c-Fos Expression of Dopaminergic and GABAergic Neurons of the Ventral Mesencephallic Tegmentum after Paradoxical Sleep Deprivation and Recovery

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SHORT COMMUNICATION
c-Fos expression in dopaminergic and GABAergic neurons of the ventral mesencephalic tegmentum after paradoxical sleep deprivation and recovery

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Keywords: rat, REM sleep, substantia nigra, ventral tegmental area

Abstract

Evidence suggests that dopaminergic neurons of the ventral mesencephalic tegmentum (VMT) could be important for paradoxical sleep (PS). Here, we examined whether dopamine (DA) and adjacent γ-aminobutyric acid (GABA)-synthesizing neurons are active in association with PS recovery as compared to PS deprivation or control conditions in different groups of rats by using c-Fos expression as a reflection of neural activity, combined with dual immunostaining for tyrosine hydroxylase (TH) or glutamic acid decarboxylase (GAD). Numbers of TH^+/c-Fos^+ neurons in the substantia nigra (SN) were not significantly different across groups, whereas those in the ventral tegmental area (VTA) were significantly different and greatest in PS recovery. Numbers of GAD^+/c-Fos^+ neurons in both VTA and SN were greatest in PS recovery. Thus, DA neuronal activity does not appear to be suppressed by local GABAergic neuronal activity during PS but might be altered in pattern by this inhibitory as well as other excitatory, particularly cholinergic, inputs such as to allow DA VTA neurons to become maximally active during PS and thereby contribute to the unique physiological and cognitive aspects of that state.

Introduction

Dopaminergic neurons of the ventral mesencephalic tegmentum (VMT) have long been known to play a role in the regulation of sleep-wake states. Lesions of the VMT decreased arousal (Ungerstedt, 1971; Jones *et al*., 1973), whereas enhancement of extracellular dopmaine (DA) promoted waking and decreased both slow wave sleep (SWS) and paradoxical sleep [PS or rapid eye movement sleep (REMS)] (Nishino & Mignot, 1997). Yet, a decrease in PS, as well as SWS, has also been reported following lesions or inactivation of the VMT (Lin *et al*., 1989; Lai *et al*., 1999). Such different effects could be due in part to different roles played by substantia nigra (SN) vs. ventral tegmental area (VTA) neurons. Indeed, in contrast to the behavioural hypoactivity produced by lesions of the SN, hyperactivity with decrements in attentive, motivational arousal was produced by lesions of the VTA (Galey *et al*., 1977). Accordingly, the DA SN nigrostriatal system is considered to be important in the initiation of motor activity, which is impaired in Parkinson's disease, and the DA VTA mesolimbocortical,system to be important in the promotion of rewarding arousal, which is impaired in attention deficit disorder and overactive in schizophrenia (Bunney *et al*., 1991). It would also appear likely that these DA cell groups could be differentially active during different sleep-wake states, and particularly during PS, when muscle atonia occurs concurrently with dreams, often likened to hallucinations (Yeomans, 1995).

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DA VMT neurons receive an excitatory input from the cholinergic neurons of the pontomesencephalic tegmentum (PMT) that is integral to reward circuits (Lacey *et al*., 1990; Corrigall *et al*., 1994; Blaha *et al*., 1996). The PMT cholinergic neurons are also critically involved in generating PS (Webster & Jones, 1988). In recent studies applying a paradigm of PS deprivation and recovery and using c-Fos immunostaining as a reflection of neural activity combined with choline-acetyltransferase (ChAT)-immunostaining (Maloney *et al*., 1999), we found that cholinergic PMT neurons were most active in association with PS. Unless inhibited by local γ-aminobutyric acid (GABA)ergic neurons, dopaminergic neurons would thus presumably be excited in tandem with the cholinergic PMT neurons during PS. In an attempt to resolve whether DA and GABAergic SN and/or VTA neurons are active during PS, we thus examined c-Fos expression in the VMT with dual immunostaining for tyrosine hydroxylase (TH) or glutamic acid decarboxylase (GAD) in brains previously processed for c-Fos and ChAT in the PMT from rats submitted to PS deprivation, recovery or control conditions (Maloney *et al*., 1999).

