Temporal memory and its enhancement by estradiol requires surface dynamics of hippocampal CA1 NMDA receptors

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Abstract

Background: Identifying the underlying cellular mechanisms of episodic memory is an important challenge since this memory, based on temporal and contextual associations among events, undergoes preferential degradation in aging and various neuropsychiatric disorders. Memory storage of temporal and contextual associations is known to rely on hippocampal NMDA receptor (NMDAR)-dependent synaptic plasticity, which depends on dynamic organization of surface NMDAR. Whether NMDAR surface trafficking sustains the formation of associative memory remains however unknown. **Methods:** We tested this hypothesis, using single nanoparticle imaging, in vivo electrophysiology and behavioral approaches in hippocampal networks challenged with a potent modulator of NMDAR-dependent synaptic plasticity and memory, 17β-estradiol (E2). **Results:** We demonstrate that E2 modulates NMDAR surface trafficking, a necessary condition for E2-induced potentiation at hippocampal CA1 synapses. Strikingly, CA1 NMDAR surface trafficking controls basal and E2-enhanced mnemonic retention of temporal, but not contextual associations. **Conclusions:** NMDAR surface trafficking controls basal and E2-enhanced mnemonic retention of temporal, but not contextual associations, opening a new and non-canonical research avenue in the physiopathology of cognition.

Text

INTRODUCTION

NMDA receptor (NMDAR)-dependent synaptic plasticity is thought to be the basis for information storage including the formation of long-term episodic memory, our capability to remember conjunctions of *what happened, when and where* (Eichenbaum, 2004 #41). In this line, it has been reported that NMDAR located in the CA1 subfield of the hippocampus, critical structure for episodic memory, are needed for long-term mnemonic retention of spatial/contextual (Tsien, 1996 #135) and temporal associations (Collingridge, 2004 #84;Huerta, 2000 #83). However, the implication of hippocampal NMDAR in long-term associative memory is still matter of questioning (Taylor, 2014 #132) and better understanding of the mechanisms by which these receptors may sustain memory is needed.

In hippocampal cultured neurons and slices, NMDAR are dynamic at the membrane surface, exploring both extrasynaptic and synaptic compartments (Tovar, 2002 #87;Bard, 2011, 2011;Dupuis, 2014 #90). Moreover, NMDAR surface trafficking tunes the plasticity of maturing glutamate synapses through the fine regulation of intracellular kinase trafficking (Dupuis, 2014 #90). Thus, NMDAR surface trafficking is an efficient mechanism to sustain synaptic plasticity and potentially associative memory formation, although this emerging possibility remains an open question.

One of the most potent physiological regulators of NMDAR-dependent plasticity and memory in the hippocampus is the sex hormone 17β-estradiol (E2; for review, (McEwen, 2010 #13)). In various hippocampus-dependent learning tasks, performance varies as a function of the estrous cycle in females and can be enhanced by exogenous E2 (Shors, 1998 #91;Sandstrom, 2001 #92;Luine, 2003 #93;Leuner, 2004 #124). Furthermore, in both males and females, E2 is produced within the hippocampus (Hojo, #95), which expresses both types of estrogen receptors (ER)α and ERβ (Mitra, #19;Mitterling, #20) and displays a robust response to E2. In the CA1 area, E2 modulates multiple aspects of morphological and functional synaptic plasticity, including dendritic spine density and

long-term potentiation (LTP)/depression (LTD) of glutamate synapses, in a direction beneficial to mnemonic storage (Good, 1999 #97;Gould, 1990 #6;Mukai, 2007 #101;Woolley, 1992 #34;Zamani, 2000 #98;Woolley, 1990 #96). At the molecular level, E2 increases NMDAR agonist binding, sensitivity to NMDAR-mediated synaptic inputs and GluN2B-NMDA excitatory post-synaptic currents (Gazzaley, 1996 #102;Woolley, 1997 #37;Smith, 2006 #23). In addition, E2-induced GluN2B-NMDAR current potentiation does not result from changes in NMDAR subunit expression levels or phosphorylation status (Snyder, 2011 #26), but could potentially be mediated by a surface redistribution of the GluN2B-NMDAR in the perisynaptic area, as such an effect was previously observed in aged rats (Adams, 2004 #1). Collectively, these studies support the possibility that E2 may affect both NMDAR synaptic plasticity and memory through a modulation of the NMDAR surface dynamics in the hippocampus.

Here, we test the hypothesis that hippocampal CA1 NMDAR surface dynamics plays a role in associative memory and its modulation by E2. We used antibodies directed against extracellular epitopes of the NMDAR, to interfere with the receptor surface trafficking without altering other intrinsic properties of hippocampal neurons as previously shown (Dupuis, 2014 #90) *in vitro* and *ex vivo*. Using a combination of single nanoparticle imaging, electrophysiology and behavioral testing, we provide direct evidence that E2 regulates NMDAR surface dynamics and this mechanism is a necessary condition for E2-induced *in vitro* and *in vivo* hippocampal synaptic plasticity and for the temporal component of basal and E2-enhanced associative memory.

