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Neuropeptide Y and 1,25-dihydroxyvitamin D3 receptors colocalize in neurons of the rat cerebral cortex

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SUMMARY

Using immunocytochemistry we studied the distribution of 1,25-dihydroxyvitamin D3 receptors (VDR) in series of vibratome sections of intact rat brains. With subsequent immunofluorescence we investigated whether calcitriol target neurons express neuropeptide Y (NPY). Single pyramidal cells in the primary motor cortex showed nuclear staining for VDR. A fraction of these cells also contained cytoplasmic NPY immunofluorescence. About 20% of the interneurons in layers 2 and 4 throughout the cerebral cortex stained for VDR, and 10% of these cells were NPY positive. Similar findings were observed in the prefrontal and cingulate cortex, the sensory cortex, the entorhinal cortex and the piriform cortex. In the CA2 and CA3 regions of the hippocampus, numerous non-pyramidal cells and basket cells were seen to be VDR immunoreactive. A fraction of these neurons stained with the NPY antibody. The hypothalamus showed numerous NPY positive perikarya and widely distributed NPY networks. However, no coexistence of VDR and NPY could be observed in this region. Our findings further emphasize that calcitriol may be another important neurosteroid with distinct target areas throughout the brain.

Key Words: Neuropeptide Y - Vitamin D receptor - Immunohistochemistry - Double staining

INTRODUCTION

Extensive mapping studies have revealed the widespread neuroanatomical distribution of Neuropeptide Y (NPY) in the rat CNS (De Quidt and Emson, 1986). Since its discovery, NPY has been found in many brain regions at relatively high concentrations. NPY systems have been shown to innervate hypothalamic and basal forebrain regions involved in neuroendocrine regulation. Hypothalamic nuclei are particularly rich in NPY expressing neurons; the paraventricular nucleus contains perhaps the densest supply of NPY in the brain (Magnuson et al., 1984). In humans, NPY immunoreactivity has been found in the basal ganglia, the nucleus accumbens and amygdala. Moderate amounts of NPY were found in the hippocampus, the septal nuclei, the cortex and the periaqueductal gray. A large number of NPY-positive neuronal cell bodies was found in the caudate putamen. Immunoreactive neuronal cell bodies were also localized in cortical areas, particularly in layers 5 and 6 (Adrian et al., 1983). The distribution of NPY in the rat parallels the findings in humans: NPY neurons and fibers were most abundant in the periaqueductal gray, the nucleus accumbens, hypothalamus, septum and amygdala. Lower amounts of NPY have been found in the basal ganglia, globus pallidus, hippocampus and cortex (Allen et al., 1983).

Throughout the CNS, NPY is widely distributed either in local interneurons of, for example, the cerebral cortex or in long neuronal projections, such as the brainstem-hypothalamus noradrenergic tract, where NPY is colocalized with catecholamines (Everitt et al., 1984). Little is known about the functional properties of NPY in the brain. Like most other neuropeptides, NPY seems to be involved in many different functions in several neuronal circuits. McShane et al. (1994) suggested that NPY neurons in the arcuate nucleus modulate gonadotropin-relea-

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sing hormone function via an innervation of the medial septum-diagonal band (MSDB). They observed large populations of NPY neurons in the ventromedial arcuate nucleus. Some of these cells seem to project to the MSDB. NPY levels were observed to increase significantly in the paraventricular nucleus and in the arcuate nucleus after 3-4 days of food deprivation, and to return to baseline levels in the paraventricular nucleus with refeeding (Calza et al., 1989). Brain NPY concentrations are reduced in olfactory bulbectomized rats (Widerlöv et al. 1988). Following bilateral removal of the olfactory bulb from rats, changes in behavior and endocrine regulation have been observed. Chronic treatment with antidepressant drugs results in increased NPY concentrations in many rat brain regions (Heilig et al., 1988; Widdowson and Halaris, 1989). Furthermore, NPY can modulate cognitive processes. NPY was localized in the hippocampus and amygdala, two regions strongly involved in normal memory processing (Mohs, 1988; Ordy et al., 1988; Flood and Morley, 1989). NPY enhances memory processing and memory retention after administration into the rostral portion of the hippocampus (Flood and Morley, 1989).

