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Kalirin-7, an important component of excitatory synapses, is regulated by estradiol in hippocampal neurons

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Abstract

Estradiol enhances the formation of dendritic spines and excitatory synapses in hippocampal neurons *in vitro* and *in vivo*, but the underlying mechanisms are not fully understood. Kalirin-7 (Kal7), the major isoform of Kalirin in the adult hippocampus, is a Rho GDP/GTP exchange factor localized to post-synaptic densities. In the hippocampus, both Kal7 and estradiol receptor (ER) α are highly expressed in a subset of interneurons. Over-expression of Kal7 caused an increase in spine density and size in hippocampal neurons. To determine whether Kalirin might play a role in the effects of estradiol on spine formation, Kal7 expression was examined in the hippocampus of ovariectomized rats. Estradiol replacement increased Kal7 staining in both CA1 pyramidal neurons and interneurons in ovariectomized rats. Estradiol treatment of cultured hippocampal neurons increased Kal7 levels at the post-synaptic side of excitatory synapses and increased the number of excitatory synapses along the dendrites of pyramidal neurons. These increases were mediated via ER α because a selective ER α agonist, but not a selective ER β agonist, caused a similar increase in both Kal7 levels and excitatory synapse number in cultured hippocampal neurons. When Kal7 expression was reduced using a Kal7-specific shRNA, the density of excitatory synapses was reduced and estradiol was no longer able to increase synapse formation. Expression of exogenous Kal7 in hippocampal interneurons resulted in decreased levels of GAD65 staining. Inhibition of GABAergic transmission with bicuculline produced a robust increase in Kal7 expression. These studies suggest Kal7 plays a key role in the mechanisms of estradiol-mediated synaptic plasticity.

Keywords

synaptogenesis; spine plasticity; estradiol; GABA

Introduction

The hippocampus, critical for learning and memory, is a major estradiol target, particularly the CA1 region (Sweatt, 2004; Spencer et al., 2008b). Dendritic spines, primary sites of excitatory synaptic transmission, constitute a crucial morphological substrate of memory (Bourne and Harris, 2008). Increases in both spine number (Engert and Bonhoeffer, 1999) and spine size (Yang et al., 2008) contribute to LTP and are associated with learning and memory mechanisms (De Roo et al., 2008). Reducing estradiol levels by ovariectomy (Ovx) causes a decrease in spine density (Gould et al., 1990), and estradiol replacement increases

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spine density and excitatory synapse number in hippocampal CA1 pyramidal neurons in Ovx rodents (Gould et al., 1990; Woolley and McEwen, 1992; Li et al., 2004; MacLusky et al., 2005) and rhesus monkeys (Hao et al., 2003). Estradiol treatment increases spine density and/or the number of excitatory synapses in cultured hippocampal neurons (Murphy et al., 1998; Gazzaley et al., 2002; Jelks et al., 2007; Mukai et al., 2007). Estradiol replacement also increases spine size and enhances LTP amplitude in CA1 pyramidal neurons of Ovx rodents (Li et al., 2004; Smith and McMahon, 2005). Spine density (Woolley et al., 1990; Gonzalez-Burgos et al., 2005; Spencer et al., 2008a), spine size (Gonzalez-Burgos et al., 2005) and LTP induction (Good et al., 1999; Warren et al., 1995) in CA1 pyramidal neurons vary across the estrous cycle, peaking during proestrus with its high estradiol levels. Reducing endogenous estradiol by treating hippocampal neurons with letrozole, an inhibitor of aromatase, causes a decrease in spine/synapse density (Prange-Kiel and Rune, 2006).

To understand the mechanism of estradiol-induced spine formation, the proteins regulating spine/synapse plasticity need to be identified. Many proteins regulate spine/synapse formation in hippocampal neurons (Tada and Sheng, 2006; Bhatt et al., 2009). Only a few of these are known to be up-regulated by estradiol: some are postsynaptic (spinophilin, PSD95, GluR1, NR1, NR2b, N-cadherin) and some are presynaptic (synaptophysin, syntaxin) (Gazzaley et al., 1996; Monks et al., 2001; Brake et al., 2001; Frick et al., 2002; Rune et al., 2002; Hao et al., 2003; Akama and McEwen, 2003; Choi et al., 2003; Li et al., 2004; Jelks et al., 2007; Liu et al., 2008). The estradiol-induced increase in PSD95 and spinophilin cannot account for estradiol-mediated spine formation since both PSD95 and spinophilin knockout mice show an increase in CA1 spine density (Feng et al., 2000; Vickers et al., 2006) and spine size is unaltered in PSD95 knockout mice (Beique et al., 2006). Reducing endogenous PSD95 expression using shRNA does not alter spine number in cultured hippocampal neurons (Elias et al., 2006). Spinophilin over-expression does not alter spine density (Futter et al., 2005) in cultured hippocampal neurons. Although estradiol activates LIM kinase (LIMK1/2) in CA1 neurons (Yildirim et al., 2008; Spencer et al., 2008a), LIMK1 knockout mice show an increase in spine size, PSD length and enhanced LTP with normal spine density and synapse number (Meng et al., 2002). Estradiol-mediated increases in NR1, NR2b and GluR1 play key roles in estradiol-mediated spine formation (Woolley and McEwen, 1994; Pozzo-Miller et al., 1999; Smith and McMahon, 2005; Smith and McMahon, 2006). Although cyclic AMP response element binding protein (CREB) (Zhou et al., 1996; Zhao et al., 2005), MAPK (Ogiue-Ikeda et al., 2008), PI3-K/Akt (Znamensky et al., 2003), CaMKII (Sawai et al., 2002), BDNF (Scharfman and MacLusky, 2006) and WAVE1 (Sanchez et al., 2009) are known estradiol targets, the molecular mechanisms through which estradiol regulates spine formation and function are still not fully understood and represent the focus of this research.

Understanding spine/synapse formation requires understanding the many factors that control the actin cytoskeleton (Sekino et al., 2007). Among these factors are Rho GTPases, which are activated by Rho GEFs (GDP/GTP exchange factors) and inactivated by Rho GAPs (GTPase activating proteins) (Negishi and Katoh, 2005). Kalirin, a Rho GEF, is expressed in hippocampal pyramidal neurons and interneurons (Ma et al., 2001). The Kalirin gene is subject to extensive alternative splicing (McPherson et al., 2002) and Kal7, which terminates with a PDZ-binding motif, is the major splice variant in the adult. Kal7, one of the few Rho GEFs identified in the post-synaptic density (PSD) (Sheng and Hoogenraad, 2007), has been identified as a downstream target of CaMKII α (Xie et al., 2007), EphB (Penzes et al., 2003), N-cadherin (Xie et al., 2008) and Cdk5 (Xin et al., 2008). Over-expression of Kal7 induces formation of spines in aspiny hippocampal interneurons (Ma et al., 2008b). Kal7 knockout (Kal7^{KO}) mice show decreases in spine density, synapse number and a deficit in LTP in CA1 pyramidal neurons (Ma et al., 2008a).

