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# THE VITAMIN D RECEPTOR IN DOPAMINE NEURONS; ITS PRESENCE IN HUMAN SUBSTANTIA NIGRA AND ITS ONTOGENESIS IN RAT MIDBRAIN

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**Abstract—There is growing evidence that vitamin D is a neuroactive steroid capable of regulating multiple pathways important for both brain development and mature brain function. In particular, there is evidence from rodent models that prenatal vitamin D deficiency alters the development of dopaminergic pathways and this disruption is associated with altered behavior and neurochemistry in the adult brain. Although the presence of the vitamin D receptor (VDR) has been noted in the human substantia nigra, there is a lack of direct evidence showing that VDR is present in dopaminergic cells. Here we confirm that the VDR is present in the nucleus of tyrosine hydroxylase (TH)-positive neurons in both the human and rat substantia nigra, and it emerges early in development in the rat, between embryonic day 12 (E12) and E15. Consistent evidence based on immunohistochemistry, real-time PCR and western blot confirmed a pattern of increasing VDR expression in the rat midbrain until weaning. The nuclear expression of VDR in TH-positive neurons during critical periods of brain development suggests that alterations in early life vitamin D status may influence the orderly development of dopaminergic neurons.** 

**Key words: vitamin D receptor, brain development, substantia nigra, rat, VDR ontogeny.**

## INTRODUCTION

The active form of vitamin D, 1,25-dihydroxyviamin $D_3$ ($1,25(OH)_2D_3$) has diverse physiological functions that extend beyond its classical role in calcium homeostasis and bone health (Jones et al., 1998). For example, several studies have postulated functions for vitamin D in both the developing (Eyles et al., 2003, 2009; Cui et al., 2007) and adult brain (McGrath, 2001; Garcion et al., 2002; McGrath et al., 2008). Low prenatal vitamin D deficiency has also been proposed as a risk-modifying factor for schizophrenia (McGrath, 1999, 2001). A recent Danish case-control study confirmed that neonatal vitamin D status was significantly associated with subsequent risk of schizophrenia (McGrath et al., 2010). In addition, cross-sectional and prospective longitudinal epidemiological studies have suggested that vitamin D deficiency is associated with an increased risk of Parkinson's disease (Newmark and Newmark, 2007; Evatt et al., 2008; Knekt et al., 2010). Abnormalities in dopamine function are implicated in both schizophrenia and Parkinson's disease.

Our group is interested in the effects of developmental vitamin D (DVD) deficiency on brain development. We have established an animal model for this purpose (Eyles et al., 2003). In this model, female rats are depleted of vitamin D prior to mating and throughout pregnancy, but returned to normal diet after the litter is born (Eyles et al., 2011). One consistent finding from this model is altered dopamine signalling. As adults, these offspring are more sensitive to the locomotor enhancing effects of the dopamine-releasing agent amphetamine (Kesby et al., 2009b). The DVD-deficient adult rat is also more sensitive to haloperidol, a dopamine receptor antagonist (Kesby et al., 2006). Of particular interest, neonates show reduced expression of the dopamine-metabolizing enzyme, catechol-O-methyltransferase (COMT), in embryonic forebrain. Consistent with this finding, the ratio of the major dopamine metabolites dihydroxyphenylacetic acid/homovanillic acid (DOPAC/HVA) is altered indicating dopamine turnover is abnormal in the neonatal forebrain (Kesby et al., 2009a). Because vitamin D is a potent pro-differentiating agent (Deeb et al., 2007), and in light of our finding of increased cellular proliferation in the absence of vitamin D (Eyles et al., 2003; Ko et al., 2004; Cui et al., 2007), we were particularly interested in the potential role of vitamin D in the orderly development of dopaminergic systems. To this end we

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*Abbreviations:* $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; DAPI, 4′,6-diamidino-2-phenylindole; DVD, developmental vitamin D; E12, embryonic day 12; EDTA, Ethylenediaminetetraacetic acid; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; NGS, normal goat serum; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; P0, postnatal day 0; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TH, tyrosine hydroxylase; VDR, vitamin D receptor.

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have recently shown factors important in the early post-mitotic specification of dopaminergic neurons, such as Nurr 1 and p57Kip2, are reduced in DVD-deficient mesencephalon (Cui et al., 2010). In summary, these findings strongly implicate a role for vitamin D in maturation of dopaminergic systems. The vitamin D receptor (VDR) has been noted in key dopaminergic brain regions in both the rat (Prufer et al., 1999) and human brain (Eyles et al., 2005). However, to date there is lack of evidence that the VDR is actually expressed in dopaminergic neurons.

