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Research report

Opposite long-term synaptic effects of 17β-estradiol and 5α-dihydrotestosterone and localization of their receptors in the medial vestibular nucleus of rats

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ABSTRACT

In brainstem slices of male rats, we examined in single neurons of the medial vestibular nucleus (MVN) the effect of exogenous administration of estrogenic (17β-estradiol, E_2) and androgenic (5α-dihydrotestosterone, DHT) steroids on the synaptic response to vestibular afferent stimulation. By whole cell patch clamp recordings we showed that E_2 induced synaptic long-term potentiation (LTP) that was cancelled by the subsequent administration of DHT. Conversely, DHT induced synaptic long-term depression (LTD) that was partially reversed by E_2. The electrophysiological findings were supported by immunohistochemical analysis showing the presence of estrogen (ER: α and β) and androgen receptors (AR) in the MVN neurons. We found that a large number of neurons were immunoreactive for ERα, ERβ, and AR and most of them co-localized ERβ and AR. We also showed the presence of P450-aromatase (ARO) in the MVN neurons, clearly proving that E_2 can be locally synthesized in the MVN. On the whole, these results demonstrate a role of estrogenic and androgenic signals in modulating vestibular synaptic plasticity and suggest that the enhancement or depression of vestibular synaptic response may depend on the local conversion of T into E_2 or DHT.

1. Introduction

Growing evidence suggests that neurosteroids generated in the brain from cholesterol (Baulieu, 1998; Mellon et al., 2001; Robel and Baulieu, 1994) may be physiological modulators of central vestibular pathways and play a role in synaptic plasticity (Okada et al., 1998; Yamamoto et al., 1998a,b; Seemungal et al., 2001; Grassi et al., 2007). Neurosteroidogenesis includes the production of sexual steroids like testosterone (T), which is converted into 17β-estradiol (E_2) by P450-aromatase (ARO), and 5α-dihydrotestosterone (DHT) by 5α-reductase (Hojo et al., 2004, 2008; Kimoto et al., 2001; Mukai et al., 2006; Naftolin et al., 1975; Simpson et al., 1994). It has been recently demonstrated that E_2 and T can rapidly modulate synaptic response to vestibular nerve stimulation in the medial vestibular nucleus (MVN) neurons by interacting with estrogen (ER, α and/or β) and androgen receptors (AR), respectively (Grassi et al., 2010a,b; Grassi et al., 2011a). E_2 induced long-term potentiation (LTP) of the field potential evoked by stimulation of the primary vestibular afferents (Grassi et al., 2010b, 2011a). Conversely, T induced either long-term depression (LTD), directly and through its neural transformation into DHT, or LTP through its conversion into E_2 (Grassi et al., 2010a, 2011a). In addition, it has been demonstrated that in the MVN and hippocampus the locally synthesized E_2 has a key role in the LTP induced by afferent high frequency stimulation (HFS) (Grassi et al., 2009, 2011b; Tanaka and Sokabe, 2012). On the basis of this evidence we proposed that LTP or LTD may depend on the local production of estrogenic or androgenic neurosteroids through activation of specific pathways.

Abbreviations: ACSF, artificial cerebrospinal fluid; ADP, afterdepolarization potential; AHP, afterhyperpolarization; ANOVA, analysis of variance; AR, androgen receptors; ARO, P450-aromatase; C, central; D, dorsal; DAB, diaminobenzidine; DHT, 5α-dihydrotestosterone; E2, 17β-oestradiol; EPSP, excitatory postsynaptic potential; ER, estrogen receptors; HFS, high frequency stimulation; IR, immunoreactive; LTD, long-term depression; LTP, long-term potentiation; MVN, medial vestibular nucleus; PBS, phosphate buffer solution; T, testosterone; V, ventral; Vm, membrane potential.

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However, in all previous studies the steroid synaptic effects were assessed through field potential recordings, resulting from activation of a variety of different neurons. Thus, up to now we do not know whether the opposite responsiveness to E_2 and T or DHT in the MVN is a characteristic of different groups of neurons or a single neuron can be influenced by both neurosteroids. This is a relevant question since in the first case, the existence of separate neuronal pathways with different sensitivity to sexual neurosteroids may be suggested, while in the second case the same neuron may be depressed or facilitated depending on the activity of local neurosteroids or the circulatory hormonal condition. To solve this question we performed an electrophysiological study using whole-cell patch clamp recordings aimed at assaying the effects of different neurosteroids on synaptic response of single MVN neurons. The estrogenic and androgenic influences were examined by administering E_2 and DHT, respectively.

