Rapid Effects of Estrogen Receptor α and β Selective Agonists on Learning and Dendritic Spines in Female Mice

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Estrogen receptor (ER) agonists rapidly affect neural plasticity within 1 h, suggesting they play a functional role in learning and memory. However, behavioral learning experiments on such a rapid time scale are lacking. Therefore we investigated whether the ERα agonist propyl pyrazole triol (PPT) and ERβ agonist diarylpropionitrile (DPN) could affect social recognition, object recognition, or object placement learning within 40 min of drug administration. At the same time, we examined their effects on CA1 hippocampal dendritic spines. Ovariectomized female CD1 mice were administered a range of PPT or DPN doses (0, 30, 50, 75, or 150 μg/mouse). PPT at the middle doses improved social recognition, facilitated object recognition and placement at a dose of 75 μg, and increased dendritic spine density in the stratum radiatum and lacunosum-moleculare. In contrast, DPN impaired social recognition at higher doses, did not affect object recognition, but slightly facilitated object placement learning at the 75-μg dose. DPN did not affect spines in the stratum radiatum but decreased spine density and increased spine length in the lacunosum-moleculare. This suggests that rapid estrogen-mediated learning enhancements may predominantly be mediated through ERα, while the effects of DPN are weaker and may depend on the learning paradigm. The role of ERα and ERβ in learning and memory may vary depending on the timing of drug administration, as genomic studies often implicate ERβ in enhancing effects on learning and memory. To our knowledge, this is the first report of estrogens' effects on learning within such a short time frame. **(*Endocrinology* 152: 1492–1502, 2011)**

Estrogens affect many physiological and behavioral processes including reproduction, feeding, mood, and learning and memory (see 1). The classical mechanism of action for intranuclear estrogen receptors (ER), ERα and ERβ, is to regulate transcription of target genes, requiring hours to affect protein expression (reviewed in Ref. 2). However, estrogens also have nongenomic actions initiated at the cell membrane that influence cell signaling cascades within minutes (reviewed in Ref. 3). While there are many studies on estrogens' genomic effects, their rapid, nongenomic effects and the functional behavioral implications thereof are not well understood.

The natural estrogen, 17β-estradiol, rapidly modulates cell signaling, synaptic transmission, and dendritic spine density within 1 h of administration. Signaling cascades (4–7) and excitatory transmission (4, 8–10) were enhanced in cultured neurons or hippocampal sections within 30 min of 17β-estradiol or estradiol benzoate application. 17β-estradiol facilitated long-term potentiation (8, 11, but see Ref. 12), affected long-term depression (12, 13), and rapidly increased dendritic spine density and synapse number as quickly as 15 min after drug application, thereby enhancing neuronal connections in brain regions critical for learning and memory (6, 12, 14, 15). Thus estrogens rapidly modulate synaptic plasticity in a way that suggests they play an important role in learning and memory (reviewed in Ref. 16).

Both ERα and ERβ were localized to neuronal cell membranes *in vivo* (17–20) and can rapidly affect synaptic

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1273 Received November 1, 2010. Accepted December 22, 2010.
First Published Online February 1, 2011

Abbreviations: DPN, Diarylpropionitrile; ER, estrogen receptor; IR, investigation ratio; KO, knock out; PPT, propyl pyrazole triol.

plasticity. ERα agonist propyl pyrazole triol (PPT) and ERβ agonist diarylpropionitrile (DPN) both affect cell signaling, synaptic transmission, and long-term depression in hippocampal sections within 1.5 h of application (4, 10, 12). Rapid estrogen-mediated spinogenesis may occur through ERα in the hippocampus, because PPT increased dendritic spines in rat CA1 hippocampal sections, while DPN produced no effect after 2 h (12, 14). However, in cultured cortical neurons, ERβ agonist WAY-200070 rapidly increased dendritic spines within 30 min of application (21). Therefore both ERα and ERβ can mediate at least some of estrogens' rapid effects, and thus may modulate learning and memory functions.

Involvement of estradiol and its receptors in learning and memory have been repeatedly shown (reviewed in Refs. 1, 22) in experiments that typically assess estrogens' effects hours to days after treatment, a time frame consistent with their genomic mechanisms of action. However, rapid behavioral effects have also been reported. Male Japanese quails, rats, and California mice displayed increased sexual behavior or aggression 15–35 min after systemic 17β-estradiol administration (16, 23–25). Enhanced object recognition and spatial memory consolidation have been observed after 17β-estradiol, 17α-estradiol, and a selective ERβ agonist when they are given immediately (but not 45 min or 2 h) after learning acquisition, with assessments of learning effects performed 4 h, 24 h, or 48 h after drug administration (26–32). These studies suggest a role for estrogens in consolidation phases of memory formation. Whether estrogens would affect the acquisition phase of memory in a time frame similar to that of rapid effects observed in neurons or for other behaviors [generally within 1 h (16)] is unknown. Therefore, we modified standard learning paradigms to be completed in 25 min (between 15 min and 40 min after drug injection), when memory maintenance is transcription independent (33–35). We chose the social recognition, object recognition and object placement paradigms because they are spontaneous (*i.e.*, do not require previous training) and assess different memory systems (social information processing, item recognition, and spatial, respectively), whose underlying neuroanatomical mechanisms do not completely overlap (36–38).

Because both ERα and ERβ can rapidly affect neurons, we tested whether ER selective agonists could rapidly affect learning within 40 min of injection. To detect both learning improvements and impairments, we developed "difficult" and "easy" versions of the three learning tasks, respectively. The easy version was only administered when improving effects were not found. In addition, we examined whether PPT and DPN produced dendritic spine changes that were consistent with drug effects on learning within 40 min.

Materials and Methods

Subjects

Subjects were 558 female CD1 mice (*Mus musculus*), purchased ovariectomized at 2 months of age (Charles River, QC), and tested 10–15 d later. An outbred mouse strain was used so results would be more generalizable to other mouse strains. Eighteen stimulus mice were randomly chosen for social recognition paradigms, and 49 animals were used for dendritic spine analyses. Remaining mice were tested in behavioral paradigms. Mice were pair housed, then individually housed for 3d. Subjects were housed with corncob bedding and environmental enrichment in clear polyethylene cages (16 cm × 12 cm × 26 cm), on a 12:12-h reversed light/dark cycle (lights off at 0800 h) and received rodent chow (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, WI) and water *ad libitum*. Ambient temperature was 21 ± 1 C. Research was conducted in accordance with the Canadian Council on Animal Care and approved by University of Guelph's Animal Care and Use Committee.

Animals were moved into the experimental room the night before testing, weighed, and vaginal smears taken and stained with Giemsa (Sigma-Aldrich, ON) to ensure completeness of ovariectomy. All behavioral tests were conducted in home cage during the dark phase of the light cycle under red light illumination.