Due to their lipophilic nature, steroids are capable of crossing the blood brain barrier. Therefore, steroids are predestined peripheral modulators of central neuroendocrine functions. McShane et al. (1994) suggested an interaction of NPY with steroid hormones. Thus, the localization of steroid receptors in NPY neurons may provide new insights into the functional properties of NPY in the brain. One of the most abundant steroid receptors in the central nervous system is the one for 1,25-dihydroxyvitamin D3 (calcitriol), the VDR. VDR has been extensively studied in the central nervous system of several species by autoradiography of radiolabelled ligand binding (Stumpf and O'Brien, 1987; Bidmon and Stumpf, 1994) as well as by immunocytochemistry for the receptor protein (Prüfer et al., 1999). With both approaches, it was established that VDR are widely distributed in brain and spinal cord, indicating that calcitriol is another important neurosteroid. Calcitriol is known to play an important role in calcium homeostasis (Kumar et al., 1994; DeLuca, 1988; Kumar et al., 1992). In addition, calcitriol interacts with its nuclear receptor (VDR) to regulate the transcription of several genes (Kumar et al., 1994; DeLuca, 1988; Kumar et al., 1992; Hannah and Norman, 1994; Carlberg, 1995). In recent studies, we observed VDR immunoreactivity in the rat olfactory system (Glaser et al., 1999), in the hypothalamus (Prüfer et al., 1997), throughout the limbic system, and in the spinal cord.

Bidmon and Stumpf (1996) observed colocalization of vasopressin and neurophysin with [3H] 1,25-dihydroxyvitamin D3 binding. In a recent study we found that a fraction of the magnocellular oxytocinergic perikarya of the rat hypothalamus contains nuclear VDR immunostaining (Prüfer and Jirikowski, 1997). These findings indicated that numerous different neuropeptidergic systems are calcitriol targets.

In the present study, we employed combined immunoperoxidase and immunofluorescence methods in order to assess the topographical distribution of VDR immunoreactive NPY neurons in the rat brain.

Materials and Methods

Three male and three female Sprague-Dawley rats, kept under normal conditions with an artificial 12 h day-night rhythm and with free access to food and water, were killed by CO_2 inhalation. The animals were immediately perfused with Bouin's fixative. Brains were postfixed for 48 hours in the above fixative and then cut on a Vibratome (Plano Instruments) into 50µm thick serial sections. Free-floating sections were collected in PBS (pH 7.4) supplemented with 0.5% NaN_3 and 0.5% Triton X-100.

Immunohistochemistry was performed with a rat anti-VDR monoclonal antibody, clone 9A7 (Chemicon) as previously described (Prüfer et al., 1997).

This antibody has been well characterized and has been shown to not cross-react with the estrogen or glucocorticoid receptors. To unmask the antigens, sections were placed in 10 mM citrate, pH 6.0, and heated twice for 2 min in a 780 W microwave oven set on high. Endogenous peroxidase activity was blocked with 5% normal rabbit serum in PBS followed by incubation with a 1:300 dilution of rat anti-VDR antibody (overnight at 4°C). After several washes with PBS, the sections were treated with a 1:200 dilution of biotinylated rabbit anti-rat IgG, (Vector, Burlingame, CA) followed by a 1:500 dilution of peroxidase-labeled streptavidin (Dako, Carpintine, CA). Diaminobenzidine and hydrogen peroxide were used for color development. For control purposes, VDR antibody that had been preadsorbed with an excess of VDR was used instead of the first antibody. After successful VDR immunostaining had been confirmed, the immunocomplexes were removed by incubating the sections for 10min at room temperature in 0.01N HCl. Thereafter, sections were incubated with rabbit anti-NPY polyclonal antibody (Chemicon) diluted 1:500 in PBS-Triton overnight at 4°C. After washing in PBS-T, sections were stained with Cy3-labeled anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc.) diluted 1:500 in PBS-T for 60 min at room temperature. Immunocytochemical controls were performed with rabbit normal serum instead of the specific NPY antiserum. Finally, sections were rinsed in tap water,

mounted onto slides, and coverslipped with Mowiol (Aldrich). The topographical distribution of immunostained cells was assessed by light microscopy or epifluorescence. The atlas by Paxinos and Watson (1985) was used for verification of anatomical structures.

