels of NMDAR are reduced in synaptoneurosomes after exposure to saccharin. Levels of different NMDAR subunits in synapto-
neurosomal fractions were assayed by using anti-NR1, anti-NR2A, and anti-NR2B antibodies, as described above. *Saccharin
versus water, $p < 0.05$. *C*, No change in presynaptic markers after novel taste exposure. Synaptoneurosomal fractions were
probed with monoclonal antibodies against the presynaptic proteins synapsin and syntaxin. *D*, Redistribution of NR2B and NR2A
after exposure to novel taste is reversed by genistein. Bilaterally cannulated animals were injected with either vehicle (0.3% DMSO
in saline) or genistein (1 μl, 30 μmol) 10 min before ingestion of fluids. Synaptoneurosomes were prepared from the insular
cortices and analyzed for NR2B, NR2A, and NR1 content, as described above. *Saccharin versus water, $p < 0.05$.

tion of memory. Several important studies have closely examined
mechanisms of NR2B cellular distribution and found that its
surface expression is oppositely regulated through interactions
with either PSD-95, which stabilizes receptor expression on the
plasma membrane, or with the adaptor protein AP-2, which pro-
motes internalization via the clathrin-coated pit machinery
(Roche et al., 2001; Barria and Malinow, 2002; Lavezzari et al.,
2003). Another recent study in neuronal primary cultures
showed that inhibition of cdk5, a serine/threonine kinase, results
in increased phosphorylation of Y1472 NR2B and decreases
activity-dependent endocytosis of NMDARs (Zhang et al., 2008).
In this regard, one of the surprising findings of our study was that
whereas the total cortex fractions showed a marked elevation in
the phosphorylation of NR2B but no changes in the overall levels
of NMDAR, the subcellular fraction of synaptoneurosomes
showed a marked reduction in the actual levels of NMDA com-
ponents. These data presumably contradict the previously re-
ported findings; however, the comparison between an *in vivo*
study using a physiological input signal at a very specific time
point and studies done in heterologous systems or *ex vivo* should
be carefully considered. The studies showing that mutation in the
Y1472 residue of NR2B block NR2B trafficking and increasing
the number of synaptic receptors (Lavezzari et al., 2003; Pryby-
lowski et al., 2005) were preformed in heterologous systems or
cultured neurons, overexpressing proteins in question. Also, in-
ternalization was monitored at much shorter time points (15–30

min) and not beyond. In our system, the changes in NR2B phosphorylation became detectable only 1 h of exposure and subsided after 2 h (data not shown); therefore, we document changes that occur much later than those reported in isolated systems. Therefore, the increased NR2B phosphorylation and association with PSD-95 in total tissue homogenate may reflect a dynamic cellular process intended to normalize a decreased amount of NMDAR in synaptic regions. The fractionation method used to produce synaptoneurosomal fractions in this study merely represents a subcellular fraction that is rich in synaptic proteins and contains both presynaptic and postsynaptic membranes. Therefore, the current findings cannot determine whether the observed reduction in NMDARs in this fraction is attributable to receptor endocytosis or to lateral diffusion away from the synapse, nor can they predict the contribution of PSD-95 to this process. Nonetheless, this process seems to be important for the formation of taste memory because its prevention by genistein is associated with impaired memory to a novel taste.

Another interesting observation in this study concerns the fate of the NMDAR subunits NR2 and NR1. When studying the levels of these components in the synaptoneurosomal fractions, we found that as NR2B, NR2A levels too were reduced. Surprisingly however, pre-exposure to genistein reversed the effect on NR2B and NR2A back to those of the control animals, whereas levels of NR1 continued to be decreased (Fig. 4*D*). A similar observation was made by Goebel et al. (2005) in adult rat hippocampal slices, where blocking of tyrosine kinase affected the concentration of NR2A and NR2B but not NR1. Furthermore, the subunit composition of NR2 was shown in several instances to direct delivery of receptors to synapses in different ways. Thus, phosphorylation of 1472-NR2B was found to be associated with enrichment of synaptic NMDARs, whereas phosphorylation of 1336-NR2B was associated with enrichment of extrasynaptic NMDARs (Goebel-Goody, 2009). Trafficking of NR2 subunits was also found to heavily depend on their specific interaction with scaffolding molecules. Association of NR2B with PSD-95, PSD 93, and SAP97 inhibited its internalization (Roche et al., 2001; Lavezzari, 2003), whereas binding of 1472-NR2B to AP-2 promoted internalization (Lavezzari, 2003). Furthermore, whereas the localization of the NR2B to the synapse was found to be dependent on functional PDZ domains within NR2B, its interactions with the scaffold protein AP-2 cause NR2B to move away from the synapse. In contrast, absence of PDZ domains, or ability to interact with AP-2, had no effect on the synaptic localization of NR2A (Prybylowski et al., 2005). Together, these studies all point to the fact that the NMDAR subunits traffic independently during endocytosis. The effect of these processes on taste learning and memory are yet to be determined.

In summary, we have demonstrated that exposure of rats to a novel taste markedly increases NR2B phosphorylation in the insular cortex, and that this phosphorylation is necessary for the formation of a memory to that taste. Although the mechanisms that underlie this process are only partially understood, we provide evidence that a natural input that is given to a live animal leads to changes in NMDAR phosphorylation and localization. One of the major challenges of this work is to determine in detail the exact patterns and locations of NMDAR movement in response to novel taste exposure, to increase our overall understanding of the connection between learning and memory and biochemical processes that take place in the brain.

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