Drugs

Mice were subcutaneously injected with 10 ml/kg of the selective ERα agonist 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT; Sigma-Aldrich, Oakville, ON, Canada) (39), or ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; Tocris Biosciences, Ellisville, MO) (40). PPT's vehicle was sesame oil. DPN′s vehicle was sesame oil with 2% ethanol. PPT and DPN experiments each included five treatment groups: vehicle, 1 mg/kg, 1.67 mg/kg, 2.5 mg/kg, 5 mg/kg, corresponding to 30 μg, 50 μg, 75 μg, and 150 μg per average 30-g mouse, respectively. Injection site was sealed using Nexcare liquid bandage (3M Canada, London, ON, Canada) to prevent leakage. Drug treatments were assigned using a random number generator.

PPT and DPN most likely produce rapid effects through intracellular and/or membrane bound ERα and ERβ. However, we cannot exclude the possibility that they may activate other putative, not fully characterized membrane ERs such as ER-X (41).

Rapid learning paradigms

Animals were injected with PPT, DPN, or their vehicles 15 min before learning paradigms. Testing was completed 40 min after injection to determine rapid effects of ER agonists on learning (paradigm-specific details below). Two versions of each learning paradigm were developed. The "difficult" paradigm to assess learning enhancements was designed such that control animals would not learn because of limited learning opportunity (*i.e.*, fewer exposures to the stimuli). The "easy" paradigm has greater learning opportunity (*i.e.*, more stimuli exposures) and was designed such that control animals would learn. Difficult paradigms had two habituations followed by test (all 5 min in

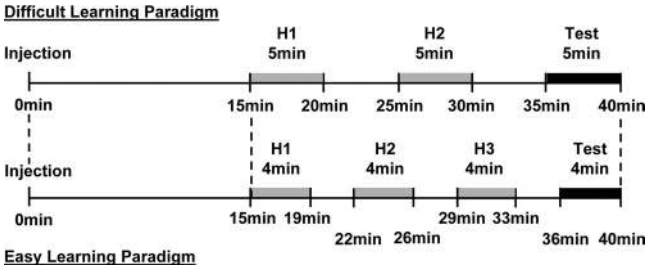

FIG. 1. A comparison of the time line of events for the difficult and easy behavioral paradigms (H indicates habituations). Both learning paradigms begin 15 min after drug injection and are completed within 40 min of injection.

duration), with 5-min intertest intervals (Fig. 1). Easy paradigms had three habituations followed by test (all 4 min in duration), with 3-min intertest intervals (Fig. 1). Even though total habituation time in the easy paradigm is only 2 min longer than in the difficult paradigm, the greater number of habituations elicits enhanced stimulus investigation, thus facilitating learning. Treatment effects were first tested using the difficult paradigm. Whenever improving effects were not observed, the easy paradigm was used with different mice to assess learning impairments. All experiments consisted of a unique set of mice; no mice were tested on more than one learning paradigm. Habituations and tests were video recorded under infrared light (8 mm Handycam Nightshot, Sony, Cambridge, ON, Canada). Between exposures objects (stainless steel drain catchers, glass cubes, plastic hairclips) and cylinders (described below) were washed using odorless detergent and baking soda to remove odor cues such that novelty of the stimuli remained consistent. Objects were held in position using Velcro and removed during intertest intervals. Pilot studies indicated mice did not prefer one type of object to any other.

Social recognition learning paradigm

This paradigm was modified from Choleris *et al.* (44). Stimulus mice (2–4 months old, ovariectomized CD1 mice) were presented to a test mouse in clear Plexiglas cylinders with holes at the bottom, allowing passage of olfactory cues, eliciting high levels of investigation (42–44). During habituations, the test mouse was exposed to the same two stimulus mice in consistent positions within home cage. During test, one of the two stimulus mice was replaced with a novel individual (the mouse that was replaced was counterbalanced). Stimulus mice were replaced with empty Plexiglas cylinders during intertest intervals.

Object recognition learning paradigm

During the difficult paradigm habituations, two different objects were used, while two identical objects were used during habituations in the easy version. (Pilot studies indicated this was necessary to make the paradigm 'easy'.) One of the objects was replaced by a novel object during test (the object replaced was counterbalanced). Objects were held in consistent positions throughout the paradigm.

Object placement learning paradigm

A test mouse was presented with two identical objects in consistent positions (position A and position B) during habituations. During test, one of the two objects was moved to a novel location (position C). The object moved was counterbalanced.

Olfaction test

Mice were weighed, moved into the experimental room, and food deprived the evening before testing (12–14 h) to increase motivation to feed. Mice were administered sesame oil (vehicle; n = 11), 50 μg (n = 11), or 75 μg (n = 9) of PPT. These doses were tested to determine whether learning effects could be explained by changes in olfaction. Mice were given ¼ chocolate chip (Hershey's, approximately 70–80 mg) 15 min after injection to familiarize them with the food item. Forty minutes after injection, the mouse was distracted and ¼ of a chocolate chip was buried in a random location in the bedding of their home cage. Latency to find the chocolate chip was recorded.

Behavioral data analysis

Numbers of mice tested in each treatment group are detailed in investigation duration figure legends. Ten behaviors (listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org/) were recorded for the learning paradigms using The Observer Video Analysis software (Noldus Information Technology, Wageningen, Netherlands) by four trained observers, blind to drug treatment.

Active sniffing of stimuli (nose twitching and within ~1–2 mm of stimulus) was considered indication of investigation (36, 43). Because mice naturally investigate novel or displaced stimuli more than familiar ones (36, 43), stimulus investigation was used to determine whether mice recognized novel or displaced stimulus during test. Therefore, for each mouse an investigation ratio (IR) was calculated: $IR = A/(A + B)$, where A is the investigation duration of the novel or displaced stimulus during test (or during habituations, of the stimulus to be replaced or moved at test), and B is the investigation duration of the other stimulus. Significant increases of investigation ratio from the average investigation ratio over all habituations (IR_{Hab}) to test (IR_{Test}) demonstrated novel stimulus recognition. Mice spending less than 5% of test duration investigating social stimuli (<15 sec in the difficult paradigm, <12 sec in the easy paradigm) or less than 3% of test duration investigating object stimuli (<9 sec for the difficult paradigm, <7 sec for the easy paradigm) were excluded from analysis (~5% of total animals excluded). Individuals that spent <5 sec investigating each of the two stimuli during habituations (~1% of animals) and IR_{Test} outliers (>2 SDs ± mean; <1% of individuals) were also excluded.

