*Neuroscience* 146 (2007) 1117–1127

# EXPLORATION OF PROSTANOID RECEPTOR SUBTYPE REGULATING ESTRADIOL AND PROSTAGLANDIN $E_2$ INDUCTION OF SPINOPHILIN IN DEVELOPING PREOPTIC AREA NEURONS

**S. R. BURKS,[a] C. L. WRIGHT[b] AND M. M. MCCARTHY[a,b,c]***

[a]*Department of Physiology, University of Maryland, School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201, USA*

[b]*Program in Neuroscience, University of Maryland, School of Medicine, Baltimore, MD 21201, USA*

[c]*Department of Psychiatry, University of Maryland, School of Medicine, Baltimore, MD 21201, USA*

**Abstract—The prostaglandin $E_2$ ($PGE_2$) mediates estradiol-induced masculinization of sexual behavior in the rat during a perinatal sensitive period. $PGE_2$ induces formation of dendritic spines on preoptic area (POA) neurons and this synaptic pattern change is associated with the ability to express male sexual behavior as an adult. Whether $PGE_2$ is released from astrocytes or neurons in the developing POA is unknown. To further understanding of how $PGE_2$ induces dendritic spine formation at the cellular level, we have explored the $PGE_2$ receptor subtype mediating this response. There are four receptors for $PGE_2$, EP1, EP2, EP3 and EP4, each having unique but interacting signal transduction profiles. Treatment of newborn female rats with the EP receptor agonists iloprost, butaprost and sulprostone indicated that stimulation of both the EP2 and EP3 receptors significantly increased spinophilin, a protein whose levels positively correlate to the presence of dendritic spines and masculinization of the POA. Use of antisense oligonucleotides against the mRNA for each receptor reveals that either EP2 or EP3 receptor knockdown reduces spinophilin in $PGE_2$- or estradiol-treated females, whereas reducing EP1 or EP4 receptor levels by the same means has a smaller but also significant effect. A developmental profile of EP receptor expression indicates EP1 in particular is elevated for the first few days of life, corresponding to the critical period for masculinization, whereas mRNA levels for the other three receptors remain relatively constant.** 

**Key words: cox-2, masculinization, male sexual behavior, sex differences.**

The preoptic area (POA) is a subdivision of the rostral hypothalamus and serves an integral role in adult behavioral patterns associated with reproduction such as sexual behavior and parenting. Neurotoxic lesions of the POA disrupt normal copulation in males (Christensen et al., 1977; Meisel and Sachs, 1997) and direct androgen stimulation of the POA elicits male-typical sexual behaviors after castration (Christensen and Clemens, 1974). The capacity to express male sexual behavior in adult inbred laboratory rats is dependent upon developmental exposure to estradiol (Booth, 1977), which is aromatized from androgen precursors in neurons. The origin of the androgens is the fetal and neonatal testis, resulting in sexually dimorphic hormonal milieus during a critical perinatal sensitive window that organizes the neural substrate(s) mediating adult behavior. Developmental exposure to estradiol results in numerous sexually dimorphic aspects of the POA including cellular morphology of both neurons and astrocytes, and synaptic patterning (Dohler et al., 1984; McCarthy et al., 2002, 2003; Amateau et al., 2004; Amateau and McCarthy, 2002a,b). Parameters seen normally in males can be mimicked in females by exogenous administration of high levels of estradiol or testosterone during a perinatal sensitive period. We recently reported that a principal action of estradiol in the developing POA is up-regulation of the enzyme, cyclooxygenase-2 (COX-2), which converts arachidonic acid into prostaglandin $G_2$, ultimately resulting in increased prostaglandin $E_2$ ($PGE_2$) via the enzyme PGE synthase acting on the substrate prostaglandin $H_2$ (Blatteis et al., 2005). Elevated $PGE_2$ in the developing POA results in increased dendritic spines via an AMPA receptor dependent mechanism and this morphological change to the neuronal network correlates with the expression of male sexual behavior in adulthood (Amateau and McCarthy, 2004). Treatment of newborn females with $PGE_2$ directly into the POA results in the capacity to express the full complement of male sexual behavior (exclusive of intromission and ejaculation) as adults and is a first ever demonstration of masculinization in the absence of exogenous hormone administration (Amateau and McCarthy, 2004). Thus, $PGE_2$ is a major mediator of estradiol-induced masculinization of male sexual behavior and increased dendritic spine density on POA neurons.

