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DISTRIBUTION OF mRNAs ENCODING CLASSICAL PROGESTIN RECEPTOR, PROGESTERONE MEMBRANE COMPONENTS 1 AND 2, SERPINE mRNA BINDING PROTEIN 1, AND PROGESTIN AND ADIPOQ RECEPTOR FAMILY MEMBERS 7 AND 8 IN RAT FOREBRAIN

K. A. INTLEKOFER AND S. L. PETERSEN[1]*

Center for Neuroendocrinology, University of Massachusetts, Amherst, MA 01003, USA

Abstract—Several lines of evidence suggest the existence of multiple progestin receptors that may account for rapid and delayed effects of progesterone in the CNS. The delayed effects have been long attributed to activation of the classical progestin receptor (Pgr). Recent studies have discovered novel progestin signaling molecules that may be responsible for rapid effects. These include progesterone receptor membrane component 1 (Pgrmc1), Pgrmc2, progestin and adipoQ receptor 7 (Paqr7) and Paqr8. The functions of these molecules have been investigated extensively in non-neural, but not in neural tissues, partly because it is unclear which are expressed in the brain and where they are expressed. To address these issues, we compared the distributions of mRNAs encoding Pgr, Pgrmc1, Pgrmc2, Paqr7 and Paqr8 using *in situ* hybridization with radiolabeled oligodeoxynucleotidyl probes in forebrain tissues of estradiol-treated female rats. We also examined the distribution of serpine mRNA binding protein 1 (Serbp1), a putative binding partner of Pgrmc1. Analyses of adjacent brain sections showed that the highest expression of mRNAs encoding Pgr, Pgrmc1, Pgrmc2 and Serbp1 was detected in several hypothalamic nuclei important for female reproduction. In contrast, expression patterns of *Paqr7* and *Paqr8* were low and homogeneous in the hypothalamus, and more abundant in thalamic nuclei. The neuroanatomical distributions of these putative progestin signaling molecules suggest that Pgrmc1 and Pgrmc2 may play roles in neuroendocrine functions while Paqr7 and Paqr8 are more likely to regulate sensory and cognitive functions.

Key words: neuroendocrine, steroid, mPR, progestin, mapping.

Progesterone (P_4) is widely recognized for its ability to regulate neural functions related to reproduction, but it also affects diverse processes such as cognition and neurogenesis (Berman et al., 1997; Giachino et al., 2003). The traditional tenet of P_4 action is that it binds the cognate progestin receptor (Pgr), and functions as a ligand-activated transcription factor to regulate gene expression. However, rapid non-genomic effects have also been reported (Meyerson, 1972; Parsons et al., 1980; Mani et al., 1994), and P_4 can act in the absence of Pgr (Frye et al., 2006). These data support the emerging concept that P_4 actions in the brain may be through the classical Pgr and also through non-classical mechanisms.

Current research suggests that there are several possible candidates for mediating the non-classical effects of P_4. One such protein is progesterone receptor membrane component 1 (Pgrmc1), but it does not appear to function as a traditional receptor because it requires a binding partner known as serpine mRNA binding protein 1 (Serbp1) (Peluso et al., 2005, 2006). Moreover, the structure of Pgrmc1 does not share homology with either classical steroid receptors or G-coupled protein receptors (Mifsud and Bateman, 2002). Nonetheless, Pgrmc1 mediates several important Pgr-independent effects. For example, P_4 acts through Pgrmc1 to activate phosphoinositide-dependent protein kinase 1 and phosphorylate Akt (Hand and Craven, 2003). In the ovary, Pgrmc1 and Serbp1 form a receptor complex required for the antiapoptotic effects of P_4 in granulosa cells (Peluso et al., 2006; Zhang et al., 2008). Pgrmc2 is a closely related isoform of Pgrmc1, differing mainly in its N-terminus, but there is virtually no information regarding Pgrmc2 function (Falkenstein et al., 1999; Peluso et al., 2005). Pgrmc1 has been localized to several brain regions (Krebs et al., 2000; Sakamoto et al., 2004; Meffre et al., 2005), but no studies have systematically mapped its distribution and the role of this protein in the brain remains unknown. Likewise, no studies have mapped neural expression of *Serbp1* or *Pgrmc2*. Despite these limitations, several lines of evidence indicate that Pgrmc1/Serbp1, and possibly Pgrmc2, may be important for non-classical P_4 actions in the brain.

