A sexually dimorphic distribution pattern of the novel estrogen receptor G-protein-coupled receptor 30 in some brain areas of the hamster

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Abstract

The isolation of the G-protein-coupled receptor 30 (GPR30), an orphan membrane receptor unrelated to nuclear estrogen receptors (ERs), has become a key factor towards the unraveling of rapid estrogen action. This membrane receptor together with cellular signaling intermediaries, i.e., extracellular signal-dependent kinases 1 and 2, may promote neuronal proliferation and differentiation activities. In the present study, an evident gene expression pattern of GPR30 characterized postnatal 7 (young) and 60 (adult) days of age hamsters as shown by its heterogeneous mRNA distribution in hypothalamic, amygdalar and cerebellar areas of both sexes. In particular, most of the brain areas considered in the adult hamster plus only the amygdala and cerebellum of young animals behaved in a sexually dimorphic fashion. This similar pattern was also detected for the ERα and β, as shown by the latter receptor prevailing in young and adult females, while the former predominated in young females. Even for the two kinases, a sexually dimorphic distribution was featured above all for young hamsters. Overall, the findings of the present study established a distinct expression pattern of the novel ER (GPR30) that may operate differently in some brain areas of the hamster and this may provide interesting insights regarding its probable neuroprotective role during the execution of some hibernating states, which are typical of our rodent model.


Introduction

A plethora of evidence has demonstrated that estrogens are involved in many physiological processes in mammals, including developmental features, cellular homeostasis and neurobiological activities (Kow et al. 2005, Simpson et al. 2005) as well as pathological conditions (Maggiolini et al. 2004). Recently, in addition to the classical genomic mechanisms of action of intracellular estrogen receptor (ER) α and β, some studies have begun to emphasize the importance of a rapid non-genomic steroid action by the binding of 17β-estradiol (E2) to a G-protein-coupled receptor 30 named GPR30. Although it is often straightforward to link the physiological effects of E2 to a genomic model, considerable controversy still exists on its ability to elicit transcriptional responses independently of the classical nuclear receptor isoforms. On the basis of such observations, growing attention is beginning to be focused on some major health conditions such as tumor formations in which estrogen-dependent activities in the absence of ERs seem to predominantly operate through GPR30 (Filardo & Thomas 2005). At the brain level, an elevated number of estrogen-dependent neuronal actions in areas such as hypothalamus (HTH), which is noted for its estrogen-enriched properties (McEwen 1991), seem to underlie GPR30 as a key factor for rapid cerebral estrogen actions (Canonaco et al. 2002). Recently, studies have pointed to the specific activity of GPR30 being tightly linked to some cellular signaling intermediaries, such as extracellular signal-related kinase (ERK) 1/2, since estrogens are able to induce their activities even in the absence of ERs (Sweatt 2004) and the inhibition of these factors by the rapid signaling cascades seems to decrease the potentiation of neuronal transcriptional activities (Vasudevan et al. 2005). Moreover, the aforementioned signaling intermediaries that seem to overlap the expression pattern of GPR30 throughout the brain have been reported to regulate neuronal proliferation, differentiation and postsynaptic processing. In this context, estrogen-induced neuronal signals seem to facilitate early ERK-dependent migration of brain elements and namely neurons and glia (Zsarnovszky & Belcher 2004).

On the basis of these neuronal characteristics, it was our intention to evaluate the sexually dimorphic distribution pattern of GPR30 in some brain areas of young postnatal day 7 (PND7) and 60-day-old (PND60, adults) golden hamster (Mesocricetus auratus), a hibernating rodent. In addition, this pattern was correlated to that
of both ERα and ERβ as well as ERK1/2 considering the protective roles exerted by these factors against excitotoxicity events in cortical and hippocampal neurons (Nielsen & Díaz 2003). Overall, the resulting sexually dimorphic distribution pattern of GPR30 expressing neurons could nicely represent a starting point regarding its role not only on estrogen functions in the absence of ERs, but also on ischemic-like events that are evoked in hibernators during arousal (Canonica et al. 2005).

