Published in final edited form as:

Dev Neurobiol. 2008 October ; 68(12): 1406–1419. doi:10.1002/dneu.20665.

Identification of Prostaglandin E2 Receptors Mediating Perinatal Masculinization of Adult Sex Behavior and Neuroanatomical Correlates

Christopher L. Wright¹, Scott R. Burks², and Margaret M. McCarthy^{1,2,3}

¹ Program in Neuroscience, University of Maryland, School of Medicine, Baltimore

² Department of Physiology, University of Maryland, School of Medicine, Baltimore

³ Department of Psychiatry, University of Maryland, School of Medicine, Baltimore

Abstract

Prostaglandin E2 (PGE2) mediates the organization of male rat sexual behavior and medial preoptic area (MPOA) neuroanatomy during a sensitive perinatal window. PGE2 is up-regulated in response to estradiol, and initiates a two-fold increase in dendritic spines densities on neurons. All the four receptors for PGE2 and EP1-4 are present in developing POA, a critical region controlling male sexual behavior. Previous studies explored that EP receptors are involved in PGE2-induction of neonatal levels of spinophilin protein, a surrogate marker for dendritic spine formation, but did not assess behavioral masculinization. Here, we used two approaches, suppression of EP receptor expression with antisense oligonucleotides and activation of EP receptors with selective agonists, to test which receptors are necessary and sufficient, respectively, for the effects of PGE2 on behavior and neuronal morphology. In female rats, neonatal treatment with antisense oligonucleotides against EP2 or EP4 but not EP1 or EP3 completely prevented the expression of adult behavior organized by PGE2 exposure. The effects of ONO-DI-004, ONO-AE-259-01, ONO-AE-248, and ONO-AE1-329 (EP1-4 agonists respectively) were equivalent to PGE2 treatment, which suggests activating any EP receptor neonatally suffices in masculinizing sex behavior. When given alone, not all EP agonists increased neonatal POA spinophilin levels; yet giving each agonist neonatally increased adult levels. Moreover, adult spinophilin levels significantly correlated with two measures of male sexual behavior. The body of evidence suggests that EP2 and EP4 are both necessary and sufficient for PGE2-induced masculinization of sex behavior, whereas EP1 and EP3 provide redundant roles.

Keywords

prostaglandin E2; dendritic spine; cyclooxygenase; masculinization; sex behavior; preoptic area; spinophilin; EP receptor

INTRODUCTION

The ability to express male sexual behavior as an adult requires the action of testicular steroids during a sensitive perinatal window to organize the appropriate neural circuits (Phoenix et al., 1959). The laboratory rat is an excellent model for determining the cellular mechanisms organizing sex differences in the brain and behavior. Research in the past 30 years has

Correspondence to: C.L. Wright (cwright003@umaryland.edu).

Michael E. Taylor, Dr. Gerald Lardner, Bridget M. Nugent and ONO Pharmaceuticals, Osaka, Japan, including Dr. Takayuki Maruyama.

established that perinatal gonadal surges of androgen and subsequent aromatization in the brain to estradiol initiate masculinization of sex behavior (Naftolin et al., 1975). A critical step in this process is the organization of the preoptic area (POA) and hypothalamus (Wersinger et al., 1997), including a two-fold increase in a type of post synaptic specializations, called dendritic spines (Amateau and McCarthy, 2002a,b; Todd et al., 2007). The POA lies rostral to the hypothalamus and dorsal to the optic chiasm. Lesion, electrostimulation, and pharmacological manipulations implicate the medial POA (MPOA) as a critical component of the neural circuitry controlling male sexual behavior (Hillarp et al., 1954; Larsson and Heimer, 1964; Fernandez-Guasti et al., 1992; Meisel and Sachs, 1994).

Our lab previously reported that prostaglandin E2 (PGE2) is necessary and sufficient to organize male sex behavior and synaptic patterning in the POA (Amateau and McCarthy, 2002b; Amateau and McCarthy, 2004). PGE2 is synthesized from arachidonic acid via cyclooxygenases (COX) and terminal PGE synthases. Developmental exposure to estradiol masculinizes the brain by up-regulating the inducible isoform COX-2 two-fold and leading to a seven-fold increase in PGE2 (Amateau and McCarthy, 2004). Subsequently, PGE2 exposure induces a two-fold increase in spinophilin protein and dendritic spine density. Spinophilin is enriched in dendritic spines (Yan et al., 1999), and we have found it to be an excellent surrogate marker for dendritic spine formation in the POA and hypothalamus. The effects of PGE2 are propagated by one or more G-coupled E-type prostanoid receptors, called EP1-4. All four EP receptors are expressed in the perinatal POA and are necessary for increases in neonatal POA spinophilin protein levels in response to either estradiol or PGE2 treatment (Burks et al., 2007). However, whether all four receptors impact on adult sexual behavior has not previously been investigated.

We have now explored which of the EP receptor subtypes are necessary and sufficient for the perinatal organization of adult male sex behavior and its neuroanatomical correlates (see Table 1). We assessed adult male sexual behavior in females neonatally treated with both PGE2 and antisense oligonucleotides against mRNA for each of the EP receptors. We also evaluated whether neonatal treatment of females with selective agonists for each of the EP receptors from ONO Pharmaceuticals (Osaka, Japan) organized adult male sexual behavior. Finally, we assessed whether EP receptor agonist treatment increased neonatal and adult POA spinophilin protein levels similar to those with PGE2 treatment. Consistent with the previous findings, the current results implicate that all of the EP receptors in the perinatal organization of male sex behavior and its neuroanatomical correlates, but EP2 and EP4 perform a critical role. Moreover, measures of male sex behavior significantly correlate with adult POA spinophilin protein levels in individual animals.

MATERIALS AND METHODS

Animals and Intracerebroventricular Injections

All procedures employed in these studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Animals were kept under a 12 h reverse light/dark cycle and allowed free access to food and water. Pregnant Sprague-Dawley dams mated in our facility or ordered from Charles River Laboratories (Wilmington, MA), two weeks before birth were allowed to deliver normally. Female pups were treated on the day of birth (DOB) and postnatal day 1 (PN1). Bilateral intracerebroventricular (ICV) injections were carried out 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 agonist, antisense oligonucleotides, or the corresponding vehicle over 60 s. The process was then repeated for the other hemisphere. This injection site targeted the lateral

ventricles and provided exposure of the POA via the cerebrospinal fluid. Injections were not given directly into the POA to avoid tissue damage.