To support the effects of sexual neurosteroid and their crucial role in inducing LTP or LTD in single MVN neurons, we need to show the presence of ER and AR and their possible co-localization at this level, being not available in the literature evidence for the MVN presence of ER and still debated that of AR (Hamson et al., 2004; Simerly et al., 1990). Therefore, we combined the electrophysiological study with an immunohistochemical analysis for evidencing these receptors and their possible co-localization. We also re-examined the distribution of ARO in the MVN (Horvath and Wikler, 1999) to support the possibility that a single neuron can develop LTP or LTD depending on the local synthesis of E_2 from T (Grassi et al., 2009).

2. Materials and methods

2.1. Ethical approval

All procedures on animals were performed in strict accordance with protocols approved by the Ethical Committees of the University of Perugia, in compliance with the guidelines of the Italian Ministry of Health, the national laws on animal research (DL 116/92) and the European Economic Community Council Directive on Animal Research (No. 86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

The study was conducted in brainstem slices prepared from young male Wistar rats (20–40 days old Harlan, Italy) to avoid the influence of the female cyclic estrogenic fluctuation on the synaptic responses and receptor expression (Grassi et al., 2011a; Pettorossi et al., 2011).

2.2. Electrophysiological analysis

Following anaesthesia with Avertin (i.p. 250 mg/kg), the animals were decapitated and brainstems rapidly removed into ice-cold modified high sucrose artificial cerebrospinal fluid (ACSF) of composition (mM): KCl, 2.5; NaH_2PO_4, 1; $MgSO_4$, 2; $CaCl_2$, 0.5; D-glucose 11; $NaHCO_3$, 26.2 and sucrose 238, saturated with 95% O_2 and 5% CO_2. Transverse slices (250–300 μm), containing the MVN, were cut using a vibratome (Series 1000 plus starter CE, Vibratome, St. Louis, MO, USA) and were incubated for at least 1 h before recording in warmed (30 ± 1 °C) ACSF containing (mM): NaCl, 124; KCl, 3; KH_2PO_4, 1.25; $NaHCO_3$, 26; $CaCl_2$, 2.1; $MgSO_4$, 1.7; D-glucose 10 and L-ascorbate 2, saturated with 95% O_2 and 5% CO_2, pH ~7.4). The submerged recording chamber was perfused with warmed (30 ± 1 °C) and oxygenated ACSF at a rate of 2 ml/min.

2.3. Whole cell patch clamp recordings

For each animal we used 2–3 slices prepared from the middle portion of the MVN (about 1.6 mm of the rostro-caudal nucleus length), corresponding to the vestibular nerve root. Neurons from the ventral part of the MVN were visualized by means of a 60× water immersion objective mounted on an upright microscope (Eclipse FN1, Nikon, Tokyo, Japan), fitted with an analogue video camera (WAT-902B, Watec, Japan). They generally had ovoid soma (~15 μm in diameter) and at least two processes visible. Whole-cell patch recordings were obtained using thick-walled borosilicate glass pipettes (Harvard Apparatus, Holliston, MA, USA) pulled on a P-97 puller (Sutter Instruments, Novato, CA, USA) with a tip resistance of 6–10 MΩ in ACSF when filled with intracellular solution containing (mM): K-gluconate, 145; $MgCl_2 6H_2O$, 2; HEPES, 5; EGTA, 0.1 and K_2ATP, 5 (pH 7.2–7.3; osmolarity adjusted to ~290 mOsm). The recordings were performed using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 3 kHz and digitized at 10 kHz with an Axon Digidata 1440 A interface controlled by Clampex 10.2 software (Axon Instruments, Foster City, CA, USA). We chose to analyze only type B neurons since we found that synaptic response in type A neurons are not influenced by E_2 (Grassi et al., 2010b). The neurons were identified as type B when exhibited a dual component afterhyperpolarization (AHP) with an afterdepolarization potential (ADP) (Fig. 1B) (Babalian et al., 1997; Johnston et al., 1994; Serafin et al., 1991).

Excitatory postsynaptic potential (EPSP) was evoked by stimulating the primary vestibular afferents with a bipolar home-made Pt/Ir-stimulating electrode placed in a narrow zone at the medial border of the lateral or descending vestibular nucleus (Fig. 1A), which is the point where a bundle of vestibular fibres enter the MVN. The EPSP was recorded in current clamp mode with the membrane potential (Vm) held at −75 mV by negative holding current to suppress spontaneous neuron discharge. All the recordings were performed under picrotoxin (100 μM) and strychnine (1 μM) (Sigma–Aldrich, St. Louis, MO, USA) to block spontaneous $GABA_A$ and glycine mediated inhibitory post-synaptic currents.