Dendritic spine analysis

Golgi-Cox staining followed methods described by Gibb and Kolb (45). Mice were injected with PPT, DPN, or vehicle (numbers per groups are in figure captions) and returned to home cage. Forty minutes after drug injection, as per institutional Animal Care Committee guidelines, animals were sedated with CO_2 then decapitated. To limit potential effects of CO_2 on brain cytoarchitecture, the asphyxiation was performed very rapidly (less than 1 min on average) and similarly for all groups. Brains were quickly extracted, placed into Golgi-Cox solution (1% potassium dichromate, 0.8% potassium monochromate, 1% mercuric chloride), then stored for 3–4 wk in the dark. Brains were placed in 20% sucrose (48 h at 4 C), sectioned (200 μm) using a vibrating microtome (Leica VT1000s, Leica Microsystems, Richmond Hill, ON, Canada), then stored in 6% sucrose (24 h at 4 C). Free-floating sections were placed in 4% paraformaldehyde (15 min), 1% NH_4OH (15 min), 1% Kodak rapid fixative

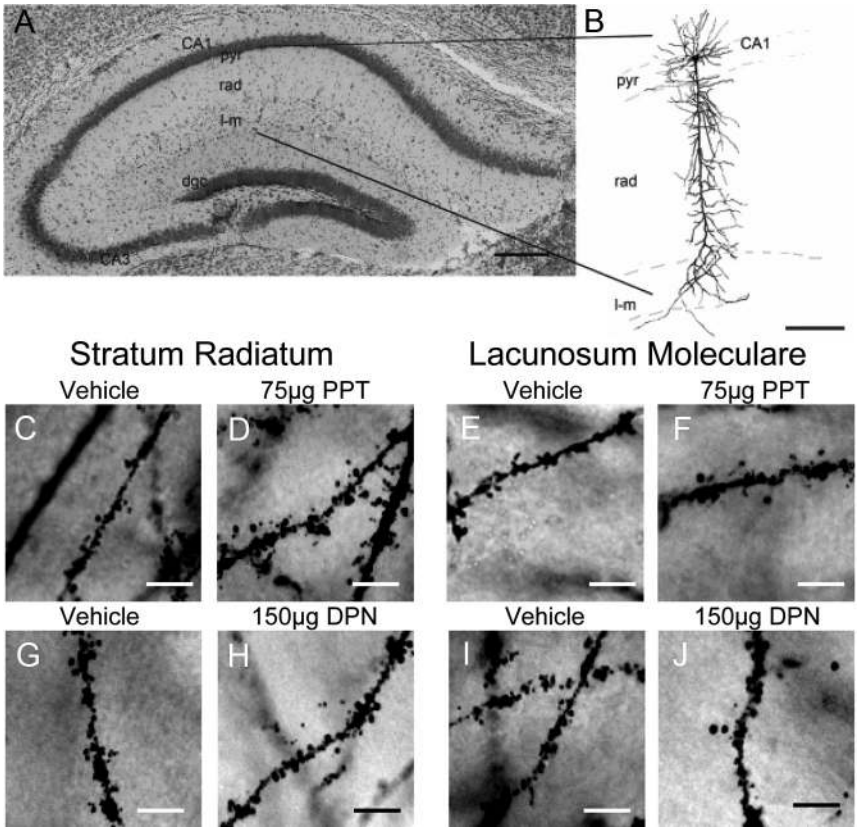

FIG. 2. Images of hippocampus or CA1 hippocampal neurons. Pyr, pyramidal cell layer; rad, stratum radiatum; l-m, lacunosum-moleculare. A, Nissl-stained hippocampus section. *Scale bar*, 400 μm. B, Camera lucida drawing of Golgi stained hippocampal primary neuron. *Scale bar*, 100 μg. C–J, Microscope images of Golgi-stained CA1 secondary dendrites in the stratum radiatum and lacunosum-moleculare from animals treated with PPT vehicle, 75 μg of PPT, DPN vehicle, or 150 μg DPN. *Scale bars*, 5 μm.

(15–60 min), then mounted on gelatin-coated slides. Slides were air-dried at room temperature (1.5–2 h), dehydrated (50%, 70%, 95%, 100% ethanol twice, xylene twice, each for 1 min), and coverslipped with Entallan.

Images of CA1 hippocampal neurons magnified with a ×63 oil objective (Fig. 2 and Supplemental Fig. 1; Axio Imager D1 microscope, captured with AxioCam MRc5 digital camera and AxioVision 4.6 software, Carl Zeiss, Toronto, ON, Canada) and analyzed using Image J software (version 1.38x, National Institutes of Health, Bethesda, MD) by an observer blind to the treatments. Completely stained CA1 pyramidal neurons with intact whole apical dendrites were chosen for analysis. Dendritic spine density and length were measured on apical and distal dendrites in the stratum radiatum and lacunosum-moleculare. Five CA1 neurons were analyzed per animal. Samples from secondary dendrites (>10 μm) between 40–60% (stratum radiatum) and from 80–100% (lacunosum-moleculare) the length of the apical dendrite were analyzed. Spine density was calculated as number of spines per 10 μm of dendrite length per dendritic subregion for each neuron. Spine lengths were measured from the distal tip of the spine head to the edge of the dendrite and calculated as average per dendritic subregion for each neuron.

Statistical analysis

Behavioral data were analyzed with two-way repeated measures ANOVAs with habituation and test as the repeated measures factor and dose treatment as a between groups factor. Specific *a priori* binary mean comparisons were planned to reduce the risk of type I errors. Paired *t* tests within each group assessed whether preference for the novel stimulus changed from habituation (IR_{Hab} expected to be at 0.5) to test (IR_{Test} expected to be greater than 0.5 if learning has occurred). One-way ANOVAs for each dose treatment compared the preference for the novel stimulus at test (IR_{Test}) to that of the control group. For statistical analysis of investigation ratios, data were arcsin-transformed (figures represent original ratio data). The two-way repeated measures ANOVA and planned comparisons were performed for all experiments. For brevity, only significant values are reported. Latency data for olfactory test was analyzed with a one-way ANOVA. One-way ANOVAs and Student-Newman-Keuls *post hoc* tests were used to analyze differences in dendritic spine density and average spine length, setting statistical significance at $P < 0.05$. For all analyses SigmaStat version 3.5, (Systat Software, Inc., Chicago, IL) was used.

Results

ERα agonist PPT

PPT improved social recognition learning at 50 μg and 75 μg, and slightly improved object recognition and placement learning at the 75 μg dose, within 40 min of injection (Fig. 3). Because PPT improved learning on all three tasks, easy versions of these learning paradigms (used to detect learning impairments) were not tested. PPT treatment did not enhance olfaction in the chocolate chip test (Supplemental Fig. 2). PPT also significantly increased dendritic spine density in the CA1 hippocampal lacunosum-moleculare (75 μg PPT) and stratum radiatum (50 μg and 75 μg PPT; Fig. 4, A and B).