EP Receptor Agonists

EP receptor specific agonists were a generous gift of ONO Pharmaceuticals (Osaka, Japan). A dose of 3.5 nmoles of EP1, ONO-DI004; EP2, ONO-AE-259-01; EP3, ONO-AE-248; and/or EP4, ONO-AE1-329 was delivered by ICV injection in 2 μ L of 0.9% saline with 1% DMSO. When administered in combination, 3.5 nmoles of each agonist was suspended in the same 2 μ L volume. Vehicle or PGE2 (2.5 μ g) was administered ICV in 2 μ L of 0.9% saline with 1% DMSO.

Antisense Deoxyoligonucleotides

Oligonucleotides were obtained from Sigma/Proligo (Boulder, CO). Oligonucleotide sequences were generated from GenBank accession numbers for prostanoid receptor mRNA sequences: EP1, (NM_013100); EP2, (NM_031088); EP3 α , (NM_012704); EP3 β , (X80133); and EP4, (D28860). A random sequence of oligonucleotides (SCRAM) served as a control for oligonucleotide infusion. The synthetic oligonucleotide sequences are as follows: SCRAM C*C*G* ATG AAC TGT CGC GAT G*G*A*, EP1 G*G*C* TCA TAT CAG TGG CCA A*G*A* (571–591 bp), EP2 A*A*G* AAT TGT CCA TGG TGG A*G*G* (35–55 bp), EP3 A*C*A* CGC CGG TAG TGG C*G*G* (78–98 bp EP3 α , 96–116 bp EP3 β), and EP4 A*C*T* CCA ACC ACC ATC CAG G*T*C* (62–82 bp). All oligonucleotides contained locked nucleic acids, denoted by asterisks (*). Sequences were queried into the BLAST database (www.ncbi.nlm.nih.gov/BLAST/) and showed significant homology only to their specific target sequences within the rat genome, except for the SCRAM sequence which had no significant homology to any sequence in the genome. The EP3 sequence was specific to each splice variant of the receptor. A dose of 1 μ g of antisense oligonucleotide was delivered in 2 μ L of 0.9% saline.

Surgery

Animals used for behavioral testing were reared to PN50, gonadectomized under ketamine/acepromazine anesthesia (75 mg/2.5 mg per kg respectively) and implanted with a silastic capsule, which is 1.58 mm inner diameter, 3.18 mm outer diameter $\times$ 30 mm containing crystalline testosterone as described previously (Amateau and McCarthy, 2004).

Behavioral Testing

Beginning at PN60–65, animals were tested for male sexual behavior as previously described (Amateau and McCarthy, 2004). In brief, animals received 3 weekly 20 min tests after a 10 min acclimation period to the testing arena (49 cm L $\times$ 37 cm W $\times$ 24 cm H), 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 estradiol benzoate in 0.1 mL sesame oil 1 and 2 days prior and 0.75 mg progesterone in 0.15 mL sesame oil 4 h before testing). Frequencies of mounts and thrusts and latencies to first mount and first thrust were quantified as described previously (Amateau and McCarthy, 2004). After behavioral testing and before tissue extraction, animals were humanely killed by placing them in a CO2 chamber.

Microdissection of POA

After removal from the cranium, the brains were 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 was to define the

dorsal boundary of the POA. The isolated tissue was frozen on dry ice and stored at -80°C until use.

Western Immunoblotting

Tissue was homogenized in a RIPA buffer containing 1% Igepal CA630 (Sigma, St. Louis, MO), 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 previously described (Amateau and McCarthy, 2002a). 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 μg of protein was then electrophoresed on a precast of 8–16% SDS-polyacryl-amide gel (Invitrogen, Carlsbad, CA), and transferred onto a polyvinyl difluoride membrane (Bio-rad, Hercules, CA). Membranes were blocked with 5% nonfat milk in 0.1% Tween supplemented tris-buffered saline. Antisera to spinophilin (1:1000, Upstate, Lake Placid, NY) were then applied followed by anti-rabbit secondary antibody (1:10,000 for adult tissue, 1:3000 for neonate tissue). A Phototope chemiluminescence system (New England Biolabs, Beverly, MA) was used to detect the 110 kDa spinophilin immunoblot by exposing the membrane to Hyperfilm-ECL (GE Healthcare, Piscataway, NJ). Integrative gray-scale pixel area densitometry captured with a CCD camera was quantified with NIH IMAGE or ImageJ software. GAPDH or Ponceau staining were used for loading controls depending on the experiment. For GAPDH, the membrane was stripped, reprobed with primary (1:100,000), anti-mouse secondary (1:10,000) antibodies, and underwent the same chemiluminescent detection system and quantification methods as outlined earlier.

Experiment 1

Effect of neonatal treatment with PGE2 or EP receptor agonist on POA spinophilin protein at PN3—Female pups were infused on PN0 and 1 with PGE2 ($n = 6$), VEH ($n = 5$), or one of the following agonists: EP1 ($n = 5$), EP2 ($n = 6$), EP3 ($n = 6$), and EP4 ($n = 6$), as earlier. A separate comparison determined if one injection of PGE2 increased POA spinophilin protein levels at PN3 similar those following two injections ($n = 5$ except VEH: $n = 7$). On PN3, animals were humanely killed, and the POA was microdissected and assayed for spinophilin protein levels as earlier.

Experiment 2

Effect of neonatal treatment with a combination of two EP receptor agonists on POA spinophilin protein at PN3—Female pups were infused on DOB and PN1 with PGE2, VEH, or a combination of two EP receptor agonists ($n = 6$ –9 per group). Four separate spinophilin western blot analyses were carried out (see Table 2). Each western blot analysis was restricted to the comparing groups relevant to only one EP agonist. Thus, when the western blot analysis was restricted to the EP1 agonist comparisons, the groups were: EP1 and 2, EP1 and 3, and EP1 and 4 agonist combinations along with PGE2 and VEH conditions. The second analysis was restricted to the EP2 agonist by comparing EP1 and 2, EP2 and 3, and EP2 and 4 agonist combinations, along with PGE2 and VEH conditions. The third contained groups: PGE2, VEH, and EP1 and 3, EP2 and 3, and EP3 and 4 agonists combined. The fourth contained groups: PGE2, VEH, and EP1 and 4, EP2 and 4, and EP3 and 4 agonists combined. The same PGE2 and VEH treatment samples were used in all the four western blot analyses, whereas samples generated from the combination of two EP agonists were only used in the two western blot analyses.

