Prostaglandin E₂-Induced Masculinization of Brain and Behavior Requires Protein Kinase A, AMPA/Kainate, and Metabotropic Glutamate Receptor Signaling

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Prostaglandin E₂ (PGE₂) mediates the masculinization of adult sex behavior in rats in response to the surge in serum testosterone at approximately birth. Measures of behavioral masculinization correlate with a twofold increase in spinophilin protein and the density of dendritic spines in the medial preoptic area (POA). Of the four receptors for PGE₂, EP₂ and EP₄ are required for the masculinization of behavior by PGE₂. EP₂ and EP₄ couple to G_s-proteins, activating protein kinase A (PKA). By using H89 (*N*-[2-(*p*-bromo-cinnamylamino)-ethyl]-5-isoquinoline-sulfonamide 2HCl) and Ht31, disruptors of PKA signaling, we have determined that PKA signaling is required for the masculinization of behavior by PGE₂. Glutamatergic signaling often mediates PGE₂ signaling; therefore, we tested whether inhibition of AMPA/kainate and metabotropic glutamate receptor (mGluR) signaling prevents PGE₂-induced behavioral masculinization and whether activation of glutamate receptors mimics PGE₂. Females treated neonatally with NBQX (2,3-dihydroxy-6-nitro-7-sulfonylbenzo[*f*]quinoxaline) plus LY341495 [(2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid] combined (AMPA/kainate and mGluR inhibitors, respectively) before PGE₂ did not exhibit as many mounts or intromission-like behaviors or initiate these behaviors as quickly as animals treated with PGE₂ alone. Animals neonatally treated with kainate, (±)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) (type I mGluR agonist), or the two combined mounted as frequently and initiated mounting behavior as quickly as those given PGE₂. Ht31 does not prevent the masculinization of behavior by ACPD plus kainate cotreatment; rather, the coadministration of NBQX plus LY341495 prevents the forskolin-induced formation of POA dendritic spine-like processes. We conclude that PKA, AMPA/kainate, and metabotropic glutamate receptor signaling are necessary for the effects of PGE₂, that each receptor individually suffices to organize behavior, and that PKA is upstream of the glutamate receptors.

Introduction

The behavioral components of mating are not only the most robust examples of sexual dimorphism in adulthood but also best represent how developmental exposure to gonadal steroids organizes behavior and the attendant neural systems. In adulthood, gonadal hormones activate the neuronal architecture differentiated during development to elicit the appropriate sex-specific behavioral response (for review, see Morris et al., 2004; McCarthy, 2008). Referred to as the sexual differentiation of the brain, these early organizational processes are initiated in males by testicular synthesis of testosterone, which gains access to the brain and is converted to its end product, estradiol, by the p450_{sc} enzyme cyp19 (Naftolin et al., 1975a; McEwen et al., 1977; Wersinger et al., 1997). The masculinizing effects of both testosterone and estradiol are particularly pronounced in the rat medial preoptic area (mPOA), a brain region critical for the dis-

play of male sexual behavior (Hillarp et al., 1954; Larsson and Heimer, 1964; Fernández-Guasti et al., 1992; Markowski et al., 1994; Meisel and Sachs, 1994; Powell et al., 2003). We have determined recently that estradiol-induced prostaglandin (PG) E₂ (PGE₂) production is an essential step in the masculinization of the brain and sex behavior (Amateau and McCarthy, 2002, 2004).

Prostaglandins are membrane-derived signaling molecules synthesized from arachidonic acid by the cyclooxygenase (COX-1 and COX-2) enzymes and a class of specific terminal synthases to produce PGE₂, PGF₂-α, PGD₂, PGI₂, and thromboxane A₂. Neonatal males have higher levels of both COX-1 and COX-2 in the preoptic area (Wright, 2009), and PGE₂ is increased up to sevenfold by an estradiol-induced upregulation of the cyclooxygenase enzymes (Amateau and McCarthy, 2004). PGE₂ induces a twofold increase in the density of mPOA dendritic spines and levels of spinophilin, a protein enriched in and integral to the functioning of dendritic spines (Amateau and McCarthy, 2002, 2004). PGE₂ signaling is propagated by four G-protein-coupled receptors, EP₁–EP₄ (Sugimoto and Narumiya, 2007). All four EP receptors are expressed in the developing POA, but only two, EP₂ and EP₄, have been found essential for the organization of male sexual behavior in response to PGE₂ (Wright et al., 2008). EP₂ and EP₄ couple to G_s-proteins, activate adenylyl cyclase, increase

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intracellular cAMP concentrations, and recruit protein kinase A (PKA), leading to phosphorylation of serine or threonine protein substrates (Honda et al., 1993; Regan et al., 1994).

Because EP₂ and EP₄ are critical for behavioral masculinization induced by PGE₂ and link to PKA and because AMPA/kainate but not NMDA receptors in part mediate PGE₂-induced dendritic spine formation in the POA (Amateau and McCarthy, 2002), we hypothesized that PKA and AMPA/kainate receptors and metabotropic glutamate receptors (mGluRs) mediate a serial pathway downstream of PGE₂ that results in the masculinization of brain and behavior. We have tested this by assessing adult male sexual behavior in females in which perinatal masculinization was either induced by neonatal treatment with glutamate receptor agonists or prevented by coadministration of glutamate receptor antagonists or Ht31, a disruptor of PKA signaling, before PGE₂. We report here that PKA signaling is necessary for the profound effects of neonatal PGE₂ exposure on both neonatal POA spinophilin protein and adult male rat sexual behavior and that these effects are mediated by the downstream signaling of AMPA/kainate and type I or II metabotropic glutamate receptors.