There were significant main effects of PPT treatment ($F_{4,59} = 3.12$, $P < 0.05$) and test number ($F_{1,59} = 48.48$, $P < 0.001$) on social recognition IRs as well as a significant interaction of the main factors ($F_{4,59} = 4.56$, $P < 0.01$). For the social recognition paradigm, planned comparisons indicated a significant main effect of PPT treatment on IR_{Test} ($F_{4,59} = 4.17$, $P < 0.01$). *Post hoc* analysis revealed IR_{Test} values for 50 μg and 75 μg PPT were significantly higher than vehicle (50 μg: q = 4.17, $df = 25$, $P < 0.05$, 75 μg: q = 3.45, $df = 28$, $P < 0.05$). In addition IR_{Test} was significantly higher than IR_{Hab} for groups treated with 50 μg (t = 4.70, $df = 8$, $P < 0.01$) and 75 μg of PPT (t = 7.09, $df = 13$, $P < 0.001$; Fig. 3A). No other groups, including

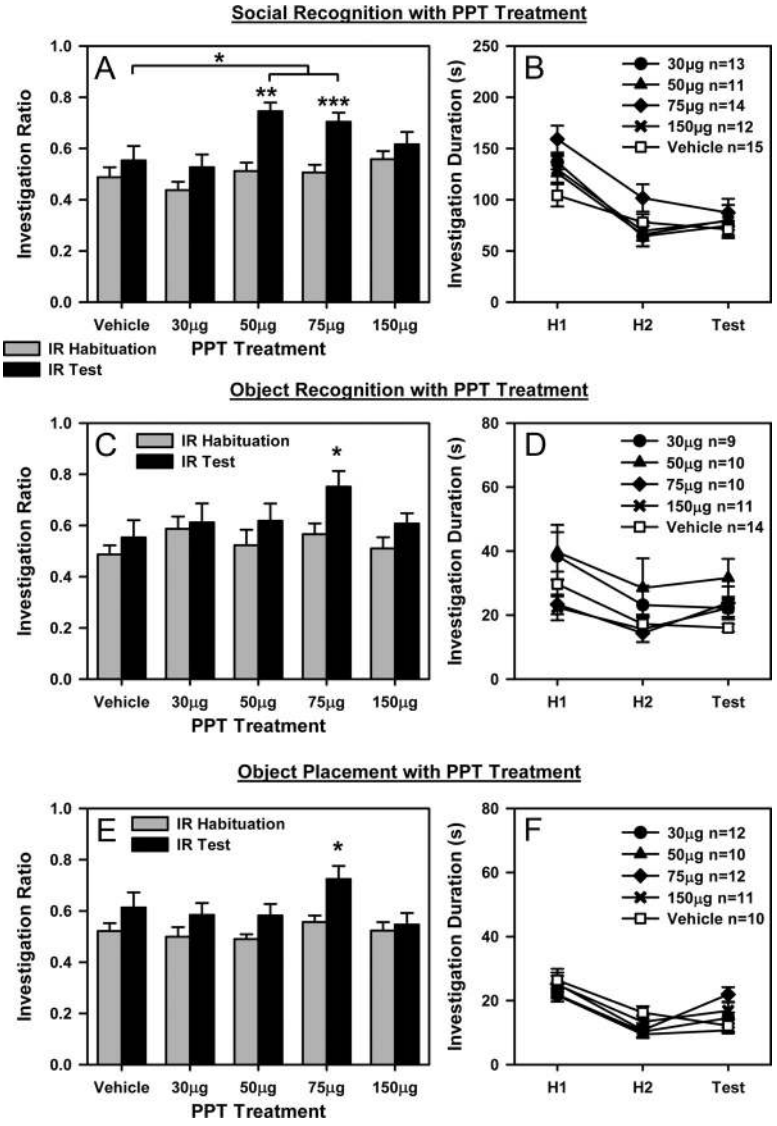

FIG. 3. Rapid effects of PPT on learning paradigms. Asterisks above *black bars* in A, C, and E represent a significant difference between the investigation ratio (IR) at habituation *vs.* the IR at test for the treatment. A, PPT at doses of 50 μg and 75 μg significantly improved social recognition above vehicle-treated animals (indicated by an asterisk above lines over the 50 μg and 75 μg PPT and vehicle controls). B, Total investigation times during the social recognition experiment were not affected by PPT treatment. C, The group receiving 75 μg of PPT was able to successfully perform the object recognition task. D, Total investigation durations for the object recognition experiment did not differ with PPT treatment. E, Mice administered 75 μg of PPT were successfully able to perform the object placement task. F, PPT treatment did not significantly affect total investigation durations for the object placement experiment. Means and SE are depicted. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vehicle controls, demonstrated a significant difference between IR_{Hab} and IR_{Test}. All other behaviors analyzed, including total investigation times (Fig. 3B), revealed no significant effects of treatment.

Planned comparisons in the object recognition and object placement paradigms indicated the 75-μg PPT group exhibited object and place recognition, because IR_{Test} was significantly increased from IR_{Hab} (object recognition: $t = 2.70$, $df = 9$, $P < 0.05$, Fig. 3C; object placement: $t = 2.82$, $df = 11$, $P < 0.05$, Fig. 3E). No other experimental group, including vehicle controls, demonstrated object recognition. PPT did not affect total investigation times (Fig. 3, D and F) or other behaviors recorded.

For all PPT learning paradigms, there was a significant main effect of test number for total investigation durations (all $P < 0.001$). *Post hoc* analyses revealed significant differences between habituation 1 and habituation 2 (all $P < 0.001$), as well as habituation 1 and test (all $P < 0.001$), indicating that animals habituated to stimuli (Fig. 3, B, D and E), as normally observed in these paradigms (22).

Administration of 50 μg or 75 μg PPT did not affect their latencies to find a buried chocolate chip in the olfaction test (Supplemental Fig. 2).

There was a significant main effect of PPT treatment on dendritic spine density in the lacunosum-moleculare ($F_{4,115} = 3.22$, $P < 0.05$) and stratum radiatum ($F_{4,115} = 3.10$, $df = 4$, $P < 0.05$). *Post hoc* analysis revealed PPT at 50 μg and 75 μg increased stratum radiatum spine density compared with vehicle (50 μg: $q = 4.22$, $df = 43$, $P < 0.05$, 75 μg: $q = 4.16$, $df = 48$, $P < 0.05$; Figs. 2, C and D, and 4A and Supplemental Fig. 3A). Dendritic spine density in the lacunosum-moleculare increased significantly with 75 μg of PPT compared with vehicle ($q = 4.60$, $df = 48$, $P < 0.05$; Figs. 2, E and F, and 4B, and Supplemental Fig. 3B). PPT did not affect dendritic spine length (Fig. 4, C and D and Supplemental Fig. 3, E and F).