METHODS AND MATERIALS

A detailed description of all methods and materials can be found in the Supplemental Information (SI) file.

Cell culture, protein expression, synaptic live staining, immunocytochemistry, single particle (Quantum dot, QD) tracking and surface diffusion were performed in live hippocampal neurons from 18 days-old rat embryos. Surface GluA1 or GluN2A and -2B subunits containing NMDAR staining was

performed, imaged and analyzed. Spine density, receptor surface staining, average intensity and explored area of fluorescently-labelled surface receptor were measured within dendritic fields. GluN2A and -2B subunits containing NMDAR surface dynamics were examined after 15 min or 24 h. To affect the surface diffusion of NMDAR, we used the cross-link (X-link) procedure (figure 2G) adapted from previous published studies (Groc, 2008 #7;Heine, 2008 #105).

In vivo studies were performed on 3-4 month-old C57Bl/6J naive male mice. Field excitatory postsynaptic potentials (fEPSP) evoked by stimulation of CA3 input in CA1 synapses were recorded, amplified, filtered, digitized and analyzed. NMDAR surface mobility was blocked using the X-link protocol 45 minutes before HFS (1 train of 1s at 100 Hz) or CA1 infusion of E2 (17β-estradiol, 10 nM, 60 nL) (figure 3A). Surgery and intra-hippocampal infusions in freely moving mice were performed as described previously (Mingaud, 2007 #108;Kaouane, 2012 #107), (figures 4A, 5A and S1). The fear-conditioning task was conducted as described previously (Kaouane, 2012 #107). Independent groups of mice were trained either with a 20 s interval between the tone and the shock (20 s; *trace* condition) or with the 2 stimuli contiguous (0 s; *delay* condition) (Misane, 2005 #18) (figures 4A and S1). Freezing behavior was used as an index of fear responses (Fanselow, 1980 #5). The object location task was performed as described previously (Assini et al, 2009) with minor modifications. General locomotor activity and time spent exploring each object was measured during the acquisition and the test phase after 24 h (figure 5A). Histological control of *cannulae* placements after behavioral testing was used as exclusion criteria. To check for intra-hippocampal X-link diffusion, anti-GluN1 was infused 45 min prior to sacrifice and immunohistochemistry on brains sections were performed using only secondary antibody (figures 4B and S1). All quantitative data are expressed as mean±sem. Statistical analyses performed are detailed in the SI file and in figure legends. Statistical comparisons are indicated as *, ** and *** for $p<0.05$, $p<0.01$ and $p<0.0001$, respectively.

RESULTS

E2 increases dendritic spine density, potentiates synaptic AMPAR and modulates surface NMDAR distribution in hippocampal neurons.

Since E2 is known to enhance dendritic spine density and promote hippocampal LTP, we first tested in our experimental conditions, *i.e.* cultured hippocampal neurons, that E2 induces morphological changes associated with plasticity. We measured dendritic spine density and size, as well as surface glutamate receptors density and distribution using immunocytochemistry. As expected from previous studies (Woolley, 1997 #37; (Waters, 2009 #111;Jelks, 2007 #110;Murphy, 1996 #109;Srivastava, 2008 #29;Woolley, 1994 #35) hippocampal neurons exposed to E2 (10 nM, 24h) exhibited an increase in the density of dendritic spines containing post-synaptic density (PSD), labelled either with the scaffold protein Homer 1c (Figure 1A, top panel; +48.1±6.4% t_{57}=5.781; p<0.0001; n=32 vehicle and n=27 E2 dendritic fields) or Shank (+28.1±2.7% t_{194}=8.125;p<0.0001; n=92 vehicle and n=104 E2 dendritic fields), reaching an average 32.4±3.9% increase without any detectable effect on the spine size (figure 1B, upper left). In addition, E2 treatment increased the surface GluA1-AMPAR content in the synaptic and extrasynaptic compartments (figures 1A-B, bottom panel and histograms). We next explored whether E2 regulates endogenous NMDAR surface distribution by focusing our attention on the two most expressed NMDAR subunits in the hippocampus, the GluN2A and GluN2B subtypes. E2 did not alter the global surface content of either of these subunits (GluN2A: +25.1±12.5%; p=0.1301; GluN2B: -0.3±3.2%; p=0.1204) but it altered their synaptic contents, increasing GluN2A-NMDAR and decreasing GluN2B-NMDAR synaptic content respectively to 183.7±25.0% and 80.9±4.6% relative to vehicle (figures 1C-D). Altogether, these data indicate that E2 increases spine density and potentiates synaptic GluA1-AMPAR. Quite remarkably, E2 redistributes surface GluN2A- and GluN2B-NMDAR in the same time window, therefore altering their synaptic ratio and possibly their related signaling.