Materials and Methods

Animals and dissections of brain regions

Sexually mature female (n = 6) and male (n = 6) golden hamsters (M. auratus) at PND60 (adults) plus female (n = 10) and male (n = 10) pups at PND7 were purchased from Charles River (Como, Italy). They were housed in the stabularium of the Cellular Biology Department (University of Calabria, Cosenza, Italy) and maintained at a 14 h light:10 h darkness schedule. All maintenance and experimental procedures were in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals. Efforts were made to minimize animal suffering and reduce the number of specimens used. The brain areas, such as amygdala (AMY), hypothalamus (HTH), thalamus (TH), and cerebellum (Cb), of both sexes at PND60, and at PND7 ages were dissected from the brain of anesthetized decapitated hamsters. For this part, a dissecting microscope was used in order to carefully isolate and remove these specific brain areas that by placing the brain dorsal side up was possible to view them. All operations were handled with sterile surgical instruments that were cleaned with RNAase-free PBS to assure no contamination of these brain areas. The Cb was first area to be removed because by making a coronal cut along the mesencephalic portion behind the inferior colliculi, it was possible to easily remove this rhombencephalic area. Along the mesencephalic portion behind the inferior colliculi, it was possible to view them. All operations were handled with sterilized surgical instruments that were cleaned with RNAase-seZap to assure no contamination of these brain areas. The Cb was the first area to be removed because by making a coronal cut along the mesencephalic portion behind the inferior colliculi, it was possible to easily remove this rhombencephalic area according to its anatomical position reported in the stereotaxic atlas of Paxinos & Watson (1982). Maintaining the brain in the same position and using this atlas, two coronal cuts were made just behind the rostral and caudal limits of the diencephalic area to assure the removal of HTH and TH, while the AMY was just behind the rostral and caudal limits of the diencephalic area. The same position and using this atlas, two coronal cuts were made just behind the rostral and caudal limits of the diencephalic area to assure the removal of HTH and TH, while the AMY was just behind the rostral and caudal limits of the diencephalic area.

Selection of primers

Primers used for RT-PCR were designed on the basis of rat gene sequences: ERα (accession AF181077) forward 5'-CCGCCTACGAGTTCACAC-3' and reverse 5'-CTC-TTAAAGAAAGCCTTGCAGCC-3'; ERβ (accession AB076607) forward 5'-CTATGCAGAAACCTCAAAAAGT CC-3' and reverse 5'-TTCGTGGCAGACAGATAATC-3'; GPR30 (accession DQ237895) forward 5'-GTGGCCGACTCCCTGA TC-3' and reverse 5'-GGCCTTTCTGATGATGATGAGTG-3'; and reverse 5'-GCTGGTGGGGTCCTTTGTTGC-3'; ERK1 (accession S46779) forward 5'-CCTCAAATCTGGCTTATCA AC-3' and reverse 5'-GGGCTTTCTGATGATGATGAGTG-3'; ERK2 (accession M64300) forward 5'-TGGTACAGAGCTCCA GAAT-3' and reverse 5'-GAGCCGTGTTCAACTTCA ATC-3'. The housekeeping gene 36B4 forward 5'-CTCA ACATCTCCCTTTCTC-3' and reverse 5'-CATAATCTCGTCC-3'.

RNA extraction and RT-PCR

Total RNA was extracted from the above-mentioned dissected brain areas of every animal using Triazol reagent as suggested by the manufacturer (Invitrogen). The purity and the integrity of RNA were checked by gel electrophoresis before carrying out any analytical procedure. To determine even low gene expression, PCR, was performed for 40 cycles using the above primers. The cycles were for ERα/β: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for GPR30: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, for ERK1/2: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, for the housekeeping gene 36B4: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, as illustrated in all figure panels of representative analyses. To check for the presence of DNA contamination, a RT-PCR was performed using 1 µg total RNA without Moloney murine leukemia virus reverse transcriptase (negative control). The PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide. At first, all fragments of DNA amplified were cloned and sequenced to verify their exact corresponding sequences, which showed a high homology (>80%) between the rat sequence and the hamster for all genes considered in this study (data not shown).