ERβ agonist DPN

DPN at 75 μg slightly improved object placement learning (Fig. 7) but did not improve social recognition or object recognition in the difficult versions of these tasks (Figs. 5A and 6A). Therefore, we tested whether DPN impaired social and object recognition using the easy versions of these paradigms. We found that DPN slightly impaired social recognition at higher doses (75 μg and 150 μg; Fig. 5C) but did not impair object recognition (Fig. 6C). DPN did not affect spines in the CA1 stratum radiatum but decreased spine density (50 μg and 150 μg) and increased spine lengths at 30 μg in the lacunosum-moleculare (Fig. 8).

No treatment groups demonstrated social recognition in the difficult social recognition paradigm, because IR_{Test}

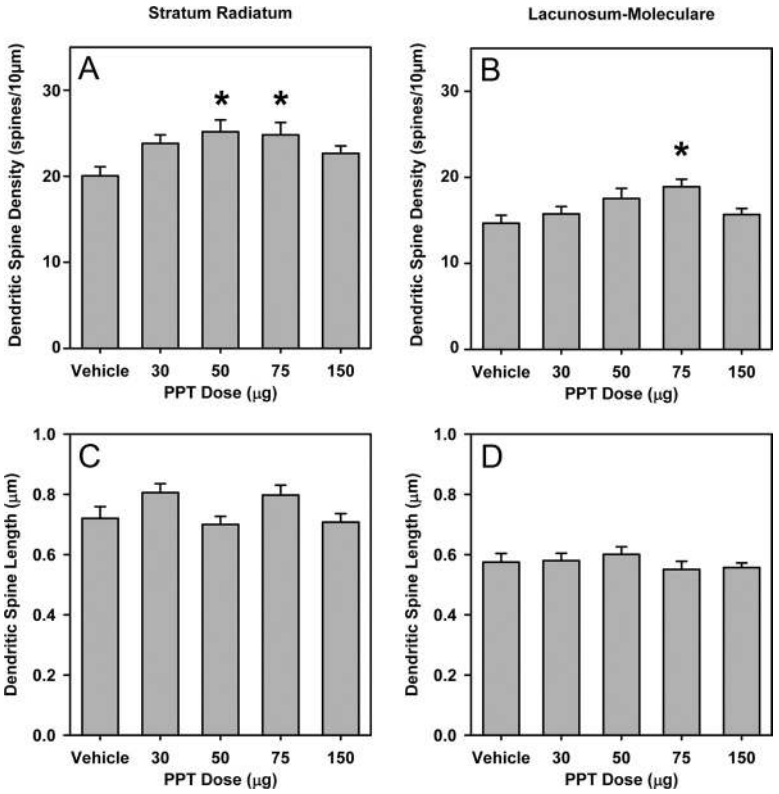

FIG. 4. Rapid effects of PPT on dendritic spine density and length in the CA1 hippocampus. All groups contain measures of 25 neurons from five animals, except the 50 μg PPT group which contain measures of 20 neurons from four animals. A, PPT at 50 μg and 75 μg increased spine density in the stratum radiatum. B, PPT at a dose of 75 μg increased spine density in the lacunosum-moleculare. C, PPT does not affect spine lengths in the stratum radiatum. D, Spine lengths in the lacunosum-moleculare are also not affected by PPT treatment. Means and SE are depicted. *, $P < 0.05$.

and IR_{Hab} were not significantly different (Fig. 5A). In the easy social recognition paradigm, planned comparisons revealed vehicle, 30 μg, and 50 μg DPN groups exhibited social recognition learning as IR_{Test} was significantly higher than IR_{Hab} (vehicle: $t = 2.65$, $df = 9$, $P < 0.05$, 30 μg: $t = 4.10$, $df = 8$, $P < 0.01$, 50 μg: $t = 2.94$, $df = 11$, $P < 0.05$; Fig. 5C). However, 75 μg and 150 μg did not demonstrate learning on this task. There was no effect of DPN treatment on any other behavior recorded, including total investigation times, during either of these experiments (Fig. 5, B and D). Hence, while DPN did not improve social recognition, it slightly impaired it at higher doses.

No groups displayed object recognition learning in the difficult object recognition paradigm (Fig. 6A). All groups exhibited learning in the easy object recognition paradigm, showing significantly higher IR_{Test} than IR_{Hab} (vehicle: $t = 2.50$, $df = 11$, $P < 0.05$, 30 μg: $t = 4.44$, $df = 11$, $P < 0.001$, 50 μg: $t = 3.43$, $df = 11$, $P < 0.01$, 75 μg: $t = 3.73$, $df = 10$, $P < 0.01$, 150 μg: $t = 4.42$, $df = 11$, $P = 0.001$; Fig. 6C). Therefore, DPN did not rapidly modulate object recognition learning. Again, DPN did not significantly affect total investigation times (Fig. 6, B and D) or other behaviors during these two experiments.

Only animals treated with 75 μg of DPN demonstrated object placement learning in the difficult object placement task, as planned comparisons revealed IR_{Test} was significantly higher than IR_{Hab} for this group ($t = 3.37$, $df = 11$, $P < 0.01$; Fig. 7A). Therefore, DPN at 75 μg slightly facilitated object placement learning. There was no significant difference in total investigation times (Fig. 7B) or other behaviors recorded, except there was a significant main effect of treatment on horizontal exploration durations ($F = 2.78$, $df = 4$, $P < 0.05$). *Post hoc* analyses revealed that the 50 μg DPN group had significantly lower horizontal exploration times than the vehicle group ($q = 4.10$, $df = 23$, $P < 0.05$). However, these differences in horizontal exploration were not paralleled by changes on object placement performance.

For all DPN difficult and easy learning paradigms, there was a significant main effect of test number for total investigation durations (all $P < 0.001$). *Post hoc* analyses revealed there were significant differences between habituation 1 and habituation 2 (all $P < 0.001$), habituation 1 and habituation 3 in the easy learning paradigms (all $P < 0.001$), as well as habituation 1 and test (all $P < 0.001$; Figs. 5, B and D, 6, B and D, and 7B) indicating normal habituation of the test animal to stimuli (22). In the easy object recognition paradigm, there was also a significant increase in investigation durations at test when compared with habituation 2 and to habituation 3 (all $P < 0.001$), indicating a dishabituation caused by the novel object during test (22). This dishabituation was not seen in the easy social recognition paradigm, because greater habituation may be necessary to reliably see dishabituation, as in the original social recognition protocol (22).