This study was designed to test the hypothesis that Kal7 plays a role in the pathway through which estradiol regulates spine/synapse formation *in vivo* and in cell culture. Our data suggest that both the ability of estradiol to inhibit GAD expression in Kal7-expressing interneurons and the ability of estradiol to affect CA1 pyramidal neurons contribute to increased spine formation. A better understanding of the roles of Kal7 in estradiol-induced synapse formation will contribute to our understanding of the underlying mechanisms of estradiol-mediated synaptic plasticity.

Materials and Methods

Animals

Timed pregnant adult female Sprague Dawley rats (1 per cage) from Charles River Laboratories and middle-aged (8-9 month-old) F-344 female rats (3 per cage) from Harlan Sprague Dawley Inc. were maintained with a 14-10 h light-dark cycle with food and water available *ad libitum*. Female Sprague Dawley rats were used for preparation of hippocampal cultures and F344 rats were used for *in vivo* experiment. All experiments were conducted in accordance with the guidelines established by the UCHC Animal Care and Use Committee. Middle-aged (8-9 month-old) F-344 female rats were studied 4 months following bilateral ovariectomized (Ovx) or sham surgery. Ovx animals received subcutaneous silastic capsule (Dow Corning: #508-006) implants containing cholesterol (placebo) or 17 β -estradiol (E2) (1:4; E2:cholesterol) 7 days before sacrifice (Yang et al., 2005). This regimen has been shown to result in serum E2 levels in the high proestrus range (Yang et al., 2005). Successful ovariectomy and estradiol replacement were established using vaginal cytology. Animals were anesthetized with ketamine and perfused transcardially with 4% paraformaldehyde. The brains were postfixed for 4 h in the same solution, then transferred to 25% sucrose in PBS overnight and stored at -80°C until they were sectioned.

Antibodies

The following polyclonal antibodies were used: affinity-purified Kal7 (JH2958, 1:200), Kalirin spectrin (JH2582, detects all major isoforms of Kalirin), GAD65/67 (1:2000, G5163, Sigma), GluR1 (1:500, AB5855, Upstate), MAP2 (1:500, Ab5622, Chemicon), PAM (JH629, 1:500). Mouse monoclonal antibodies were: PSD95 (clone28/43, 1:300, gift from Dr. Matt Rasband, Baylor University), NR1 (1:300, 556308, BD Pharmingen), NR2b (1:200, N59/20, NeuroMab, Davis, CA), Shank3 (1:200, Clone 69/46, NeuroMab, Davis, CA), GAD65 (GAD6, 1:200, Developmental Studies Hybridoma Bank, University of Iowa), spinophilin (neurabin II, 612166, 1:200, BD Transduction Laboratories), GABA_A receptor (β -chain, MAB341, 1:200, Chemicon), Myc (9E10, 1:20), GFP (1:200, Chemicon), ER α (clone ID5, KakoCytomafion) and α -adaptin (610501, BD Transduction Laboratories). Guinea pig antibody to Vglut1 (1:3000, AB5909, Chemicon) was used where indicated.

Immunohistochemistry (brain sections)

Coronal sections (12 μ m) were cut and mounted on gelatin-coated slides. The specificity of the Kal7 antibody was evaluated by replacement of antibody with pre-immune serum and pre-incubation of antibody with its antigen (10 μ g/ μ l) as described (Ma et al., 2003; Ma et al., 2008b); staining was eliminated in both controls. Sections were blocked in PBS containing 1% BSA/10% normal goat serum/0.25% Triton X-100 (pH 7.4) for 1 h at room temperature. Sections were stained with polyclonal Kal7 antibody or simultaneously with Kal7 and monoclonal ER α antibodies at 4°C overnight. Primary antibodies were visualized with Cy3-labeled donkey anti-rabbit IgG (Jackson Lab) and FITC-labeled goat anti-mouse IgG (Jackson Lab). Images were taken with a Zeiss LSM510 confocal microscope (University of Connecticut Health Center for Cell Analysis and Molecular Modeling).

Western blot analysis

After dissection, each hippocampus was homogenized in 10 volumes of SDS lysis buffer (Ma et al., 2008a) with five strokes of a glass/Teflon homogenizer; samples were heated at 95°C for 5 min and centrifuged. Proteins were fractionated on 4–20% polyacrylamide gels (BioRad) and transferred to Immobilon-P membranes (Millipore). Kalirin was visualized with rabbit antiserum specific to the spectrin domain (1:1000, JH2582) and the Amersham Pharmacia Biotech ECL Kit (Ma et al., 2003). α -adaptin was used as a loading control.

Primary cultures of rat hippocampal neurons and drug treatments

Cultures were prepared from P1 female Sprague Dawley rats as described (Ma et al., 2003). The rationale for using female neurons are based on the facts that male and female rats have different spine densities on their CA1 hippocampal pyramidal neurons (Shors et al., 2001), and estradiol treatment fails to cause an increase in spine density in male rat CA1 pyramidal neurons *in vivo* (Romeo et al., 2005; Lee et al., 2004). Female pups were identified by comparing the distance between the genital papillae and the anus (male > female). Briefly, the hippocampi were digested with 0.25% trypsin for 25 min at 37°C. Cultured cells were plated in Neurobasal A medium containing 7% heat-inactivated horse serum, and maintained at 37°C in 5% CO₂. Three hours later, plating medium was replaced with fresh medium containing only 3% horse serum (heat inactivated). Three days after plating, the culture medium was exchanged with maintenance medium [Neurobasal A medium containing 2% B27 supplement, 0.5mM glutamine, 25 units/ml penicillin, 25 µg/ml streptomycin]. Thereafter, 50% of the medium was replaced twice a week for up to 4 weeks. Freshly dissociated P1 neurons ($1-2 \times 10^6$ neurons) were nucleofected (Amaxa GmbH, Germany) using the rat neuron kit and 6 µg plasmid as described (Ma et al., 2008b). Following nucleofection, neurons were plated and allowed to recover in DMEM containing 10% FCS for 3 hours; nucleofected neurons were kept in Neurobasal A maintenance medium for up to 4 weeks. Where indicated, after 19 days *in vitro* (DIV), hippocampal neurons were treated with E2 (10 nM, Sigma), vehicle (same working solution without E2) or E2 plus estradiol receptor antagonist, ICI182,780 (1 µM, TOCRIS, Avonmouth, UK) for 48 h. In independent experiments, primary cultures of female hippocampal neurons (DIV21) were treated with vehicle, E2 (10 nM), ER α selective agonist, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, 10 nM, TOCRIS, Bristol, UK), and ER β selective agonist, diarylpropionitrile (DPN, 10 nM, TOCRIS, Bristol, UK), for 48h. Neurons were fixed with cold methanol for visualization of Kal7, Vglut1 and MAP2 as described (Ma et al., 2008b; Ma et al., 2008a). In brief, a concentrated stock solution (E2 or ICI182,780 in ethanol, PPT or DPN in DMSO) was diluted with Neurobasal A medium and added to cultures to produce the working concentration. At DIV21, cultures were treated with 20 µM bicuculline (Fluka) or vehicle for 24 h and fixed for immunostaining.