Materials and Methods

Animals and intracerebroventricular injections. All procedures used in these studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Animals were kept under a reverse 12 h light/dark cycle and allowed *ad libitum* access to food and water. Pregnant Sprague Dawley dams mated in our facility or ordered from Charles River Laboratories 2 weeks before birth were allowed to deliver normally. Female pups were treated on the day of birth (DOB) and/or postnatal day 1 (P1). Bilateral intracerebroventricular injections were performed under cryoanesthesia and were made 1 mm caudal to bregma and 1 mm lateral to the midline over each hemisphere. A 23 gauge 1 μ l beveled Hamilton syringe attached to a stereotaxic manipulator was lowered 3.0 mm into the brain and then backed out 1 mm before infusion of 1 μ l of drug or vehicle over 60 s. The process was then repeated for the other hemisphere. Injections were not given directly into the POA to avoid tissue damage. Ht31, control protein (CP), PGE₂, H89 (N-[2-(*p*-bromo-cinnamylamino)-ethyl]-5-isoquinoline-sulfonamide 2HCl), and appropriate vehicles were injected intracerebroventricularly; all others were injected subcutaneously.

Treatment with Ht31, H89, and glutamate receptor agonists and antagonists. Ht31 and a CP were obtained from Promega, and 7 nmol were injected intracerebroventricularly 45–60 min before injection of either 2.5 μ g of PGE₂ intracerebroventricularly (Sigma) or 0.9% w/v PBS vehicle (VEH) intracerebroventricularly or subcutaneously depending on the experiment. Both the Ht31 and CP are steared to promote cellular uptake; however, the CP differs from the Ht31 by having a proline substitution in the midpoint of the peptide that disrupts the formation of α -helix secondary structure and prevents binding to PKA. The PKA inhibitor H89 (2 nmol; Sigma) was injected intracerebroventricularly 45–60 min before PGE₂. Kainate (2.5 μ g), the AMPA/kainate antagonist 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX) (1 μ g), the type I and II mGluR agonist ($\pm$)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) (2.28 μ g), and the type I–III mGluR antagonist LY341495 [(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid] (4.65 μ g) (Tocris Bioscience) were injected subcutaneously in 0.1 ml of 0.9% w/v saline 45 min after the intracerebroventricular injection of 2.5 μ g of PGE₂, 0.9% w/v PBS vehicle, or Ht31. Doses were based on those found previously to be effective (Todd et al., 2007).

Surgery. Animals used for behavioral testing were reared to P50, gonadectomized under ketamine/acepromazine anesthesia (75 and 2.5 mg/kg, respectively) and implanted with a SILASTIC capsule 1.58 mm inner diameter, 3.18 mm outer diameter $\times$ 30 mm containing crystalline testosterone, producing adult levels of testosterone (Amateau and McCarthy, 2004).

Behavioral testing. Beginning at P60–P65 animals were tested for male sexual behavior as described previously (Amateau and McCarthy, 2004). In brief, animals received three weekly 20 min tests after a 10 min acclimation period to the testing arena (49 cm length $\times$ 37 cm width $\times$ 24 cm height) during the dark phase of the light cycle and under red-light illumination. Testing began with the addition to the arena of a hormonally primed receptive female (10 μ g of estradiol benzoate in 0.1 ml of sesame oil 1 and 2 d before testing and 0.75 mg of progesterone in 0.15 ml of sesame oil 4 h before testing). Frequencies of mounts and intromission-like behaviors and latencies to first mount and first intromission-like behavior were quantified. A mount was counted when the subject placed both forepaws on the haunches of the stimulus female and thrust against her. A mount could include an intromission-like behavior. An intromission-like behavior was counted only when the subject made pelvic contact with the genitals of the stimulus female by quickly shifting its weight, engaging in extreme flexion of the haunches, and then reflexively disengaging. The mean latency to mount, mean latency to intromission-like behavior, mean number of mounts, and mean number of intromission-like behaviors were generated by averaging the respective measures across all three trials.

Microdissection of POA. Animals were killed by CO₂ inhalation, and brains removed and placed in a Zivic Miller brain block, dorsal surface down. The rostral and caudal boundaries of the optic chiasm were used as margins for making a coronal section containing the POA. For adult brains, a 2 mm coronal section was made, and for neonates, a 1 mm section was made. The anterior commissure defined the dorsal boundary of the POA. The isolated tissue was frozen on dry ice and stored at -80°C until use.