Results

VDR immunostaining was observed mostly within neuronal nuclei. At higher magnification it was often possible to determine distinct immunostained patterns within the nuclei. Some of the magnocellular neurons of the hypothalamo-neurohypophysial system also contained cytoplasmic VDR immunoreactivity. NPY immunofluorescence was confined to the perinuclear cytoplasm. In many regions we observed widely distributed networks of NPY-positive fibers. This was especially true for the hypothalamus, the preoptic region and the CA2 and CA3 portions of the hippocampus. Neurons containing both NPY and VDR were visualized and counted with combined epifluorescence and interference contrast illumination. Immunocytochemical controls were devoid of either immunostains. The distribution of VDR- and NPY- like immunoreactivity in the rat brain is shown in Fig.1. VDR immunoreactivity was observed in numerous neurons throughout the neocortex. In the motor cortex a small fraction of the pyramidal cells contained VDR staining. Single pyramidal cells in layers 3 and 5 showed NPY immunofluorescence (Fig. 2/1) and most of them were in addition VDR-positive (Fig. 2/2). About 20% of the granular cells in layers 2 and 4 throughout the motor cortex stained for VDR (Fig. 2/4). About 10% of these cells were NPY-positive (Fig. 2/3). Similar observations were made in the prefrontal and cingulate cortex, the primary sensory cortex, the entorhinal cortex and the piriform cortex.

In the hippocampus, nuclear VDR staining was visible in many of the pyramidal and non-pyramidal cells, including basket cells. NPY immunofluorescence was confined to basket cells in the CA2 and CA3 region (Fig. 2/5). Most of these neurons also stained for VDR (Fig. 2/6). Single NPY-positive neurons were visible in the molecular layer. These neurons were in all cases devoid of VDR-staining. VDR-positive neurons were visible in the magnocellular portion of the paraventricular nucleus (PVN), the supraoptic nucleus (SON), the suprachiasmatic nucleus (SCN) and the arcuate nucleus (ARC). The PVN, the periventricular nucleus (PEV) and the preoptic area (POA) showed numerous perikarya with cytoplasmic NPY staining. Many of these cells extended long processes with specific NPY immunofluorescence. Dense networks of NPY-positive fibers were abundant in the thalamus and hypothalamus. However, no coexistence of VDR and NPY could be observed in these regions. The approximate numerical distribution of VDR-positive and NPY-immunostained neurons is given in Table 1.

Table 1.- Percentage distribution of VDR-positive and double stained neurons in rat cortex and hippocampus.

	pirifom cortex cingulate cortex prefrontal cortex entorhinal cortex prim. motor cortex prim. sensory cortex		hippocampus region CA2 and CA3	
	VDR	VDR+NPY	VDR	VDR+NPY
pyramidal cells	10%	6%	8%	4%
interneurons	20%	10%		
non pyramidal incl. basket cells			36%	25%

Discussion

VDR immunoreactivity has been shown to be broadly distributed throughout the rat brain, indicating that the VDR is involved in a multitude of neuronal and neuroendocrine functions. Topographical assessment of calcitriol targets in the brain has been performed in several species by in vivo autoradiography using [^{3}H] 1,25-dihydroxyvitamin D3 (Stumpf and O'Brien, 1987; Stumpf et al., 1992; Musiol et al., 1992; Bidmon and Stumpf, 1994; Stumpf, 1995; Bidmon and Stumpf, 1996). Several different neuronal and behavioral functions have been linked to calcitriol, including the central control of reproduction (Stumpf, 1988; Stumpf, 1995). Light-induced production of calcitriol is thought to be partly responsible for the therapeutic action of phototherapy in the treatment of depression (Stumpf and Privette, 1989).

We observed VDR immunoreactivity in numerous neuronal cells throughout the rat neocortex, hippocampus and diencephalon. The various locations of VDR immunoreactivity in the CNS suggest that calcitriol might be involved in several neuropeptidergic systems (Prüfer et al.,