Immunocytochemistry

Immunostaining of cultures was performed as described (Ma et al., 2008b). Briefly, for visualization of NR1, NR2b, Vglut1, GluR1, Shank3, spinophilin, GABA_A receptor and Kal7, neurons were fixed with methanol for 12 min at -20°C, except where indicated. After 10 min in blocking buffer [1% BSA/1xPBS/5% normal goat and/or donkey serum, pH7.4] containing 0.20% Triton X-100 followed by 50 min in blocking buffer without Triton X-100 at room temperature, cells were doubly or triply stained with appropriate primary antibodies overnight at 4°C. Primary antibodies were visualized with appropriate secondary antibodies: Cy3-donkey anti-rabbit IgG (Jackson Lab), FITC-donkey anti-mouse IgG (Jackson Lab), FITC-donkey anti-guinea pig IgG (Jackson Lab), Alexa Fluor 633-goat anti-rabbit IgG (Molecular Probes), Alexa Fluor 633-goat anti-mouse IgG (Molecular Probes). Coverslips were mounted with Prolong Gold antifade reagent (Molecular Probes). Images were visualized with a Zeiss LSM510 confocal microscope.

DNA constructs

pSIREN-Dsred-Kal7-3'UTR shRNA constructs: the RNAi-Ready-pSiren-DNR-DsRed-Express vector (Clontech) places shRNA expression under control of the human U6 promoter and DsRed-monomer expression under control of the CMV promoter; the Kal7-3'UTR and PAM shRNAs have been described (Ma et al., 2008b). pSIREN-shRNA-scramble encodes a randomized sequence with no matches among murine transcripts in GenBank (AATGCTCGCACAGCACAAG); all shRNAs have the general composition G₃₋₄A₇₋₈T₂₋₄C₄₋₆. pEAK vectors encoding Kal7GFP were described previously (Ma et al., 2008b). pwiWII-pmGFP encodes N-terminally extended GFP (pmGFP) under control of the chicken β actin promoter; the N-terminal extension allows myristoylation and palmitoylation of pmGFP, attaching it to the inner leaflet of the plasma membrane (Koster and Fraser, 2001).

Image analysis and quantification—For *in vitro* studies, triplicate cultures were examined in each condition (e.g. vehicle, E2). At least 4 cells per culture and at least two dendrites per cell were randomly imaged. Images captured using a Zeiss LSM510 confocal microscope were analyzed as described (Ma et al., 2008b). For quantification of spine density and synaptic clusters, a stack of images (Z step, 0.3 μ m) was acquired using a 63 $\times$ objective (2.5 digital zoom factor) and dendrites were visualized in 3-dimensions (Ma et al., 2003). For visualization of Kal7 clusters and synaptic markers, single plane images through the brightest point were taken. For each experiment, all images were taken with identical settings under the same conditions with a calibrated scale bar. Spine density and synaptic clusters were counted using Metamorph (Molecular Devices, Downingtown, PA) and were limited to dendrites within 100 μ m of the cell soma.

For quantification of Kal7 staining in the hippocampal CA1 region of tissue sections, all images were randomly taken with identical settings under the same conditions. Sections were coded so that the image analyzers were blinded to the treatment group during all imaging and analysis. Four sections (12 μ m) were analyzed for each animal (4 animals each group); the mean number for each section was used to calculate group means. Data are presented as mean $\pm$ SEM. Five sets of sections were made through the hippocampus of each rat; the first section in each set was used for Kal7 immunostaining. Since the minimum distance between sections analyzed using the same antiserum was 60 μ m, the same neuron was not measured twice. The integrated intensity, which combines size and immunofluorescence intensity, was evaluated after images were calibrated using the scale bar and the intensity thresholds were set to ensure that background signal was eliminated and all interesting structures were included in the analysis. Quantification was performed using Metamorph as described (Ma et al., 2008b). Quantification of estradiol-induced changes in the immunoreactivity of synaptic proteins in the hippocampus was reported before (Spencer et al., 2008a; Liu et al., 2008). Data are presented as average $\pm$ SEM. Statistical analyses were performed with JMP6 software (SAS Institute, Cary, NC) using one way analysis of variance (ANOVA) followed by Dunnett's test to assess statistical significance between groups (Ma et al., 2003; Ma et al., 2008b); * $p < 0.05$ or ** $p < 0.01$ was considered statistically significant.

Results

Expression of Kal7 in the CA1 region of the hippocampus is regulated *in vivo* by estradiol

Kal7 is expressed in pyramidal neurons and interneurons in the adult rat hippocampus (Ma et al., 2003). To explore the hypothesis that Kal7 is involved in the mechanism through which estradiol-induces spine formation in hippocampal neurons, we first determined whether estradiol affected expression of Kal7 in the hippocampus of middle-aged female

rats. Ovx rats were examined after receiving E2 or vehicle for 7 days (Fig. 1). E2 replacement caused an increase in Kal7 staining in the soma of pyramidal neurons and interneurons in the stratum oriens (SO) and stratum radiatum (SR) of the CA1 region (Fig. 1A-B and A1-B1). When staining intensities were quantified, it was clear that expression of Kal7 in interneurons, pyramidal neurons and the SR of the CA1 region increased approximately two-fold following E2 replacement in Ovx rats compared to vehicle-treated Ovx controls (Fig. 1C-E, $P < 0.01$). Hippocampal tissue from Ovx+vehicle and Ovx+E2 rats was subjected to Western blot analysis using an antibody specific for the spectrin region of Kalirin, which reveals Kal7 and lesser amounts of Kal9 (Fig. 1F). For quantification, levels of Kal7 were normalized to α -adaptin levels on the same blot; seven days of E2 replacement increased Kal7 levels in the hippocampus about two-fold (Fig. 1F-G). In cultured hippocampal neurons, Kal7 is exclusively localized to the postsynaptic side of excitatory synapse Like contacts (ESLCs) and is co-localized with postnaptic proteins including PSD95, NR1, NR2b, GluR1, spinophilin and Shank3 (Fig. S1). These results showed that Kal7 is exclusively localized to the postsynaptic side of excitatory synapses by confocal analyses, ultrastructural analyses were not performed. As in cortical neurons, over-expression of Kal7 caused an increase in spine density and spine size in cultured hippocampal neurons (Fig. S2). Reducing endogenous Kal7 expression by expressing a Kal7-specific shRNA caused a decrease in spine density compared with control neurons transfected with an empty vector encoding DsRed only, shRNA to PAM or a scrambled shRNA (**not shown**).