Experiment 3

Effect of neonatal treatment with PGE2 or EP receptor agonists on adult male sex behavior and adult POA spinophilin protein—Female pups were infused with either PGE2 ($n = 5$), VEH ($n = 5$), or one of the following agonists: EP1 ($n = 3$), EP2 ($n = 6$), EP3 ($n = 4$), and EP4 ($n = 5$), on DOB and PN1. Animals were grown to adulthood, gonadectomized, and implanted with testosterone capsules. Ten to 14 days later animals were assessed for male sexual behavior with a hormonally-primed, sexually-receptive, stimulus female. Following the three behavioral trials, experimental animals were humanely killed by CO₂ inhalation, and the POA was immediately microdissected. Adult POA spinophilin protein levels were then assessed by western blot.

Experiment 4

Effect of neonatal treatment with antisense oligonucleotides (AS) against EP receptor mRNA on PGE2-induced masculinization of adult sexual behavior—Female pups were injected on DOB and PN1 with AS against mRNA for one of the four EP receptors and 4 h later with PGE2 ($n = 4$ –7 per group for EP1–4 AS conditions respectively). Three other groups on 2 consecutive days (DOB and PN1) were given a scrambled oligonucleotide control (SCRAM) then 4 h later either: PGE2, each day ($n = 6$); VEH, each day ($n = 7$); or PGE2 on the first and VEH on the second day ($n = 4$). Animals were grown to adulthood, gonadectomized, and implanted with testosterone capsules. Ten to 14 days later, animals were assessed for male sexual behavior.

Statistics

One-way ANOVAs were used for data obtained by western blot analyses. For adult POA spinophilin western blot analysis, a Dunnett's post hoc test was used with the VEH condition as the control. For the western blot analysis of neonatal tissue a Tukey's HSD post hoc test was used to allow for multiple comparisons between groups. For the measures of male sexual behavior, data were analyzed by a two-way ANOVA with repeated-measures across the three trials. In the event of a significant interaction between the treatment and trials, a post hoc Dunnett's test was used within each trial to determine the significant differences. If there was no interaction, the same test was used on treatment group means collapsed across trials. The Dunnett's test control groups were the VEH and SCRAM + PGE2 treatment animals for the effects of EP agonist and AS, respectively. Regression analyses were performed between the POA adult spinophilin levels and measures of male sexual behavior.

RESULTS

Experiment 1

Effect of neonatal treatment with PGE2 or one EP receptor agonist on POA spinophilin protein at PN3—Two injections of PGE2 significantly increased the POA spinophilin protein by ~50% when compared with VEH treatment, consistent with previous findings [$F_{2,16} = 5.77$, $p = 0.015$, Fig. 1(A)]. One injection of PGE2 also significantly increased the POA spinophilin protein when compared with VEH group ($p < 0.05$). Of the four receptor agonists, only treatment with EP4 agonist significantly increased the spinophilin levels above those from VEH treated animals and equivalent to those from PGE2 treated animals [$F_{5,33} = 2.51$, $p < 0.05$, Fig. 1(B)]. Treatment with either EP1 or EP3 agonists induced intermediate levels of spinophilin that were not different from levels in either PGE2 or VEH treated animals. Treatment with EP2 agonist did not increase neonatal POA spinophilin levels above those from the VEH treatment and was significantly lower than the levels after PGE2 treatment ($p < 0.05$).

Experiment 2

Effect of neonatal treatment with a combination of two EP receptor agonists on POA spinophilin protein at PN3—Any combination of two EP agonists increased POA spinophilin levels above VEH and equivalent to PGE2 treatment [EP1 comparisons: $F_{4,37} = 6.32$; EP2 comparisons: $F_{4,37} = 9.81$; EP3 comparisons: $F_{4,31} = 7.38$; EP4 comparisons: $F_{4,35} = 11.03$, $p < 0.001$ for all; Fig. 2(A–D)].

Experiment 3

Effect of neonatal treatment with PGE2 or EP receptor agonists on adult male sex behavior and adult POA spinophilin protein—In animals treated with either PGE2 or EP1, 2, or 3 agonist neonatally, adult POA spinophilin protein levels increased 2 to 3-fold over levels in VEH treated animals [$F_{5,26} = 4.39$, $p = 0.007$; post hoc $p < 0.05$; Fig. 3(A,B)]. In animals treated with the EP4 agonist, the mean level of POA spinophilin protein trended higher ($p = 0.068$) than the level from VEH treated animals. None of the EP agonist treatments differed from each other or PGE2 treatment in effects on spinophilin levels.

Neonatal treatment with any one of the selective ONO receptor agonists induced masculinization of adult sex behavior with measures equivalent to those observed in animals treated neonatally with PGE2 [Fig. 4(A–D)]. There was a significant difference in the latency to onset of first mount across treatment groups [$F_{5,26} = 9.74$, $p < 0.001$, Fig. 4(A)]. All animals neonatally treated with PGE2 or an EP agonist exhibited significantly shorter latencies to mount than VEH treated animals ($p < 0.05$). The latency to first thrust also showed a similar response pattern [$F_{5,26} = 11.26$, $p < 0.001$, Fig. 4(B)] with measures for PGE2 and EP agonist treated animals being shorter than VEH treated ($p < 0.01$). The number of mounts exhibited in a 20 min trial significantly increased in response to neonatal treatment [$F_{5,26} = 9.45$, $p < 0.001$, Fig. 4(C)] such that PGE2 treated and all EP agonist treated animals mounted more often than VEH treated animals ($p < 0.05$). The number of thrusts was affected by the treatment, [$F_{5,26} = 6.61$, $p < 0.001$, Fig. 4(D)]; however, only the EP1 or EP2 agonist treated animals displayed more thrusts than the VEH animals ($p < 0.05$).