Materials and methods

Animals

The mesencephalon was taken from 12 rats previously employed in PS deprivation and recovery experiments (Maloney *et al*., 1999). Briefly, male Wistar rats (Charles River Canada, St. Constant, Quebec) were submitted to procedures approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care. They were operated under barbiturate anaesthesia

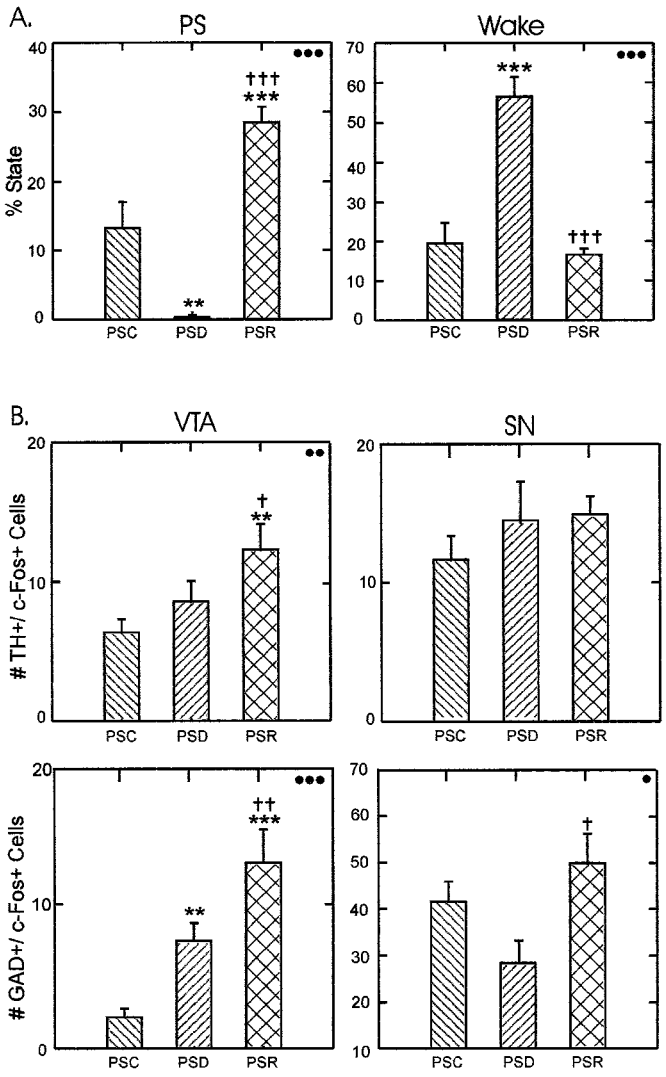

FIG. 1. Sleep-wake states and c-Fos expressing cells (mean ± SEM) in the three groups of animals submitted to PS control (PSC), deprivation (PSD) or recovery (PSR) conditions (4 rats per group). (A) Percentage of time spent in PS and Wake during the ~3 h recording period preceding perfusion. (B) Average numbers of TH^+/c-Fos^+ and GAD^+/c-Fos^+ cells counted per side per level in the VTA and SN. By ANOVA, ·,·· or ··· indicates a significant main effect of condition between groups; *, ** or ***, a significant difference with respect to PSC, and †, †† or ††† with respect to PSD

(Somnotol, 67 mg/kg, i.p.) for implantation of chronically indwelling electrodes to permit recording and scoring of sleep-wake states.

PS deprivation

PS deprivation was performed using the flowerpot technique as described previously in detail (Maloney *et al*., 1999). Following one baseline day, the 'condition' was varied for three different groups (with four animals per group): PS control (PSC); PS deprivation (PSD); and PS recovery (PSR). (i) Each PSC animal remained on the dry floor of its recording box for 4 days, and on the fourth day was anaesthetized for perfusion (at ~15.00 or 15.30 h). (ii) Each PSD animal was placed on a flowerpot surrounded by water in its recording box for the second, third and fourth days, when it was anaesthetized for perfusion (at ~15.30 h), having been in the