Statistical analysis

DNA quantity in each lane was analyzed by scanning densitometry and optical density (OD; ± S.E.M.) were evaluated via computer-assisted image analyzer system (National Institutes of Health-Scion Image 2.0). The different expression levels were compared between the two animal sexes of the same PND age using one-way ANOVA plus post hoc Student's t-test. *P < 0.05 (moderately significant); **P < 0.01 (significant); ***P < 0.001 (very robust).

Results

Distribution of ERα/β and GPR30

The application of primers for ERα/β and GPR30 supplied an age-dependent sexually dimorphic pattern of their gene expression. In particular, the notable OD for both ERs (Fig. 1a–d) when compared with housekeeping gene (Fig. 1e) allowed us to show that an early (PND7) moderately significant (P < 0.05) expression capacity characterized both
Sexually dimorphic gene expression of (a and c) ERβ and (b and d) ERα was performed in some brain areas of (a and b) PND7 and (c and d) PND60 *Mesocricetus auratus* by RT-PCR. The control (e) for this and other signaling factors was determined by the application of the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (f) ERβ and (g) ERα in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of *Mesocricetus auratus*. Data obtained by RT-PCR were calculated as optical density (O.D. ± S.E.M.) via computer-assisted image analyzer system (National Institutes of Health – Scion Image 2.0). Statistical analysis: ANOVA plus post hoc test (Neuman–Keul’s multiple range test) were used, *P<0.05; **P<0.01; ***P<0.001.

**Figure 1** Sexually dimorphic gene expression of (a and c) ERβ and (b and d) ERα was performed in some brain areas of (a and b) PND7 and (c and d) PND60 *Mesocricetus auratus* by RT-PCR. The control (e) for this and other signaling factors was determined by the application of the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (f) ERβ and (g) ERα in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of *Mesocricetus auratus*. Data obtained by RT-PCR were calculated as optical density (O.D. ± S.E.M.) via computer-assisted image analyzer system (National Institutes of Health – Scion Image 2.0). Statistical analysis: ANOVA plus post hoc test (Neuman–Keul’s multiple range test) were used, *P<0.05; **P<0.01; ***P<0.001.
ERβ (Fig. 1f) and α (Fig. 1g) of the female AMY with respect to that of the male. In adult males, a significant ($P < 0.01$) expression capacity was detected in HTH for both ERs, apart moderately significant expression levels of ERα in TH, while significant expression levels were typical of ERβ and ERα in the female AMY and Cb respectively.

Interestingly enough, even GPR30 supplied a heterogeneous expression pattern in the different hamster brain areas (Fig. 2a and b), as reported for PND7 male which exhibited a very robust ($P < 0.001$) GPR30 expression level in Cb, while a significant expression signal was detected in AMY of the female hamster (Fig. 2d). In line with the expression pattern of the two ERs, also the greater expression differences of GPR30 appeared to preferentially occur in adult hamsters as shown by the very robust and moderately significant mRNA levels of this membrane receptor in HTH and Cb of the female respectively. On the other hand, a very robust expression signal was instead obtained in both TH and AMY of male adults (Fig. 2d).