E2 modulates GluN2-NMDAR surface trafficking, a process necessary for E2-induced increase in

spine density *in vitro*

To investigate the cellular mechanism responsible for the E2 effects on GluN2-NMDAR synaptic content, we used the single nanoparticle (Quantum Dot, QD) imaging approach to track surface GluN2A- and GluN2B-NMDAR in live hippocampal neurons. We first examined the acute effect of E2 on GluN2A- or GluN2B-NMDAR surface dynamics (10 nM, 15 min; figures 2A-C). While E2 does not affect GluN2A-NMDAR, it decreases GluN2B-NMDAR surface diffusion and increases its synaptic dwell-time (time spent in the PSD area; figure 2C), leading to an overall decreased synaptic GluN2A/2B ratio.

Since a 24 h-treatment with E2 increases the spine density and potentiates synapses (figure 1), we next investigated the associated dynamic rearrangement of surface NMDAR. After 24 h exposure, E2 reduces GluN2A-NMDAR and enhances GluN2B-NMDAR surface diffusion (figures 2D-F). The GluN2A-NMDAR cumulative distribution of the membrane diffusion coefficient was significantly shifted, the relative fraction of immobile receptors (diffusion coefficient <0.005 $\mu m^2/s$) was increased from 52% to 75% (figure 2E) and consistently, the synaptic dwell-time was increased by 29% (data not shown). On the opposite, E2 increased membrane diffusion coefficient and mobile fraction of GluN2B-NMDAR receptors (figure 2E) and decreased the synaptic dwell-time of 18% (data not shown). Thus, E2 acutely decreases synaptic GluN2B-NMDAR surface dynamics and GluN2A/2B synaptic ratio, but after 24 h exposure, when E2-induced synaptic potentiation/spinogenesis has occurred, strong anchoring of GluN2A-NMDAR and lateral displacement of synaptic GluN2B-NMDAR, *i.e.* high synaptic GluN2A/2B ratio, is observed.

This observation prompted us to test whether this surface redistribution of GluN2-NMDAR plays an instrumental role in E2 effects on the glutamate synapse adaptation. For this purpose, we artificially reduced the surface GluN2A- and GluN2B-NMDAR trafficking using an antibody-based cross-linking protocol (Groc, 2008 #7;Heine, 2008 #105;Dupuis, 2014 #90) (figure 2G). Remarkably, both GluN2A- and GluN2B-NMDAR cross-links (X-link) fully prevented E2 effect on spine density (figures 2H-I). These data provide thus the first demonstration that GluN2A/B-NMDAR surface dynamics is required

for E2 to mediate its regulatory action on the spine number in cultured hippocampal neurons.

GluN1-NMDAR surface X-linking impairs electrically- and E2-induced potentiation of CA1 synapses *in vivo*

To further explore the interplay between hippocampal NMDAR dynamics and synaptic adaptations, we tested the impact of interfering with surface NMDAR trafficking on two different protocols of hippocampal synaptic potentiation. *In vivo* electrophysiological recordings of fEPSP evoked by stimulation of CA3 input in CA1 synapses were performed in anesthetized mice (figures 3A-B). We first established the NMDAR-dependent LTP paradigm by controlling that i) basal fEPSP were abolished by the AMPAR antagonist, CNQX (figure 3C), ii) high frequency stimulation (HFS, 1 s, 100 Hz) induced a long-lasting enhancement of fEPSP (+160.3±38.7% compared to baseline) and iii) this LTP was completely blocked by NMDAR antagonists pre-treatments (-37.7±8.8% and -11.1±5.3% compared to baseline for AP5 and MK801, respectively; figure 3D).

Since GluN2A-NMDAR and GluN2B-NMDAR surface dynamics is required for E2-mediated action on the spine density, to interfere with surface trafficking of all NMDAR subtypes, we infused locally into hippocampal CA1, the GluN1-NMDAR X-link, as previously described (Dupuis, 2014 #90). When injected alone, GluN1 X-link did not alter basal synaptic transmission (figure 3E) but it completely prevented the HFS-induced LTP (figure 3F). When GluN1 X-link was injected in combination with E2 into hippocampal CA1, it prevented the long-lasting potentiation of synaptic transmission evoked by E2 alone (figure 3G). Thus, NMDAR surface trafficking is a physiological mechanism contributing to *in vivo* synaptic plasticity since it is required for HFS- and E2-evoked potentiation of CA1 synaptic transmission.

Reducing NMDAR surface dynamics in the CA1 area selectively impairs temporal associative memory and its enhancement by E2.