Western blot immunoblotting. Tissue was homogenized in a radioimmunoprecipitation assay buffer containing 1% Igepal CA630 (Sigma), 0.25% deoxycholic acid (Sigma), 1 mM EDTA, 154 mM NaCl, and 65 mM Trizma Base (Sigma) containing protease and phosphatase inhibitors (1:1000 each; Sigma) and was subjected to Western blot analysis as described previously (Amateau and McCarthy, 2002). In short, protein supernatant was extracted after a 3000 rpm centrifugation with a 6.5–7.0 cm radius at 4°C for 30 min, and protein concentration was standardized by a Bradford assay. Ten micrograms of protein was then electrophoresed on a precast 8–16% SDS polyacrylamide gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% nonfat milk in 0.1% Tween-supplemented Tris-buffered saline. Antisera to spinophilin (1:1000; Millipore Corporation) was then applied, followed by anti-rabbit secondary antibody (1:3000). A Phototope chemiluminescence system (New England Biolabs) was used to detect the 110 kDa spinophilin immunoblot by exposing the membrane to Hyperfilm-ECL (GE Healthcare). Integrative grayscale pixel area densitometry captured with a CCD camera was quantified with NIH Image or ImageJ software. Ponceau or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) staining were used for loading controls depending on the experiment. For GAPDH, the membrane was stripped, reprobed with primary (1:100,000) or anti-mouse secondary (1:10,000) antibodies, and underwent the same chemiluminescent detection system and quantification methods outlined above.

POA neuronal culture. The POA was microdissected from female pups on the day of birth, placed into 2 ml of HBSS with EDTA, and trypsinized with 250 μ l of 0.25% trypsin (Invitrogen) for 15 min at 37°C . The supernatant was removed, and cells were washed twice, once with 2 ml of HBSS and again with cell culture media consisting of DMEM/F-12 (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 4% horse serum, and 1% antibiotics/antimycotics (Invitrogen); 2 ml of cell culture media was then added to the tissue. The POA cells were then dispersed under gentle trituration through a Pasteur pipette, and 450,000 cells were plated on 2.5 cm round poly-lysine pretreated glass coverslips. Cell culture media (2 ml) was added to each 3.5 cm Petri dish (BD Biosciences) containing one coverslip and were allowed to acclimate for 24 h before treatment.

Immunocytochemistry for microtubule-associated protein-2. Coverslips with adhered POA cells were fixed with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 min and then placed into 0.5 M potassium/PBS (KPBS) until use. Coverslips were washed three times in KPBS, and cells

were permeabilized with 50% ethanol for 1 h and blocked with 10% normal goat serum in 0.1% Triton X-100-supplemented KPBS (0.1% KPBST). After three 15 min KPBS washes, coverslipped cells were then incubated overnight in primary anti-microtubule-associated protein-2 (MAP-2) monoclonal antibody (Sigma) at 1:1000 dilution in 0.1% KPBST at 4°C. Three washes of 0.1% KPBST was then followed by immersion for 2 h in secondary anti-mouse antibody conjugated to biotin (Vector Laboratories) diluted 1:500 in 0.1% KPBST, washed in 0.1% KPBST, and visualized using Vectastain avidin–biotin horseradish peroxidase complex (Vector Laboratories) at 1:1000 dilution in 0.1% KPBST for 1 h before staining with 0.05% (w/v) 3,3'-diaminobenzidine tetrachloride (Sigma), 0.005% (v/v) H₂O₂, and 2.5% (w/v) nickel (II) sulfate in 0.175 M sodium acetate. MAP-2 protein is not localized in dendritic spines but allows for visualization of the neuron in culture and for accurate quantification of dendritic spine-like processes attributable to sufficient diffusion of the reaction product from the neurites into the spine-like process. MAP-2 immunocytochemistry does not stain astrocytes.

Experiment 1: effect of neonatal treatment with H89 on neonatal POA spinophilin protein induction by PGE₂ exposure. On the DOB, animals were treated with VEH + PGE₂ ($n = 6$), H89 + VEH ($n = 5$), VEH + VEH ($n = 4$), or H89 + PGE₂ ($n = 5$). On P3, animals were killed, and the POA were microdissected, flash frozen, and stored at -80°C until Western blot analysis for spinophilin protein.

Experiment 2: effect of neonatal treatment with Ht31 on neonatal POA spinophilin protein induction by PGE₂ exposure. On the DOB, animals were treated with PGE₂ ($n = 6$), VEH ($n = 7$), Ht31 + VEH ($n = 5$), CP + VEH ($n = 4$), or Ht31 + PGE₂ ($n = 5$). POAs were collected and analyzed in a manner identical to experiment 1.

Experiment 3: effect of neonatal treatment with Ht31 on the organization of adult male sexual behavior by PGE₂ exposure. We used Ht31 instead of H89 to neonatally disrupt PGE₂-induced behavioral masculinization to further implicate PKA bound to A-kinase anchoring protein (AKAP) because Ht31 is a 24 aa peptide that specifically disrupts AKAP/PKA binding. On the DOB, animals were treated with CP + PGE₂ ($n = 10$), CP + VEH ($n = 9$), Ht31 + VEH ($n = 8$), or Ht31 + PGE₂ ($n = 8$). Between P45 and P50, animals were ovariectomized and implanted with testosterone-releasing SILASTIC capsules. On P60, P67, and P74, animals were assessed for male sexual behavior.