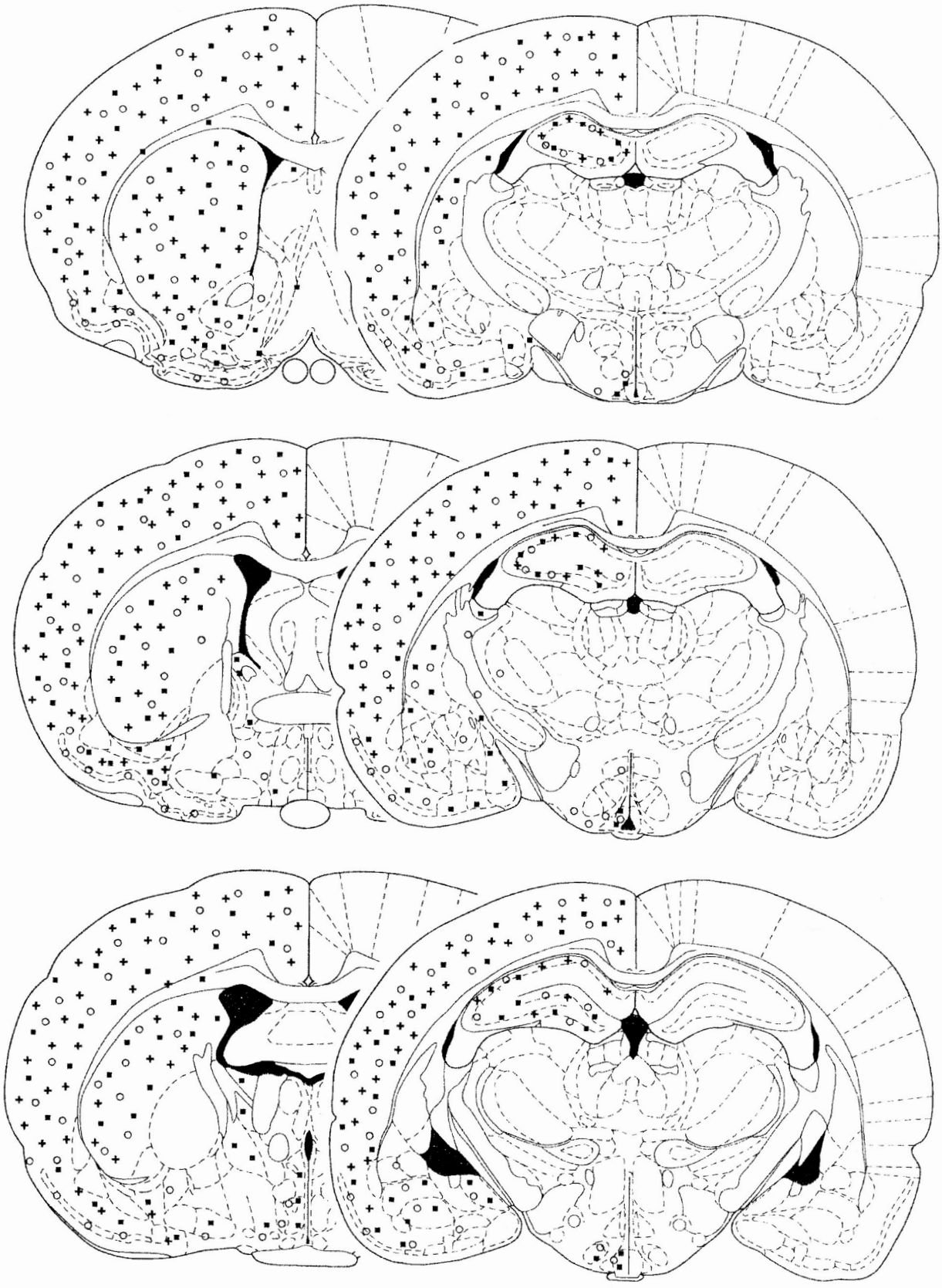

Fig. 1.- Distribution of the VDR and NPY immunoreactive neurons in the rat brain. Frontal sections in rostro-caudal sequence showing the approximate localization of VDR-positive neurons (dots), NPY neurons (squares) and coexistence of both antigens (+). Abbreviations and outlines of structures, according to the atlas of Paxinos and Watson.

