5-HT₂ receptor immunoreactivity on cholinergic neurons of the pontomesencephalic tegmentum shown by double immunofluorescence

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(Accepted 8 June 1993)

Key words: Laterodorsal tegmental nucleus; Pedunculopontine nucleus; Receptor antibody; Serotonin-2 receptor

INTRODUCTION

The physiological effects of serotonergic neurotransmission in the central nervous system (CNS) are mediated by several serotonin (5-HT) receptor subtypes, designated 5-HT₁, 5-HT₂, 5-HT₃ and 5-HT₄. Of particular interest is the 5-HT₂ receptor subtype, which may play a role in the pathophysiology of a number of neuropsychiatric disorders such as schizophrenia and depression, and which has been implicated in the mechanism of action of hallucinogenic drugs. To gain a better understanding of the mechanisms underlying the physiological and pharmacological actions and regulation of the 5-HT₂ receptor, we are attempting to define the specific neuronal elements in the CNS that express 5-HT₂ receptors.

In a previous study using a polyclonal antibody raised against the N-terminal portion of the rat 5-HT₂ receptor protein, we have described the localization of 5-HT₂ receptor-bearing neurons in the adult rat brain. In a number of regions, the distribution and morphology of 5-HT₂ immunolabelled neurons suggested that they may be cholinergic. A moderate number of medium-sized multipolar neurons were labelled in regions of the basal forebrain, including the olfactory tubercle, ventral pallidum and nucleus accumbens. We also observed a relatively sparse and even distribution of medium-sized bi- and multipolar neurons in the neostriatum. Most striking, however, was the dense labelling of neurons in two pontomesencephalic regions, the pedunculopontine tegmental nucleus (PPT), and the laterodorsal tegmental nucleus (LDT). The 5-HT₂-immunopositive neurons in these two regions showed a strong similarity to the distribution and morphology of cholinergic neurons in the Ch5 and Ch6 cell groups, respectively. In the present study, we have investigated the possible cholinergic nature of the 5-HT₂ immunopositive neurons in these regions of the rat brain by double-immunofluorescence, using the 5-HT₂ receptor antibody in conjunction with an antibody against the synthetic enzyme for acetylcholine, choline acetyltransferase (ChAT).

MATERIALS AND METHODS

Five adult Sprague–Dawley rats (Simonsen, Gilroy, CA) of either sex were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the heart with phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde–PBS. The brains were
post-fixed for 1 h and cryoprotected in 30% sucrose–PBS before freezing in isopentane cooled on dry ice and storage at −80°C. One brain was used for a preliminary titration of optimal antibody concentrations and for tests of antibody specificity. These included incubation of adjacent sections with each primary antibody alone to test for bleed through of fluorescence, and incubation of the fluorescent second antibodies with the inappropriate primary antibody to test for cross-reactivity.

For the remaining four brains, sections through the pontomesencephalic region containing the LDT and PPT nuclei, and through the forebrain from the frontal pole of the cortex through the rostral portion of the dorsal hippocampus were processed free-floating for double immunofluorescence. One of five adjacent series of 50 μm frozen sections was washed in PBS and pre-incubated for 60 min in blocking buffer (5% normal donkey serum in PBS with 0.3% Triton X-100), then co-incubated in anti-5-HT₂ and anti-ChAT primary antisera for 4 h at 24°C and then 60 h at 4°C. Affinity-purified 5-HT₂ antibody, raised in rabbit against a unique portion of the N-terminal region of the rat 5-HT₂ receptor, was diluted 1/25 (approximately 5 μg/ml final protein concentration), and the goat anti-ChAT antibody (Chemicon) was diluted 1/1500 in blocking buffer containing 0.05% thimerosal. After removing primary antisera and washing in PBS, sections were incubated for 3 h at 24°C in fluorescein-conjugated donkey anti-rabbit IgG and rhodamine-conjugated donkey anti-goat IgG (Jackson Immunoresearch), both diluted 1/100 in blocking buffer. Sections were then rinsed, mounted on poly-lysine coated slides, air-dried overnight in the dark, and coverslipped using a 90% glycerol/PBS/antifadent solution (CitiFluor). Sections were viewed and photographed using epifluorescence on a Nikon Microphot SA microscope. Photomicrographs for both color and black-and-white prints were taken immediately to minimize loss of the fluorescent signals. Numbers of single- and double-labelled neurons were determined from matched photographic exposures taken using the fluorescein and rhodamine filter blocks.

RESULTS

Preliminary experiments and processing of alternate series of sections in the present study verified that the 5-HT₂ and ChAT antibodies did not cross-react with each other (see below), and that the secondary antibodies were specific for the appropriate primary antisera. No autofluorescence or non-specific reactivity were evident in the absence of appropriate primary antibody. Likewise, there was no bleed through of fluorescence using the opposite filter block when either antibody was used alone.