Experiment 4: effect of neonatal treatment with NBQX and/or LY341495 on PGE₂-induced masculinization of adult sexual behavior. On the DOB, female pups were injected with VEH + PGE₂ ($n = 8$), VEH + VEH ($n = 9$), NBQX + PGE₂ ($n = 10$), LY341495 + PGE₂ ($n = 8$), NBQX + LY341495 + PGE₂ ($n = 7$), or NBQX + LY341495 + VEH ($n = 5$). Animals were raised to adulthood and tested in the same manner as in experiment 3.

Experiment 5: effect of neonatal treatment of kainate and/or ACPD on the masculinization of sexual behavior. On the DOB and P1, female pups were injected with PGE₂ + VEH ($n = 8$), VEH + VEH ($n = 8$), VEH + kainate ($n = 9$), VEH + ACPD ($n = 7$), or VEH + kainate + ACPD ($n = 8$). Six females were given Ht31 intracerebroventricularly and then kainate + ACPD 45–60 min later. Animals were raised to adulthood and tested in the same manner as in experiments 3 and 4.

Experiment 6: effect of the adenylyl cyclase stimulator forskolin, and NBQX and LY341495 on dendritic spine density in vitro. Cultures were treated for 48 h with 10 nM PGE₂ ($n = 5$), vehicle ($n = 6$), forskolin ($n = 6$), 10 μM NBQX and 50 μM LY341495 ($n = 5$), or 10 μM forskolin combined with 10 μM NBQX and 50 μM LY341495 ($n = 6$), followed by immunocytochemistry for MAP-2. Ten to 11 individual neurons from each coverslip were visualized under 100 $\times$ magnification on a Nikon Eclipse E600 microscope attached to a MicroBrightField DX9000 camera and a computer running Neurolucida version 9 (MicroBrightField). Neurons were chosen for characterization, and parameters were quantified as described previously (Amateau and McCarthy, 2002); in short, these parameters included the size of the cell body, length of the primary neurite from the cell body to the farthest tip, and number of branches and spine-like processes, defined as any protrusion from the neurite $<5\ \mu\text{m}$ long.

Statistics. One-way ANOVA was used to assess data generated by Western blot and immunocytochemistry analyses, and two-way mixed

Figure 1. Inhibition of PKA blocks PGE₂-induction of spinophilin in the POA. **A**, PGE₂ treatment significantly increased neonatal POA spinophilin protein levels almost twofold over VEH + VEH, whereas treatment with the PKA inhibitor H89 + PGE₂ or H89 + VEH had no effect on neonatal spinophilin levels compared with vehicle (* $p < 0.05$ compared with all other groups, ANOVA; $n = 4–6$; error bars indicate SEM). **B**, Western immunoblot for spinophilin (above) and Ponceau-stained proteins (below) in which each lane is tissue from one animal representing the group mean.

ANOVAs with trial as a repeated measure were used for behavioral data. Tukey's honestly significant difference (HSD) tests were used for all *post hoc* analyses between treatment groups, and Dunnett's was used for comparison with controls (when stated) with a $p < 0.05$ to conclude significant differences.

Results

Experiment 1: the PKA inhibitor H89 blocks PGE₂-induced spinophilin in the neonatal POA

There was an overall effect of treatment on neonatal POA spinophilin protein levels ($F_{(3,24)} = 13.9$, $p < 0.001$) (Fig. 1). Consistent with previous findings, VEH + PGE₂ treatment significantly increased spinophilin protein above VEH + VEH controls, but in animals coadministered H89 + PGE₂ or H89 + VEH, spinophilin levels were equivalent to VEH + VEH controls and significantly lower than VEH + PGE₂ ($p < 0.05$ by Tukey's test).

Experiment 2: AKAP inhibitor Ht31 also blocks PGE₂-induced spinophilin

PGE₂ again significantly increased neonatal POA spinophilin ($F_{(4,21)} = 6.43$, $p < 0.001$) (Fig. 2), but in animals coadministered Ht31 + PGE₂, levels did not increase above VEH-treated controls and were significantly lower than after PGE₂ treatment alone ($p < 0.05$). There was no effect of Ht31 treatment alone or CP on spinophilin protein compared with VEH treatment.

Experiment 3: Ht31 treatment blocks PGE₂-induced masculinization of adult sexual behavior

In tests of adult male sexual behavior, there was an overall effect of neonatal treatment on the latency to mount ($F_{(3,34)} = 20.33$, $p < 0.0001$), the latency to intromission-like behavior ($F_{(3,34)} = 4.32$, $p = 0.011$), the number of mounts ($F_{(3,34)} = 19.29$, $p < 0.0001$), and the number of intromission-like behaviors ($F_{(3,34)} = 3.08$, $p = 0.042$). Specifically, animals treated neonatally

Figure 2. The PKA/AKAP binding inhibitor Ht31 blocks PGE₂ induction of spinophilin in the POA. PGE₂ treatment significantly increased neonatal POA spinophilin protein levels almost twofold over VEH, whereas treatment with Ht31 + PGE₂, Ht31 alone, or control protein had no effect on neonatal spinophilin levels compared with vehicle (* $p < 0.05$ compared with all other groups, ANOVA; $n = 4–6$; error bars indicate SEM).