To demonstrate the implication of local surface NMDAR trafficking in CA1 NMDAR-dependent associative memory, we used a classical *trace* fear conditioning procedure (figure 4A). This task enables assessment of both temporal and contextual fear memories, based on associations of the shock with the tone (20 s interval) and with the surrounding cues of conditioning context, respectively. We used low or high electric foot-shock intensity (0.3 and 0.6 mA) to induce weak or strong associative memories, respectively. Restricting NMDAR surface trafficking by intra CA1-infusions of GluN1 X-link (figure 4B) before a conditioning normally leading to strong fear memories (0.6 mA) has no effect on the acquisition of the fear conditioning, (figure 4C), but selectively impairs the retention of temporal associative memories (figures 4D-E). When GluN1 X-link was infused into the whole dorsal hippocampus, instead of dorsal CA1, before conditioning (figures S1A-B), it impairs acquisition of fear conditioning (figure S1C) and both retention of temporal and contextual associative memories (figures S1D-E), whereas, infusion into the Dentate Gyrus (DG) does not impair neither acquisition nor retention of the tone and context (supplemental results, SI file).

Taken together, these findings indicate selective involvement of CA1 NMDAR surface trafficking in the temporal component of associative memory.

To provide further evidence supporting this functional selectivity, we performed intra CA1-infusion of GluN1 X-Link in another prototypical hippocampal dependent -task with no requirement for temporal association, the object location task (Assini et al, 2009; Barker and Warburton 2011).

As expected, restricting NMDAR surface trafficking by intra CA1-infusions of GluN1 X-link (figure 5A) spares both the acquisition and the retention (figures 5B-C; table S1) of object location memory. Overall, our results show that CA1 NMDAR surface trafficking is required during learning to form long-term memory of temporal, but not contextual associations.

To further demonstrate the implication of CA1 NMDAR surface trafficking in the formation of long-term associative memory, we examined whether this trafficking was required for associative memory modulation by E2 using combinations of intra-CA1 infusions of vehicle/E2 and control/GluN1 X-link before conditioning. As we expected E2 to enhance memory retention, conditioning was performed

under the low shock intensity condition (0.3 mA), normally leading to weak fear memories. In addition to *trace* conditioning, we also used the *delay* conditioning procedure (*i.e.*, 0 s tone-shock interval) evaluating elementary fear memory, to further assess selectivity of GluN1 X-link effects for the temporal component of associative memories (figure 6). We found that E2 enhanced the retention of both temporal and elementary fear memories and that only the enhancement of temporal memory was prevented by GluN1 X-link (figures 6A-B). While in *trace* or *delay* conditioning, neither the acquisition of conditioning (data not shown) nor contextual memory retention (figures 6C-D) were modified by E2 and/or GluN1 X-link (figures 6C-D).

Altogether, our findings indicate that NMDAR surface trafficking in hippocampal CA1 is a critical mechanism in the formation of long-term fear memories based on associations across time and their enhancement by E2.

DISCUSSION

We here provide the first demonstration that NMDAR surface trafficking in the dorsal CA1 of the hippocampus is a crucial cellular mechanism for long-term plasticity of glutamate synapse and contributes to certain NMDAR components of associative memory and their modulation by E2 in the adult rodent. Using a X-linking procedure that restricts NMDAR surface mobility without altering other intrinsic receptor properties (Dupuis, 2014 #90) and a set of high-resolution live imaging, *in vivo* electrophysiology and behavioral testing, we demonstrate that i) E2 regulates the surface trafficking of GluN2A- and GluN2B-NMDAR, ii) NMDAR surface dynamics is required for E2-induced increase in spine density, HFS- and E2-induced LTP of CA1 synapses and iii) NMDAR surface dynamics in the dorsal CA1 is required for 24 h mnemonic retention of temporal associative fear memory and its enhancement by E2, but is not necessary for contextual and elementary fear memories. This study sheds a new light on the key role of NMDAR cellular distribution in memory since it indicates that the NMDAR surface dynamics and not only the channel activity *per se* (Dupuis, 2014 #90;Nabavi, 2013 #112) regulates the LTP of glutamate synapses *in vitro* and *in vivo* and, in CA1, sustains the formation

of long-term memory of associations across time, an important component of episodic memory (Eichenbaum, #133).