E_2 (1 nM) and DHT (1 nM) (Sigma–Aldrich, St. Louis, MO, USA) were perfused at a rate of 2 ml/min for 15 min and drug administration was considered to have commenced when the drug reached its steady concentration in the recording chamber (about 3.2 min after the start of drug infusion).

Test stimulation consisted of current pulses (intensity 0.5–40 μA, duration 0.1 ms) delivered at a frequency of 0.06 Hz using a home-made stimulus isolation unit driven by the computer. The stimulus intensity was chosen so that the amplitude of evoked EPSP was 40–60% of the maximum at both stimulus polarities, as determined by an input–output curve.

2.4. Electrophysiological data analysis

All data analysis was performed with Clampfit 10.2 (Axon Instruments, Foster City, CA, USA) and Origin 7.0 (Microcal Software, Northampton, MA, USA) software. The recordings were analyzed when the height of neuron spike was ≥50 mV and the resting Vm did not change more than 2–3 mV throughout the baseline. Recordings where series resistance changed by more than 20% over the duration of the experiment (30–40 min) were rejected. To characterize the effects of E_2 and DHT on the EPSP, vestibular nerve stimulation was applied every 15 s. We measured the peak EPSP amplitude and used the average response recorded during a stable period (10 min) at the beginning of each experiment as the baseline. Neurons in which the EPSP amplitude varied by more than 20% during the baseline period, were not studied further. Changes in the EPSP amplitude induced by drugs were expressed as a percentage of the baseline. Within a single experiment, we consider the mean ± SD over 5 min intervals from the start to the end of drug perfusion and during drug washout. Potentiation and depression induced by drugs were established when the EPSP amplitude was significantly increased or decreased compared to the baseline (Student's paired t test, $P < 0.05$). The steady state was considered reached when at least three consecutive mean values were not statistically different.

2.5. Immunohistochemical analysis of ERβ, ERα, AR, and ARO

Qualitative analysis of the ER and AR occurrence and ARO distribution in the MVN was carried out from 3 rats at three different levels (~250 μm apart) along the cranio-caudal axis of the nucleus. Two slices, 10 μm apart, were examined at each level (total six slices for each animal). In each single slice, three zones of about the same vertical dimension [dorsal (D), central (C) and ventral (V)] were identified and, in these zones, immunoreactive (IR) cells were counted within four square areas of 0.0025 mm^2 randomly distributed along the medio-lateral axis (unit area). The most peripheral part of the nucleus (50–100 μm from the border) was not examined, since the density of the IR cells was less uniform and remarkably reduced with respect to the remaining areas of the nucleus.

For the analysis of labelled cell density we took into account only the neuronal cell bodies, excluding astrocytes, oligodendrocytes or microglia. A neuronal cell body was recognized if the cell nucleus could clearly be identified and its shape fulfilled the criteria of a neuron (shape of cell and nucleus; ratio of cytoplasm and nucleus; process arborization and shape). The neurons were classified as small- or large-sized if their diameter was lower or greater than 12 μm, respectively.

After the transcardiac perfusion with normal saline followed by fixative (4% paraformaldehyde) in phosphate buffer (PBS, 0.1 M, pH 7.4) the brain was carefully dissected. The brainstem part containing the vestibular nucleus complex that extends rostrally to the juncture of the cerebellar floor and brainstem roof was removed and post-fixed by immersion in the same fixative for 24 h at room temperature and subsequently processed for embedding in paraffin, following routine tissue preparation procedures for later immunohistochemical detection of ERα, ERβ, ARO, and AR.

In detail, according to the coordinates from the "Paxinos & Watson Stereological Atlas" (Paxinos and Watson, 1986), the area of sectioning was enclosed between the bregma at −10.04 (10.3) mm and −12.30 (12.8) mm. The obtained sections (5 μm thickness) alternatively underwent histological (haematoxylin and eosin staining) and immunohistochemical procedures. In particular, for immunohistochemistry we chose those sections where the MVN was identified on the basis of its position (dorsal to the nucleus of the solitary beam) and size of its neurones. Briefly, sections were mounted on poly-L-lysine coated glass slides, and, after completing the process of dewaxing, they were microwaved for 15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. All subsequent steps were carried out in a moist chamber at room