with Ht31 + PGE₂ exhibited longer latencies to mount than animals treated with CP + PGE₂ ($p < 0.05$) (Fig. 3A), such that their latencies were equivalent to those of animals treated with CP + VEH and Ht31 + VEH. The same pattern was seen in the latency to intromission-like behavior ($p < 0.05$) (Fig. 3B). Animals treated with Ht31 + PGE₂ also mounted the sexually receptive female significantly less often than CP + PGE₂-treated animals ($p < 0.05$) (Fig. 3C), and measures were equivalent to those of CP + VEH-treated and Ht31 + VEH-treated animals. CP + PGE₂-treated animals also exhibited intromission-like behaviors more often than all other animals [$p < 0.05$ by Tukey's least significant difference (LSD) test] (Fig. 3D).

Experiment 4: combined neonatal antagonism of AMPA/kainate receptors and mGluRs blocks PGE₂-induced masculinization of adult sexual behavior

There was an overall effect of neonatal treatment on the latency to mount (Fig. 4A) ($F_{(5,41)} = 19.1$, $p < 0.001$), the latency to intromission-like behavior (Fig. 4B) ($F_{(5,41)} = 10.9$, $p < 0.001$), the number of mounts (Fig. 4C) ($F_{(5,41)} = 24.9$, $p < 0.001$), and the number of intromission-like behaviors (Fig. 4D) ($F_{(5,41)} = 7.14$, $p < 0.001$). Animals neonatally treated with NBQX + LY341495 before PGE₂ exhibited fewer mounting and intromission-like behaviors and longer latencies to onset of both behaviors compared with animals treated with VEH + PGE₂ ($p < 0.05$ by Tukey's HSD test). All four measures were equivalent to those from animals treated with either VEH + VEH or NBQX + LY341495 without PGE₂, demonstrating that combined antagonism of AMPA/kainate and metabotropic glutamate receptors resulted in a complete block of PGE₂-induced masculinization. In contrast, animals treated neonatally with either NBQX + PGE₂ or LY341495 + PGE₂ exhibited mounting and intromission-like behaviors as frequently and initiated these behaviors as quickly as the animals treated with VEH + PGE₂, such that the number of mounts and latency to onset differed significantly from those of VEH + VEH-treated, NBQX + LY341495-treated, or NBQX + LY341495 + PGE₂-treated animals ($p < 0.05$ by Tukey's HSD test), demonstrating that the antagonism of either AMPA/kainate or metabotropic glutamate receptors alone is incapable of blocking PGE₂-induced masculinization.

Figure 3. Effect of neonatal coadministration of Ht31 and PGE₂ on measures of male sexual behavior. The mean $\pm$ SEM of the latency to mount (A), latency to intromission-like behavior (B), number of mounts (C), and number of intromission-like behaviors (D) collapsed across three behavioral trials. Females treated neonatally with Ht31 + PGE₂ exhibited diminished male sexual behavior compared with those treated with CP + PGE₂, and their behavior was on par with VEH + VEH-treated and Ht31 + VEH-treated females (* $p < 0.05$ compared with all others by Tukey's HSD test for numbers of mounts and latency measures and Tukey's LSD test for number of intromission-like behaviors, repeated measures ANOVAs; $n = 8–10$).

Experiment 5: stimulation of either AMPA/kainate or mGluR receptors neonatally mimics the masculinizing effects of PGE₂ on adult sex behavior

There was a significant effect of neonatal treatment with glutamate agonists ACPD and kainate on the adult latency to mount (Fig. 5A) ($F = 6.47$, $p < 0.001$), the latency to intromission-like behavior (Fig. 5B) ($F = 2.73$, $p < 0.05$), the number of mounts (Fig. 5C) ($F = 8.65$, $p < 0.001$), and the number of intromission-like behaviors (Fig. 5D) ($F = 4.35$, $p < 0.003$). Animals neonatally treated with VEH + kainate initiated mounting behavior more quickly ($p < 0.05$ by Dunnett's test) and trended toward increased number of mounts ($p = 0.05$) compared with VEH + VEH-treated animals. Similarly, animals treated with VEH + ACPD exhibited mounting and intromission-like behaviors more often and initiated these behaviors more quickly than VEH + VEH-treated animals ($p < 0.05$ by Dunnett's test). Animals treated with VEH + ACPD + kainate mounted more often and initiated mounting more quickly than VEH + VEH-treated animals ($p < 0.05$).