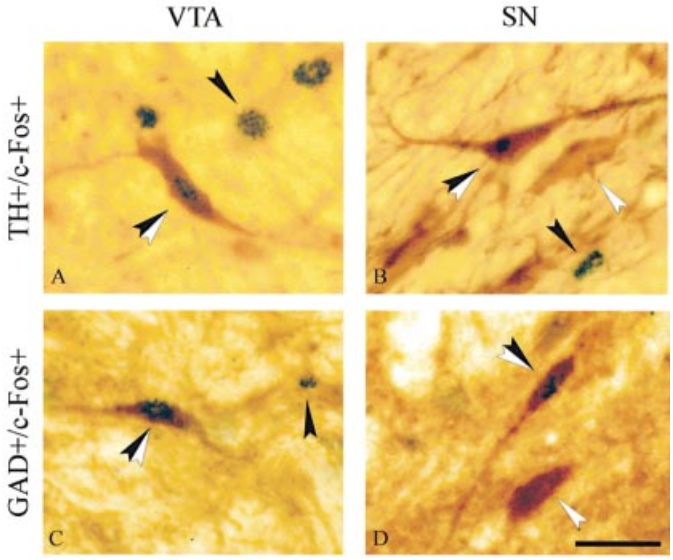

FIG. 2. Photomicrographs of sections through the VTA and SN with dual immunostaining for c-Fos (blue, BDHC) and either TH (A and B) or GAD (C and D) (brown, DAB). Black arrowheads indicate single-immunostained c-Fos^+ cells; white arrowheads, single-immunostained TH^+ or GAD^+ cells; and paired black and white arrowheads, dual-immunostained cells. Scale bar, 25 μm.

deprivation condition for ~53 h. (iii) Each PSR animal was placed on a flowerpot surrounded by water for the second, third and fourth days, when after ~50 h of PS deprivation, it was removed from the flowerpot, returned to the cleaned, dry floor of its recording box and anaesthetized for perfusion (at ~15.00 h), having been in the recovery condition for ~3 h.

Immunostaining and analyses

The animals were killed under barbiturate anaesthesia (Somnotol, ~100 mg/kg) by perfusion with a fixative as published previously (Maloney *et al*., 1999). On adjacent series of sections, single immunostaining for c-Fos or dual immunostaining for TH and c-Fos or GAD and c-Fos was performed according to the previously published procedures (for noradrenergic and GABAergic neurons). Briefly, a sheep anti-c-Fos antiserum (at 1 : 3000, Cambridge Research Biochemicals, Cheshire, UK), a rabbit anti-TH antiserum (1 : 15 000, Eugene Tech International, Allendale, NJ, USA) and a rabbit anti-GAD 67 antiserum (1 : 3000, Chemicon International, Temecula, CA, USA) were employed. In all brains, one series of sections was immunostained for c-Fos alone using 3,3′ diaminobenzidine (DAB). In other adjacent series, dual immunostaining was performed using a sequential procedure that involved for the major series, staining of the enzyme first using DAB and c-Fos second using benzidine dihydrochloride (BDHC). Using a computer-based image analysis system (Biocom, Les Ulis, France), profiles of c-Fos-immunopositive nuclei were mapped and tabulated for cell counts. At one level (~A 2.9; Paxinos & Watson, 1986), single-immunostained c-Fos^+ nuclei were measured for their large diameter in order to confirm that the counts were not biased by a difference in nuclear size across conditions. The TH^+/c-Fos^+ cells and GAD^+/c-Fos^+ cells were mapped and tabulated on each side every 400 μm at ~A 2.9, A 2.5 and A 2.1. An analysis of variance (ANOVA), followed by Fisher's *post hoc* pair-wise comparisons, was employed to determine if numbers of cells counted over multiple levels per animal varied as a function of condition in the SN and VTA (using Systat, Evanston, IL,