After assessing male sex behavior and spinophilin in these animals, we performed correlation/regression analyses between individuals' POA spinophilin levels and two measures of behavior: the average number of mounts across trials and the shortest latency to onset of first mount observed from one of three trials. POA spinophilin levels positively correlated by linear regression with the average number of mounts [$R = 0.643$, $R_{\text{crit}} = 0.381$, $p < 0.001$, Fig. 5(A)] and negatively correlated by exponential regression with the shortest observed latency to mount in one of the three twenty minute trials [$R = 0.715$, $R_{\text{crit}} = 0.381$, $p < 0.001$, Fig. 5(B)]. An exponential regression and log10 y-axis scale was used for the latency to mount because this value can only approach but never be less than zero.

Experiment 4

Effect of neonatal treatment with antisense oligonucleotides against EP receptor mRNA on PGE2-induced masculinization of adult sexual behavior—

Western blot analysis for EP receptor protein levels 24 h after AS treatment was previously performed in Burks, Wright and McCarthy (2007), and the results demonstrate that administration of each AS oligonucleotide significantly decreased the protein for the targeted receptor 20–30%. Treatment with each specific AS did not decrease the expression of the other EP receptors protein [Fig. 6(F)], demonstrating that the AS oligos were both effective and specific.

When assessing male sex behavior in animals neonatally treated with AS oligos against mRNA for each of the EP receptors, there was a significant difference in the latency to onset of first

mount across the treatment groups [$F_{6,30} = 4.06$, $p = 0.004$, Fig. 6(A)]. Animals neonatally treated with SCRAM + VEH, EP1 AS + PGE2, EP2 AS + PGE2, or EP4 AS + PGE2 expressed longer latencies to mount than animals administered SCRAM + PGE2 ($p < 0.05$). No difference was observed in latencies to mount for EP3 AS + PGE2 when compared with SCRAM + PGE2 treated animals. A similar treatment effect was observed with latencies to thrust [$F_{6,30} = 4.21$, $p < 0.005$, Fig. 6(B)]; animals treated with SCRAM + VEH, EP1 AS + PGE2, EP2 AS + PGE2, or EP4 AS + PGE2 but not EP3 AS + PGE2 exhibited longer latencies than SCRAM + PGE2 treated animals ($p < 0.05$). The numbers of mounts significantly decreased in response to AS treatment [$(F_{6,30} = 12.05$, $p < 0.001$, Fig. 6(C)], such that SCRAM + VEH, EP2 AS + PGE2, and EP4 AS + PGE2 but not EP3 AS + PGE2 treatment groups exhibited fewer mounts across all three trials than SCRAM + PGE2 treated animals ($p < 0.05$). Animals neonatally treated with EP1 AS + PGE2 displayed increasing number of mounts across trials; although number of mounts was lower on the first trial than from the positive control group receiving SCRAM + PGE2 ($p < 0.05$), the number of mounts increased by the third trial to become equivalent [Fig. 6(D)]. The numbers of thrusts decreased due to the neonatal AS treatment [$F_{6,30} = 5.98$, $p < 0.01$, Fig. 6(E)] and were lower for animals given SCRAM + VEH, EP2 AS + PGE2, and EP4 AS + PGE2 when compared with SCRAM + PGE2 across all three trials. Animals given EP1 AS + PGE2 displayed increasing numbers of thrusts across trials, being different from levels displayed by SCRAM + PGE2 treated animals on the first trial ($p < 0.05$) and equivalent on the third trial (data not shown). We also tested whether one neonatal injection of PGE2 sufficed to organize adult male sexual behavior. Animals treated with only one injection of PGE2 exhibited more mounts and thrusts and shorter latencies than those from VEH treated animals ($p < 0.05$), and measures did not differ when compared with those from animals given two daily PGE2 injections (by Tukey's HSD).

DISCUSSION

Before this study, the question remained as to which of the EP receptors mediate the organization of the neuroarchitecture controlling sex behavior in response to PGE2 exposure. We have now observed that the activation of the EP4 receptor by a selective agonist increased neonatal spinophilin protein, a surrogate marker for dendritic spine formation, whereas activation of EP1–3 did not. Yet, EP1–4 activation can still induce neonatal spinophilin via signaling involving receptor subtype cross-talk or synergism because any combination of two agonists for EP1–4 increased spinophilin. Moreover, neonatal activation of any four of the EP receptors can have enduring effects because we observed significant increases in adult POA spinophilin levels and expression of male sex behavior. We further explored the potential involvement of particular EP receptors by knock down with AS oligonucleotides. Administration of AS oligos against EP2 or EP4 mRNA completely prevented the perinatal masculinization of adult sex behavior in response to PGE2. EP3 receptor-AS-treated animals showed no impairments in behavioral measures when compared with PGE2 treated animals, and EP1 receptor AS treated animals were impaired on the first trial but gradually increased the performance by the third trial. Taken together, the data are consistent with an interpretation that neonatal activation of EP2 and EP4 are both necessary and sufficient for the organization of male sexual behavior, but that EP1 and EP3 provide redundant support to ensure that PGE2-mediated masculinization is achieved.

A second interesting observation from the current study is that the adult POA spinophilin levels significantly correlated with behavioral measures, such as the average number of mounts across trials and the shortest latency to mount. This further supports the contention that spinophilin is an accurate proxy for dendritic spines and attendant synapses. A critical limitation is that adult brain spinophilin levels can only be measured post-hoc and cannot predict behavioral outcomes. Nonetheless, the high degree of correlation between behavioral performance and

spinophilin further substantiates the vital involvement of PGE2 and EP receptors in behavioral masculinization.

Previously, our lab has shown that PGE2 exposure initiates dendritic spine formation in POA cultured neurons, suggesting that the POA responds directly to PGE2 and other brain regions are not needed to detect PGE2 then convey its effects to the POA (Amateau and McCarthy, 2002a). Here, we administered PGE2 and analogs to the lateral ventricles, thereby exposing a wide range of brain areas. In using this paradigm, we cannot preclude the possibility that PGE2 has an impact elsewhere in the brain. Taken as a whole, the evidence here suggests that the organization of the neuroarchitecture controlling male sexual behavior is very sensitive to EP receptor manipulations.