DPN treatment did not significantly affect spines in the stratum radiatum (Fig. 8, A and C, and Supplemental Fig. 3, C and G). However, there were significant main effects of DPN on spine density ($F_{4,120} = 5.78$, $P < 0.001$) and length ($F_{4,120} = 3.742$, $P < 0.01$) in the lacunosum-moleculare. *Post hoc* analysis revealed mice treated with 50 μg or 150 μg of DPN had fewer spines compared with vehicle controls (50 μg: $q = 4.49$, $df = 48$, $P < 0.01$, 150 μg: $q = 4.73$, $df = 48$, $P < 0.01$; Fig. 8B and Supplemental Fig. 3D). Injection of 30 μg of DPN increased dendritic spine length in the lacunosum-moleculare ($q = 4.11$, $df = 48$, $P < 0.05$; Fig. 8D and Supplemental Fig. 3H).

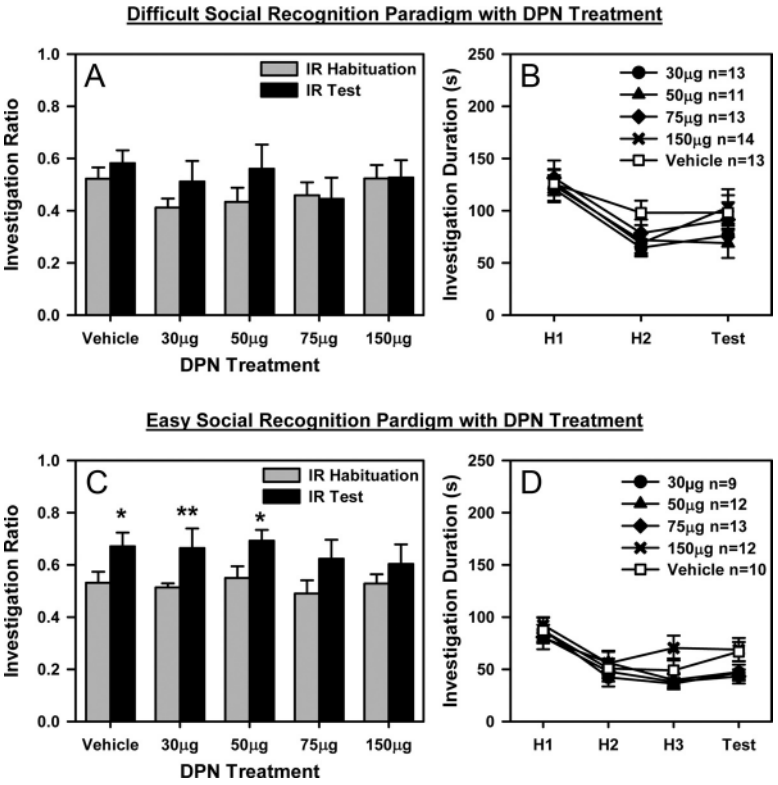

FIG. 5. Rapid effects of DPN on social recognition. Asterisks above *black bars* in A and C represent a significant difference between the investigation ratio (IR) at habituation *vs.* the IR at test for the treatment. A, DPN treatment had no improving effects on social recognition as assessed by the difficult social recognition task. B, Treatment with DPN also did not affect total investigation durations for the difficult social recognition experiment. C, Animals treated with vehicle, 30 μg, or 50 μg of DPN were able to successfully recognize the novel conspecific at test in the easy version of the social recognition paradigm. D, Investigation durations during the easy social recognition experiment were not affected by DPN treatment. Means and SE are depicted. *, $P < 0.05$; **, $P < 0.01$.

Discussion

Rapid effects via ERα

ERα activation through PPT treatment improved social recognition at doses of 50 μg or 75 μg within 40 min of administration (Fig. 3A). Only groups administered 75 μg of PPT exhibited object recognition and placement learning (Fig. 3, C and E). Therefore, 75 μg of PPT may facilitate novel object and novel place recognition. These results appear specific to learning, as PPT treatment did not significantly affect any other behavior recorded from these mice (*e.g.*, horizontal exploration, rearing, total time spent sniffing stimuli), nor did it affect their olfactory capabilities (Supplemental Fig. 2). It also seems unlikely that drug treatments increased interest in novelty *per se*, because PPT did not increase investigation times during the first habituation session when both stimuli were novel (Fig. 3, B, D, and F).

Overall, effects of PPT appear greater for social recognition than for object recognition or placement. This may be attributable to greater salience of social stimuli, leading to enhanced learning about the stimuli (22). This explanation is supported by the fact that investigation durations were approximately doubled for stimulus mice compared with object stimuli in all groups (Fig. 3, B, D, and F). Because PPT rapidly improved performance on all three behavioral paradigms, rapid ERα activation may improve learning in general. Because we did not examine whether PPT impaired learning using the easy paradigms, we cannot exclude the possibility that higher doses of PPT may impair performance on these tasks.

Our results with PPT are consistent with previous studies reporting 17β-estradiol and 17α-estradiol enhance object recognition and placement memory consolidation when administered immediately (but not 45 min or 2 h) after the learning trial, when effects were assessed 4–48 h after drug administration (28–32). To our knowledge, this is the first study including the acquisition phase in PPT's rapid effects. The timing of our experiments (40 min after drug injection and within 25 min of acquisition during the transcription-independent phase of learning; 33–35) suggests that PPT affects early memory mechanisms. Our recent data indicate that, like PPT, 17β-estradiol improves performance on all three learning paradigms described here (46), suggesting that estradiol's rapid effects during this timeframe may predominantly be mediated through ERα.

Within 40 min of treatment, PPT (50 μg or 75 μg) increased dendritic spine density in the CA1 stratum radiatum, while 75 μg PPT increased spine density in the CA1 lacunosum-moleculare (Fig. 4, A and B). PPT was reported to increase spine density 2 h after drug application in hippocampal slices from male rats (12, 14). In cultured cortical neurons, increases in spine density occurred after 15 min of incubation with 17β-estradiol (6), suggesting that estrogens may affect spine density within an even shorter time frame than the 40 min used here. At the same time, the later effects observed in hippocampal sections (2 h) (12, 14) suggest that PPT's enhancing effects on dendritic spines may be of sufficient duration to mediate the effects on learning observed in the present study. Increases in spine density may reflect an increase in the number of synaptic connections (47) and therefore provide new sites at which learning can occur.