The regulation of NMDAR signaling and NMDAR-dependent synaptic plasticity by E2 has long been reported (Woolley, #33;McEwen, #13). We here show that E2 increases AMPAR synaptic content and dendritic spine density through a surface redistribution of the two key GluN2A- and GluN2B-NMDAR subtypes in hippocampal neurons. Acutely, E2 rapidly increases the synaptic anchoring of GluN2B-NMDAR without notably changing GluN2A-NMDAR surface trafficking. This effect is transitory since after 24 h of E2 exposure, when synaptic AMPAR contents and spine density are already and stably increased, we reported an increased synaptic GluN2A/2B ratio. The description of the regulated NMDAR membrane dynamics supports the well-established enhancing effects of E2 on various forms of hippocampal NMDAR-dependent LTP (reviewed in (Smith, 2009 #24)). The acute change in GluN2B-NMDAR surface trafficking leading to reduced synaptic GluN2A/2B ratio was predictive of a LTP induction facilitation at excitatory synapses (Lau, 2007 #11;Yashiro, 2008 #113). This suggestion is supported since an *in vivo* intra-hippocampal infusion of E2, at a physiological dose (Hojo, #95), acutely potentiates CA3-CA1 synaptic transmission (see also, (Smith, 2006 #23)) in a surface NMDAR trafficking-dependent manner. In line with the present findings, E2 has been shown to enhance GluN2B-dependent excitatory postsynaptic currents independently of GluN2B-NMDAR expression or phosphorylation status (Snyder, 2011 #26). Also, the surface redistribution of both GluN2A and 2B subunits 24 h after E2 exposure may contribute to the maintenance of the synaptic potentiation. Even if different effects of E2 have been reported on GluN2A/2B regulation depending on the animal models, timing and stimulation protocols (Woolley, 2007 #33;Zamani, 2000 #98), the present findings are consistent with a two-step model in which E2 exposure first favors LTP induction through an increased GluN2B-NMDAR synaptic retention and signaling, and second E2 contributes to LTP maintenance through the control of a GluN2A/2B ratio in the newly-potentiated synapses that prevents further plasticity (Srivastava, 2008 #29). At the cellular level, our data identify the surface dynamics of NMDAR as a primary target of E2-mediated signaling in hippocampal neurons. How

these effects interplay with the role of the receptor current and intracellular signaling remains to be thoroughly explored. Nevertheless, one can speculate that these effects would implicate some of the non-genomic mechanisms of E2 action, *i.e.* E2 action at or near the plasma membrane on either metabotropic glutamate receptor or G-protein estrogen receptor to transduce second messenger cascades and elicit ERK/CREB, mTOR or JNK signaling (for in depth reviews, see Tuscher 2014 and Frick 2015).

Our behavioral results indicate that surface NMDAR trafficking in the dorsal CA1 subfield of the hippocampus contributes to experience-dependent changes in integrative properties of hippocampal synapses that sustain certain, but not all forms of NMDAR-dependent associative memory. In dorsal CA1, whether in basal condition or in presence of E2, local surface trafficking of NMDAR is selectively required for long-term retention of temporal association between events separated by a brief interval (20 s, *trace fear conditioning*), but neither for contextual/spatial (fear conditioning/object location task) nor elementary (0 s, *delay fear conditioning*) associative memories.

Present selectivity of the surface trafficking manipulation in dorsal CA1 is in agreement with a functional dissociation among dorsal hippocampal subfields, and preferential implication of the CA1 subfield and local NMDAR in the temporal component of memory function (Huerta 2000; Reviewed in Kesner 2004), while spatial and contextual components would rely more on CA3 and DG (Rogers 2006; Hunsaker 2008; Mc Hugh 2009, Place 2012). Indeed, while restricting surface trafficking of NMDAR in dorsal CA1 had a selective impact on temporal fear memory, infusion of GluN1 X-link targeting the whole dorsal hippocampus produces an unselective impairment of acquisition of trace fear conditioning and retention of both temporal and contextual associative memories *i.e.* all components previously shown to rely on hippocampal NMDAR (Bast, 2003 #115;Czerniawski, 2012 #120;Gao, 2010 #121;Misane, 2005 #18;Quinn, 2005 #116;Schenberg, 2008 #118;Stiedl, 2000 #117;Wanisch, 2005 #119)

However, several lines of evidence suggest that the selective effect of GluN1 X-link infusion into the dorsal CA1 for temporal memory cannot be explained entirely by functional regionalization

of the hippocampus. First, we found no impact of the infusion of GluN1 X-link in the CA1-dependent object location task (Assini et al, 2009) while NMDAR besides their role in temporal memory have also been involved in spatial memory (Tsien, 1996 #135; Place 2012). Second, GluN1 X-link infusion into the DG has no effect on contextual discrimination whereas this capability was previously shown to rely on DG NMDAR (Eadie et al Hipp 2012; Kheirbeck et al, J Neurosci 2012, Neuron 2013). Hence it appears that restricting NMDAR surface trafficking in a particular subfield of the hippocampus, does not affect all associative memory functions of local NMDAR. The behavioral selectivity of GluN1 X-link effects is indicative of GluN1 X-link specificity, which echoes with previous electrophysiological demonstration that GluN1 X-link does not prevent agonist binding or alter functional properties of NMDAR besides lateral mobility (Dupuis, 2014 #90). Since GluN1-X link only interferes with NMDAR surface trafficking, our data support the conclusion that in hippocampal CA1 NMDAR surface diffusion around the synapse is selectively needed to form long-term associative memories of events occurring with a brief temporal interval.