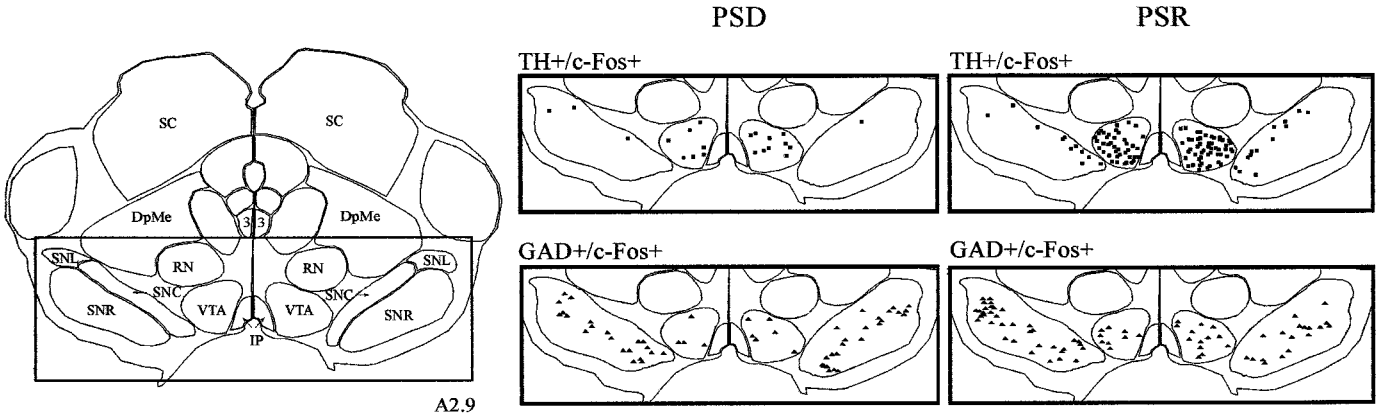

FIG. 3. Computerized atlas figure through the mesencephalon (at ~A 2.9). TH^+/c-Fos^+ cells (squares) and GAD^+/c-Fos^+ cells (triangles) were mapped in the VTA and SN in representative animals from PSD (left) and PSR (right) groups. DpMe, deep mesencephalic nucleus; IP, interpeduncular nucleus; RN, red nucleus; SC, superior colliculus; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; SNL, substantia nigra, pars lateralis; VTA, ventral tegmental area; 3, oculomotor nucleus.

USA). With significant differences across groups, a general linear model was employed to determine by an interactive forward stepwise method if the cell counts varied as a function of %PS or, if not, %Wake with %PS, across animals. With significant linear models for %PS and %Wake, the correlation with %PS was subsequently examined after partialing out the correlation with %Wake.

Results

As previously reported in detail (Maloney *et al*., 1999), there were significant changes in the amount of time spent in PS when the PSD, PSR and PSC groups were compared (Fig. 1A). PS represented 0% in the PSD group and ~28% in the PSR group, as compared to ~15% in the PSC group during the 3 h recording prior to perfusion. Wake was significantly higher in the PSD group than in the PSC and PSR groups.

In both the SN and VTA, c-Fos expression was evident within neurons in sections dual-immunostained for c-Fos and TH or for c-Fos and GAD (Fig. 2). Across the VMT, it appeared that TH^+/c-Fos^+ cells and GAD^+/c-Fos^+ cells were more numerous in brains from the PSR group than in those from the PSD group (Fig. 3) and also from the PSC group (not shown).

In the SN, numbers of TH^+/c-Fos^+ cells did not vary significantly across groups (Fig. 1B). GAD^+/c-Fos^+ cells did and were more numerous in the PSR than in the PSD group (Fig. 1B). In a linear regression model, the numbers of GAD^+/c-Fos^+ cells covaried positively with the percentage PS across animals ($F = 4.03$; d.f. = 1, 46; $P = 0.05$).

In the VTA, numbers of TH^+/c-Fos^+ cells and GAD^+/c-Fos^+ cell counts differed significantly across groups (Fig. 1B). TH^+/c-Fos^+ cells were more numerous in the PSR group than in both the PSD and PSC groups. They were also more numerous in the PSD than in the PSC group, though not significantly so ($P = 0.12$). In a linear regression model, the numbers of TH^+/c-Fos^+ cells did not covary significantly with %PS alone. However, when %Wake was included in the model, the numbers of TH^+/c-Fos^+ cells covaried positively with %PS together with %Wake ($F = 7.71$, d.f. = 2, 63; $P = 0.001$). After partialing out the correlation due to %Wake, %PS was significantly positively correlated with TH^+/c-Fos^+ cells ($F = 14.972$; d.f. = 1, 63; $P = 0.000$). GAD^+/c-Fos^+ cells were also more numerous in the PSR group than in the PSD and PSC groups and also more numerous in the PSD group than in the PSC group (Fig. 1B). As with the TH^+/c-Fos^+ cells, the numbers of GAD^+/c-Fos^+ cells covaried significantly with %PS and %Wake together ($F = 11.29$; d.f. = 2, 45; $P = 0.000$) and with %PS, after partialing out the correlation due to %Wake ($F = 22.639$; d.f. = 1, 45; $P = 0.000$).

Discussion

The present results show greater c-Fos expression and thus postulated neural activity with PS in dopaminergic VTA neurons and in GABAergic VTA and SN neurons.

In the SN, enhanced PS during recovery was found to be associated with an increase in the number of c-Fos expressing neurons, as also reported in a pharmacological study (Sastre *et al*., 2000), but here found specifically for GABAergic neurons. These results corroborate electrophysiological data showing an increase in average discharge rate by presumed nondopaminergic neurons during PS (Miller *et al*., 1983; Steinfels *et al*., 1983). With long projections to the thalamus and mesencephalon, GABAergic SN neurons might influence the discharge pattern of other distant neurons during PS (Datta *et al*., 1991). In contrast to the apparent influence of pontine GABAergic neurons upon neighbouring noradrenergic locus coeruleus neurons, which decrease c-Fos expression and firing with PS (see Maloney *et al*., 1999), the GABAergic SN neurons do not appear to suppress discharge of the DA SN neurons during PS, as these do not decrease c-Fos expression, or average discharge (Miller *et al*., 1983), with PS.