Symbols: ■ NPY-reactive neurons
○ VDR-targets
\+ NPY-reactive VDR-targets

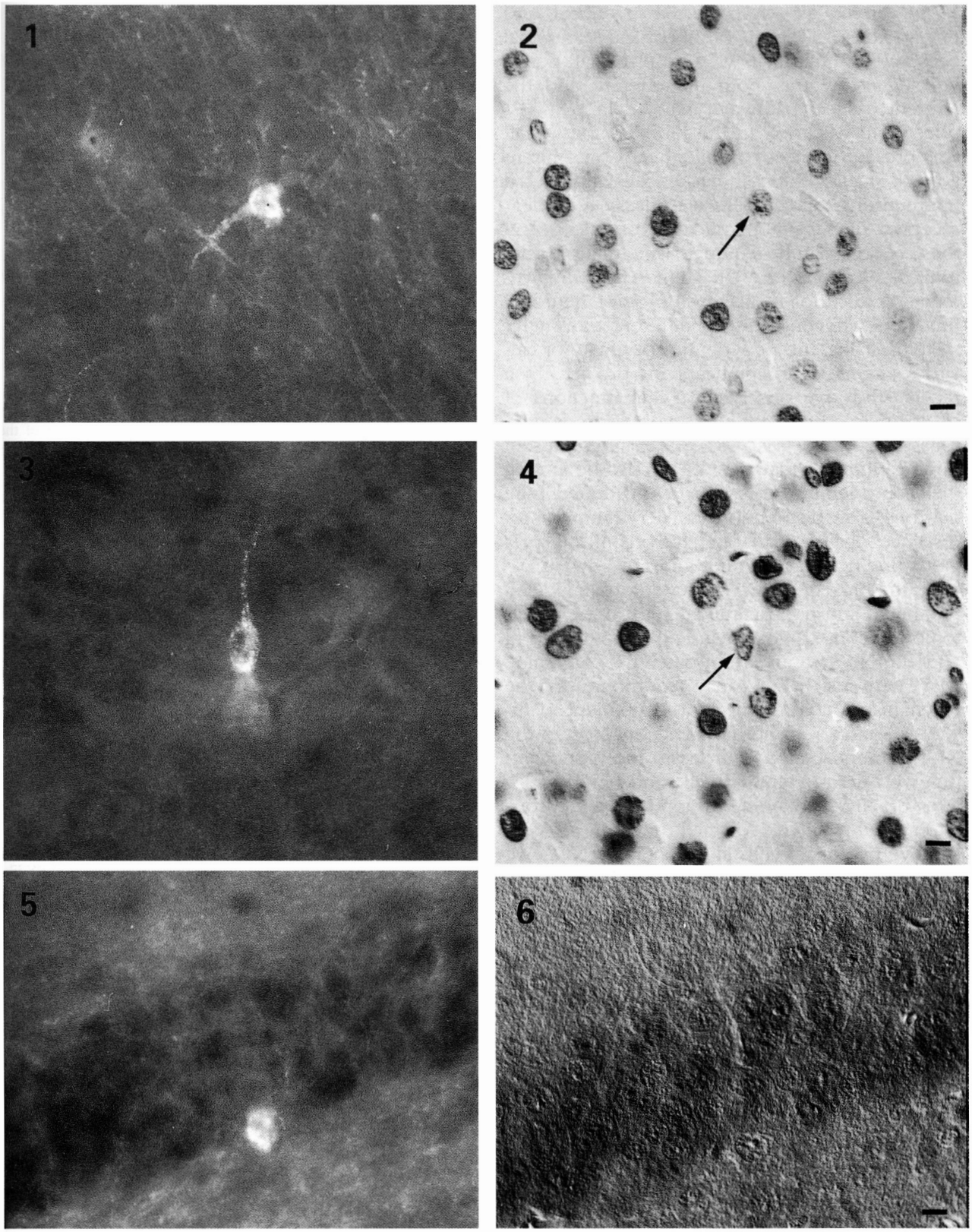

Fig. 2.- Coexpression of VDR and NPY. NPY immunofluorescence of a multipolar neuron in the temporal cortex (**1**). This cell also shows nuclear VDR immunostaining (**2**). NPY immunostained neurons represent only a small fraction of the total number of VDR-positive neurons. Pyramidal cell in the postcentral gyrus with cytoplasmic NPY fluorescence (**3**). This cell is one of many VDR-positive neurons in this region (**4**, arrow). Numerous neurons in the CA3 region of the hippocampus show nuclear VDR immunostaining (**6**), single cells within this region also contain NPY immunofluorescence (**5**). Scale bar = 5 μm.

