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Dopamine transporter immunoreactive terminals in the bovine pineal gland

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Abstract

The dopaminergic system has been proposed to be one of the innervations in controlling the mammalian pineal gland function. The dopamine receptors have been characterized in the pineal and the biphasic effects of dopamine on melatonin production have been demonstrated. Recently, the site of dopamine transporter (DAT), a plasma membrane transport protein of dopaminergic neuron, also has been characterized in the bovine pineal gland. The aim of the present study was to identify the dopaminergic innervation in the bovine pineal gland. The localization and expression of DAT have been performed by using an immunohistochemical method and a reverse transcriptase polymerase chain reaction (RT–PCR) technique. DAT-immunoreactivity was found in the nerve terminals throughout the gland, but not in pinealocytes or neuronal-like cells. Some DAT-immunoreactive nerve fibers were observed along the pineal stalk. DAT mRNA product from RT–PCR was found in the bovine substantia nigra, but not in the pineal gland. The colocalization of DAT with tyrosine hydroxylase (TH) or dopamine beta hydroxylase (DBH) immunoreactivities was observed in nerve terminals. However, no colocalization of DAT with DBH was found in some terminals/fibers. The present results showed the central dopaminergic innervation in the bovine pineal gland distinctively from noradrenergic nerve fibers, and their perikarya origin was located possibly outside of the gland.
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Keywords: Dopamine transporter; Pineal gland; Tyrosine hydroxylase; Dopamine beta-hydroxylase

The major neuronal innervation controlling melatonin synthesis in the mammalian pineal gland is the noradrenergic sympathetic system from the superior cervical ganglion [4]. In addition, nerve fibers containing a number of neurotransmitters/neuropeptides from the brain and other peripheral ganglia have been demonstrated to innervate the gland [11]. Many neurotransmitters such as acetylcholine, gamma-aminobutyric acid (GABA), glutamate and dopamine in the pineal glands influenced the melatonin synthesis [18]. The presence of dopaminergic system in the pineal gland has been of interest. The function of dopamine in the pineal gland was first described by Axelrod et al. [1] that it activated ^{14}C melatonin production in the rat pineal gland culture. Then, dopamine has been detected in bovine pineal glands using high performance liquid chromatography and exhibited a dual effect on serotonin *N*-acetyltransferase (NAT) activity in cultured rat pineal glands, inhibiting activity at 0.1 μM and stimulating activity at 10 μM [6]. Psychoactive substances that block D_2 dopamine receptors such as haloperidol increase the concentration of melatonin [6]; and psychoactive agents, which block the neuronal uptake of dopamine such as nomifensine, alter the level of melatonin [5]. D_2-dopamine receptors have been characterized in the bovine pineal gland by radioligand binding technique [7] and the presence of dopamine and noradrenergic receptors density have been analyzed by receptor autoradiography in the bovine pineal gland [16]. The result showed that the density order of the receptor was D_1-dopamine receptor > alpha$_1$-adrenergic receptor > D_2-dopamine receptor > beta$_1$-adrenergic

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receptor. Furthermore, both D_1- and D_2-dopamine receptor mRNAs have been expressed in the bovine pineal gland by using a reverse transcriptase polymerase chain reaction (RT–PCR) technique [13]. Moreover, dopamine transporter (DAT) has been characterized in the bovine pineal gland by a radioligand technique using [^{3}H] GBR 12935 as a ligand [8]. However, the dopaminergic nerve fibers have never been possible to demonstrate in the mammalian pineal gland. Therefore, in the present study, we investigated the localization of these fibers in the bovine pineal gland by an immunohistochemical study using the monoclonal antibody against DAT, a good marker for dopaminergic neurons and their projections [2]. In addition, the expression of DAT has been determined by the RT–PCR in order to serve for perikarya origin of these dopaminergic fibers. Immunofluorescence double-labeling studies have been also performed between DAT and tyrosine hydroxylase (TH), a marker catecholamine system/dopamine beta hydroxylase (DBH), a marker of noradrenergic nerve fibers, for studying the relationship among these three markers in the bovine pineal gland.