In the VTA, enhanced PS during recovery resulted in an increase in the numbers of both GABAergic and dopaminergic cells expressing c-Fos. Their numbers were higher in the recovery than in the deprivation condition and somewhat higher in the deprivation than in the control condition, suggesting increases in association with PS during recovery but also with Wake during deprivation. Using linear regression models, their numbers were found to covary significantly with the amount of time spent in PS and Wake and to correlate with PS after partialing out the correlation with Wake. The present results therefore suggest that both the GABAergic and dopaminergic neurons in the VTA are more active during PS and Wake than during SWS and most active in association with PS under the present conditions. These results are corroborated by recent electrophysiological studies

showing that fast-spiking VTA neurons, which can be immunohistochemically identified as GABAergic, discharge at their highest rates during PS (Lee *et al*., 2001). As mesolimbocortical projection neurons, these GABAergic neurons could influence neurons in the forebrain. They would not appear to impose a tonic suppression upon activity of the adjacent dopaminergic VTA neurons in view of the current c-Fos results and previous recording results showing no significant change in average firing rate of presumed dopaminergic VTA neurons during PS (Miller *et al*., 1983; Trulson & Preussler, 1984).

According to our assumption that increases in c-Fos expression reflect increases in neural activity, we posit that the present results also indicate an increase in activity by the immunohistochemically identified DA VTA neurons during PS. The question then arises why single unit recording studies have not revealed increases in firing by presumed DA VTA neurons during PS relative to SWS. In the original recording study (Miller *et al*., 1983), it was reported that although there was not an increase in the average firing rate, there was an increase in the variance of the interspike interval, such as to indicate that DA VTA neurons might discharge in a different pattern during PS as compared to SWS. During waking, presumed dopaminergic neurons tend to discharge in bursts of spikes and do so especially during behaviourally significant and rewarding conditions (Schultz, 1986; Mirenowicz & Schultz, 1996). Here, the increased c-Fos expression in the DA VTA neurons in association with PS could be due to a bursting discharge, which is associated with increased calcium influx and other signals that stimulate immediate early gene expression (Morgan & Curran, 1986; Fields *et al*., 1997; Overton & Clark, 1997). In anaesthetized rats, a much larger percentage of presumed DA VTA neurons than SN neurons discharge in bursts (Grenhoff *et al*., 1988). Because such bursting appears to depend upon inhibitory and/or excitatory inputs (Overton & Clark, 1997), we speculate that through differential inputs, the DA VTA, but not the SN, neurons become maximally active by altering their pattern of discharge during PS.

The burst discharge of VTA neurons has been shown to depend upon excitatory input, which comes from glutamatergic afferents (Overton & Clark, 1997) and also cholinergic input originating in the PMT (Grenhoff *et al*., 1986; Gronier & Rasmussen, 1998). Acetylcholine (ACh) induces bursting through nicotinic receptors in DA VTA and SN neurons, and notably, however, also induces bursting through muscarinic receptors in DA VTA and not SN neurons. Hence, cholinergic PMT neurons could stimulate burst firing, particularly in DA VTA neurons, during PS, when cholinergic neurons are maximally active (Maloney *et al*., 1999).

As in rewarding brain stimulation during waking (Flores *et al*., 1997), DA VTA neurons could activate limbic forebrain areas during PS (Sastre *et al*., 2000). As in learning during waking (Ljungberg *et al*., 1992), they could promote memory consolidation during PS (Karni *et al*., 1994), and, as in abnormal hallucinations during waking (Yeomans, 1995), they could also stimulate the special cognitive elements of PS, namely dreams.

Acknowledgements

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Abbreviations

ChAT, choline acetyl transferase; DA, dopamine; GAD, glutamic acid decarboxylase; PMT, pontomesencephalic tegmentum; PS, paradoxical sleep; PSC, PS control; PSD, PS deprivation; PSR, PS recovery; REM, rapid eye movement; SN, substantia nigra; SWS, slow wave sleep; TH, tyrosine hydroxylase; VMT, ventral mesencephalic tegmentum; VTA, ventral tegmental area.

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