1999). Here, we were able to confirm and extend these findings: both cortical pyramidal cells and granular cells were in part VDR-positive, suggesting that both the efferent and the afferent cortical pathways may be in part responsive to calcitriol. In the hippocampus, VDR was present in most pyramidal cells but only to a much smaller extent in basket cells and related interneurons. Since VDR is a classical nuclear receptor, direct genomic actions of calcitriol in these regions can be assumed. The multiple anatomical locations of VDR suggest the involvement of calcitriol in a large range of neuronal functions, affecting various different transmitter systems. Thus, the peptidergic specificity of calcitriol targets is of significant importance. In a recent study we observed VDR in fractions of oxytocinergic magnocellular hypothalamus neurons (Prüfer and Jirikowski, 1997). Our present findings indicate that NPY might also be among the neuropeptides present in VDR-containing neurons. The distribution of NPY immunofluorescence observed in our study confirms previous findings by De Quidt and Emson (1986).

In the present study we found double-labeled pyramidal and non-pyramidal cells in the somatomotory cortex. We conclude that both afferent and efferent systems are in part VDR containing NPY neurons, which may contribute to the mechanisms controlling light-dependent pathways for motion or hormonal action. Most of the NPY immunostained neurons in the hippocampus were non-pyramidal cells and basket cells; 70% of NPY-containing hippocampal neurons were VDR-positive. A small fraction of VDR positive pyramidal cells also contained NPY. We were able to show in this paper that two widely expressed proteins, - the VDR and NPY -, colocalize in specific areas of the CNS. Further studies are necessary to assess the physiological importance of this colocalization.

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Abbreviations

ARC	arcuate nucleus
CNS	central nervous system
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MSDB	medial septum-diagonal band
NPY	neuropeptide Y
PBS	phosphate-buffered saline
PEV	periventricular nucleus
POA	preoptic area
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
VDR	1,25 dihydroxyvitamin D3 receptor

References

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K and Polak JM (1983). Neuropeptide Y distribution in human brain. *Nature,* 306: 584-586.

Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR and Polak JM (1983). Neuropeptide Y distribution in the rat brain. *Science,* 221: 877-879.

Bidmon HJ and Stumpf WE (1994). Distribution of target cells for 1,25-dihydroxyvitamin D3 in the brain of the yellow bellied turtle *Trachemys scripta. Brain Res,* 640: 277-285.

Bidmon HJ and Stumpf WE (1996). Vitamin D target systems in the green lizard *Anolis carolinensis. Anat Embryol,* 193: 145-160.

Calza L, Giardino L, Battistini N, Zanni M, Galetti S, Protopapa F and Velardo A (1989). Increase of neuropeptide Y-like immunoreactivity in the paraventricular nucleus of fasting rats. *Neurosci Lett,* 104: 99-104.

Carlberg C (1995). Mechanisms of nuclear signalling by vitamin D3. *Eur J Biochem,* 231: 517-527.

Deluca HF (1988). The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J,* 2: 224-236.

De Quidt ME and Emson PC (1986). Distribution of neuropeptide-like immunoreactivity in rat central nervous system II. Immunohistochemical analysis. *Neuroscience,* 18: 545-618.

Everitt BJ, Hökfelt T, Terenius L, Tatemoto K, Mutt V and Goldstein M (1984). Differential coexistence of NPY-like immunoreactivity with catecholamines in the central nervous system of the rat. *Neuroscience,* 11: 443-462.

Flood JF and Morley JE (1989). Dissociation of the effects of neuropeptide Y on feeding and memory: evidence for pre- and postsynaptic mediation. *Peptides,* 10: 963-966.

Glaser SD, Veenstra TD, Jirikowski GF and Prüfer K (1999). Distribution of 1,25-Dihydroxyvitamin D3 receptor immunoreactivity in the rat olfactory system. *Cell Mol Neurobiol,* 19: 613-624.

Hannah SS and Orman AW (1994). 1alpha,25(OH)2 vitamin D3-regulated expression of the eukaryotic genome. *Nutr Rev,* 52: 376-382.

Heilig M, Wahlestedt C and Widerlöv E (1988). Neuropeptide Y (NPY) induced suppression of activity in the rat: evidence for NPY receptor heterogeneity and for interaction with alpha adrenoceptors. *Eur J Pharmac,* 157: 205-213.