ER α co-localizes with Kal7 in hippocampal neurons *in vivo*

In hippocampal neurons, the effects of estradiol on spine density are thought to occur through ER α (Szymczak et al., 2006; Jelks et al., 2007; Mukai et al., 2007). To determine whether the two proteins were co-localized, adult female rat hippocampal sections were stained simultaneously with antibodies specific to Kal7 and ER α . All ER α positive interneurons in the CA1 region of the hippocampus were stained for Kal7 (Fig. 2A1-C1). About half of the Kal7 positive interneurons were strongly stained for ER α (200 interneurons were counted). Kal7 positive pyramidal neurons did not express nuclear ER α , in agreement with previous studies (Weiland et al., 1997; Orikasa et al., 2000; Hart et al., 2001; McEwen, 2002). Based on these analyses, we next asked whether estradiol regulated Kal7 expression in cultured hippocampal neurons.

Expression of Kal7 is regulated by estradiol in cultured hippocampal neurons

Strong evidence shows that estradiol treatment increases spine density and/or the number of excitatory synapses in cultured hippocampal neurons (Murphy et al., 1998; Gazzaley et al., 2002; Jelks et al., 2007). To determine whether estradiol altered Kal7 expression, primary cultures of hippocampal neurons (DIV21) prepared from female P1 rat pups were treated with vehicle (Fig. 3A, D), 10 nM E2 (Fig. 3B, E) or 10 nM E2 plus 1 μ M ER α antagonist ICI 182,780 (Fig. 3C, F) for 48 h. GAD65, a marker for GABA-producing interneurons, is down-regulated by estradiol treatment (Murphy et al., 1998; Gazzaley et al., 2002) and was visualized as a control for treatment efficacy. GAD65 was largely localized to inhibitory nerve terminals outlining the soma and dendrites of target cells (Fig. 3A, D). Methanol fixation facilitates visualization of Kal7 in excitatory synapses (Ma et al., 2008b), but is incompatible with visualization of GAD65; cultures were therefore fixed with 4% paraformaldehyde. As expected, GAD65 staining was down-regulated following estradiol treatment ($p < 0.05$, Fig. 3B, E, G). Estradiol treatment caused an increase in the intensity of Kal7 staining in neuronal processes (arrows) ($P < 0.05$, Fig. 3B, E, G). The estradiol-induced decrease in GAD65 staining and increase in Kal7 staining were both partially blocked by ICI 182,780 treatment (Fig. 3A-F, G). Levels of Kal7 in the cell soma were not changed significantly by estradiol-treatment. Estradiol-mediated increase in Kal7 levels in

the processes, but not soma showed that increased Kal7 protein was transported to the processes and suggested that E2 may regulate Kal7 levels through transcription, protein translation and/or protein stability, which remain to be determined. These data raised the possibility that the estradiol-induced increase in spine density observed in hippocampal neurons might occur through the ability of estradiol to up-regulate Kal7 expression.

Estradiol increases the number of Kal7-positive ESLCs on hippocampal neurons

To determine whether estradiol treatment produced the expected increase in the number of ESLCs in our cultures and whether these ESLCs contained Kal7, DIV21 hippocampal neurons prepared from female P1 rat pups were treated with vehicle or 10 nM E2 for 48h. To optimize synaptic staining (Ma et al., 2008b), cultures were fixed with cold methanol for triple-staining with antibodies to Kal7, Vglut1 and MAP2. Kal7 paired with Vglut1 serves as a reliable marker for ESLCs (Fig. S1), so we quantified ESLC number by measuring Kal7/Vglut1 clusters. Estradiol treatment resulted in a greater than 50% increase in the number of Kal7-Vglut1 positive ESLCs ($P<0.01$) and an increase in Kal7 staining intensity ($P<0.01$) in these ESLCs compared with vehicle-treated control (Fig. 4A-B, E, F). The estradiol-induced increase in the number of Kal7-Vglut1 positive ESLCs and in Kal7 staining intensity at these ESLCs was partially blocked by ICI 182,780 treatment (Fig. S3).

ER α and ER β are both found in hippocampal neurons (Shughrue et al., 1997; Mitra et al., 2003). To determine the roles of ER α and ER β in estradiol-mediated Kal7 expression and synapse density, neurons were treated with either the ER α -specific agonist PPT (10 nM) or the ER β -specific agonist DPN (10 nM) (Jelks et al., 2007; Mukai et al., 2007). Similar to estradiol, PPT treatment caused a significant increase in both Kal7 staining intensity ($P<0.01$) and the number of Kal7-Vglut1 clusters ($P<0.01$) compared with vehicle-treated control (Fig. 4C, E, F). However, DPN treatment did not cause a significant increase in the number of Kal7-Vglut1 ESLCs ($P>0.05$) or Kal7 staining intensity ($P>0.05$) compared with vehicle-treated controls (Fig. 4D, E, F). The ratio of Kal7/Vglut1 clusters was not altered by any of these treatments (Table 1). Estradiol treatment caused a decrease in both percentage of free Kal7 clusters and free Vglut1 terminals which were not formed ESLCs with Vglut1 or Kal7 ($P<0.05$) (Table 1).

It is well documented that spinophilin expression in hippocampal neurons is up-regulated by both estradiol replacement of Ovx rats and estradiol treatment of cultured hippocampal neurons (Hao et al., 2003; Brake et al., 2001; Zhao et al., 2006; Lee et al., 2004). Kal7 is not only colocalized with spinophilin (Fig. S1F) but interacts with spinophilin (Penzes et al., 2001) at the postsynaptic density.

To verify our estradiol treatment efficacy, sister female hippocampal neuronal cultures were fixed with cold methanol for triple-staining with antibodies to spinophilin, Vglut1 and MAP2 after 48h treatment with vehicle, E2 (10 nM), PPT (10 nM) or DPN (10 nM). Similar to Kal7, both E2 and PPT, but not DPN treatment caused an increase in spinophilin staining intensity at Vglut1-spinophilin clusters compared with vehicle-treated neurons (Fig. S4).