Two other candidates for mediating the non-genomic effects of P_4 are progestin and adipoQ receptor 7 (Paqr7) and Paqr8. These are G-protein coupled receptors first discovered in spotted sea trout, and subsequently in mammalian tissues (Zhu et al., 2003a,b). Although activation of these receptors by P_4 regulates cAMP levels and mitogen-activated protein kinase activity in fish (Hanna et al., 2006), there is some debate about whether they function as bona fide P_4 receptors in mammals (Fernandes et al., 2008). Recent reports detected Paqr7 and Paqr8 mRNAs in hy-

[1] Present address: Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003, USA.
*Corresponding author. Tel: +1-413-545-1808; fax: +1-413-577-1665.
E-mail address: spetersen@vasci.umass.edu (S. L. Petersen).
Abbreviations: Arc, arcuate nucleus; ISHH, *in situ* hybridization histochemistry; LH, luteinizing hormone; MPN, medial preoptic nuclei; PAG, periaqueductal gray; Paqr7/8, progestin and adipoQ, receptor 7 or 8; Pgr, progestin receptor; Pgrmc1/2, progesterone receptor membrane component 1/2; PVN, paraventricular nucleus; P_4, progesterone; SCN, suprachiasmatic nuclei; Serbp1, serpine mRNA binding protein 1; SON, supraoptic nucleus; QPCR, quantitative polymerase chain reaction; VMH, ventromedial nucleus.

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pothalamic tissue of mice (Sleiter et al., 2009), but the exact anatomical localization is unknown. Collectively, these findings raise the possibility that Paqr7 and Paqr8 also mediate P_4 neural actions.

Although there is abundant evidence that these signaling molecules participate in P_4 signaling, it is unclear which are important in the nervous system. Moreover, while many neural functions are modulated by P_4, there is little information about which functions require Pgr, non-nuclear receptors or both. One obstacle to resolving this question is that neither the classical Pgr nor any of the non-classical P_4 receptor candidates have been systematically mapped in the brain. To address this issue, we used *in situ* hybridization histochemistry (ISHH) to map the expression of mRNAs encoding Pgr, Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8. In these studies, we used female rats because of the important role P_4 plays in regulation of female-specific physiological functions.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Six adult female Sprague–Dawley rats (225–250 g; approximately 95 days of age; Harlan Sprague–Dawley, Madison, WI, USA) were individually housed in the Animal Care Facility on a 14:10 light:dark cycle with food and water provided *ad libitum*. To achieve a similar hormonal milieu among rats, we ovariectomized them and implanted two Silastic (Dow Corning, Midland, MI, USA) capsules containing E_2 (150 μg/ml 17β-estradiol in sesame oil) a week later as described previously (Petersen and LaFlamme, 1997). Twenty-four hours later, we collected brains and rapidly froze and stored them at −80 °C until they were cryosectioned (Leica CM3000, Nussloch, Germany).

For three animals, 14 μm coronal forebrain sections were obtained and thaw-mounted onto gelatin-coated slides and stored at −80 °C until ISHH was performed. The remaining three animals were used for RNA isolation in validation studies described below.

Oligodeoxynucleotidyl probe preparation

In these studies, we used oligodeoxynucleotidyl probes of the same length and specific activity. Antisense oligodeoxynucleotide sequences used for end-tailing are provided in Table 1. Both sense and antisense sequences were produced by an automated DNA synthesizer and purified by reverse-phase HPLC by Integrated DNA Technologies (Coralville, IA, USA). Oligodeoxynucleotides were 3′-end labeled with [α^{33}P]-dATP (PerkinElmer, Waltham, MA, USA) using terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN, USA) as described previously (Petersen et al., 1989). Incubation was halted by addition of TE (10 mM Tris–HCl; pH 8.0, 1 mM EDTA), and the probe was purified by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was washed with 70% ethanol and resuspended in 25 μl TE.

