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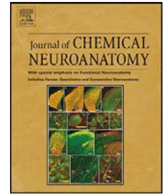

Dopaminergic neurons expressing Fos during waking and paradoxical sleep in the rat

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ABSTRACT

Formerly believed to contribute to behavioural waking (W) alone, dopaminergic (DA) neurons are now also known to participate in the regulation of paradoxical sleep (PS or REM) in mammals. Indeed, stimulation of postsynaptic DA1 receptors with agonists induces a reduction in the daily amount of PS. DA neurons in the ventral tegmental area were recently shown to fire in bursts during PS, but nothing is known about the activity of the other DA cell groups in relation to waking or PS. To fulfil this gap, we used a protocol in which rats were maintained in continuous W for 3 h in a novel environment, or specifically deprived of PS for 3 days with some of them allowed to recover from this deprivation. A double immunohistochemical labeling with Fos and tyrosine hydroxylase was then performed. DA neurons in the substantia nigra (A9) and ventral tegmental area (A10), and its dorsocaudal extension in the periaqueductal gray (A10dc), almost never showed a Fos-immunoreactive nucleus, regardless of the experimental condition. The caudal hypothalamic (A11) group showed a moderate activation after PS deprivation and novel environment. During PS-recovery, the zona incerta (A13) group contained a significant number and percentage of double-labeled neurons. These results suggest that some DA neurons (A11) could participate in waking and/or the inhibition of PS during PS deprivation whereas others (A13) would be involved in the control of PS.
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1. Introduction

In mammals, dopaminergic (DA) neurons are contained in several cellular groups distributed from the caudal mesencephalon to the rostralmost levels of the brain (Hökfelt et al., 1984; Lindvall and Björklund, 1983). These neurons participate in physiological processes as diverse as locomotor activity, sensorimotor integration, motivation and reward, and sexual function (Giuliano and Allard, 2001; Le Moal, 1995). Since the experimental lesioning made by Jones et al. (1973) in the substantia nigra and ventral tegmental area (VTA), several attempts have been made to disclose the exact role of dopamine in the regulation of sleep and waking (for review see Monti and Monti, 2007). An overwhelming number of data indicate that dopamine is a key transmitter in the modulation of behavioural arousal, but its role in the regulation of sleep is less clear. For example, depending on the dose, agonists and antagonists of the DA receptors either decrease or increase slow-wave sleep and paradoxical (REM) sleep (PS) (Monti and Monti, 2007). Interestingly, it has been suggested that dopamine could participate in dreaming occurring during PS (Solms, 2000). Early electrophysiological recordings showed that DA neurons in the substantia nigra and VTA do not change their mean firing rate across vigilance stages (Trulson et al., 1981; Trulson and Preussler, 1984). However, recent

Abbreviations: A8, A8 dopaminergic group (retrorubral field); A9, A9 dopaminergic group (substantia nigra); A10, A10 dopaminergic group (ventral tegmental area); A10dc, dorsocaudal A10 (ventral periaqueductal gray); A10vr, ventrorostral A10 (supramammillary nucleus); A11, A11 dopaminergic group (caudal hypothalamus); A12, A12 dopaminergic group (arcuate nucleus); A13, A13 dopaminergic group (medial zona incerta); A14, A14 dopaminergic group (rostral third ventricle); AH, anterior hypothalamic area; Cp, cerebral peduncle; DLG, dorsal lateral geniculate nucleus; DM, dorsomedial hypothalamic nucleus; DpMe, deep mesencephalic nucleus; f, fornix; ic, internal capsule; IP, interpeduncular nucleus; LH, lateral hypothalamic area; Ml, medial lemniscus; MM, medial mammillary nucleus; NEv, novel environment; Opt, optic tract; Ox, optic chiasm; Pa, paraventricular hypothalamic nucleus; PAG, periaqueductal gray; Pc, posteriror commissure; PVP, paraventricular thalamic nucleus posterior part; PH, posterior hypothalamic area; PnO, pontine reticular nucleus oral part; PS, paradoxical sleep; PSC, paradoxical sleep control condition; PSD, paradoxical sleep deprivation condition; PSR, paradoxical sleep recovery condition; SC, superior colliculus; SI, substantia innominata smstria medullaris thalami; SuM, supramammillary nucleus; VLG, ventral lateral geniculate nucleus; VMH, ventromedial nucleus; VTA, ventral tegmental area; Xscp, decussation of the superior cerebellar peduncle; ZI, zona incerta.