In the laterodorsal and pedunculopontine tegmental nuclei, there was extensive colocalization of ChAT and 5-HT₂ receptor immunoactivity (Figs. 1–3). In both the LDT and PPT, there was 94–99% concordance between the two populations of cells. These cells were medium to large multipolar neurons, as described pre-
Fig. 2. Graphic representation of single- and double-labelled 5-HT₂ and ChAT-immunopositive cells in the laterodorsal tegmental nucleus. This figure shows the distribution of labelled cells in the LDT pictured in the right half of Fig. 1. Cells in the LDT that were immunopositive for the 5-HT₂ receptor only (open circles), for ChAT only (open squares) or that were double-labelled (filled circles) were marked on a transparency laid over matched color exposures taken of the same field using the FITC and TRITC filter blocks.

Previously²⁴. In the same sections, we also observed many ChAT-positive cells in cranial nerve motor nuclei (e.g. trigeminal and abducens) that did not show fluorescein-labelled 5-HT₂ immunoreactivity, suggesting that the anti-5-HT₂ antibody did not label cholinergic neurons non-specifically, and that there was no cross-reactivity between the two primary antibodies. Likewise, very little double-labelling was seen in the forebrain. In the basal forebrain, including the olfactory tubercle, ventral pallidum and nucleus accumbens, the ChAT and 5-HT₂-immunopositive neurons overlapped extensively, showing similar distribution and cell numbers. However, only approximately 10% of both the 5-HT₂ and the ChAT-immunopositive neurons in these regions were double-labelled (Fig. 4). A similarly low degree of double-labelling was observed in the neostriatum (not shown), though in limited samples, up to 20% of immunoreactive neurons were double-labelled.

Fig. 3. Co-localization of 5-HT₂ and ChAT immunoreactivity in the pedunculopontine tegmental nucleus. Panel A shows FITC immunofluorescence of 5-HT₂-positive neurons. Panel B shows TRITC immunofluorescence of ChAT-positive neurons. Again, cells were labelled more intensely and extensively by the ChAT antibody. Nonetheless, 94–100% of the labelled cells in the PPT were double-labelled. Medial is to the left; dorsal is up; fibers of the lateral lemniscus are to the right. Scale bar = 100 μm.

Fig. 4. 5-HT₂ and ChAT immunoreactivity in the basal forebrain. The region shown is in the olfactory tubercle. Panel A shows FITC immunofluorescence of 5-HT₂-positive neurons. Panel B shows TRITC immunofluorescence of ChAT-positive neurons. Solid arrowheads in both panels indicate 5-HT₂-immunopositive cells that were negative for ChAT reactivity, while open arrowheads indicate ChAT-positive neurons that were negative for 5-HT₂ reactivity. Only 2–14% of either 5-HT₂- or ChAT-labelled cells in the basal forebrain were double-labelled. Asterisk indicates 5-HT₂ immunoreactivity in an Island of Calleja (cf. refs. 9,24). Medial is left; dorsal is up. Scale bar = 100 μm.
However, these accounted for very few cells, and represented a substantial overestimate, since these analyses were purposely conducted in areas showing cells that reacted positively to both primary antisera for the sake of comparison.

DISCUSSION

In this study, we have examined the co-localization of immunoreactivity for the 5-HT$_2$ receptor and ChAT, a marker for cholinergic neurons, in the forebrain and dorsal pontine tegmentum. While very little co-localization was observed in cholinergic neurons of the neostriatum and basal forebrain, there was extensive double-labelling in cells of the laterodorsal and pedunculopontine tegmental nuclei, suggesting that nearly all 5-HT$_2$-positive neurons in the LDT/PPT are cholinergic, and nearly all cholinergic neurons in these two brainstem nuclei carry the 5-HT$_2$ receptor. We are aware of only one previous study using radioligand binding in which 5-HT$_2$ receptors have been shown in the region of the LDT$^2$.

The PPT and LDT nuclei, representing the Ch5 and Ch6 cell groups, have been postulated to play an important role in the initiation and regulation of paradoxical, or rapid eye movement (REM) sleep, and are believed to be essential to the brainstem regulation of arousal states. A recent electrophysiological investigation has shown that the primary response of cholinergic neurons in the PPT to administration of 5-HT was inhibition, mediated by a 5-HT$_1$ receptor mechanism$^{18}$, consistent with the presumed inhibitory role of serotonin in REM sleep control. Withdrawal of this serotonergic inhibition of brainstem cholinergic neurons is thought to be permissive for the occurrence of REM sleep, and for the initiation and transmission of pontogeniculo-occipital (PGO) waves to the thalamus$^{30}$. These PGO waves are phasic electrical potentials recorded in the brainstem, lateral geniculate and visual cortex that are temporally associated with REMs and with visual aspects of dreaming. Depletion or lesioning of the inhibitory serotonergic input to the LDT/PPT induces spontaneous PGOs in waking and non-REM sleep, as well as in REM sleep$^{29}$. This brainstem-thalamo-cortical sensory transmission associated with REM sleep may not be limited to visual phenomena, as the cholinergic neurons in the brainstem also project to the medial geniculate nucleus, and phasic auditory-related muscle activity has been recorded in the middle ear during REM sleep, analogous to the occurrence of rapid eye movements$^{27,32}$. Thus, the thalamic projections of the brainstem cholinergic cell groups, when released from 5-HT$_{1A}$ inhibition, may be generally involved in transmitting internally generated sensory signals of many modalities through the thalamus, and ultimately to cortex, during REM sleep$^{15,19,30}$.