Many PGE2-mediated responses depend upon the activation of two or more receptors. Differentiation of chondrocytes and activation of osteoblasts by PGE2 involves both EP2 and EP4 (Suzawa et al., 2000; Miyamoto et al., 2003). EP1 and EP4 activation can phosphorylate signal transducer and activator of transcription-3 (STAT-3) in human carcinomas (Han et al., 2006a). EP1 and EP3 together stimulate melanocyte dendrite formation (Scott et al., 2007). Uterine contraction during parturition involves EP1 and EP3, whereas relaxation involves EP2 and EP4 together (Ma et al., 1999). Receptor cross-talk or feed forward mechanisms often contribute to the involvement of multiple EP receptors in a single system (Wise et al., 2002). This is consistent with our current observation that activation of EP1-3 alone did not increase neonatal spinophilin levels but that activating any two receptors did.

For these reasons, studying PGE2 signaling often involves delineating complex pathways due to the propensity for EP receptor cross-talk. EP2 and EP4 were initially identified as $G_{s\alpha}$ -protein coupled which can stimulate production of cAMP by adenylyl cyclase and then recruit protein kinase A (PKA) (Regan et al., 1994; Regan, 2003). In the immune system activation of EP2 and EP4 sequentially stimulates PKA, inhibits glycogen synthase kinase-3 α (GSK-3 α), releases the tonic inhibition of the transcriptional factor β -catenin and finally transcribes the COX-2 gene (Fujino et al., 2002; Regan, 2003). Activation of EP4 can also inhibit GSK through its sequential coupling to PI3 kinase and akt, again resulting in β -catenin disinhibition (Fujino et al., 2002). Finally, EP4 activation of PI3 kinase can phosphorylate Erks leading to induction of early growth response-1 transcriptional factor and transcription of PGE synthase, tumor necrosis factor- α , synapsin, and cyclin D1 (Fujino et al., 2003). In turn, tumor necrosis factor- α can have synergistic effects with PGE2 in the brain (Domercq et al., 2006).

Of the four EP receptors, EP3 has the most diverse signaling, in part, because of the differential splicing of its mRNA. In the rat, there are three splice-variants of the EP3 gene that differ at the c-terminal region, an area important for G-protein coupling. As such, EP3 was originally identified as coupled to G_i , which can inhibit the production of cAMP by adenylyl cyclase, but later studies also described G_s and G_{13} coupling (Aoki et al., 1999; Sugimoto et al., 2003). The G_{13} coupling can in turn activate Rho in the family of small GTPases (Katoh et al., 1998). EP3 can augment the production of cAMP initiated by EP2 or EP4 signaling in cell lines (Hatae et al., 2002, 2003). In the case of EP4 signaling in mouse mastocytoma P-815 cell lines, giving pertussis toxin prevents EP3-induced augmentation of cAMP production suggesting EP3 couples to G_i -protein signaling for increased $[cAMP]_i$ (Hatae et al., 2003). We observed that neonatal EP3 activation, like EP2 or EP4, masculinized adult behavior, and combined activation of EP2 and EP3 increased the neonatal spinophilin. The convergent and synergistic effects of EP3 with the other receptors suggest that EP3 may positively couple to adenylyl cyclase in the neonatal rat POA.

The current findings also suggest EP1 is involved in modulating signal transduction pathways that mediate the organization of male sexual behavior. During the first 6 days of life,

corresponding to the sensitive period when estradiol can organize male sexual behavior, EP1 mRNA is elevated in the POA (Burks et al., 2007). Downregulating EP1 with AS treatment prevents the up-regulation of neonatal spinophilin after PGE2 or estradiol exposure. However, when tested for behavior as adults, animals treated with EP1 AS oligos as neonates only show impaired performance during the first trial; the animals recover by the third trial. Males exposed to a low dose aspirin, a COX inhibitor, in utero and early in maternal lactation, display a similar initial impairment of male sex behavior, which is overcome with experience (Amateau and McCarthy, 2004). The reasons for these partial (or weak) effects are unknown but are consistent with the capacity of males to improve sexual performance with experience.

The signal transduction pathway activated by EP1 is distinct from the other EP receptors in that EP1 couples to a G_q -protein, producing IP3 and DAG via PLC, and subsequently activating among other proteins, protein kinase C (PKC) (Kozawa et al., 1998). In cholangiocarcinoma and hepatocellular carcinoma cells, PGE2 signaling through EP1 activates Erk and phosphorylates the epidermal growth factor subtype of tyrosine kinase receptors (EGFR) (Han et al., 2006b; Zhang et al., 2007). Moreover, EGFR signaling can also initiate PGE2 production in multiple systems, including the rostral POA and median eminence (Ma et al., 1997; Ojeda and Ma, 1998; Prevot et al., 2005). EP1 activation can augment dopamine receptor-1 and -2 (D1 and D2, respectively) signaling in the striatum, which is involved in reward seeking and hyperlocomotion (Kitaoka et al., 2007) but also can inhibit aggressive and impulsive behavior during stress (Matsuoka et al., 2005). Because D1 receptors are G_s coupled, EP1 activation can modulate other G_s -mediated g-protein coupled receptor (GPCR) signaling in the brain despite its G-protein transduction being distinct. Moreover, D1 and D2 receptor activation initiates PGE2 synthesis in the striatum (Kitaoka et al., 2007), providing support for PGE2 signaling in the brain involving a feed-forward mechanism with GPCRs. Such interactions between PGE2 and GPCRs are further demonstrated in (Bezzi et al., 1998) where activating metabotropic glutamate receptors (mGluRs), in part, initiates PGE2 production, which then activates mGluRs through continued release of glutamate.

The current results confirm a previous observation that neonatal PGE2 exposure increases POA spinophilin that endures into adulthood (Amateau and McCarthy, 2004). The vast majority of spinophilin protein within a neuron is enriched in dendritic spines (Yan et al., 1999; Sarrouilhe et al., 2006). Spinophilin protein expression correlates superbly with other analyses of dendritic spines, proving it to be an excellent proxy marker for spines. Dendritic spines are the major site of excitatory neurotransmission in the brain, and AMPA/Kainate and NMDA receptors are enriched within them (Korkotian and Segal, 2007). During the expression of male sex behavior, extracellular glutamate levels increase and NMDA receptors are activated in the medial POA (Dominguez et al., 2006, 2007). Discernible impairments in sex behavior are observed when males are administered NMDA receptor antagonists directly into the POA (Dominguez et al., 2007). In this study, we found strong correlations between adult spinophilin protein expression in the POA and two measures of male sex behavior, the frequency of mounts averaged across trials, and the shortest latency to mount observed in one of the three trials. Although the relationship does not necessarily imply a causative one, these correlations are consistent with the hypothesis that excitatory neurotransmission in the MPOA underlies the expression of the male sex behavior.