Fresh bovine pineal glands, substantia nigra and striatum, were dissected from the brains of 6–12-month-old bulls (*Bos taurus*) between 9.00 and 10.00 am at a local slaughterhouse. They were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After 2 days of fixation, the tissues were cryoprotected and cut on a cryostat at a thickness of 20 μm and affixed to gelatin-coated glass slides. The immunolocalization of DAT was performed by using a rat monoclonal antibody against *N*-terminal of human DAT (# 24041179, Chemicon, Temecula, CA, USA). The method for immunofluorescence was as follows: after washing sections of bovine pineal glands, substantia nigra and striatum with phosphate buffer saline (PBS, pH 7.4) and PBS-B (PBS containing 0.1% Triton X-100, 0.25% bovine serum albumin), they were treated with 10% normal donkey serum (Chemicon, Temecula, CA, USA) in PBS-A (PBS containing 0.3% Triton X-100, 1% bovine serum albumin) for 30 min at room temperature. They were then incubated in rat anti-DAT diluted 1:50 in PBS-A for 48 h at 4 °C followed by incubation in donkey anti-rat IgG rhodamine (Chemicon, Temecula, CA, USA) diluted 1:200 in PBS-B, for 2 h at room temperature. Finally, these sections were mounted with antifade reagent (Vector, Burlingame, CA, USA) in glycerol buffer. The immunoperoxidase study for DAT was also performed on bovine pineal sections connected to an epithalamic area by using biotinylated rabbit IgG against rat antiserum (Chemicon, Temecula, CA, USA) as a secondary antiserum at a dilution of 1:400 in PBS-B. The sections were processed by the avidin-biotin method using a Vectastain ABC kit (Vector, Burlingame, CA, USA) as described previously by our group [12]. For immunofluorescence double-labeling, the mixture of primary antisera and their corresponding secondary antisera was used as follows: (1) 1:50 rat anti DAT and 1:25 mouse anti TH, a marker catecholamine system (Sigma, Saint Louis, MI, USA); and (2) 1:50 rat anti DAT and 1:1000 rabbit anti DBH, a marker of noradrenergic nerve fibers (Chemicon, Temecula, CA, USA). The secondary antisera used in this study were donkey anti-mouse IgG fluorescein (FITC), and donkey anti-rabbit IgG

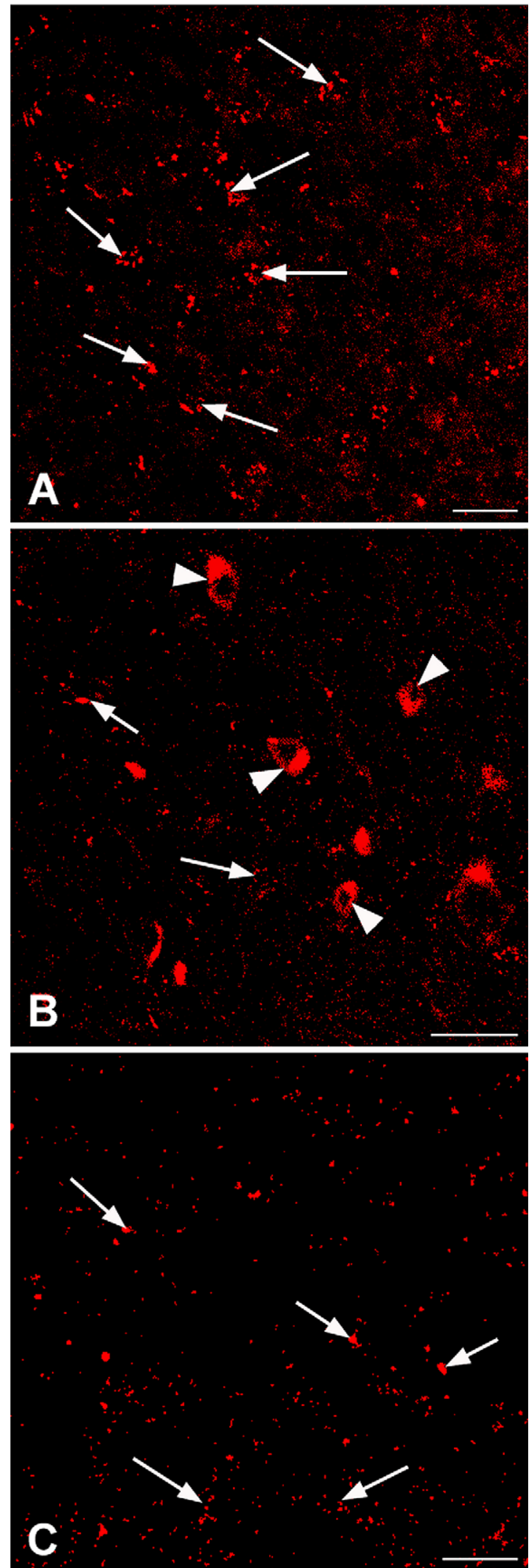

Fig. 1. Confocal laser scanning photomicrographs of DAT-immunoreactivity in the bovine pineal gland (A); bovine substantia nigra (B); and bovine striatum (C). The DAT-immunoreactive nerve terminals (arrows) were observed in all these structures, but only DAT-immunopositive neurons (arrow heads) were found in substantia nigra. Scale bars represent (A) 50 μm; (B) 100 μm; and (C) 25 μm.

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