The present results suggest that additional effects of serotonin on the LDT and PPT cholinergic neurons may be transduced by 5-HT$_2$ receptors. In many CNS regions where 5-HT$_{1A}$ and 5-HT$_2$ receptors have been found to be co-extensive, including the neocortex, periaqueductal grey, and pontine reticular formation, they exert opposing influences, with 5-HT$_{1A}$ receptors mediating inhibition and 5-HT$_2$ receptors eliciting excitation$^{3,5,6,7,28,31}$. Thus, it is possible that 5-HT$_2$ receptors may elicit an excitatory effect on cholinergic neurons of the dorsal tegmentum that is normally masked by the inhibitory 5-HT$_{1A}$ receptors. This effect may be evident only in the presence of 5-HT$_{1A}$ down-regulation or 5-HT$_2$ up-regulation, or upon selective pharmacological activation of 5-HT$_2$ receptors. Alternatively, the relative effect on neuronal activity may be concentration dependent. The affinity of 5-HT$_2$ receptors for serotonin is relatively low compared to that of the high-affinity 5-HT$_{1A}$ receptors$^{17,25}$. Thus, endogenous 5-HT$_2$-mediated effects may be evident only at high levels of serotonin release, or in the presence of a receptor imbalance wherein the 5-HT$_2$ receptors predominate.

The fact that 5-HT$_2$ receptors are localized in regions important in regulation of REM sleep and dreaming may also be relevant to the presumed involvement of 5-HT$_2$ receptors in pharmacological hallucinogenesis. 5-HT$_2$ receptors have been postulated to be the site of action of indole- and phenylalkylamine hallucinogens$^{12,14}$. While investigators of hallucinogenic action have often focused on cortical 5-HT receptors as potential mediators of hallucinogenic phenomena, it is possible that activation of 5-HT$_2$ receptors in the LDT/PPT nuclei may initiate the same internally-generated sensory processes that normally occur during dreaming, and that this may in part be the basis of pharmacological hallucinogenesis. It has been suggested that the hallucinatory, delusional and disordered thought associated with dreaming is an attempt to interpret the internally generated sensory perceptions induced by brainstem cholinergic activity during REM sleep$^{19}$.

It is also possible that a disturbance in the balance between inhibitory 5-HT$_{1A}$ receptors and excitatory 5-HT$_2$ receptors on cholinergic neurons in the brainstem may underlie in part the serotonergic involvement in schizophrenia or depression. Many atypical antipsychotic agents share a high antagonist affinity for the 5-HT$_2$ receptor$^{22}$, and an increase in the number of cholinergic neurons in the dorsal pontine tegmentum...
has recently been shown in some schizophrenics. It is tempting to speculate that, in a subset of schizophrenic disorders, some of the symptoms, such as sensory hallucinations, delusions, disordered thought, autonomic disregulation or sleep disruption may be related to serotonergic dysfunction in the cholinergic brainstem circuitry that normally regulates the manifestation of these same phenomena in REM sleep.

Serotonergic dysfunction also appears to be a central feature of many forms of depressive illness. Common neurobiological mechanisms have been suggested to regulate arousal and mood, implying that the systems involved in sleep regulation may also underlie depression. A number of sleep disorders associated with depression, including shortened REM sleep latency, decreased δ-sleep (non-REM sleep), and supersensitivity to cholinergic REM sleep induction may be partly attributable to disruptions of normal serotonergic/cholinergic interactions in the LDT/PPT.

Thus, the localization of 5-HT₂ receptors on the Ch5 and Ch6 cholinergic neurons of the dorsal pontine tegmentum may provide the rationale for future studies on the regulation of arousal and sleep, and may also provide insight into some of the cognitive and behavioral disfunctions arising from imbalances in these systems. Further identification of other cell types expressing 5-HT₂ receptors may also contribute to an understanding of the interactions between neurotransmitter systems, and of serotonergic modulatory processes in the brain.

Acknowledgements. This work was supported by a grant from the NIMH (MH 39437), and by the endowment to the Nancy Pritzker Laboratory. D.A.M. is the recipient of support from the Edward D. and Marjorie R. Gray Endowment. R.D.C. is the recipient of a Research Scientist Award from the NIMH (MH 00219).

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