To determine whether PGE₂/EP receptor signaling activated PKA to recruit glutamate receptors to masculinize behavior versus a scenario in which signaling of PGE₂/EP receptors influences glutamate receptors to activate PKA which then masculinizes behavior, Ht31 was administered 45 min before VEH + kainate + ACPD. We predict that, if PKA is downstream of glutamate receptors, then neonatal administration of Ht31 would prevent the organization of male sexual behavior in response to VEH + kainate + ACPD treatment. Animals neonatally treated with Ht31 before VEH + kainate + ACPD initiated mounting and intromission-like behavior as quickly as animals treated with VEH + kainate + ACPD or PGE₂ + VEH. Moreover, animals treated with Ht31 + kainate + ACPD exhibited shorter latencies to mount than those of animals treated with VEH + VEH ($p < 0.05$ by Tukey's HSD test). The animals treated with Ht31 + kainate + ACPD also mounted as often as the animals treated with VEH + kainate + ACPD or PGE₂ + VEH but significantly more often than the VEH + VEH-treated animals ($p < 0.05$ by Tukey's HSD test). There were no differences in the number of intromission-like behaviors or the latencies to onset this behavior in animals treated with Ht31 + kainate + ACPD compared with those treated with VEH + VEH, PGE₂ + VEH, or VEH + kainate + ACPD. We, there-

Figure 4. Effect of neonatal administration of inhibitors for the AMPA/kainate and metabotropic glutamate receptors before PGE₂ on measures of adult male sexual behavior. The mean $\pm$ SEM of the latency to mount (A), latency to intromission-like behavior (B), number of mounts (C), and number of intromission-like behaviors (D) collapsed across three behavioral trials. Neonatal females coadministered inhibitors of AMPA/kainate and metabotropic glutamate receptors (NBQX and LY341495, respectively) before PGE₂ did not exhibit mounting or intromission-like behaviors as often or initiate mounting and intromission-like behaviors as quickly as animals treated with VEH + PGE₂ ($*p < 0.05$ by Tukey's HSD test), demonstrating a complete block of PGE₂-induced masculinization by combined antagonism of AMPA/kainate and metabotropic glutamate receptors. In contrast, masculinized females neonatally treated with only one glutamate receptor inhibitor before PGE₂ expressed high levels of sexual behavior in adulthood ($*p < 0.05$ by Tukey's HSD test, repeated measures ANOVAs; $n = 5-10$).

Figure 5. Effect of neonatal administration of agonists for AMPA/kainate or metabotropic glutamate receptors (kainate and ACPD, respectively) on measures of adult male sexual behavior. The mean $\pm$ SEM of the latency to mount (A), latency to intromission-like behaviors (B), number of mounts (C), and number of intromission-like behaviors (D) collapsed across three behavioral trials ($n = 7-9$). Neonatal activation of either the AMPA/kainate or metabotropic glutamate receptors masculinizes sex behavior because neonatal females treated with at least one glutamate receptor agonist, such as VEH + kainate, VEH + ACPD, or VEH + kainate + ACPD, exhibited male sexual behavior in adulthood on par with those of VEH + PGE₂-treated females ($*p < 0.05$, § trend by Dunnett's test). Females treated neonatally with Ht31 before kainate + ACPD did not exhibit any reductions in the number of mounting or intromission-like behavior, nor did they exhibit longer latencies to initiate these behaviors when compared with measures from VEH + kainate + ACPD ($*p < 0.05$ by Tukey's HSD test, repeated measures ANOVAs; $n = 7-9$), demonstrating that PKA signaling is not downstream of the signaling of the glutamate receptors.

Figure 6. Effect of forskolin, NBQX, and LY341495 on the formation of dendritic spine-like processes on POA cultured neurons. Forskolin and PGE₂ treatment both induced a threefold increase in the number of dendritic spine-like processes per neurite (**A**) and per unit length of neurite (**B**) compared with vehicle treated, whereas cotreatment of NBQX and LY341495 with forskolin prevented the increase such that these measures were equivalent to those of vehicle-treated cells. Photomicrographs of neurites and dendritic spine-like processes after MAP-2 immunocytochemistry representing the means of the following treatment conditions: PGE₂ (**C**), vehicle (**D**), forskolin (**E**), forskolin + NBQX + LY341495 (**F**), and NBQX + LY341495 (**G**).

fore, conclude that glutamate receptor activation is downstream from PKA activation.

Experiment 6: activating PKA induces spine-like processes on neurites of cultured POA neurons, and this effect is blocked by antagonism of AMPA/kainate or mGluR

To further test whether AMPA/kainate and metabotropic glutamate receptor signaling is downstream of PKA, we used a stimulator of adenylyl cyclase, forskolin, to activate PKA directly in cultured POA neurons to test (1) whether forskolin treatment would mimic PGE₂ to initiate dendritic spine formation and (2) whether NBQX and LY341495 would prevent the forskolin-induced dendritic spine formation. The expression of male sex behavior correlates with the formation of POA dendritic spines, making it an excellent inferential proxy for behavioral masculinization. We observed significant effects of treatment on both the number of dendritic spine-like processes per neurite ($F_{(4,27)} = 34.63$, $p < 0.0001$) (Fig. 6A) and per length of neurite ($F_{(4,27)} = 25.41$, $p < 0.0001$) (Fig. 6B). Replicating previous findings, we observed that POA cultured neurons treated with PGE₂ (Fig. 6C) have a 3.5-fold increase in the number of dendritic spine-like processes per neurite and per length of neurite compared with vehicle treated (Fig. 6D) ($p < 0.01$). Treatment with forskolin mimicked PGE₂ treatment such that neurons had an increase in both the number of dendritic spine-like processes per neurite and per length of neurite compared with vehicle treatment (Fig. 6E) ($p < 0.01$). Neurons from POA cultures coadministered forskolin, NBQX + LY341495 (Fig. 6F), or NBQX + LY341495 (Fig. 6G) did not have increased dendritic spine-like processes per neurite or per length of neurite, such that these measures were equivalent to vehicle treated and lower than measures from either the PGE₂-treated or the forskolin-treated neurons ($p < 0.001$).