Then, how can one explain the critical implication of CA1 NMDAR surface trafficking in temporal but not contextual/spatial associative memory? The present selectivity of GluN1 X-link effects suggests that surface redistribution of NMDAR around the synaptic area may sustain the detection of co-occurrence between stimuli separated by a brief temporal interval. Although speculative, this proposal is based on the traditional view of NMDAR function according to which these receptors act as detectors of coincident synaptic activations leading to synaptic strengthening that sustain the associative memory of coincident events. In the specific case of temporally discontinuous stimuli (*i.e.* in *trace* fear conditioning), the detection of tone and shock co-occurrence implies that some of synaptic activation induced by the former (tone) persists until the second (shock) occurs (*i.e.* during the *trace* interval). We suggest that surface NMDAR mobility and its modulation by E2 may contribute to maintain persistent synaptic activation across brief temporal intervals.

In conclusion, our study provides evidence that CA1 NMDAR surface trafficking and its modulation by the sex hormone 17β-estradiol, is a cellular mechanism critical for a major component of episodic

memory. As this memory is essential to a normal life and is highly susceptible to degradation in aging and various neuropsychiatric disorders, identifying underlying mechanisms is crucial and the present discovery may have important outcome regarding therapeutic development.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

References

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Footnotes

Table/Figure legends

FIGURE LEGENDS

Figure 1. E2 increases synaptic density, surface GluA1-AMPAR content and regulates surface distribution of GluN2-NMDAR. (A) E2 treatment increases the postsynaptic density (PSD) and the surface GluA1-AMPAR content. *Top panel:* Dendritic segment of a Homer 1c-DS-Red expressing hippocampal neuron treated with vehicle or E2 (10 nM, 24h). Scale bar=2 µm. *Bottom panel:* Surface GluA1-AMPAR were immunolabelled after vehicle (open bars) and E2 (black bars) treatment. Note the increase in surface staining intensity (color-coded: low value in blue and high value in red-white). Scale bar=300nm. **(B)** Comparison of the synaptic density and synaptic size (*top*), synaptic and total surface GluA1-AMPAR content (*bottom*) after vehicle (open bars) and E2 (black bars) treatment. Data are derived from at least 3 independent experiments. Histograms show mean±sem of percent of vehicle (for synaptic density: N=47/41; t_{86}=7.212; p<0.0001; for synaptic size N=18/17; t_{33}=0.1513; p=0.4403; for synaptic GluA1 N=8/10; t_{16}=2.393; p=0.0147 and for total GluA1 N=14/14; t_{26}=2.506; p=0.0094, for vehicle/E2-treated hippocampal neurons, respectively). **(C)** Immunocytochemical detections of endogenous surface GluN2A- and GluN2B-NMDAR in vehicle and E2-treated hippocampal neurons. PSD were identified by Shank staining. Some surface GluN2 staining co localize with Shank staining (overlay in yellow). Scale bar=2 µm. **(D)** Comparisons of the synaptic content of surface GluN2A- and GluN2B-NMDAR between vehicle (open bars) and E2 (black bars) conditions. Histograms show mean±sem of percent of vehicle (GluN2A-NMDAR: N=14/14; t_{26}=2.813; p=0.0046 and GluN2B-NMDAR: N=37/37, t_{72}=2.847; p=0.0029 for vehicle/E2-treated neurons, respectively). Student's *t*-tests were performed for comparisons between vehicle- and E2-treated hippocampal neurons. *, ** and *** indicate p<0.05, 0.01 and 0.001, respectively.

Figure 2. E2 regulates surface diffusion of GluN2-NMDAR and reduction of NMDAR surface diffusion impairs E2-induced increase in dendritic spine density *in vitro*. (A) Schematic representation of the QD single nanoparticle tracking approach. **(B)** E2 acutely regulates the surface

diffusion of GluN2B-NMDAR. Representative trajectories of surface GluN2B-NMDAR-QD before (basal) and after E2 treatment (10 nM, 15min). The grey areas correspond to synaptic sites labelled with Mitotracker. Scale bar=100nm. **(C)** Comparisons of the instantaneous diffusion coefficient (left panel) and of synaptic dwell-time (right panel) of synaptic GluN2A and GluN2B-NMDAR (n=6-7 dendritic fields; 23-26 trajectories/group; respectively) before and after E2 treatment (diffusion coefficient: p=0.102 and p=0.041; dwell time: p=0.21 and p=0.0140 for GluN2A- and GluN2B-NMDAR, respectively). **(D)** Representative trajectories of surface GluN2A- and/or GluN2B-NMDAR in hippocampal neurons treated with vehicle solution or E2 (scale bar=200 nm). In vehicle condition, GluN2A-NMDAR are less mobile and more synaptic than GluN2B-NMDAR. E2 treatment (10 nM, 24h) favours the synaptic anchoring of GluN2A-NMDAR and lateral escape of GluN2B-NMDAR to extrasynaptic areas. **(E)** Cumulative distribution of the instantaneous diffusion coefficient of GluN2A- and GluN2B-NMDAR in the synaptic area of vehicle (GluN2A-NMDAR: 127; GluN2B-NMDAR: 153 trajectories) and E2-treated neurons (GluN2A-NMDAR: 117; GluN2B-NMDAR: 237 trajectories). **(F)** Relative comparisons of the instantaneous diffusion coefficient of GluN2A- and GluN2B-NMDAR in synapses between vehicle and E2 conditions (p=0.0029 and 0.0051 for GluN2A- and GluN2B-NMDAR respectively) **(G)** Schematic representation of the GluN1 NMDAR X-link procedure. **(H)** Shank clusters fluorescently-detected in dendritic fields of hippocampal neurons following vehicle-, E2 (10 nM) with GluN2A X-link or GluN2B X-link treatments. Scale bar=5 µm. (**I**) Comparison of the Shank cluster density in the different conditions (vehicle/E2, n=124/131; GluN2A-NMDAR: n=22/21; GluN2B-NMDAR: n=53/44 dendritic fields, respectively; one way ANOVA: $F_{5,389}$=32.3; p<0.0001). Error bars represent sem. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 respectively (Non-parametric Mann-Whitney test, Student *t*-test and One way ANOVA followed by Bonferroni *post-hoc* comparisons where applicable).