The role of prostaglandins in neuronal functioning appears highly varied and poorly understood, particularly in regard to brain development. In adults, $PGE_2$ levels vary in response to nociceptive events such as inflammation (Bar et al., 2004) and cerebral ischemia (McCullough et al., 2004). $PGE_2$ signaling is propagated by interaction with the EP class of receptors identified as EP1-4 (Boie et al.,

*Correspondence to: M. M. McCarthy, Departments of Physiology and Psychiatry, University of Maryland, Baltimore, 655 West Baltimore Street, Baltimore, MD 21201, USA. Tel: +1-410-706-2655; fax: +1-410-706-8341.
E-mail address: mmccarth@umaryland.edu (M. M. McCarthy).

*Abbreviations:* COX-2, cyclooxygenase-2; GSK-3$\alpha$, glycogen synthase kinase; iad, integrative grayscale pixel area density; oligo, oligonucleotide; $PGE_2$, prostaglandin $E_2$; PI3 kinase, phosphatidylinositol 3-kinase; PN, postnatal day; POA, preoptic area; RT-PCR, real-time polymerase chain reaction; SCRAM, scrambled; TBS, tris buffered saline.

0306-4522/07$30.00+0.00 © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.02.006

1997). Contrary to our previous findings it is clear that all four prostanoid receptors are present in the developing POA, but which are responsible for estradiol mediated masculinization of the region remains unknown. A reliable and robust marker for $PGE_2$-mediated masculinization of the developing POA is the upregulation of spinophilin, a signaling protein preferentially localized to the necks of dendritic spines and strongly and positively correlated with the presence of spines themselves (Amateau and McCarthy, 2004).

In the present study we use selective agonists to stimulate EP receptor activity, as well as reduce receptor protein levels with antisense oligonucleotide (oligo) sequences to gain insight into which EP receptor subtypes mediate $PGE_2$ effects on spinophilin levels. The results indicate that endogenous expression levels of all receptors are necessary for the full induction of POA spinophilin by estradiol and/or $PGE_2$ but also indicate the combination of selective EP2 or EP3 activation produces a result identical to that of the hormones.

## EXPERIMENTAL PROCEDURES

### Animals

Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were mated in our animal facility. Pregnancy was confirmed by presence of sperm in a vaginal smear and pregnant dams were isolated and allowed to deliver normally. Animals were given free access to food and water and kept on a reverse 12-h light/dark cycle. On the day of birth [postnatal day 0 (PN0)], pups from multiple litters were assigned to experimental groups All procedures were approved by the IACUC of the University of Maryland, Baltimore, MD, USA.

### I.c.v. injections

Injections were performed by hand after pups were anesthetized by cold and placed under bright light where the cranial landmark Bregma is visible underneath the skin, and can be used to locate the ventricles. Injections were performed bilaterally with a 23 gauge 1 $\mu$l Hamilton syringe (Hamilton Co., Reno, NV, USA) stereotaxically lowered to a depth of 4 mm. A volume of 1 $\mu$l was infused over a period of 60 s.

### Experiment 1

*EP receptor agonist manipulations.* Female pups were treated within 6 h of birth, and again ~24 h later. Pups were assigned to one of six treatment groups and given either (1) vehicle alone ($n$=5), (2) 2.5 $\mu$g $PGE_2$ (Sigma, St. Louis, MO, USA) ($n$=5), (3) 22.7 $\mu$g iloprost, an EP1 agonist ($n$=6), (4) 25 $\mu$g butaprost, an EP2 agonist ($n$=6), (5) 7.14 $\mu$g sulprostone, an EP1/EP3 agonist ($n$=6), (6) 7.14 $\mu$g sulprostone and 25 $\mu$g butaprost ($n$=6). The 2.5 $\mu$g dose of $PGE_2$ is consistent with previous studies in our laboratory and shown to be sufficient to induce masculinization (Amateau and McCarthy, 2002b). Doses of the synthetic agonists necessary to mimic the control dose of $PGE_2$ were calculated by a ratio of disassociation constants ($K_d PGE_2$/$K_d$agonist) and then multiplied by 2.5. All EP receptor agonists were obtained from Cayman Chemical (Ann Arbor, MI, USA). All drugs were diluted in 100 $\mu$l 0.9% saline vehicle and administered by i.c.v. injection. Brains were collected on PN2, ~24 h after the second treatment.