Kal7 plays an essential role in the maintenance of excitatory synapses in hippocampal neurons

Antisense-mediated reductions in the expression of all of the major isoforms of endogenous Kalirin in cultured hippocampal pyramidal neurons revealed an essential role for Kalirin in the maintenance of dendritic spines (Ma et al., 2003). Kal7 was shown to play an essential role in the formation of excitatory synapses onto the dendritic shafts of interneurons in culture (Ma et al., 2008b). Knockout mice engineered to lack Kal7 show a modest decrease in the density of excitatory synapses in the stratum radiatum of the CA1 region (Ma et al.,

2008a), emphasizing the complexity of *in vivo* spine formation and maintenance. We wanted to verify that Kal7 was the isoform required for the formation of excitatory synapses in hippocampal pyramidal neurons using an shRNA approach. Having again validated the specificity of our Kal7-UTR shRNA (Fig. S5) (Ma et al., 2008b), we next explored its effects on ESLC number by visualizing MAP2 and Vglut1 in neurons expressing DsRed-Kal7-UTR shRNA (Fig. 5A and A1) or DsRed alone (Fig. 5B and B1). Interneurons were excluded from this analysis since hippocampal interneurons lack spines (Ma et al., 2008b). Expression of Kal7-UTR shRNA caused a decrease in the density of Vglut1 clusters along the pyramidal cell dendrites when compared to control DsRed expressing neurons (Fig. 5E). About 93% of Vglut1 positive endings formed ESLCs with Kal7 clusters (Fig. 4, Table 1). Kal7 is co-localized with PSD95, NR1 and GluR1 (Fig. S1). Therefore, Vglut1 positive clusters can be used as an index to evaluate the formation of ESLCs. The effect of DsRed-Kal7-UTR shRNA on expression of Kal7 was verified by staining cells simultaneously for MAP2 and Kal7 (Fig. 5C, D). Quantification of the Kal7 staining intensity revealed a greater than four-fold decrease in neurons expressing DsRed-Kal7-UTR shRNA (Fig. 5F). Thus Kal7 plays an essential role in the formation of excitatory synapses in hippocampal pyramidal neurons (Ma et al., 2008a).

Estradiol-induced increase in the number of ESLCs is attenuated in Kal7 shRNA-expressing hippocampal neurons

Expression of exogenous Kal7 causes an increase in both spine density and size in cultured hippocampal neurons and Kal7 is required for the maintenance of ESLCs in hippocampal neurons *in vitro*. If the estradiol -induced increase in the number of ESLCs required up-regulation of Kal7 expression, no increase would be apparent in the absence of Kal7. To test this prediction, levels of endogenous Kal7 were reduced by expressing the Kal7-UTR shRNA in hippocampal neurons and then treating these cultures with vehicle alone or with 10 nM E2 for 48h; control neurons expressing DsRed alone were treated in the same manner. Cultures were fixed on DIV25 and simultaneously visualized with antibodies to MAP2 and Vglut1 or antibodies to MAP2 and Kal7. Transfected neurons were identified based on their expression of DsRed, limiting staining to two antigens. As expected, the number of Vglut1-positive clusters along the dendrites of DsRed positive neurons increased substantially when cultures were treated with 10 nM E2 (Fig. 6A, B). This effect of estradiol was eliminated in Kal7-UTR shRNA-expressing hippocampal neurons (Fig. 6C-F). Estradiol treatment did not significantly increase Kal7 levels in the dendrites of Kal7-UTR shRNA-expressing neurons compared with vehicle-treated Kal7-UTR shRNA-expressing neurons (**not shown**).

Quantification of Vglut1-positive clusters demonstrated an approximately 5-fold decrease in synapse density in neurons expressing Kal7-UTR shRNA (Fig. 6G). While estradiol treatment caused a two-fold increase in synapse density in control DsRed-expressing neurons, it had no effect on synapse density in Kal7-UTR shRNA-expressing neurons. These results indicate that Kal7 is required for estradiol to increase the number of excitatory synapses in hippocampal neurons.

GAD65 levels are decreased by increased expression of Kal7 in hippocampal interneurons

When we studied Kal7 expression in hippocampal interneurons (Ma et al., 2008b), we noted that GAD65 staining intensity tended to decrease in the soma of hippocampal interneurons expressing exogenous Kal7. To explore this possibility, dissociated rat hippocampal neurons prepared from P1 were transfected with a vector encoding GFP alone or a vector encoding the Kal7GFP fusion protein; at DIV16, cultures were fixed for simultaneous staining with antibodies to GAD65 and GFP. Kal7GFP was as potent as Kal7 and expression of Kal7GFP induced the formation of spine-like structures in hippocampal aspiny interneurons, as

expected (Fig. 7A-C vs. D-F) (Ma et al., 2008b). Higher power images of the dendrites of GFP-expressing (Fig. 7G) and Kal7GFP-expressing (Fig. 7H) GAD-positive interneurons illustrate the complex shapes of the spine-like structures formed in response to expression of Kal7GFP. In comparison to expression of GFP, expression of Kal7GFP resulted in a decrease in the levels of GAD65 staining in the soma of hippocampal interneurons (Fig. 7B, E). Quantification revealed a 34% decline in levels of GAD65 staining in interneurons expressing Kal7GFP compared with interneurons expressing GFP ($P < 0.01$, Fig. 7I). These results show that there is a reciprocal relation between GAD65 levels and Kal7 levels in hippocampal interneurons.

Kal7 levels in excitatory synapses are regulated by bicuculline

The estradiol-mediated increase in Kal7 expression in pyramidal neurons could contribute to the observed increase in dendritic spines. We next explored the possibility that estradiol-mediated down-regulation of GAD65 expression and GABA inhibitory tone might also contribute to the increase in dendritic spines. To determine whether Kal7 localization in hippocampal pyramidal neurons were affected by the inhibitory action of GABA, bicuculline, a competitive antagonist of the GABA_A receptor, was used. Hippocampal neurons (DIV25) were treated with bicuculline for 24 hours and then fixed for simultaneous visualization of Kal7, Vglut1 and MAP2. MAP2 staining was used to highlight the dendritic shaft. Hippocampal interneurons are free of spines and Vglut1 positive excitatory presynaptic terminals terminate on the surface of their dendritic shaft (Ma et al., 2008b) making it possible to distinguish interneurons from pyramidal neurons. In comparison to vehicle-treated pyramidal neurons (Fig. 8A-C), exposure to bicuculline increased the intensity of Kal7 staining at the postsynaptic side of ESLCs onto pyramidal neurons (Fig. 8D-F; 8G, $P < 0.01$). Kal7 staining at the postsynaptic side of ESLCs onto pyramidal neurons in control cultures, where GABAergic transmission was intact, was not as intense (Fig. 8G). Kal7-Vglut1 positive clusters along the dendrites of hippocampal pyramidal neurons were quantified as a measure of excitatory synaptic contacts (Fig. 8A-F, H). The number of ESLCs increased significantly following 24 h of exposure to bicuculline compared with vehicle-treated neurons ($P < 0.01$). The ratio of Kal7/Vglut1 clusters, the percentage of free Kal7 clusters and percentage of free Vglut1 terminals that are not formed ESLCs were not altered by bicuculline (right 2 columns of Table 1).