Figure 3. Surface cross-linking of GluN1-NMDAR impairs electrically and E2-induced NMDAR-dependent LTP *in vivo* in adult mice. (A) Experimental design of electrophysiological *in vivo* recording of CA1 fEPSP in anesthetized mice. CA3 fibers from the contralateral hippocampus were stimulated (STIM) and field potentials were recorded (REC) in CA1 before and after high frequency stimulation (HFS, 100 Hz, 1s). **(B)** Representative histological controls of REC and STIM sites. **(C)** Representative fEPSP traces before (1) and after (2) CNQX application. **(D)** NMDAR antagonists (AP5, 100 μM, 60 nL intra-CA1, 5 min pre-HFS; MK801, 3 mg/kg, *ip* 45 min pre-HFS) blocked the HFS-induced LTP (*Treatment*: $F_{2,10}$=8.8; p=0.006). **(E)** Representative fEPSP traces before (1) and after (2) GluN1 X-link infusion. Neither control nor GluN1 X-link infusions change basal synaptic transmission at least for 40 min after infusions (*Time*: $F_{9,50}$=2.7; p=0.0128; *Treatment:* $F_{1,50}$=2.8; p=0.0994 and *Treatment x Time*: $F_{9,50}$=0.4; p=0.9426). GluN1 X-link impairs HFS-induced LTP **(F)** and E2-induced LTP **(G)**. Representative fEPSP traces before (1) and after (2) HFS/E2 after control (open circles) or GluN1 X-link (filled circles) infusions and corresponding plots (*Time*: $F_{9,110}$=3.2; p=0.0018; *Treatment:* $F_{1,110}$=82.0; p<0.0001; *Treatment x Time*: $F_{9,110}$=3.3, p=0.0013 **(F)** and *Time*: $F_{9,110}$=3.8; p=0.0003; *Treatment:* $F_{1,110}$=20.3; p<0.0001; *Treatment x Time*: $F_{9,110}$=2.3; p=0.0212 **(G)**. All graphs show mean±sem of change from baseline. Sample sizes (number of animals) are indicated in legends/within bars. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 respectively (one or two-way ANOVA followed by Bonferroni *post-hoc* comparisons, where applicable).

Figure 4. Reduction of NMDAR surface dynamics in the dorsal hippocampus (CA1) selectively impairs temporal associative memory. (A) Schematic representation of the experimental procedure. Bilateral intra-CA1 infusions (GluN1 X-link/control, 300 nL per side) were performed 45 min before *trace* fear conditioning (five pairings of tone (65 dB, 1 kHz, 30 s) and foot shock (0.6 mA, 50 Hz, 1 s) with a 20 s interval. Retention of temporal and contextual fear memories, respectively based on a 20 s *trace* tone-shock association and a context-shock association, were measured during re-exposure to the tone alone in a neutral context (context B, tone, 24 h after conditioning) and to the

conditioning context (context A, context, 2 h after tone test). Graphs show the percentage of time spent freezing **(C)** during the tone relative to the no tone periods (*i.e.*, pre- and post-tone in context B and **(D)** during re-exposure to conditioning context (A) relative to the neutral context (B).

(B) Representative photography showing location of intra-CA1 *cannulae* and the extent of GluN1 X-link diffusion 45 min after infusion in living mice and revealed with only secondary antibody. (Scale bar=250µm). **(C) Conditioning:** GluN1 X-link spares the acquisition of *trace* fear conditioning. The increase of time spent freezing over repeated tone presentations is not different between groups (*Tone repetition*: $F_{4,64}$=25.2; p<0.0001; *Treatment* $F_{1,16}$=0.5; p=0.5063 and *Interaction*: $F_{4,64}$=0.5; p=0.7114). **(D-E) Retention:** GluN1 X-link diminishes the 24h retention of fear memory based on a temporal association (**D, Tone:** *Tone*: $F_{1,32}$=23.0; p<0.0001; *Treatment:* $F_{1,32}$=2.2; p=0.1496; *Interaction*: $F_{1,32}$=4.7; p=0.0381) but not contextual association (**E, Context:** *Context*: $F_{1,32}$=43.7; p<0.0001; *Treatment*: $F_{1,32}$=1.0; p=0.3178 and *Interaction*: $F_{1,32}$=1.1; p=0.3043). All graphs show mean±sem of percent of time spent freezing (% freezing); n=numbers of animals. Statistical comparisons to control condition or within experimental groups are indicated by * and *** for p<0.05 and p<0.001, respectively (two-way ANOVA and Bonferroni *post-hoc comparisons*).