*Microdissections.* Under aseptic conditions, animals were killed on PN2. Brains were removed and placed in a Zivic Miller brain mold (Zivic-Miller Laboratories, Portersville, PA, USA) and sectioned at 1 mm. The optic chiasm which appears at the rostral portion of the diencephalon, served as the marker to guide rostral-to-caudal sectioning within the mold. The POA was then dissected out using the anterior commissure as the perimeter for both dorsal and lateral incisions. The next most caudal 1 mm section was isolated, the cortex peeled from the dorsal end of the slice, and the 1 mm thick section of both hippocampi was dissected. The dissected tissue was immediately collected, flash frozen in isopentane, and stored at −80 °C.

*Western immunoblots.* The microdissected POA was homogenized in radio-immunoprecipitation assay buffer and then centrifuged at −9 °C at 3000 r.p.m. for 30 min. Supernatant was removed and used for subsequent experiments. For standardization, protein concentration of samples was determined by Bradford protein assay. Aliquots of 15 $\mu$g of each protein sample were electrophoresed through an 8–16% precast SDS–polyacrylamide gel (Novex, San Diego, CA, USA) and then transferred to a polyvinyl difluoride membrane (Bio-Rad, Hercules, CA, USA). Blocking was done in 5% non-fat milk in 0.1% Tween tris-buffered saline (TBS) for 1 h at room temperature for hybridizations with anti-spinophilin. Membranes were then incubated with anti-spinophilin/neurabin II rabbit polyclonal IgG at 1:3000 (Upstate Biotechnology, Lake Placid, NY, USA). Secondary incubations for 30 min at room temperature were done in either goat anti-rabbit HRP conjugated IgG at 1:10,000 dilution (New England Biolabs, Beverley, MA, USA). The Phototope chemiluminescence system (New England Biolabs) was used to detect protein. All blots were exposed on Hyperfilm-ECL (Amersham, Arlington Heights, IL, USA) for 30 s.

### Experiment 2

*Antisense oligo interference with EP receptor mRNA translation.* On the day of birth, female pups were assigned to six experimental groups. SCRAM+saline ($n$=6), SCRAM+$PGE_2$ ($n$=6), EP1AS+$PGE_2$ ($n$=6), EP2AS+$PGE_2$ ($n$=6), EP3AS+$PGE_2$ ($n$=6), or EP4AS+$PGE_2$ ($n$=5). All oligo treatments were administered at a concentration of 0.5 $\mu$g/$\mu$l saline via i.c.v. injection as outlined above, followed ~4 h later by $PGE_2$ infusion (2.5 $\mu$g/$\mu$l saline via i.c.v.). This procedure was repeated on PN1 for a total of four infusions (two of oligo, two of $PGE_2$). Animals were killed and brains dissected on PN2. Oligo sequences were generated from GenBank accession numbers for prostanoid receptor sequences: EP1 (NM_013100); EP2 (NM_031088); EP3$\alpha$ (NM_012704); EP3$\beta$ (X80133); EP4 (D28860). A random sequence of oligos (SCRAM) served as a control for oligo infusion. The synthetic oligo sequences are as follows: SCRAM C*C*G* ATG AAC TGT CGC GAT G*G*A*, EP1AS G*G*C* TCA TAT CAG TGG CCA A*G*A* (571–591), EP2AS A*A*G* AAT TGT CCA TGG TGG A*G*G* (35–55), EP3AS A*C*A* CGC CGG TAG TGG C*G*G* (78–98 EP3$\alpha$, 96–116 EP3$\beta$), EP4AS A*C*T* CCA ACC ACC ATC CAG G*T*C* (62–82). All oligos contained locked nucleic acid (LNA) bases that are 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 relevant genome. The sequence SCRAM had no significant homology to any sequence in the genome. Oligos were obtained from Proligo (Boulder, CO, USA).

*Treatment with estradiol.* To investigate the effects of antisense with estradiol, the previous experiment using antisense with $PGE_2$ treatment was repeated in an identical manner with the exception of using estradiol in place of $PGE_2$. Female pups were injected s.c. with estradiol benzoate (100 $\mu$g) in 0.1 mL sesame seed oil or oil alone on PN0 and again on PN1. This dose is routinely used in our laboratory for induction of masculinization of the brain (Amateau and McCarthy, 2004; Mong and McCarthy,