There was no effect of treatment on mean soma size or the number of neurites per neuron. There was an effect on the average length of the primary neurite ($F_{(4,27)} = 3.2$, $p = 0.032$); neurites were ~16% shorter if cultures were treated with NBQX + LY341495 regardless of whether forskolin was also administered ($p < 0.05$ by Tukey's LSD test) (Table 1).

Discussion

Our previous observation that PGE₂ mediates estradiol-induced masculinization of sexual behavior in the rat was incomplete in the absence of any information about the signaling pathways through which PGE₂ was acting. The PGE₂ receptors EP₂ and EP₄ are both critical for PGE₂-mediated masculinization (Wright et al., 2008). These receptors can couple to G_s-proteins, activate adenylyl cyclase, and recruit PKA signaling. PKA signaling is localized to discrete subcellular microdomains by AKAPs that sequester PKA coupled to the regulatory-2 subunits (type II PKA) (Rubin, 1994; Hausken et al., 1996). By binding to PKA, AKAPs restrict the phosphorylation of PKA targets to macromolecular signaling complexes and allow for specific and diverse signaling pathways within a cell (Michel and Scott, 2002). Using a disruptor of PKA signaling,

Ht31, and a pharmacological inhibitor, H89, we have now determined that the signaling of this kinase and downstream glutamate receptors are the critical determinants of PGE₂-induced masculinization of brain and behavior. PKA can be displaced from macromolecular complexes when it comes into contact with a small mimetic analog of AKAP, Ht31, because it contains the PKA-binding domain of AKAP but does not include the motifs that allow AKAP to localize to these macromolecular complexes (Hausken et al., 1996). Thus, Ht31 not only inhibits AKAP/PKA binding but it also prevents the phosphorylation of specific targets within a cell (Ruehr et al., 2004). AKAP-79/150 and PKA are enriched in dendritic spines (Snyder et al., 2005) and are implicated in membrane trafficking of AMPA receptors by regulation of receptor subunit phosphorylation (Snyder et al., 2005; Dell'Acqua et al., 2006). Females neonatally treated with Ht31 before PGE₂ failed to express male sexual behavior, whereas females treated with a control peptide before PGE₂ exhibited increased mounts, intromission-like behaviors, and shorter latencies to onset of both behaviors (Amateau and McCarthy, 2004). PKA signaling is not only necessary for organization of behavior but also the induction of POA dendritic spines by PGE₂ because treatment with either Ht31 or H89 prevented the increase in POA spinophilin by PGE₂. Moreover, forskolin, a stimulator of PKA, mimicked PGE₂ by increasing the density of dendritic spine-like processes on POA neurons threefold to fourfold. Dendritic spine formation and POA spinophilin levels correlate positively and strongly with measures of male rat sexual behavior, including in individual animals (Amateau and McCarthy, 2004; Wright et al., 2008). The induction of dendritic spine-like processes by forskolin is consistent with the hypothesis that the signaling of PKA

Table 1. No effect of treatment on several measures of neuronal morphology, including soma area of neurons and the number of neurites per neuron

	PGE ₂	Vehicle	Forskolin	Forskolin + NBQX + LY341495	NBQX + LY341495
Mean ± SEM soma area (μm ²)	50.42 ± 0.96	65.97 ± 2.77	59.73 ± 7.77	58.62 ± 5.60	62.99 ± 3.90
Mean ± SEM number of neurites per neuron	2.34 ± 0.108	2.14 ± 0.132	2.62 ± 0.170	2.48 ± 0.153	2.51 ± 0.046
Mean ± SEM neurite length (μm)	64.34 ± 5.05	64.73 ± 2.10	61.44 ± 3.45	53.25 ± 2.28*	53.18 ± 2.91*

There was an effect of treatment on mean length of the primary neurite, which were ~16% shorter in NBQX + LY341495-treated groups (ANOVA, **p* < 0.05), but this did not affect the total number of dendritic spine-like processes per neurite.

mediates that of PGE₂ to affect dendritic spine formation and potentially the organization of male rat sex behavior.

We also determined that both AMPA/kainate and metabotropic glutamate receptor signaling are required and each individually suffices for the organization of male sexual behavior by PGE₂. Newborn females treated with either an AMPA/kainate or a metabotropic glutamate receptor antagonist were fully masculinized by PGE₂, but females given both antagonists together before PGE₂ exhibited little or no masculine behavior in adulthood. Conversely, females treated neonatally with kainate, the type I/II metabotropic agonist ACPD, or both agonists combined showed adult male sexual behavior that was equivalent to that seen in PGE₂-masculinized females and males in previous reports. Neonatal treatment of ACPD influenced all measures of adult behavior, whereas kainate treatment affected a few. We confirmed that AMPA/kainate and metabotropic glutamate receptor signaling are not upstream of PKA because animals treated neonatally with Ht31 before ACPD and kainate still expressed male rat sexual behavior in adulthood. Rather, glutamate receptor signaling is downstream of PKA. We conclude that PGE₂ signaling through the EP₂ and EP₄ receptors likely activates PKA to recruit AMPA/kainate and metabotropic glutamate receptor signaling that then concurrently initiates POA dendritic spine formation and organizes the neuroarchitecture controlling rat sexual behavior.