When the fitting of GPR30 and the two ERs were handled on representative schemes of medial-posterior telencephalic and diencephalic areas (Fig. 3), it was possible to observe a sexually dimorphic distribution pattern of these two classes of receptors as early as the developmental stages. In a first case, overlapping dense quantities of GPR30 plus ERα and β were demonstrated in AMY and HTH of both animal sex at PND7, aside the lack of ERβ in the latter brain area of the male hamster (Fig. 3a and b). However, an evident sexually dimorphic pattern was mostly typical of adults. Indeed, GPR30 was densely located in the female HTH, while moderate and low levels were instead observed in the male AMY and HTH respectively (Fig. 3c and d). Concerning the ERs, the α isoform seemed to be densely located in the different brain areas of the male adult hamster, despite

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**Figure 2** Sexually dimorphic gene expression of GPR30 was investigated in the same brain areas as for the ER study in (a) PND7 and (b) PND60 Mesocricetus auratus by RT-PCR. Similarly the control (c) for this receptor was determined by applying the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (d) GPR30 in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of Mesocricetus auratus. Data obtained by RT-PCR were calculated as O.D. ($\pm$ S.E.M.) via computer-assisted image analyzer system. Statistical analysis: ANOVA plus post hoc test (Neuman–Keul’s multiple range test) were applied, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. 

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the moderate and low levels being typical of the female AMY and HTH respectively. In this same animal sex, dense ERβ levels are favorably located in TH and AMY.

**Distribution of ERK1/2**

Given that GPR30 functions are tightly linked to ERK1/2, the distribution pattern of these two factors was also taken into consideration. Overall, a heterogeneous pattern was detected in both developmental stages. In particular, elevated ERK1/2 signals were shown in male and female PND7 animals, while lower levels characterized both adult animal sexes (Fig. 4a–d). In spite of the very robust signal registered for ERK1 in the PND7 male Cb with respect to the same brain areas of the female (Fig. 4f), it was the diencephalic areas such as HTH and TH of PND7 female that supplied very robust and moderately significant expression levels respectively. This feature was still maintained in the adult female hamster, as demonstrated by very robust expression levels in HTH and TH of this sex with respect to the same male brain areas (Fig. 4f). In a comparable manner, also the expression of the other signal factor ERK2 exhibited an evident sexually dimorphic pattern in PND7 animals, as shown by very robust levels in the male HTH and AMY (Fig. 4g). On the other hand, very robust and moderately significant signals were typical of female TH and Cb respectively. Nevertheless, a consistent sexually dimorphic expression pattern of ERK2 like ERK1 was not a very common feature of adults since a very robust signal was only observed in TH of male with respect to that of the female (Fig. 4g).

**Discussion**

This first comprehensive study of GPR30, ERα/β, and ERK1/2 in some brain areas of young and adult hamsters displayed an evident sexually dimorphic expression pattern, suggesting that hibernators could very well be a useful model for unveiling the sexually differentiated neurobiological role of the classical action of estrogens, i.e., classical versus rapid non-genomic actions. At present, mechanisms dealing with estrogen-mediated cellular responses are becoming increasingly complex, especially if we consider the recent involvement of GPR30 activity in human cancer cells of reproductive organs (Maggiolini et al. 2004, Vivacqua et al. 2006, Albanito et al. 2007). The functional role of GPR30 has recently been also extended to a wide range of neuronal functions, namely gene expression, differentiation, neuroprotection plus neuroendocrine secretion (Qiu et al. 2006). In line with these observations, GPR30 may be considered a novel ER, which could readily elicit responses to estrogens during ischemia crisis observed during neurodegenerative syndromes.