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findings indicate that DA cells in the VTA switch to a prominent bursting pattern of discharge during PS, similarly to the activity they display during rewarding processes (Dahan et al., 2007). Moreover, dopamine concentrations, as measured by microdialysis in the prefrontal cortex, are higher during PS than during slow-wave sleep (Léna et al., 2005). In the VTA, DA neurons express Fos during the recovery period following a selective PS deprivation (Maloney et al., 2002). In addition, there are indications that the DA neurons located in the caudal periaqueductal gray express Fos during sleep deprivation (Lu et al., 2006). The activity of the DA neurons in the hypothalamic cell groups in relation to the sleep-waking cycle is unknown. The present work was undertaken in order to re-examine, in the ventral mesencephalic groups, and study in the hypothalamic groups the distribution of the DA neurons activated when enhancing waking or suppressing PS.

To achieve this goal, we took advantage of the elevated expression of the immediate-early gene *c-fos* in neurons after a stimulation (Dragunow and Faull, 1989; Kovacs, 1998; Morgan and Curran, 1991), a method widely used to map neurons activated in different experimental situations. This method has been successfully used in the rat by our and other groups to localize, with the immunohistochemical detection of the protein Fos, the brainstem neurons active during PS and/or W (Léger et al., 2009b; Maloney et al., 1999, 2000; Sapin et al., 2009; Shiromani et al., 1995; Verret et al., 2003, 2005, 2006). In the present work, rats either remained awake and active by placing them in a novel environment (NEv) or were specifically deprived of PS, with some of them allowed to recover from this deprivation. The expression of the protein Fos in DA neurons was visualized by dual immunostaining combining Fos and tyrosine hydroxylase (TH) immunohistochemistry. The DA A8 to A14 groups were examined. This work already appeared in abstract form (Léger et al., 2006, 2007, 2009a) and is complementary of that describing the expression of Fos in the noradrenergic neurons in the same experimental conditions (Léger et al., 2009b).

2. Experimental procedures

2.1. Animals

OFA adult male rats (250–350 g) (Charles River France, L'Arbresle, France) were used in this study. Before and during the experiments they were housed with a 12-h light dark cycle (lights on between 7.00 h and 19.00 h). Four series of three animals were submitted to the protocol consisting in a selective deprivation/recovery of PS. Four additional animals were submitted to the NEv protocol to maintain them awake. All experiments were performed according to the European Community Council Directive (86/609/EEC). The protocol was approved by the Institutional Animal Care and Use Committee of our University (BH 2006-09).

2.2. Novel environment and PS deprivation-recovery protocols

The NEv protocol was used to induce a continuous period of alert waking at a time when rats are spending most of their time asleep. The animals were placed two-by-two and free to move during 3 h (13.00 h to 16.00 h) in a square 1.5 m × 1.5 m × 0.5 m black open-field with numerous coloured plastic toys. The light intensity above the open field was the same as that of the animal room. The animals had free access to water and food. They were continuously observed by the experimenter and the toys were moved when the animals seemed to get sleepy.

In the deprivation/recovery protocol, each series consisted in one control rat remaining on a bed of woodchips in its individual container during the whole experiment (PSC condition), a second rat deprived of PS during 75 h with the flowerpot technique on a platform (6.5 cm in diameter) surrounded by water, and sacrificed at the end of the deprivation period (PSD condition), and a third rat deprived of PS during 72 h, but allowed to recover during 3 h on a dry bed of woodchips (PSR condition). During the deprivation period, the three rats were placed next to each other, which reduced the stress associated to social isolation. They were regularly watched over through an eyepiece. During the daily cleaning of the deprivation container (starting at 10 a.m.), the rats were transferred for 30 min to a clean container with dry woodchips.

2.3. Histological and immunohistochemical procedures

Rats were perfused at the same time of the day, i.e. 16.00 h to avoid interference of the nycthemeral rhythm with the results. They were deeply anesthetized with pentobarbital and perfused with a Ringer-lactate solution containing 0.1% heparin, followed by 400 mL of a fixative composed of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were postfixed overnight in the same fixative, stored for at least 2 days in 0.1 M PB with 30% sucrose and frozen with expanded CO_2 gas. Coronal 25-μm-thick sections were obtained with a cryostat (HM550 Microm) and stored free-floating at 4 °C in 0.02 M PB containing 0.9% NaCl, 0.3% Triton X-100 (PBST) and 0.1% sodium azide (PBST-Az).

When Fos and TH immunostaining were combined, Fos immunostaining was performed first in order to obtain black nuclei and TH second to obtain brown cytoplasms. Sections from one animal in each series (PSC, PSD, PSR and NEv) were run simultaneously. The sequence of incubations was: (i) a rabbit antiserum to Fos (1: 10,000; Ab-5, Calbiochem) in PBST-Az for 3 days at 4 °C; (ii) a biotinylated goat anti-rabbit IgG (1: 2,000; Vector) overnight at 4 °C and (iii) an ABC-HRP solution (1: 1,000; Elite kit, Vector) for 90 min at room temperature. The reaction was developed in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.025% 3,3′-diamino-benzidine-4HCl (DAB; Sigma), 0.003% H_2O_2 and 0.6% nickel ammonium sulphate. The sections were kept not less than one night in PBST-Az at 4 °C. They were then incubated in a rabbit antiserum to TH (1:5000; Institut J. Boy, France) in PBST-Az for 3 days at 4 °C, followed by the same sequence of antibodies and reagents as before, except that DAB was prepared without nickel ammonium sulphate. The sections were mounted on gelatin-coated slides, dried, dehydrated and coverslipped with DePeX.