Kumar R, Schäfer J and Wieben E (1992). The expression of milligram amounts of functional human 1,25 dihydroxyvitamin D3 receptor in a bacterial expression system. *Biochem Biophys Res Commun,* 189: 1417-1423.

Kumar R, Schäfer JS, Grande JP and Roche PC (1994). Immunolocalisation of the calcitriol receptor, 24-hydroxylase cytochrome P-450 and calbindin D28K in human kidney. *Am J Physiol,* 266: F477-F485.

Magnuson DJ, O'donohue TL and Gray TS (1984). Neuropeptide 'Y' innervation of hypothalamic and amygdala

neurons projecting to the nucleus tractus solitarius/dorsal vagal nuclei. *Abstract Soc Neurosci,* 10: 128.9.

MCSHANE TM, WISE PM and JENNES L (1994). Neuropeptide Y neurons projecting to the medial septum-diagonal band do not have access to fenestrated capillaries in the rat brain. *Mol Cell Neurosci,* 5: 459-465.

MOHS RC (1988). Memory impairment in amnesia and dementia: implications for the use of animal models. *Neurobiol Aging,* 9: 465-468.

MUSIOL IM, STUMPF WE, BIDMON HJ, HEISS C, MAYERHOFER A and BARTKE A (1992). Vitamin D nuclear binding to neurons of the septal, substriatal and amygdaloid areas in the Siberian hamster (*Phodopus sungorus*) brain. *Neuroscience,* 48: 841-848.

ORDY JM, THOMAS GJ, WOLPE BT, DUNLAPS WP and COLOMBO PM (1988). An animal model of human-type memory loss based on aging, lesion, forebrain ischaemia and drug studies with the rat. *Neurobiol Aging,* 9: 667-683.

PAXINOS G and WATSON C (1985). The rat brain in stereotaxic coordinates, 2nd ed. Academic Press.

PRÜFER K and JIRIKOWSKI GF (1997). 1,25-Dihydroxyvitamin D3 receptor is partly colocalized with oxytocin immunoreactivity in neurons of the male rat hypothalamus. *Cell Mol Biol,* 43: 543-548.

PRÜFER K, PLATZER C and JIRIKOWSKI GF (1997). Distribution of vitamin D receptor immunoreactive neurons in the male rat hypothalamus. *Eur J Anat,* 1: 145-150.

PRÜFER K, VEENSTRA TD, JIRIKOWSKI GF and KUMAR R (1999). Distribution of 1,25dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. *J Chem Neuroanat,* 16: 135-145.

PUCHACZ E, STUMPF WE, STACHOWIAK EK and STACHOWIAK MK (1996). Vitamin D increases expression of the tyrosin hydroxylase gene in adrenal medullar cells. *Mol Brain Res,* 36:193-196.

STUMPF WE and O'BRIEN LP (1987). 1,25(OH)2D3 sites of action in the brain, an autoradiographic study. *Histochemistry,* 87: 393-406.

STUMPF WE (1988). Vitamin D-soltriol. The heliogenic steroid hormone: somatotrophic activator and regulator. Discoveries from histochemical studies lead to new concepts. *Histochemistry,* 89: 247-251.

STUMPF WE and PRIVETTE TH (1989). Light, vitamin D and psychiatry. *Psychopharmacology,* 97: 285-297.

STUMPF WE, BIDMON HJ, LI L, PILGRIM C, BARTKE A, MAYERHOFER A and HEISS C (1992). Nuclear receptor sites for vitamin D, soltriol in midbrain and hindbrain of Siberian hamster (*Phodopus sungorus*) assessed autoradiography. *Histochemistry,* 98: 155-164.

STUMPF WE (1995). Vitamin D sites and mechanisms of action: a histochemical perspective. Reflections on the utility of autoradiography and cytopharmacology for drug targeting. *Histochem Cell Biol,* 104: 417-427.

WIDDOWSON PS and HALARIS AE (1989). Increased levels of neuropeptide Y immunoreactivity in rat brain limbic structures following antidepressant treatment. *J Neurochem,* 52: (Suppl.): 77.

WIDERLÖV E, HEILIG M, EKMAN R and WAHLESTEDT C (1988). Possible relationship between neuropeptide Y (NPY) and major depression: evidence from human and animal studies. *Nord J Psychiat,* 42:131-137.