Acknowledgments

Contract grant sponsor: National Institutes of Mental Health R01 grant; contract grant number: MH052716.

References

- Amateau SK, McCarthy MM. A novel mechanism of dendritic spine plasticity involving estradiol induction of prostaglandin-E2. *J Neurosci* 2002a;22:8586–8596. [PubMed: 12351732]
- Amateau SK, McCarthy MM. Sexual differentiation of astrocyte morphology in the developing rat preoptic area. *J Neuroendocrinol* 2002b;14:904–910. [PubMed: 12421344]
- Amateau SK, McCarthy MM. Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. *Nat Neurosci* 2004;7:643–650. [PubMed: 15156148]
- Aoki J, Katoh H, Yasui H, Yamaguchi Y, Nakamura K, Hasegawa H, Ichikawa A, et al. Signal transduction pathway regulating prostaglandin EP3 receptor-induced neurite retraction: Requirement for two different tyrosine kinases. *Biochem J* 1999;340(Pt 2):365–369. [PubMed: 10333476]
- Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, et al. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 1998;391:281–285. [PubMed: 9440691]
- Burks SR, Wright CL, McCarthy MM. Exploration of prostanoid receptor subtype regulating estradiol and prostaglandin E2 induction of spinophilin in developing preoptic area neurons. *Neuroscience* 2007;146:1117–1127. [PubMed: 17408863]
- Domercq M, Brambilla L, Pilati E, Marchaland J, Volterra A, Bezzi P. P2Y1 receptor-evoked glutamate exocytosis from astrocytes: Control by tumor necrosis factor- α and prostaglandins. *J Biol Chem* 2006;281:30684–30696. [PubMed: 16882655]
- Dominguez JM, Balfour ME, Lee HS, Brown JL, Davis BA, Coolen LM. Mating activates NMDA receptors in the medial preoptic area of male rats. *Behav Neurosci* 2007;121:1023–1031. [PubMed: 17907833]
- Dominguez JM, Gil M, Hull EM. Preoptic glutamate facilitates male sexual behavior. *J Neurosci* 2006;26:1699–1703. [PubMed: 16467517]
- Fernandez-Guasti A, Escalante AL, Ahlenius S, Hillegaart V, Larsson K. Stimulation of 5-HT1A and 5-HT1B receptors in brain regions and its effects on male rat sexual behaviour. *Eur J Pharmacol* 1992;210:121–129. [PubMed: 1534765]
- Fujino H, West KA, Regan JW. Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *J Biol Chem* 2002;277:2614–2619. [PubMed: 11706038]
- Fujino H, Xu W, Regan JW. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J Biol Chem* 2003;278:12151–12156. [PubMed: 12566441]
- Han C, Demetris AJ, Stolz DB, Xu L, Lim K, Wu T. Modulation of Stat3 activation by the cytosolic phospholipase A2 α and cyclooxygenase-2-controlled prostaglandin E2 signaling pathway. *J Biol Chem* 2006a;281:24831–24846. [PubMed: 16790433]
- Han C, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J Cell Physiol* 2006b;207:261–270. [PubMed: 16331686]
- Hatae N, Kita A, Tanaka S, Sugimoto Y, Ichikawa A. Induction of adherent activity in mastocytoma P-815 cells by the cooperation of two prostaglandin E2 receptor subtypes, EP3 and EP4. *J Biol Chem* 2003;278:17977–17981. [PubMed: 12637575]
- Hatae N, Yamaoka K, Sugimoto Y, Negishi M, Ichikawa A. Augmentation of receptor-mediated adenylyl cyclase activity by Gi-coupled prostaglandin receptor subtype EP3 in a Gbetagamma subunit-independent manner. *Biochem Biophys Res Commun* 2002;290:162–168. [PubMed: 11779148]
- Hillarp NA, Olivecrona H, Silfverskiold W. Evidence for the participation of the preoptic area in male mating behaviour. *Experientia* 1954;10:224–225. [PubMed: 13173472]
- Katoh H, Aoki J, Yamaguchi Y, Kitano Y, Ichikawa A, Negishi M. Constitutively active G α 12, G α 13, and G α 14 induce Rho-dependent neurite retraction through different signaling pathways. *J Biol Chem* 1998;273:28700–28707. [PubMed: 9786865]
- Kitaoka S, Furuyashiki T, Nishi A, Shuto T, Koyasu S, Matsuoka T, Miyasaka M, et al. Prostaglandin E2 acts on EP1 receptor and amplifies both dopamine D1 and D2 receptor signaling in the striatum. *J Neurosci* 2007;27:12900–12907. [PubMed: 18032663]

