

1,25(OH)₂ Vitamin D₃ sites of action in spinal cord and sensory ganglion*

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Summary. Autoradiographic studies revealed concentration of ³H 1,25(OH)₂ vitamin D₃ in nuclei of certain neurons in the spinal cord of adult and neonatal mice, fed a normal or a vitamin D deficient diet. Nuclear uptake and retention was strongest in motor neurons in lamina IX. Nuclear concentration also existed in neurons of lamina II, lamina VIII, lamina X and intermediate nucleus of the lateral column. The results indicate that these neurons are target neurons which contain nuclear receptors for 1,25(OH)₂ vitamin D₃. This suggests that 1,25(OH)₂ vitamin D₃ has direct genomic actions on the innervation of skeletal muscle by exerting related trophic, secretory, and electrophysiological effects. In addition, these data point to direct genomic actions of 1,25(OH)₂ vitamin D₃ on spinal sensory perception, and on certain autonomic functions. Nuclear binding in certain neurons in the peripheral ganglion of the trigeminal nerve further suggests that sensory perception is influenced by 1,25(OH)₂ vitamin D₃ not only at the level of the substantia gelatinosa, but also at the level of spinal ganglia.

Key words: Vitamin D – Receptor – Spinal cord – Autoradiography – Motor neuron – Substantia gelatinosa – Spinal ganglion

Materials and methods

Adult male JAX C57/6J mice (*n*=2), 2-day (*n*=1), 10-day (*n*=1), 15-day (*n*=5), and 22-day (*n*=2) Swiss Albino mice, and adult female Swiss Webster (*n*=1) mice were used. The animals, or their mothers prior to parturition, were maintained on a vitamin D-deficient diet (D–), supplemented with vitamins A, E, and K, except the Swiss-Webster mouse, which received a normal vitamin D replete rodent chow (D+). [26,27-³H] 1,25(OH)₂ vitamin D₃, specific activity 160 Ci/mMol, dissolved in ethanol-isotonic saline was injected intraperitoneally to the JAX C57 BL/6J mice and neonatal Swiss Albino mice, 0.10 µg/100 g bw, or subcutaneously to the Swiss-Webster mouse, 0.38 µg/100 g bw, which was divided into 2 doses administered at one hour intervals. Three to four hours after the injection of the radiolabeled hormone, the animals were sacrificed by decapitation.

Cervical, thoracic, and lumbar spinal cord and sensory ganglion of the trigeminal were dissected and frozen onto tissue holders in liquefied propane at –180 C and subsequently stored in liquid nitrogen. Four µm sections were cut in a cryostat (Jung Frigocut, Heidelberg). The frozen sections were thaw-mounted onto photographic emulsion (Kodak NTB 3)-coated slides and exposed in lightproof desiccator boxes at –20 C for different lengths of time. After the exposure, slides were fixed for 30 sec in 2.5% paraformaldehyde in 0.01 M phosphate-buffered saline. The slides were then rinsed in tap water, developed in Kodak D-19 developer (diluted 1:1) for 1 or 2 min, rinsed, and then fixed in Kodak fixer for 4 min, rinsed again and then stained with methylgreen-pyronin. The thaw-mount autoradiographic technique, which was developed in our laboratory, has been described in detail (Stumpf 1976). Nuclear concentration of ³H 1,25(OH)₂ vitamin D₃ was quantitated as described (Clark et al. 1987).

