Neuroscience 166 (2010) 94–106

MEMBRANE PROGESTERONE RECEPTORS LOCALIZATION IN THE MOUSE SPINAL CORD

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Abstract—The recent molecular cloning of membrane receptors for progesterone (mPRs) has tremendous implications for understanding the multiple actions of the hormone in the nervous system. The three isoforms which have been cloned from several species, mPRα, mPRβ and mPRγ, have seven-transmembrane domains, are G protein-coupled and may thus account for the rapid modulation of many intracellular signaling cascades by progesterone. However, in order to elucidate the precise functions of mPRs within the nervous system it is first necessary to determine their expression patterns and also to develop new pharmacological and molecular tools. The aim of the present study was to profile mPR expression in the mouse spinal cord, where progesterone has been shown to exert pleiotropic actions on neurons and glial cells, and where the hormone can also be locally synthesized. Our results show a wide distribution of mPRα, which is expressed in most neurons, astrocytes, oligodendrocytes, and also in a large proportion of NG2$^+$ progenitor cells. This mPR isoform is thus likely to play a major role in the neuroprotective and promyelinating effects of progesterone. On the contrary, mPRβ showed a more restricted distribution, and was mainly present in ventral horn motoneurons and in neurites, consistent with an important role in neuronal transmission and plasticity. Interestingly, mPRβ was not present in glial cells. These observations suggest that the two mPR isoforms mediate distinct and specific functions of progesterone in the spinal cord. A significant observation was their very stable expression, which was similar in both sexes and not influenced by the presence or absence of the classical progesterone receptors. Although mPRγ mRNA could be detected in spinal cord tissue by reverse transcriptase–polymerase chain reaction (RT–PCR), *in situ* hybridization analysis did not allow us to verify and to map its presence, probably due to its relatively low expression. The present study is the first precise map of the regional and cellular distribution of mPR expression in the nervous system, a prior requirement for *in vivo* molecular and pharmacological strategies aimed to elucidate their precise functions. It thus represents a first important step towards a new understanding of progesterone actions in the nervous system within a precise neuroanatomical context.

Key words: membrane progesterone receptor, progesterone, spinal cord, motoneuron, oligodendrocyte, astrocyte.

Recent studies have identified the spinal cord as an important target for the actions of progesterone. After injury and in a genetic model of spontaneous motoneuron degeneration, progesterone has been shown to exert marked protective effects on motoneurons and to regulate the expression of genes involved in neuronal functions and viability (Labombarda et al., 2002; De Nicola et al., 2006; Schumacher et al., 2007). In addition, progesterone regulates the synthesis of astrocyte-specific proteins, including the intermediate filament glial fibrillary acidic protein (GFAP), and it targets cells of the oligodendrocyte lineage, promoting the maturation of oligodendrocytes and myelin formation (De Nicola et al., 2006; Labombarda et al., 2009). In a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), progesterone treatment attenuated disease severity and reduced inflammatory responses and the occurrence of demyelination (Garay et al., 2007).

In spite of the multiple effects of progesterone at the level of the spinal cord, its signaling mechanisms remain poorly understood. It is likely that its transcriptional effects, also named genomic effects, involve intracellular progesterone receptors (PR), as their presence in the rodent spinal cord has been demonstrated (Labombarda et al., 2000a, 2003). However, the precise functions of the two PR isoforms, PR-A and PR-B, which have different transcriptional activities, still remain to be elucidated in the nervous system (Conneely and Jericevic, 2002). Furthermore, direct actions of progesterone on specific membrane receptors may also play an important role. Progesterone is indeed the first steroid hormone for which membrane receptors have been cloned and well characterized, thus opening up completely new perspectives for understanding its actions in the nervous system. Progesterone receptor membrane component 1 (PGRMC1), previously also named 25-Dx, was a first membrane progesterone receptor candidate to be cloned, but the capacity of the protein to bind progesterone and its precise functions remain controversial (Cahill, 2007; Losel et al., 2008). In the rat spinal cord, PGRMC1-immunoreactivity has been located to dor-

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Abbreviations: ANOVA, analysis of variance; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; HTZ, heterozygous; mPRs, progesterone membrane receptors; PGRMC1, progesterone receptor membrane component 1; PR, progesterone receptors; RT–PCR, reverse transcriptase–polymerase chain reaction.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2009.12.012

sal horn and central canal ependymal cells, but is absent in ventral horn motoneurons, which are important targets of progesterone (Labombarda et al., 2003; Guennoun et al., 2008).