- Korkotian E, Segal M. Morphological constraints on calcium dependent glutamate receptor trafficking into individual dendritic spine. *Cell Calcium* 2007;42:41–57. [PubMed: 17187855]
- Kozawa O, Suzuki A, Tokuda H, Kaida T, Uematsu T. Interleukin-6 synthesis induced by prostaglandin E2: Cross-talk regulation by protein kinase C. *Bone* 1998;22:355–360. [PubMed: 9556135]
- Larsson K, Heimer L. Mating behaviour of male rats after lesions in the preoptic area. *Nature* 1964;202:413–414. [PubMed: 14152848]
- Ma X, Wu WX, Nathanielsz PW. Differential regulation of prostaglandin EP and FP receptors in pregnant sheep myometrium and endometrium during spontaneous term labor. *Biol Reprod* 1999;61:1281–1286. [PubMed: 10529275]
- Ma YJ, Berg-von der Emde K, Rage F, Wetsel WC, Ojeda SR. Hypothalamic astrocytes respond to transforming growth factor- α with the secretion of neuro-active substances that stimulate the release of luteinizing hormone-releasing hormone. *Endocrinology* 1997;138:19–25. [PubMed: 8977380]
- Matsuoka Y, Furuyashiki T, Yamada K, Nagai T, Bito H, Tanaka Y, Kitaoka S, et al. Prostaglandin E receptor EP1 controls impulsive behavior under stress. *Proc Natl Acad Sci USA* 2005;102:16066–16071. [PubMed: 16247016]
- Meisel, RL.; Sachs, BD. The physiology of male sexual behavior. In: Knobil, E.; Neill, JD., editors. *The Physiology of Reproduction*. Vol. 2. New York, NY: Raven Press; 1994. p. 3-107.
- Miyamoto M, Ito H, Mukai S, Kobayashi T, Yamamoto H, Kobayashi M, Maruyama T, et al. Simultaneous stimulation of EP2 and EP4 is essential to the effect of prostaglandin E2 in chondrocyte differentiation. *Osteoarthritis Cartilage* 2003;11:644–652. [PubMed: 12954235]
- Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z, Kuhn M, et al. The formation of estrogens by central neuroendocrine tissues. *Recent Prog Horm Res* 1975;31:295–319. [PubMed: 812160]
- Ojeda SR, Ma YJ. Epidermal growth factor tyrosine kinase receptors and the neuroendocrine control of mammalian puberty. *Mol Cell Endocrinol* 1998;140:101–106. [PubMed: 9722176]
- Phoenix CH, Goy RW, Gerall AA, Young WC. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology* 1959;65:369–382. [PubMed: 14432658]
- Prevot V, Lomniczi A, Corfas G, Ojeda SR. erbB-1 and erbB-4 receptors act in concert to facilitate female sexual development and mature reproductive function. *Endocrinology* 2005;146:1465–1472. [PubMed: 15591145]
- Regan JW. EP2 and EP4 prostanoid receptor signaling. *Life Sci* 2003;74:143–153. [PubMed: 14607241]
- Regan JW, Bailey TJ, Pepperl DJ, Pierce KL, Bogardus AM, Donello JE, Fairbairn CE, et al. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. *Mol Pharmacol* 1994;46:213–220. [PubMed: 8078484]
- Sarrouilhe D, di Tommaso A, Metaye T, Ladeveze V. Spinophilin: From partners to functions. *Biochimie* 2006;88:1099–1113. [PubMed: 16737766]
- Scott G, Fricke A, Fender A, McClelland L, Jacobs S. Prostaglandin E2 regulates melanocyte dendrite formation through activation of PKC ζ . *Exp Cell Res* 2007;313:3840–3850. [PubMed: 17850789]
- Sugimoto Y, Nakato T, Kita A, Hatae N, Tabata H, Tanaka S, Ichikawa A. Functional domains essential for Gs activity in prostaglandin EP2 and EP3 receptors. *Life Sci* 2003;74:135–141. [PubMed: 14607240]
- Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, Ichikawa A, et al. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: An analysis using specific agonists for the respective EPs. *Endocrinology* 2000;141:1554–1559. [PubMed: 10746663]
- Todd BJ, Schwarz JM, Mong JA, McCarthy MM. Glutamate AMPA/kainate receptors, not GABA(A) receptors, mediate estradiol-induced sex differences in the hypothalamus. *Dev Neurobiol* 2007;67:304–315. [PubMed: 17443789]
- Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor α gene. *Horm Behav* 1997;32:176–183. [PubMed: 9454668]
- Wise H, Wong YH, Jones RL. Prostanoid signal integration and cross talk. *Neurosignals* 2002;11:20–28. [PubMed: 11943879]

- Yan Z, Hsieh-Wilson L, Feng J, Tomizawa K, Allen PB, Fienberg AA, Nairn AC, et al. Protein phosphatase 1 modulation of neostriatal AMPA channels: Regulation by DARPP-32 and spinophilin. *Nat Neurosci* 1999;2:13–17. [PubMed: 10195174]
- Zhang L, Jiang L, Sun Q, Peng T, Lou K, Liu N, Leng J. Prostaglandin E2 enhances mitogen-activated protein kinase/Erk pathway in human cholangiocarcinoma cells: Involvement of EP1 receptor, calcium and EGF receptors signaling. *Mol Cell Biochem* 2007;305:19–26. [PubMed: 17551669]

Figure 1.

Effect of neonatal treatment with PGE2 or one EP receptor agonist on POA spinophilin protein at PN3 assessed by western blot analysis. (A) Either one or two injections of PGE2 significantly increased POA spinophilin protein above vehicle (VEH, standardized to 1) (ANOVA; $n = 5-7$; $* = p < 0.05$ when compared with VEH). (B) Treatment with EP4 agonist increased POA spinophilin protein above VEH (standardized to 1, lower dashed line) and equivalent to PGE2 treatment conditions (upper dashed line) (ANOVA; $n = 5-6$; $* = p < 0.05$ when compared with VEH). Treatment of EP1, 2, and 3 agonists do not increase POA spinophilin protein above VEH treatment conditions. (C) Representative images of western immunoblots for spinophilin (above) and GAPDH (below) from EP agonist treated animals where each lane is tissue from one animal representing the mean value.

Figure 2.

Effect of neonatal treatment with PGE2 or a combination of two EP receptor agonists combined on POA spinophilin protein at PN3 assessed by western blot analysis. Treatment with PGE2 or a combination of any two agonists increased PN3 POA spinophilin protein above VEH (ANOVA; $n = 6-9$; $* = p < 0.05$ when compared with VEH). (A) EP1 agonists combined with others. (B) EP2 agonists combined with others. (C) EP3 agonists combined with others. (D) EP4 agonists combined with others.

Figure 3. Effect of neonatal treatment with EP receptor agonists on adult POA spinophilin protein assessed by western blot analysis. (A) Treatment with either EP1, EP2, or EP3 agonist increased adult POA spinophilin protein above VEH, equivalent to PGE2 treatment conditions (ANOVA; $n = 3-6$; $* = p < 0.05$ when compared with VEH). Treatment with an EP4 agonist resulted in a strong trend toward a significant difference from VEH ($n = 5$; $§p = 0.068$). (B) Representative western immunoblot for spinophilin (above) and GAPDH loading control (below) where each lane is tissue from one animal representing the mean value.