These data demonstrate that Kal7 levels at ESLCs onto hippocampal pyramidal neurons in culture are tonically diminished by the inhibitory actions of GABA acting through GABA_A receptors.

Discussion

In vivo studies

Since the effects of ovariectomy and estradiol replacement on spine/synapse number and size in hippocampal pyramidal neurons are well described (Gould et al., 1990; Woolley and McEwen, 1992; Hao et al., 2003; Choi et al., 2003; Li et al., 2004; MacLusky et al., 2005; Gonzalez-Burgos et al., 2005), we evaluated their effects of Kal7 expression. After 7 days of estradiol replacement, Kal7 levels doubled in CA1 pyramidal neurons, in a substantial subset of the interneurons in CA1 and throughout the stratum radiatum. This is the first report identifying Kal7 as an estradiol target. Although further studies will be required to determine whether these changes reflect increased gene expression or regulation at the post-transcriptional level, our initial evaluation of changes in the hippocampus of Kal7^{KO} mice support a role for Kal7 in spine formation and maintenance. Spine density, spine length, PSD thickness and synapse number all decreased in CA1 pyramidal neurons of Kal7^{KO} mice, which were deficient in LTP and exhibited a decrease in spontaneous EPSC

frequency (Ma et al., 2008a). Additional studies will be required to determine whether both ER α and ER β contribute to the effects of estradiol replacement on Kal7 expression.

***In vitro* studies**

Although the underlying circuitry is absent, *in vitro* studies serve as an essential tool for exploring the molecular mechanisms underlying the *in vivo* response to OvX and estradiol replacement. Estradiol treatment of cultured hippocampal neurons causes an increase in spine/synapse number (Murphy et al., 1998; Gazzaley et al., 2002; Jelks et al., 2007; Mukai et al., 2007). This work demonstrated that estradiol increases Kal7 expression in cultured hippocampal neurons, and previous work demonstrated that increased Kal7 levels increase spine/synapse numbers. These data make Kal7 a potentially obligatory player in estradiol actions to increase excitatory signaling.

ER α , the major ER in the hippocampus (Orikasa et al., 2000; McEwen, 2002; Mitra et al., 2003), supports the effects of estradiol on spinogenesis and synapse formation (Mukai et al., 2007; Jelks et al., 2007; Szymczak et al., 2006). Consistent with a role for Kal7 in this response, ICI 182,780, an ER antagonist, partially blocked the ability of estradiol to increase Kal7 expression. This is in agreement with two previous reports (Mukai et al., 2007; Jelks et al., 2007), although an earlier study is in disagreement (Zhao et al., 2006). Since PPT, an ER α selective agonist, but not DPN, an ER β selective agonist, mimicked the effects of estradiol on both Kal7 expression and excitatory synapse formation in cultured hippocampal neurons, we can conclude that estradiol exerts these effects through ER α . Similar conclusions about the effects of estradiol on synapse formation have been reported previously (Mukai et al., 2007; Jelks et al., 2007).

The ability of estradiol to increase the number of ESLCs was eliminated when endogenous Kal7 expression was reduced using Kal7-UTR shRNA. Exogenous Kal7 increases ESLCs in several cultured neuronal types (Penzes 2001; Ma 2003; Ma2008), but selective reduction of only Kal7, with preservation of other Kalirin isoforms, has only been examined in hippocampal interneurons (Ma 2008) and now pyramidal neurons. A five-fold drop in Kal7 levels led to a similar decline in Vglut1-positive clusters. Neurons lacking normal levels of Kal7 were unable to respond to bath applied estradiol by increasing ESLC formation. A number of estradiol -responsive genes have been identified in hippocampal neurons; despite this, in the absence of Kal7, hippocampal neurons are unable to form spines/ESLCs in response to estradiol treatment. This result indicates that Kal7 plays an essential role in the pathway(s) through which estradiol stimulates spine/synapse formation. The ability of Kal7^{KO} mice to respond to estradiol has not yet been tested, but synaptic density and PSD size were both reduced in the CA1 pyramidal neurons of adult male Kal7^{KO} mice (Ma et al., 2008a).

Mechanisms through which estradiol regulates synaptic plasticity could contribute to Kal7 actions

Extensive studies on hippocampal estradiol targets and initial studies of pathways involving Kal7 identify several ways in which Kal7 could participate in estradiol-mediated regulation of spines. Increased GluR1 levels and the synaptic insertion of GluR1 cause an increase in spine size (Kopec et al., 2007). Kal7 interacts with GluR1, controls a rapid activity-induced increase of spine size and GluR1 content and an shRNA-mediated decrease in Kalirin expression reduces the GluR1 content of spines (Xie et al., 2007). NMDA receptor activation and expression induced by estradiol are essential for estradiol-mediated spine formation (Woolley and McEwen, 1994; Murphy and Segal, 1996; Smith and McMahon, 2005; Smith and McMahon, 2006; Mukai et al., 2007). Kal7 interacts with and is co-localized with NR1 and NR2b in excitatory synapses. Levels of NR2b, which is required for LTP

induction (Smith and McMahon, 2006), decrease in the Kal7^{KO} hippocampus (Ma et al., 2008a). Estradiol activates CaMKII in hippocampal neurons (Sawai et al., 2002). Activated CaMKII is reported to phosphorylate Kal7, increasing its GEF activity and thus facilitating spine formation (Xie et al. 640-56). NMDAR activation reportedly causes CaMKII-dependent phosphorylation of Kal7, leading to activation of Rac1 and rapid enlargement of existing spines (Xie et al., 2007).

Expression of N-cadherin, which colocalizes with Kal7 at excitatory synapses, is up-regulated by estradiol in CA1 pyramidal neurons (Monks et al., 2001). N-cadherin-dependent spine enlargement requires Kal7 (Xie et al., 2008), suggesting that Kal7 functions downstream of N-cadherin. Phosphorylation of Kal7 by Cdk5 increases its ability to cause spine formation (Xin et al., 2008). Since estradiol increases Cdk5 activity (Sanchez et al., 2009), this pathway may also play a role in estradiol-mediated synaptic plasticity. Consistent with this suggestion, hippocampal Cdk5 levels were decreased in Kal7^{KO} mice (Ma et al., 2008a). For both the CaMKII and Cdk5 pathways, an increase in Kal7 GEF-mediated activation of Rac1 may contribute to altered spine formation (Nakayama et al., 2000; Penzes et al., 2001). Estradiol treatment increases pyramidal neuron levels of PSD95, spinophilin and GluR1 (Akama and McEwen, 2003; Lee et al., 2004; Liu et al., 2008), each of which is known to interact with the PDZ binding motif of Kal7 (Penzes et al., 2001; Xie et al., 2007); interactions of this type could stabilize Kal7 or alter its interactions with other proteins.