Figure 5: Reduction of NMDAR surface dynamics in the dorsal hippocampus (CA1) does not impairs object location memory. (A) Schematic representation of the experimental procedure. Bilateral intra-CA1 infusions (GluN1 X-link/control, 300 nL per side) were performed 45 min before *Acquisition* phase (10 minutes of freely behaving in an open field containing 2 identical objects). Retention of object location was measured during the *Test* phase 24h after acquisition. *Test* Phase consists in re-exposure to the open field in which one of the objects (A') was moved to a new location (displaced object = DO). Graphs show the location index, defined as the *ratio* between the time (T) spent exploring DO (A') and non-displaced object (NDO, A) over the time spent exploring both objects [T_{DO}/ (T_{DO}+T_{NDO})] and [T_{NDO}/ (T_{DO}+T_{NDO})] during the *acquisition* phase **(C)** and during the *test* phase **(D).** GluN1 X-link spares the acquisition of *object location memory* and the 24 h retention of this memory.

Indeed, whichever the treatment condition, in the *Acquisition* phase the location index is neither different from chance (0.5, dashed line) nor different between objects (**B,** *Object*: $F_{1,21}$=0.0; p=0.9829; *Treatment* $F_{1,21}$=4,2; p=0.0531 and *Interaction*: $F_{1,21}$=0.25 p=0.6222). In the *Test* phase, location indexes are different from chance (0.5 dashed line) and between objects for both treatment groups and with no difference between the 2 groups (**C,** *Object*: $F_{1,21}$=13.18; p=0.00016; *Treatment:* $F_{1,21}$=1.1 p=0.3071 *Interaction*: $F_{1,21}$=0.21; p=0.6486). All graphs show mean±sem of location index; n=numbers of animals. Statistical comparisons to control condition or within experimental groups are indicated by * and *** for p<0.05 and p<0.001, respectively (two-way ANOVA and student's t tests *post-hoc* comparisons).

Figure 6. Reduction of surface NMDAR surface dynamics in the dorsal (CA1) hippocampus selectively impairs E2 enhancement of temporal associative memory. Bilateral intra-CA1 E2 infusions prior to conditioning (0,3 mA shock intensity) enhances the 24 h retention of tone shock association in both *trace* (20 s, **A**) and *delay* (0 s, **B**) fear conditioning and GluN1 X-link prevents the E2-induced enhancement of associative memory only in *trace* conditioning. **(A) Tone (*trace*, 20 s)**: Overall interaction: $F_{3,40}$=7.45 p=0.0004; treatment effect: $F_{3,40}$=1.9; p=0.1410; tone effect: $F_{1,40}$=23.7; p<0.0001; **Control:** *E2 treatment x Tone* $F_{1,21}$=4.9; p=0.00373; **GluN1 X-link***: E2 treatment* and *Interaction p>0.05*; **E2:** *GluN1 X-link treatment x Tone* $F_{1,17}$=5.9; p=0.0264) but not in *delay* (0s) conditioning. **(B): Tone (*delay*, 0 s):** Overall interaction: $F_{3,44}$=3.3; p=0.0287; Treatment effect: $F_{3,44}$=3.5; p=0.0233; Tone effect: $F_{1,44}$=52.8; p<0,0001; **E2***, GluN1 X-link treatment x Tone*: p>0.05; **GluN1 X-link***, E2 treatment x Tone:* $F_{1,23}$=5.21; p=0.0321). **(C-D)** There is no effect on context retention whatever the conditioning condition and all groups exhibit higher freezing levels in the conditioning context (A)- than the neutral one (B). Overall interaction: $F_{3,40}$=1.8 p=0.1608 and $F_{3,44}$=1.6 p=0.2006; Treatment effect: $F_{3,40}$=1.9; p=0.1479 and $F_{3,44}$=1.35; p=0.2703; Context effect: $F_{1,40}$=71.4; p<0.0001 and $F_{1,44}$=39.4; p<0.0001, for *trace* **(C)** and *delay* **(D)** condition, respectively). All histograms show mean±sem; n=number of animals. Comparisons *vs.* no-tone **(A-B)** or neutral context

(C-D) are indicated by *, ** and *** for $p<0.05$, $p<0.01$ and $p<0.001$, respectively (two-way ANOVA followed by *post-hoc* student's t tests).