Glutamate receptor signaling is also critical for the expression of male rat sex behavior in adulthood. Glutamate is released in the POA and activates NMDA receptors during male sexual behavior (Dominguez et al., 2006). We have determined previously that NMDA receptors do not play a role in estradiol- or PGE₂-induced dendritic spine formation in the neonatal POA (Amateau and McCarthy, 2002), indicating a shift in the role of glutamate between the developmental process of behavioral masculinization and the expression of male rat sex behavior in adulthood. However, ensuring proper AMPA/kainate and metabotropic signaling early in life could guarantee that the excitation of these same receptors later in life would remove the Mg²⁺ blockade of NMDA receptors (Anwy, 2009) to then regulate male sexual behavior (Dominguez et al., 2006, 2007). This dual role for glutamate transmission in both neonatal masculinization and adult

Figure 7. Neurochemical signaling pathway organizing male rat sexual behavior and neuroanatomical correlates. Masculinization of male rat sexual behavior is initiated perinatally when testosterone is converted to estradiol by aromatase in the diencephalon. Estradiol binds to estrogen receptor- α , which increases COX-1 and COX-2 levels twofold in the POA. A subsequent sevenfold increase in PGE₂ activates EP₂ and EP₄ receptors and recruits PKA signaling. PKA activity may induce AMPA/kainate and metabotropic glutamate receptor signaling, which then concurrently initiates dendritic spine formation in the POA. A higher density of excitatory dendritic spines synapses on POA neurons is highly correlated with expression of adult male sexual behavior in individual animals (Wright et al., 2008).

behavior may also be the neurologic underpinnings of plasticity in adult sexual behavior. Adult female rats will display mounting and intromission-like behaviors toward other females if they are treated with a combination of high-dose and long-duration estradiol (Emery and Sachs, 1975) or testosterone (Södersten and Ahlenius, 1972). Dendritic spines are readily induced by steroids in other brain regions, such as the hippocampus, and the potential that this could also occur in the preoptic area but with a much higher threshold for induction would explain how high-dose, long-duration exposure to steroids could eventually induce male sex behavior in otherwise normal females. Put another way, the difference between a masculinized versus feminized brain is a matter of sensitivity, not of absolutes (Södersten, 1984). Our

data suggest that the key variable in determining sensitivity is the density of POA neuron dendritic spines, but this supposes a causal relationship between dendritic spine density and behavior, which has not yet been established. However, we have established strong correlations between measures of behavior and spinophilin content, a proxy marker for POA dendritic spine levels (Wright et al., 2008).

There are multiple cellular mechanisms by which PGE₂ signaling could regulate glutamatergic neurotransmission. PGE₂ initiates release of glutamate from telencephalic astrocytes (Bezzi et al., 1998). In the hippocampus, PGE₂ enhances long-term potentiation and is attenuated by genetic deletion of EP₂ or inhibition of PKA (Yang et al., 2009a). The signaling of EP₂, EP₄, and PKA can increase the levels of the cell-adhesion molecule β -catenin (Regan, 2003). β -Catenin expression can regulate the number of dendritic spine synapses (Yu and Malenka, 2004; Takeichi and Abe, 2005), and β -catenin also binds AKAPs to the actin cytoskeleton of the dendritic spine and thus provides localization of type II PKA therein (Gorski et al., 2005). β -Catenin/AKAP/PKA binding can regulate the conductance and surface expression of the AMPA/kainate receptors by phosphorylation of the serine 845 residue on the GluR1 subunit (Swope et al., 1999; Yasuda et al., 2003; Gorski et al., 2005; Snyder et al., 2005; Dell'Acqua et al., 2006) in a manner that could initiate synaptogenesis (Tominaga-Yoshino et al., 2008) and is potentially sensitive to developmental age (Yang et al., 2009b). Of all the cellular mechanisms in which PGE₂ signaling modulates or is mediated by glutamatergic signaling, ours is, to our knowledge, the first report of such a mechanism affecting behavior.

Combining the current results with those generated previously by us and others, we have identified a critical signal transduction pathway and neurotransmitter system as essential components of the cellular processes organizing male sexual behavior in the rat (Fig. 7). Work completed 50 years ago identified the initial step as fetal and neonatal production of testosterone by the testes (Phoenix et al., 1959), followed by aromatization to estradiol in the diencephalon (Naftolin et al., 1975a,b), the induction of COX-1 and COX-2 in the POA (Wright, 2009), the synthesis of PGE₂ (Amateau and McCarthy, 2002, 2004), and the activation of EP₂ and EP₄ receptors (Burks et al., 2007; Wright et al., 2008), which induces PKA activity to modulate the signaling of the AMPA/kainate receptors and mGluRs. Glutamate receptor signaling then induces the formation and/or stabilization of the dendritic spine synapses on POA neurons and also organizes adult sexual behavior in the male rat so that the behavior is expressed under the appropriate hormonal and social circumstances.

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