CREB activation is involved in estradiol-induced spine formation in hippocampal neurons (Murphy and Segal, 1997). In PC12 cells, Kalirin potentiated the ability of NGF to stimulate phosphorylation of CREB (Chakrabarti et al., 2005). Estradiol-induced spinogenesis is abolished by PD98059, an inhibitor of Erk/MAP kinase (Ogiue-Ikeda et al., 2008) and Kalirin is known to facilitate growth factor mediated activation of MAPK (Chakrabarti et al., 2005). Estradiol-mediated activation of the PI3K/Akt pathway is also involved in regulation of synaptic plasticity (Znamensky et al., 2003) and could have an impact on Kal7 function.

It is not yet clear whether estradiol affects levels of Kal7 mRNA. The promoter regions of the two major full-length isoforms of mouse Kalirin were screened for the presence of estradiol responsive elements (EREs) using MatInspector, TFBIND and Promo(Algen). Based on all three analysis programs, the KalB promoter contains two EREs; one precedes the AUG translational start codon by 570 nts and the other precedes it by 1000 nt. Both sites are conserved in human *KALRN*. While MatInspector identified an ERE 1085 nt upstream of the KalC translational start codon, the other analysis programs did not.

Roles for estradiol in interneurons and pyramidal neurons

Glutamatergic synapses occur on the dendritic spines and dendritic shafts of pyramidal neurons as well as on the dendritic shafts of hippocampal aspiny interneurons (Rao et al., 1998; Ma et al., 2008b). In mature hippocampal pyramidal neurons, almost all Kal7 positive clusters are aligned with Vglut1 positive, excitatory presynaptic terminals; Kal7 clusters never overlap with the GABA_A receptor clusters aligned with GAD65 positive inhibitory presynaptic terminals. In agreement with previous reports (Weiland et al., 1997; Milner et al., 2001; Hart et al., 2001), ER α was observed in the nuclei of hippocampal GABAergic interneurons, but not in pyramidal neuron nuclei. Since ER α has been detected in the axons, axonal terminals and dendritic spines of hippocampal CA1 pyramidal neurons (Milner et al., 2001), genomic and non-genomic contributions from both cell types must be considered (Spencer et al., 2008b; Levin, 2005). ER α immunoreactivity has been reported in the soma/nuclei of CA1 pyramidal neurons (Solum and Handa, 2001; Mukai et al., 2007), perhaps reflecting differences in the antibodies or methodologies. Although a recent *in vivo* study

suggested a role for ER β in the effects of estradiol on spine density and levels of synaptic proteins (Liu et al., 2008), it is clear that ER α plays the primary role (Szymczak et al., 2006; Mukai et al., 2007; Jelks et al., 2007). At least *in vitro*, our inhibitor studies identified ER α as the receptor affecting Kal7 expression. ER β is found in the somal membrane, spines and axon terminals in the CA1 region (Mittra et al., 2003; Milner et al., 2005) and is not found in CA1 interneurons (Milner et al., 2005).

In our hands, estradiol treatment (10nM) decreased GAD65 staining and increased Kal7 staining in cultured hippocampal neurons; expressing exogenous Kal7 also decreased GAD65 staining, raising the possibility that estradiol is acting through or in concert with Kal7. Interestingly, estradiol replacement by injection of OvX rats causes a 3.5 fold decrease in GAD65 levels in the CA1 region (Gazzaley et al., 2002). In hippocampal cultures (Murphy et al., 1998), treatment with a much higher dose of estradiol (370 nM) also caused a decrease in total GAD levels (GAD 65 and GAD67) at 24 h, but this was followed by an increase at 48h. This discrepancy may reflect differences in the estradiol concentrations used, the use of mixed sex vs. female pups for the cultures or the different analytical methods used.

The effects of estradiol on interneurons are thought to account for many of its effects on hippocampal synaptic plasticity (Murphy et al., 1998; Rudick and Woolley, 2001; Hart et al., 2001; Ledoux and Woolley, 2005). CA1 pyramidal neurons are innervated by multiple GABAergic interneurons (Freund and Buzsaki, 1996). On the apical dendrites of CA1 pyramidal neurons, there is a reciprocal relationship between spine number and the number of GABAergic synapses (Papp et al., 2001). Blocking GABA_A receptors with bicuculline caused an increase in the levels of postsynaptic Kal7, which was paralleled by an increase in the number of excitatory synapses onto pyramidal neurons. Papa and Segal previously reported a bicuculline-induced increase in excitatory synapse number in cultured hippocampal neurons (Papa and Segal, 1996). These data suggest that Kal-7 may be a link between estradiol regulation of GABAergic inhibition and dendritic spine density.

The most parsimonious interpretation of our data is that estradiol, acting through ER α in interneurons, turns on expression of Kal7 and decreases GAD65 expression. The resulting decrease in inhibitory tone onto pyramidal neurons results in an increase in pyramidal cell Kal7 expression and excitatory synapse formation. This hypothesis is strongly supported by previous studies (Murphy et al., 1998; Rudick and Woolley, 2001; Hart et al., 2001; Ledoux and Woolley, 2005). In addition, expression of ER α in dendritic spines of CA1 pyramidal neurons suggests that estradiol may directly regulate Kal7 expression in these neurons via non-genomic effects (Milner et al., 2001). Determining the underlying mechanisms and cellular localization of estradiol-mediated Kal7 expression and function will be the focus of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Kal7 levels are regulated by estradiol in Ovx female rats

Adult female rats were ovariectomized and treated with vehicle (Veh)-containing (A, A1) or E2-containing (B, B1) pellets as described in Methods; Ovx occurred 4 months before sacrifice and E2 replacement took place during the final 7 days. Coronal sections taken through the dorsal hippocampus were stained with polyclonal antibody specific to Kal7 and visualized with Cy3-conjugated donkey anti-rabbit IgG. Z-step images (6 images; 1 µm Z-step) from all experimental groups were collected using the same conditions. Pyramidal neurons and interneurons in the stratum radiatum (SR) and stratum oriens (SO) were identified by their locations. Scattered interneurons are marked by arrows. Image analysis and quantification of staining in CA1 pyramidal layer (C), interneurons in the SR and SO of CA1 region (D) and CA1 apical dendrites in the SR (A1-B1, E) were performed with MetaMorph. Student's T test (C, D and E), *P<0.01. SP, stratum pyramidal; IN, interneurons. Scale bar=50 µm. Kal7 levels in hippocampal lysates (25 µg protein) prepared from individual rats subjected to these same experimental manipulations were determined by Western blot analysis with a Kalirin spectrin antibody which detected all major Kalirin isoforms; α -adaptin was used as an internal control (F) because we found that α -adaptin was not regulated by estradiol; Western blot data were quantified using GeneSnap software (Syngene) (G); data shown are the average of duplicates (Ovx+vehicle) or triplicates (Ovx+E2).