The VDR is a member of the nuclear receptor superfamily. Like other nuclear receptors, such as the thyroid hormone receptor and the retinoic acid receptor, the VDR has been shown to shuttle between the cytoplasm and the nucleus (Racz and Barsony, 1999; Prufer and Barsony, 2002; Sunn et al., 2005; Peleg and Nguyen, 2010). The intracellular location of the VDR is indicative of receptor function and only nuclear VDR is capable of initiating transcription (Prufer et al., 2000; Prufer and Barsony, 2002). Reduced VDR expression in the cell nucleus is associated with reduced transcriptional activity, reduced differentiation and a decrease in the ability of vitamin D to inhibit cell proliferation (Yang et al., 2001; Humeniuk-Polaczek and Marcinkowska, 2004; Garay et al., 2007), whereas increased cytoplasmic translocation has been associated with increased proliferation (Zhi et al., 2011). In brain cells, immunohistochemical studies have reported the presence of VDR in both the nucleus and cytosol (Bidmon et al., 1991; Musiol et al., 1992; Prufer and Jirikowski, 1997; Veenstra et al., 1998; Prufer et al., 1999; Langub et al., 2001; Walbert et al., 2001; Eyles et al., 2005). Given the role of vitamin D signalling via this receptor to potently affect differentiation, we wished to investigate whether the subcellular location of VDR would vary with developmental age.

To date, two studies have explored the ontogeny of VDR expression in the embryonic rodent brain. An earlier study indicated the immunohistochemical presence of VDR could be detected quite early in the developing brain (embryonic day 12, E12) and, of central relevance to dopaminergic neurons, VDR staining was particularly abundant in developing midbrain (Veenstra et al., 1998). The second study was a semi-quantitative examination of both VDR transcript and protein. This study indicated that, in general, VDR expression increased with embryonic age (Burkert et al., 2003). However this study only examined whole brain homogenates and the earliest embryonic age assessed in this study was E15 which represents a period after most mesencephalic dopaminergic cells are born.

This study had three main aims; (a) to demonstrate that the VDR is present in cells expressing the dopaminergic marker, tyrosine hydroxylase (TH) in both the adult rat and human substantia nigra, (b) to describe the subcellular distribution of the VDR in these cells, and (c) to describe the ontogeny of VDR expression in dopaminergic neurons in the developing rat midbrain.

## EXPERIMENTAL PROCEDURE

### Materials

VDR antibodies N-20, D-6, H-81, and C-20 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The monoclonal VDR antibody 9A7, used for immunohistochemical studies in the human brain was purchased from Abcam (Sapphire Bioscience Pty Ltd., Australia). A monoclonal anti-TH antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA) and a polyclonal TH antibody from Abcam. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-histone 3 antibodies were purchased from Chemicon (Billerica, MA, USA). Alexa Fluor® 488 goat anti-mouse immunoglobulin G (IgG) and Alexa Fluor® 488 conjugated to streptavidin were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Cy3-conjugated goat anti-rabbit antibody was purchased from Jackson Laboratories (West Grove, PA, USA). Biotinylated anti-rabbit secondary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse were purchased from Vector Laboratories (Burlingame, CA, USA). Recombinant human VDR protein was purchased from Sigma–Aldrich and recombinant rat VDR protein was purchased from Bioclone Inc. (San Diego, CA, USA). Recombinant rat VDR was tagged with (His)6 at its N-terminus. This consisted of 26 amino acid sequences containing two protease cleavage sites for the removal of His-tag. Thus the recombinant rat VDR molecular weight was increased to approximately 53 kDa.

### Human and animal tissues

Human formalin-fixed brain blocks (3 mm) containing the substantia nigra were obtained from four control males (age 18–59; max PMI 43 h; brain pH range 6.3–6.7) who were free of any neurodegenerative condition and had no history of psychosis or drug or alcohol abuse. Samples were obtained from the Tissue resource Centre, University of New South Wales. Female Sprague–Dawley rats were housed in controlled lighting conditions on a 12-h light–dark cycle (lights on at 0700) (Herston Medical Research Centre, Brisbane, Australia). Rats received water and food *ad libitum*. After mating, a positive plug was considered embryonic day 0 (E0). For embryonic tissue, dams were euthanized with sodium pentabarbitone (Virbac Animal Health, Milperra, Australia) and the uterus containing embryos rapidly dissected. Mesencephalic tissue was collected according to standard landmarks (Altman, 1995). Mesencephalic tissue was collected at the following time points – E12, E15, E18 and postnatal day 0 (P0). Midbrain tissue was obtained from male Sprague–Dawley rats at P21 and P70. Whole kidney was also obtained from P70 rats as a reference tissue for VDR expression. Muscle was also collected as a tissue considered negative for VDR (Wang and DeLuca, 2010; Wang et al., 2012b). After dissection, tissue was either stored at −80 °C for protein analysis; in RNAlater® for mRNA studies; or embryos were fixed in formalin for immunohistochemistry studies. In all studies representative tissue samples came from at least three independent litters. All experiments were conducted with the ethical approval of the University of Queensland and the University of New South Wales.

### Choice of antibodies

There are at least 10 available commercial antibodies for the VDR. Two recent studies have called in to question the specificity of some of these (Wang et al., 2010; Wang and DeLuca, 2010). The 9A7 clone (generated against the C terminal of ligand binding domain of the VDR) was chosen because of our previous immunohistochemical experience with detection of the VDR in the human brain (Eyles et al., 2005).