To the best of our knowledge, these data provide the most rapid *in vivo* evidence for a PPT effect on dendritic spines and a PPT dose response relationship. Interestingly, both learning and dendritic spine experiments demon-

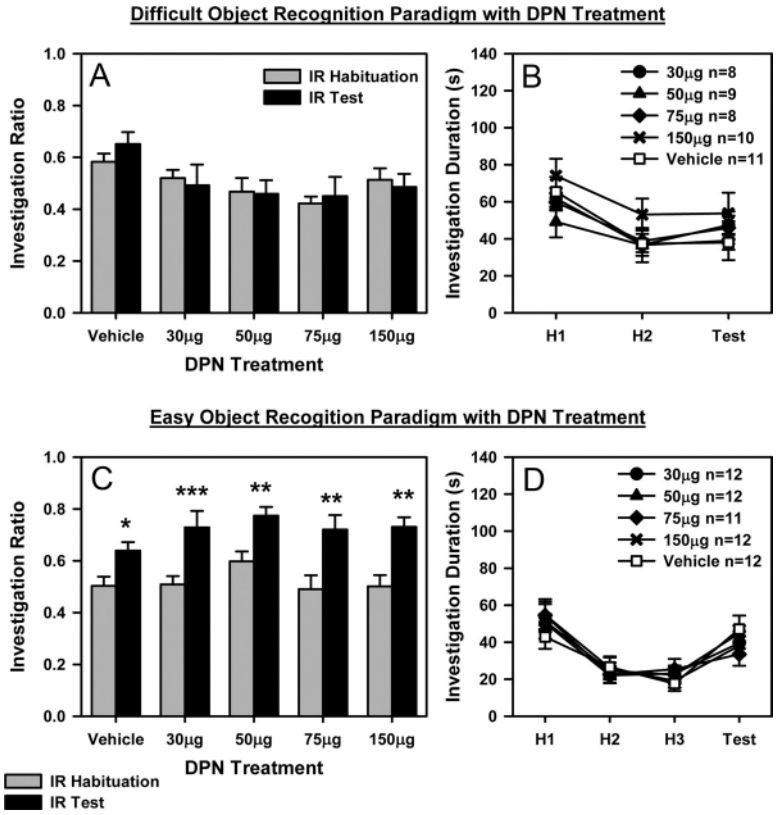

FIG. 6. Rapid effects of DPN on object recognition. Asterisks above *black bars* in A and C represent a significant difference between the investigation ratio (IR) at habituation *vs.* IR at test for the treatment group. A, DPN treatment did not rapidly improve object recognition in the difficult version of this paradigm. B, DPN treatment also did not affect the total investigation durations during the difficult object recognition experiment. C, In the easy object recognition experiment, all groups (vehicle, 30 μg, 50 μg, 75 μg, and 150 μg of DPN) were able to successfully perform the task. D, Investigation durations for the easy object recognition experiment were not affected by DPN treatment. Means and SE are depicted. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

strated PPT effects at middle doses of 50 μg and 75 μg, but not at lower or higher doses forming an inverted U-shaped dose-response curve, which has also been reported for PPT's longer term responses (*e.g.*, Ref. 48). Thus, both in terms of drug dose and timing, ERα-mediated rapid learning improvements parallel those of ERα-mediated increases in dendritic spine density.

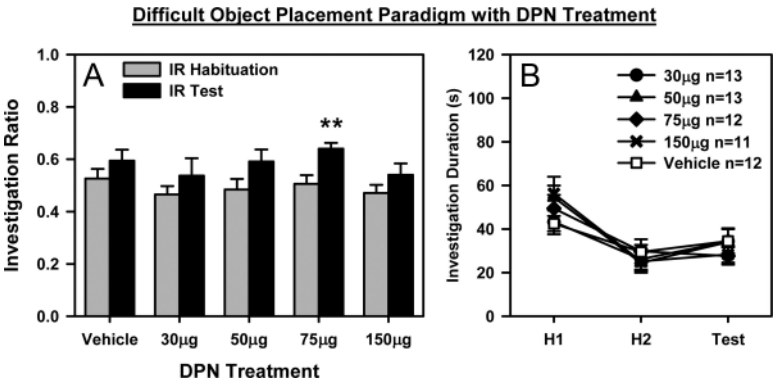

FIG. 7. Rapid effects of DPN on object placement. Asterisks above *black bars* in A represent a significant difference between the investigation ratio (IR) at habituation *vs.* IR at test for the treatment group. A, The group administered 75 μg of DPN was able to successfully distinguish the novel object placement in the difficult version of the object placement task. B, DPN treatment did not affect total investigation durations during the object placement experiment. Means and SE are depicted. **, $P < 0.01$.

Rapid effects via ERβ

Unlike ERα, rapid ERβ activation had mixed effects on learning. ERβ agonist DPN did not facilitate social recognition learning at any dose (Fig. 5A). However, when tested using the easy social recognition paradigm, animals treated with vehicle or 30 μg or 50 μg of DPN exhibited social recognition learning, while those administered 75 μg or 150 μg of DPN did not (Fig. 5C). This may indicate that DPN at higher doses impairs social recognition. Conversely, treatment with DPN neither improved nor impaired object recognition learning (Fig. 6, A and C), while it facilitated object placement at a dose of 75 μg (Fig. 7A). All treatment groups, including vehicle controls, failed to demonstrate object placement learning, with the exception of those animals treated with 75 μg of DPN (Fig. 7A). Thus, the middle dose of DPN enhanced object placement learning, indicating a possible inverted U-shaped dose-response curve similar to that reported for the long-term effects of another ERβ agonist (48). As with PPT, it seems unlikely that DPN's effects are attributable to changes in interest in novelty *per se*, because investigation durations were unaffected during the first habituation, when all stimuli were novel (Fig. 7B). Also, they do not seem to be secondary to changes in other behaviors, because DPN treatment did not significantly affect other recorded behaviors. This effect of DPN is unlikely to be attributable to changes in olfaction because preacquisition DPN and WAY 200070 did not impair performance on olfactory-based tasks (48). Therefore, the small improvement of DPN at 75 μg in the object placement paradigm suggests a specific role for ERβ in spatial learning, while higher doses of DPN may impair social recognition.

DPN did not rapidly affect dendritic spines in the CA1 stratum radiatum, but it decreased spine density in the CA1 lacunosum-moleculare at middle and higher doses (50 μg and 150 μg; Fig. 8B) and increased lacunosum-molecu-