Fig. 2. Kal7 is co-localized with ERα in hippocampal interneurons

Coronal sections through the dorsal hippocampus of adult F344 female rats were stained simultaneously for Kal7 (FITC-conjugated donkey anti-rabbit IgG) and ERα (Cy3-conjugated goat anti mouse IgG). ERα staining (fluorescence not saturated) was only found in the nuclei of a subset of interneurons that were strongly positive for Kal7 staining (long arrows, A-C). With longer exposure time, ERα staining became detectable in the nuclei of a second subset of interneurons (short arrows) and Kal7 staining in the first set of interneurons was saturated (long arrow). All staining was eliminated when the primary ERα antibody was replaced with PBS (not shown) and ERα staining was not found in the glia cells in the stratum radiatum (SR), supporting the specificity of ERα staining. Kal7 staining was observed in all interneurons in the CA1 region of the hippocampus (A-C, A1-C1). About half of the 200 Kal7 positive interneurons examined in the SR contained a high level of ERα (long arrows); the other half contained low levels of ERα (short arrows).

Fig. 3. Levels of Kal7 are regulated by estradiol in cultured hippocampal neurons

Primary cultures of female hippocampal neurons (DIV19) were treated with vehicle (**A, D**), 10 nM E2 (**B, E**) or 10 nM E2 plus 1 μ M ICI 182,780, an ER antagonist (**C, F**), for 48h; ICI 182,780 was added 1h before adding E2. Neurons were fixed with 4% paraformaldehyde for staining simultaneously with antibodies to Kal7 (Red) and glutamic acid decarboxylase (GAD65) (Green) as described in Methods. GAD65, a marker for GABA positive interneurons, was mainly localized to nerve terminals. E2 treatment caused an increase in the intensity of Kal7 staining in the processes (arrows) and a decrease in the intensity GAD65 staining along processes; the effects of E2 were blocked by ICI 182,780 (**G**). Kal7 staining in the cell soma was not altered by E2 (**H**). Scale bar: A-C=35 μ m; D-F= 5 μ m. n=15-18 cells. One way analysis of variance (ANOVA) followed by Dunnett's test, * $P<0.05$, # $P<0.01$.

Fig. 4. Estradiol increases the number of ESLCs and Kal7 content of ESLCs in hippocampal neurons

Primary cultures of female hippocampal neurons (DIV21) were treated with vehicle (A), 10 nM E2 (B), ER α agonist PPT (10 nM, C) and ER β agonist DPN (10 nM, D) for 48h. Neurons were fixed with cold methanol for optimal visualization of Kal7 (red), Vglut1 (green) and MAP2 (blue) (A-D). Kal7-Vglut1 positive clusters were taken as a measure of ESLCs and quantified (E); Kal7 staining intensity along dendrites was quantified (F). n=14-16 neurons. One way analysis of variance (ANOVA) followed by Dunnett's test, * P < 0.05.

Fig. 5. Expression of Kal7shRNA decreases the number of excitatory presynaptic terminals Hippocampal neurons were transfected at the time of plating with vectors encoding DsRed-Kal7-UTR shRNA (**A**, **A1**, **C**) or DsRed only (**B**, **B1**, **D**). At DIV 28, neurons were fixed with cold methanol to allow visualization of MAP2 (blue) and Vglut1 (**A-B**, **A1-B1**, green) or MAP2 (blue) and Kal7 (**C-D**, green). The processes of DsRed-expressing neurons were analyzed for quantification. **A1** and **B1**, high power images from boxed dendrites in **A** and **B**, respectively. Vglut1 positive clusters (excitatory presynaptic endings) within 5 μ m along MAP2 positive dendrites were quantified (**E**), as was the level of Kal7 staining (**F**). Student T test ** $p < 0.01$.

Fig. 6. Estradiol cannot increase the number of excitatory presynaptic terminals when Kal7 expression is reduced

Hippocampal neurons prepared from P1 female rat pups were transfected with vectors encoding DsRed alone (A-B) or DsRed-Kal7-UTR shRNA (C-F) at the time of plating. Cultures (DIV20) were treated with vehicle (A, C, E) or 10 nM E2 (B, D, F) for 48h before fixation with cold methanol and visualization of DsRed, Vglut1 (green) and MAP2 (blue). E and F are high power images from C and D, respectively. (G) Vglut1 positive clusters along dendrites were quantified as described; n=12 neurons. One way analysis of variance (ANOVA) followed by Dunnett's test, *P < 0.05.

Fig. 7. Expression of exogenous Kal7 decreases GAD65 levels in hippocampal interneurons
Dissociated hippocampal neurons were transfected with vector encoding GFP alone (A-C, G) or Kal7GFP (D-F, H) at the time of plating. Cultures were visualized simultaneously with antibodies to GFP (green, A, D, G, H) and GAD65 (red, B, E); merged images are shown in C and F. G and H are high power images from selected dendrites in A and D, respectively. Double dashed lines in H outlined dendritic shaft. Arrows pointed GAD65 positive cell soma. GAD65 staining intensity in the soma of hippocampal interneurons was quantified (I); n=12 neurons for GFP control; n=12 for Kal7GFP. Student T test, * P<0.01.

Fig. 8. Kal7 levels at the postsynaptic side of ESLCs are regulated by bicuculline
Cultures of hippocampal neurons (DIV21) were treated with vehicle (Control, A-C) or 20 μ M bicuculline (D-F) for 24 h. Kal7 (red, B, E), Vglut1 (green, C, F) and MAP2 (blue) were visualized simultaneously (A, D). Kal7 staining intensity in dendrites (G) and Kal7-Vglut1 clusters (ESLCs) along dendrites (H) were quantified. Bicuculline treatment caused an increase in both the intensity of Kal7 staining at the postsynaptic side of ESLCs (G) and in the number of Kal7-Vglut1 positive ESLCs (H). n=10-14 neurons. Student T test, $P < 0.05$.

Table 1

Ratio of Kal7 and Vglut1 positive clusters

	Control	E2	PPT	DPN	Control	Bicuculline
Kal7/Vglut1 cluster ratio	0.99± 0.01	1.00± 0.00	1.02± 0.01	1.02± 0.01	1.03± 0.01	0.99± 0.04
Kal7% not aligned with Vglut1 clusters	7.47± 0.47	3.36± 0.34 *	5.77± 0.48	8.08± 0.37	9.34± 0.43	9.53± 0.81
Vglut1% not aligned with Kal7 clusters	8.16± 1.28	3.55± 0.25 *	5.24± 0.67	6.91± 0.85	6.17± 0.086	5.52± 0.29

* p < 0.05 vs. control