Controls with omission of the primary or secondary antibodies were run to check for the absence of nonspecific staining and cross-reactions in the dual immunostainings performed with two primary antibodies made in rabbit. The clear-cut presence of three populations of immunoreactive (IR)-neurons, i.e. single Fos, single TH and double-labeled Fos–TH neurons, together with a perfect match of the first two populations with the descriptions reported in the literature (Chan-Palay et al., 1984; Hökfelt et al., 1984; Lindvall and Björklund, 1983; Sapin et al., 2009; Verret et al., 2003, 2005, 2006) strongly argued against any cross-reaction. The antiserum to Fos was made against a synthetic peptide corresponding to the N-terminal part (residues 4–17) of human Fos. This part of the protein displays 100% homology between human and rat and no homology with Fos-related antigens such as Fos B, Jun B, Fra-1 and Fra-2 (Blast 2 sequences, NCBI). The antiserum to TH was made against enzyme isolated and purified from rat pheochromocytoma (Arluison et al., 1984).

2.4. Analysis and quantification

The DA neurons form a continuum of cells from the ventral midbrain to the preoptic area and extend dorsally up in the periaqueductal gray. To delineate the different groups we followed the nomenclature of Dahlström and Fuxe (1964), Hökfelt et al. (1984) and Lindvall and Björklund (1983), updated by German and Manaye (1993) and Ikemoto (2007) for the ventral midbrain. The A8 group lies in the retrorubral field, occupying the ventral part of the caudal mesencephalic reticular formation. The A9 group encompasses the substantia nigra pars compacta, pars reticulata and pars lateralis in the ventral mesencephalon. The A10 group covers the whole VTA and adjacent nuclei on the midline, namely the interfascicular and rostral linear nuclei (Ikemoto, 2007). The DA cells distributed in the ventral periaqueductal gray, from the dorsal raphe nucleus to the rostralmost periaqueductal gray, were denominated A10 dorsocaudal (A10dc), according to Hökfelt et al. (1984). The DA neurons in the central linear nucleus and rostral to it were included in this DA cell group. The small round DA neurons located in the supramammillary nucleus were counted separately (Shepard et al., 1988) and denominated ventrorostral A10 (A10vr). The A11 group extends from the rostralmost periaqueductal gray to the dorsal and caudal hypothalamus, medially to the mammillothalamic tract. The caudally located A11 neurons were differentiated from the rostral A10dc neurons by their larger size and higher immunoreactivity, both parameters being similar to the A11 neurons in the hypothalamus. The A12 group is located in the hypothalamic arcuate nucleus. The A13 group is restricted to the medial part of the zona incerta, at mid-rostrocaudal level of this nucleus. The A14 group is located in the rostral hypothalamus, along the third ventricle and extends dorsally into the posterior part of the paraventricular nucleus.

Sections, taken at 600 μm intervals across the DA groups on both sides of the brain, were entirely drawn and plotted in one animal from each experimental condition. In the other 3 animals, only the DA cell groups were drawn and plotted. The selection of sections was as follows, according to Paxinos and Watson (1997): A8 (2 sections at −7.2 and −6.6 from Bregma,); A9 and A10 (4 sections at −6.6, −6.0, −5.4 and −4.8); A10vr (2 sections −4.8 and −4.2); A10dc (5 sections at −7.2, −6.6, −6.0, −5.4 and −4.8); A11 (3 sections at −4.8, −3.9 and −3.3); A12 (3 sections at −3.9, −3.3 and −2.7); A13 (1 section at −2.7) and A14 (2 sections at −2.1 and 1.5). The delineation of each DA group was made by tracing a broken line enclosing all the TH-IR cell bodies and passing 50–100 μm apart from them, usually across their dendritic fields. The delineation and naming of all the other nuclei or areas were made according to Paxinos and Watson (Paxinos and Watson, 1997) (Fig. 1).

Drawings of double immunostained sections were made with an image analysis system (Mercator, ExploraNova, La Rochelle, France) coupled to a Zeiss Axioskop microscope equipped with a motorized X–Y sensitive stage. The three categories of neurons (single Fos, single TH and double Fos/TH) were plotted and, once plotted, were automatically counted by the software Mercator. Fos-IR nuclei were

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