In 2003, a new family of progesterone membrane receptors (mPRs) was been cloned from seatrout ovaries (Zhu et al., 2003a,b). The *mPRs,* which have been subdivided into three subtypes *(mPRα, mPRβ* and *mPRγ)*, *are* unrelated to the known nuclear receptors. However, they display characteristics of true receptors, including the structure of a membrane-spanning protein with seven-transmembrane domains, plasma membrane localization, expression in steroid target tissues, selective steroid binding, regulation of intracellular signaling pathways and biological functions (Zhu et al., 2003b; Thomas, 2008). The best characterized subtype is mPRα, which has been cloned from both fishes and mammals, including seatrout and humans (Zhu et al., 2003a). When expressed in a human breast cancer cell line, mPRα from both species displayed high affinity for progestin hormones, with a Kd≈5 nM, which is however less than the affinity reported for the intracellular PRs (kd≈0.3 nM) (MacLusky and Ewen, 1980). The mPRαs showed a limited binding capacity for progestins, and they appear to be directly coupled to Gi proteins. Most importantly, competitive binding assays have revealed that recombinant mPRα exhibits a particular pharmacological profile, and that it does not bind a series of synthetic progestins designed to target intracellular PRs and used in contraception or hormone replacement therapy (HRT) (Thomas et al., 2007). mPRα, mPRβ and mPRγ have recently been shown to mediate progesterone-dependent gene regulation at physiologically relevant concentrations (EC50 in the nM range) in a heterologous (yeast) expression system that was also used to confirm the unique progestin binding characteristics of the three receptors (Smith et al., 2008). Together, these findings raise hope for a new pharmacology of progestins, with the development of selective ligands for the membrane receptors.

Whereas nothing is currently known concerning the expression patterns and regulation of the mPRs in the nervous system, they have begun to be explored in the mammalian reproductive tract. A recent study has provided evidence for the presence of mPRs in the rat corpus luteum, a tissue which responds to progesterone, but interestingly does not contain detectable levels of intracellular PR (Cai and Stocco, 2005). Both mPRα and mPRβ are also expressed in human myometrium. In cultured human myometrial cells, mPR activation lead to the transactivation of PR-B and to a decrease in steroid receptor coactivator-2 (SRC-2) expression, thus suggesting a cross-talk between mPR and PR signaling (Karteris et al., 2006). Both mPRα and mPRβ subtypes are also present in the preoptic anterior hypothalamic region of the rodent brain and in immortalized GnRH-secreting neurons (GT1–7 cells). Treatment with mPRα siRNA attenuated specific progesterone binding to GT1–7 cell membranes and reversed the progesterone inhibition of cAMP accumulation. These results suggest a role for mPRα in mediating progesterone feedback on GnRH secretion (Thomas, 2008; Sleiter et al., 2009).

However, understanding the physiological significance of mPRs *in vivo* will have to await the development of specific pharmacological tools (selective mPR agonists and antagonists) and the generation of transgenic mouse models of mPR deletion, in particular with targeted inactivation of the mPR genes in specific tissues or cell types. The use of antisense probes or *siRNA* for target-specific gene silencing offers another option for functional studies. However, a first requirement for all these experimental strategies to unmask mPR functions is to define the precise expression patterns of the different mPR types to cellular resolution, both at the mRNA and protein levels, and to study their regulation. Because of the well-documented and multiple effects of progesterone in the spinal cord, some of which are likely to involve membrane actions, the aims of the present study were to determine whether mPRα, mPRβ and mPRγ are expressed and regulated by estradiol and progesterone in the mouse spinal cord, and to precisely map their regional and cellular distributions.

EXPERIMENTAL PROCEDURES

Animals

All procedures concerning animal care and use were carried out in accordance with the European Community Council Directive (86/609/EEC). Efforts were made to minimize the number of animals used and their suffering. Either 10-week-old male C57BL6 mice (Janvier, France), or adult male and female PR knockout (PRKO, $PR^{-/-}$), heterozygous (HTZ, $PR^{+/-}$) or the corresponding wild-type (WT, $PR^{+/+}$, C57BL6/129SvEv hybrid background) mice were used in this study. Original breeders were provided by J. P. Lydon (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, USA). The development of PRKO mice has been previously described (Lydon et al., 1995). The WT, HTZ and PRKO mice were housed separately, four animals per cage, with food and water available *ad libitum*.

Genotyping. Each mouse was identified for its PR genotype by using a validated protocol (Lydon et al., 1995). Briefly, genomic DNA from mouse tails was extracted by the phenol/chloroform method and 1 μg of DNA was subjected to PCR amplification using taq DNA polymerase (Invitrogen, Inc.). PCR was performed by denaturing the DNA at 94 °C for 5 min, followed by 37 cycles of amplification: 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The following PR-specific primers were used: P1 (5′-TAG ACA GTG TCT TAG ACT CGT TGT TG-3′), P3 (5′-GAT GGG CAC ATG GAT GAA ATC-3′), and a neo gene-specific primer, N2 (5′-GCA TGC TCCAGA CTG CCT TGG GAA A-3′). The presence of primer-amplified PCR product was detected on agarose gel and visualized by ethidium bromide fluorescence. We observed the presence of a 589 bp DNA band for $PR^{+/+}$ mice (corresponding to the *PR* gene, P1/P3 primers), a 473 bp band for $PR^{-/-}$ mice (P1/N2 primers), or both bands for $PR^{+/-}$ mice.

Analysis of mPRα, mPRβ, and mPRγ mRNA expression

RNA isolation, cDNA synthesis and PCR. Mice were sacrificed by decapitation, and the lumbar region of the spinal cord was dissected out, frozen on dry ice and stored at −80 °C until use. Brain, testis and kidney were also sampled and used as positive