Indeed, the results of the current study highlighting a differentiated distribution pattern of this orphan membrane
RT-PCR was also used to evaluate the sexually dimorphic gene expression of (a and b) ERK1 and (c and d) ERK2 in the same brain areas as for the ER study of (a and c) PND7 and (b and d) adult Mesocricetus auratus. The control (e) for these kinases was determined by the application of the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (f) ERK1 and (g) ERK2 in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of Mesocricetus auratus. Data obtained by RT-PCR were expressed as O.D. (±S.E.M.) and elaborated via computer-assisted image analyzer system. Statistical analysis: ANOVA plus post hoc test (Neuman–Keul’s multiple range test) were used, *P<0.05; **P<0.01; ***P<0.001.
receptor in estrogen–dense brain sites and areas lacking ERs (Simerly et al. 1990) such as HTH and AMY (McEwen 1991) of young hamsters suggest an early non-genomic type of estrogen effects occurring via GPR30 sites. It is not surprising that GPR30 is operating during developmental stages because recently some authors have begun to link its activity to stressful conditions which may lead to eventual neurogenic and apoptotic effects at an early biological stage (Filardo & Thomas 2005). The notable expression levels occurring in HTH and AMY is in a good agreement with the dense levels observed in the former brain area of male and female rats (Brailoiu et al. 2007) and this could begin to point to GPR30 as an alternative mediator of ERα/β-dependent biological functions (Pérez et al. 2003) plus its involvement on the regulation of energy homeostasis at the HTH (Qu et al. 2006) of hibernators. Moreover, the response of some major hypothalamic nuclei, namely the ventromedial hypothalamic nucleus to rapid estrogen signals via the activation of GPR30 might be responsible for the triggering of specific cell factors (kinases) which in turn decreases the potentiation of transcriptional activities thus accounting for altered lordosis behavior (Vasudevan et al. 2005). In the case of AMY, a great density of GPR30 expressing neurons was detected in young female hamsters, while this effect subsequently predominated in male adults. The fact that this limbic area displays such sexually dimorphic differences in an age-dependent manner tends to support amygdalar neurosteroidal mechanisms of GPR30 provoking greater mood disorders in females (Walfl et al. 2006).

It is worthy to note that an overlapping distribution pattern of ERK1/2 to that of GPR30 occurred in most brain areas of our hibernating model. This relationship should not be considered an astonishing phenomenon especially since these two kinases mediate signals in mostly all estrogenic functions (Zsarnovszky & Belcher 2004), such as the remodeling and protection of vascular cells (Chambliss et al. 2005) and during anti-apoptotic processes in neuronal cells (Koriyama et al. 2003). A very great expression for both kinases in HTH was typical of AMY and Cb of young animals and appeared to be markedly lower in adults, in agreement with the early ERKs high embryonic levels reported in rat Cb (Simerly et al. 1990). On the other hand, elevated levels of the two kinases in adult HTH and TH seem to fit well with their role on endocrine-related activities in hamsters such as satiety and on visual functions respectively (Coogan & Piggins 2005).

In a comparable fashion, even the distribution of the classical ERs exhibited an evident sexually dimorphic pattern for both developmental periods. Interestingly, the prevalence of ERβ in most female brain areas, while α predominates in males are, to a large extent, supported by the pattern obtained for the rat (Pérez et al. 2003). However, despite the different developmental periods in which these isoforms prevail, they definitely seem to have a great bearing on brain morpho-functional features as suggested by ERα promoting a numerically elevated quantity of dendritic spines and axospinous synapses (Simerly et al. 1990). AMY is the other brain area that demonstrated a sexually dimorphic pattern, i.e., the prevalence of ERβ in young females, which is an overlapping condition of high levels detected in the rat lateral amygdalar area (Zhang et al. 2002). It is very likely that the distribution discrepancy of these two subtypes could be of a species-specificity nature as supported by male ewe hypothalamic areas containing greater densities of α isoform (Scott et al. 2000). However, independently of the different developmental periods in which the two ERs interact, they seem to play a key role on brain morpho-functional features as indicated by ERα being responsible for the elevated number and density of dendritic spines and axospinous synapses (Adams et al. 2002).

Overall, the sexually dimorphic distribution of GPR30 in some hamster limbic areas proposes this receptor as an important mediator of estrogen-dependent biological events. This feature is sustained by the expression of GPR30 not only in ER-enriched areas and so tends to further corroborate its critical role in the estrogen actions above all in facultative hibernators. Studies in our animal model are only at the beginning and further interests especially during the different hibernating states could prove to be useful for the unveiling of pathological processes such as ischemia that occurs during the arousal phase (Canonaco et al. 2005) of this particular physiological condition.

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Disclosure

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