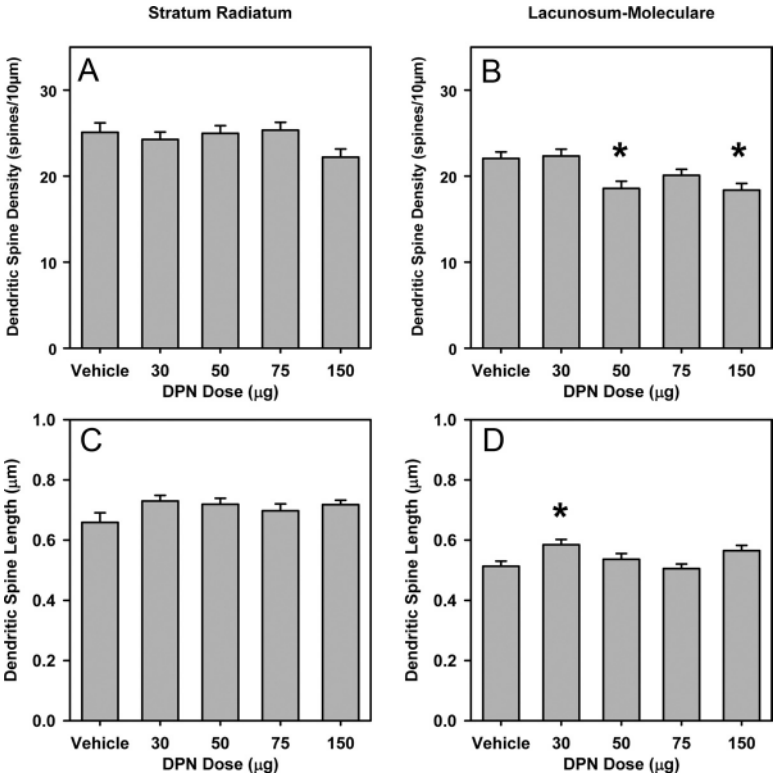

FIG. 8. Rapid effects of DPN on dendritic spine density and length in the CA1 hippocampus. All groups contain measures of 25 neurons from five animals. A, DPN did not affect dendritic spine density in the stratum radiatum. B, Treatment with 50 μg and 150 μg of DPN decreased spine density in the lacunosum-moleculare. C, DPN treatment did not significantly affect spine lengths in the stratum radiatum. D, Treatment with 30 μg of DPN significantly increased spine lengths in the lacunosum-moleculare. Means and SE are depicted. *, $P < 0.05$.

lare spine length at the lowest drug dose (30 μg; Fig. 8D). There does not appear to be any parallel between this increased spine length and performance on learning paradigms, because groups of mice treated with 30 μg of DPN did not demonstrate learning effects. Similarly, the decrease in dendritic spine density caused by 50 μg and 150 μg of DPN does not seem to coincide generally with any learning effects. It seems as though DPN′s effects on spine density and length may be more complicated than those of PPT. The ERβ agonist WAY-200070 rapidly increased spines in cultured cortical neurons (21), whereas DPN did not rapidly affect male rat CA1 hippocampal dendritic spines (12, 14). Thus, the roles of ERα and ERβ in rapid spinogenesis may differ depending on the brain region, the species, or sex of the subjects. Clearly, literature on rapid ERβ-mediated dendritic spine changes and their consequences for learning is incomplete and further investigations are needed.

Rapid *vs.* long-term effects

Rapid effects of PPT appear to be very different than those of DPN. Whereas PPT slightly improved performance on all three learning paradigms, DPN′s learning effects were weaker and depended on the type of learning paradigm used. PPT also increased hippocampal spine density, whereas DPN either had no effect or decreased spine density. These differences resulting from selective ERα and ERβ activation are not surprising, because differences in behavioral effects are commonly seen for PPT and DPN′s long-term effects (reviewed in Refs. 1, 22). Studies with knock out (KO) mice showed ERαKO mice were completely impaired in social recognition whereas ERβKO mice, while impaired compared with wild-type controls (43, 44), could still distinguish a novel from a familiar conspecific (44). These KO studies and present results suggest a greater role for ERα than ERβ in social recognition. In general, however, long-term experiments implicate ERβ in learning and memory improvements, while ERα activation generally has no effect or impairs learning and memory (1, 49, 50). Thus the respective role of ERα and ERβ in rapid *vs.* longer-term effects on learning and memory may be different.

Previous work on rapid effects of 17β-estradiol or ER agonists on memory consolidation appears consistent with long-term effects of estrogens. Postacquisition 17β-estradiol and DPN administration to wild-type but not ERβ KO mice enhanced both object recognition and placement memory consolidation when tested 4 h or 48 h after drug administration (29, 31, 32). Conversely, postacquisition PPT did not improve memory consolidation in animals tested 48 h after administration (32). Our study is the first to include learning acquisition when examining the rapid effects of ER agonists and to test animals during a time when memory is transcription-independent (33–35). Our results suggest the role of ERα and ERβ rapid effects may be different during different stages of memory processing.

The long-term effects of ERα and ERβ agonists on dendritic spine density and synapses are inconsistent. PPT, but not DPN, increased synapses 48 h after application to hippocampal cultures (51, 52; although DPN produced a trend towards an increase in 51), while selective ERβ agonist WAY-200070 increased hippocampal spine density of shorter, mushroom-type spines 48 h after drug administration (49). Increases in mushroom spines are consistent with observations that ERβ tends to improve learning and memory in longer-term behavioral studies (1, 53, 54). Our results and Kawato's group reported PPT (but not DPN) rapidly increases spine density in the CA1 hippocampus (12, 14), mimicking the rapid effects of 17β-estradiol on

spine density (6, 12, 14, 15, 47). This rapid effect of PPT increased thin and filopodia spine types (12, 14), thought to be sites at which new memories are formed and stored (53, 54). Therefore, while ERα seems to mediate estradiol's rapid effects on hippocampal spinogenesis, both ERα and ERβ may be involved in estradiol's longer-term effects.

Conclusions

To the best of our knowledge, this is the first report demonstrating *in vivo* effects of estrogen receptor agonists on CA1 dendritic spines and learning only 40 min after drug injection. This time frame is consistent with the rapid effects of estrogens on neuronal electrophysiology and cell signaling mechanisms. Our results suggests both ERα and ERβ activation can rapidly influence learning and synaptic connections but that effects are dependent on the estrogen receptor and the type of learning studied. At the time point studied, ERα seems to have a greater role in promoting estrogen-mediated enhancements in learning and memory processes and CA1 hippocampal dendritic spine increases than ERβ.

Studies on rapid learning effects of estradiol and ER agonists may help to explain the often inconsistent literature on estradiol's learning and memory effects (reviewed in Ref. 1), because physiologically, estradiol would exert rapid and genomic effects at the same time (3). It has been hypothesized that differential activation of ERα and ERβ, their involvement in different types of learning, different stages in memory processing, could all contribute to estrogen effects on learning and memory (1). Our study suggests that timing of estrogen exposure (short- *vs*. long-term) and the specific cellular mechanisms involved (nongenomic, genomic, and their interactions; 3) may also be important determinants of learning effects.

Acknowledgments

We thank Skot Koshowski, Alexandra Muller, Dana Munroe, and Dima Saab for their help with data collection.

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This work was supported by Natural Sciences and Engineering Research Council of Canada Grants 400212 and 197293. J.N.A. was the recipient of a National Alliance for Research on Schizophrenia and Depression Young Investigator Award.

Disclosure Summary: The authors have nothing to declare.

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