ISHH

The distribution pattern for each mRNA was determined in a separate ISHH run, and tissue sections were prehybridized as previously described (Ottem et al., 2004). Radioisotopic probes (0.5×10^6 cpm) were applied directly to brain tissue in 20 μl hybridization buffer. This buffer contained 4×SSC (1×SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.2), 50% (v/v) formamide, 10% (w/v) dextran sulfate, 250 μg/ml yeast tRNA, 1× Denhardt's solution, 500 μg/ml heparin sodium salt, 0.1% sodium pyrophosphate and 0.05 M dithiothreitol added freshly before use. Sections were covered with glass coverslips and hybridized overnight at 37 °C in humidified plastic boxes. Slides were removed from 37 °C and allowed to cool, and coverslips were floated off in 1×SSC. Sections were washed four times for 15 min each in 2×SSC-50% formamide solution at 40 °C, followed by four washes, 15 min each, in 1×SSC. Finally, slides were rinsed in water and briefly dehydrated in 70% ethanol. The slides were air-dried and apposed to Kodak BioMax MR film (Rochester, NY, USA) for signal detection. In order to acquire optimum signal, autoradiograms were developed at 1, 3 and 6 weeks by an X-ray film processor and images were acquired using BioQuant Imaging Software (Bio-Quant Inc., Nashville, TN, USA) and a CCD videocamera (QImaging QICAM *FAST* color, Surrey, BC, Canada).

Validation of probe specificity

To determine the specificity of the hybridization signal, sense strand probes to each target of interest were hybridized to representative sections. To verify specificity of each antisense probe, subsets of adjacent slides were treated with RNAse A solution (100 μg/ml RNAse A in 0.5 M NaCl, 0.05 M EDTA and 0.01 M Tris–HCl) for 1 h at 37 °C following prehybridization. An additional set of slides was used for Nissl staining in order to provide reference material for identification of specific brain regions.

Regardless of exposure time, Paqr7 and Paqr8 antisense probes produced diffuse and homogeneous signal, therefore multiple probes (Table 2) were used for each gene to verify signal specificity. To ensure specificity in regions that displayed low ISHH signal for Paqr7, quantitative polymerase chain reaction (QPCR) was performed using cDNA derived from RNA of the diagonal band of Broca and striatum, regions with two different signal intensities. RNA was isolated from tissue punches using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA) and reverse transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen) and manufacturer's protocol. Reactions were performed in a Stratagene Mx3000P instrument programmed as follows: 95 °C, 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Reactions contained reagents from QuantiTect SYBR Green Kit and manufacturer's protocols were used (Roche Diagnostics, Indianapolis, IN, USA). Specific primer sets were obtained from

Table 1. Sequences for oligodeoxynucleotidyl probes used in ISHH studies

NCBI gene name and accession #	Oligodeoxynucleotide sequences 5′–3′	Antisense to bases:
Pgrmc1 NM_021766	TGTAGTTCCAACCCAATTACCAGGTGTGTGAGAGTTACTGTGTGTGGG	1354–1305
Pgrmc2 NM_001008374	CAAAGTTCAGTCCTGTTTACTGTGATCCTTGGTGTCCTCCTCGTCTGT	667–619
Serbp1 NM_145086	AAGGAACCAGTGTTGTATTATGGCATCCAGTTAGGCCAGAGCGGGGAA	1274–1227
Paqr7 NM_001034081	ATAGTCCAGCGTCACAGCTTCTAGCTGGGCTAAAGTGCACAGCACC	888–843
Paqr8 NM_001014099	GCCAGCCCCGCTGGTACCACTTGACAGATCTTCCGCATAACTGGATAA	1112–1065
PgR NM_022847	CACATGGTAAGGCACAGCGAGTAGAATGACAACTCCTTCATCCTCTGC	2375–2328