Figure 4.

Effect of neonatal treatment with EP receptor agonists on adult male sexual behavior. No differences were observed across trials for any of the treatment groups within each behavioral measure. (A) The average latency to first mount collapsed across trials was significantly shorter in animals treated neonatally with PGE2 or any EP receptor agonist when compared with VEH (ANOVA; $n = 3-6$; $* = p < 0.05$ when compared with VEH). (B) The average latency to first thrust collapsed across trials was significantly shorter in animals treated neonatally with PGE2 or any EP receptor agonist when compared with VEH (ANOVA; $* = p < 0.05$ when compared with VEH). (C) The average number of mounts collapsed across trials was higher in animals treated with PGE2 or any EP receptor agonist when compared with VEH (ANOVA; $* = p < 0.05$ when compared with VEH). (D) The average number of thrusts collapsed across trials was significantly higher in animals treated with EP1 or EP2 but not EP3 or EP4 agonists (ANOVA; $* = p < 0.05$ when compared with VEH).

Figure 5.

Regression analyses of POA spinophilin levels against measures of male sexual behavior from animals neonatally treated with either PGE2 and VEH or an EP agonist. (A) Linear regression analysis (solid line) of adult POA spinophilin and average number of mounts (gray diamonds) revealed a significant positive correlation between the two endpoints ($R = 0.653$, $p < 0.001$). (B) Exponential regression analysis (solid line) of adult POA spinophilin and shortest latency to mount observed from one of three trials (gray squares) also revealed a significant but negative correlation between the two endpoints ($R = 0.715$, $p < 0.001$, Y-axis transformed to logarithmic scale).

Figure 6.

Effect of neonatal co-administration of AS against EP1–4 mRNA and PGE2 on measures of adult male sexual behavior. (A) The average latency to first mount collapsed across trials was significantly longer in animals treated with either VEH + SCRAM, EP1 AS + PGE2, EP2 AS + PGE2, or EP4 AS + PGE2 (ANOVA; $n = 4-7$; $* = p < 0.05$ when compared with SCRAM + 2 daily injections of PGE2). There was no difference between animals treated with SCRAM + 2 daily injections of PGE2 and either: EP3 AS + PGE2, or SCRAM + 1 injection of PGE2. (B) The average latency to first thrust collapsed across trials was significantly longer for animals treated with VEH + SCRAM, EP1 AS + PGE2, EP2 AS + PGE2, and EP4 AS + PGE2. (ANOVA, $* = p < 0.05$ difference from SCRAM + two daily injections of PGE2). There was no difference between animals treated with SCRAM + 2 daily injections of PGE2 and either: EP3 AS + PGE2, or SCRAM + 1 injection of PGE2. (C) The average number of mounts collapsed across trials was significantly lower for animals treated with VEH + SCRAM, EP2 AS + PGE2, and EP4 AS + PGE2 (ANOVA, $* = p < 0.05$ difference from SCRAM + 2 daily injections of PGE2). There was no difference for animals treated with SCRAM + 2 daily injections of PGE2 and either: EP3 AS + PGE2 or SCRAM + 1 injection of PGE2. (D) The average number of thrusts collapsed across trials was similarly lower for the animals treated with VEH + SCRAM, EP2 AS + PGE2, and EP4 AS + PGE2 (ANOVA, $* = p < 0.05$ difference from SCRAM + 2 daily injections of PGE2). There was no difference for animals treated with SCRAM + PGE2 and either: EP1 AS + PGE2, EP3 AS + PGE2 or SCRAM + 1 injection of

PGE2. (E) On the first trial, EP1 AS + PGE2 treated animals mounted less often than SCRAM + 2 daily injections of PGE2 control but by the second and third trial numbers of mounts were equivalent. (F) Representative western immunoblot from (Burks et al., 2007) demonstrating treatment of AS against specific EP receptor decreases only protein for the targeted receptor and not others. Each row represents the one EP receptor being probed. Columns are the AS oligo treatment groups.

Table 1

Experimental Groups for Assessing Neonatal and Adult Spinophilin Levels and Behavior Following Neonatal Manipulation of EP Receptors Signaling

Neonatal Treatment	Groups	Neonatal POA Spinophilin (PN3)	Adult POA Spinophilin and Male Sex Behavior
EP agonists alone		Experiment 1, <i>n</i> =	Experiment 3, <i>n</i> =
	EP1 agonist	5	3
	EP2 agonist	6	6
	EP3 agonist	6	4
	EP4 agonist	6	5
	PGE2	6	5
	Vehicle	5	5
Two EP agonists combined		Experiment 2, <i>n</i> =	
	EP1 & EP2 agonists	8	
	EP1 & EP3 agonists	8	
	EP1 & EP4 agonists	9	
	EP2 & EP3 agonists	8	
	EP2 & EP4 agonists	8	
	EP3 & EP4 agonists	8	
	PGE2	6	
	Vehicle	8	
Antisense oligonucleotides against EP1-4 mRNA combined with PGE2		See Burks et al. (2007)	Experiment 4, <i>n</i> =
	EP1 AS + PGE2		5
	EP2 AS + PGE2		7
	EP3 AS + PGE2		5
	EP4 AS + PGE2		4
	SCRAM + 2 injections of PGE2		6
	SCRAM + 1 injections of PGE2		4
	SCRAM + Vehicle		7

Table 2

Design of Western Blot Analysis in Experiment 2

Western Blot:	Treatment Groups						
	PGE2 <i>n</i> = 6	Vehicle <i>n</i> = 8	EP1 and 2 Agonists <i>n</i> = 8	EP1 and 3 Agonists <i>n</i> = 8	EP1 and 4 Agonists <i>n</i> = 9	EP2 and 3 Agonists <i>n</i> = 8	EP2 and 4 Agonists <i>n</i> = 8
No. 1: EP1 agonist	X	X	X	X	X		
No. 2: EP2 agonist	X	X	X			X	X
No. 3: EP3 agonist	X	X		X		X	X
No. 4: EP4 agonist	X	X			X		X

Columns are the treatment groups. Rows are the four different western blot analyses designed to assess the contribution of one receptor when activated with another. Experiment 2: Each of the four western blot analyses (rows) only included the treatment groups (columns) relevant to one EP receptor agonist.