Results

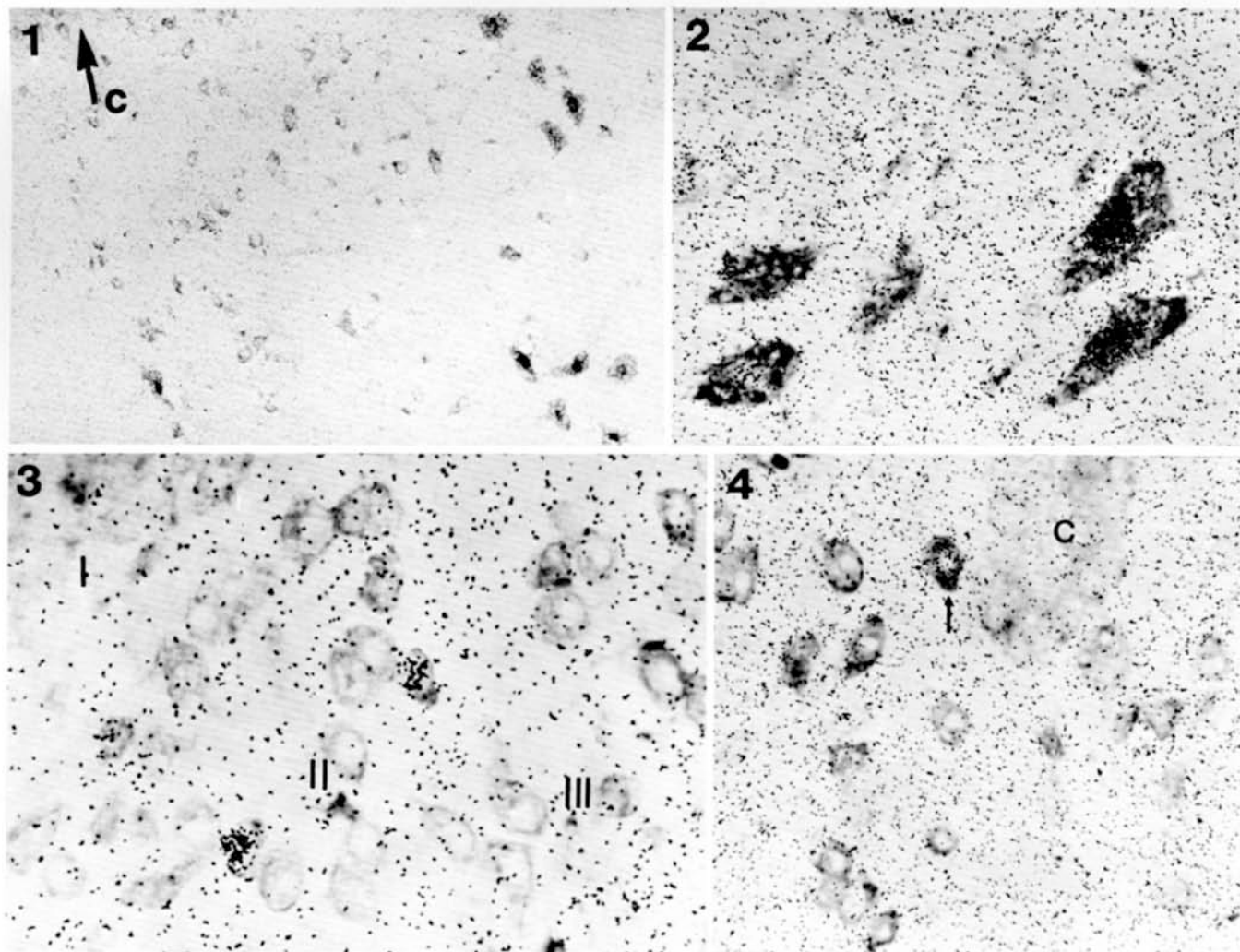
In autoradiograms of adult as well as of 10-, 15-, and 22-day old mice, nuclear concentration of radioactivity is seen in certain neurons in cervical, thoracic and lumbar spinal cord. No nuclear concentration of radioactivity was found in neurons of the spinal cord from the 2-day old mouse, although in the same animal cells of the intestine and kidney had nuclear labeling. In the adult and juvenile animals, the in-

Results from autoradiographic studies have provided evidence for nuclear receptors for 1,25(OH)₂ vitamin D₃ (1,25 D₃) in pituitary and brain (Stumpf et al. 1979, 1980, 1982). Strong nuclear uptake of ³H 1,25 D₃ was demonstrated in cranial and certain spinal motor nerve nuclei. This suggested that cranial and spinal motor neurons, and perhaps other spinal cord neurons, are targets for the hormone, and that effects of vitamin D on skeletal muscle physiology are mediated in part, or predominantly, through neurotrophic influences (Stumpf and O'Brien 1987).

The present studies were designed to investigate, where in the spinal cord nuclear receptors for 1,25 D₃ exist. Autoradiography is the method of choice (Stumpf 1976), because of its high sensitivity and cellular resolution.

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Figs. 1-4. Autoradiograms of mouse spinal cord after injection of ^3H 1,25(OH) $_2$ vitamin D_3 , showing strong nuclear concentration of radioactivity in motor neurons of lamina IX (lumbar region) in its dorsolateral, ventrolateral, and ventromedial cell groups (1 Central canal above C. 2 Ventrolateral group). Scattered labeled neurons exist in lamina II (3) and in lamina X (4 arrow) adjacent to central canal (C). Exposure 495 days. Magnification $\times 140$ (1), $\times 875$ (2), $\times 550$ (3, 4)

tensity and extent of the nuclear labeling varies, depending on the dose of radiolabeled hormone administered and the length of autoradiographic exposure. In animals that received a relatively low dose, nuclear labeling is distinct in motor neurons of lamina IX, with less obvious and lower nuclear uptake at other sites. In the mouse that received the highest dose, a more intense nuclear labeling is apparent, providing a more readily discernible localization pattern of nuclear target cells for ^3H 1,25 D_3 . When excess of unlabeled 1,25 D_3 is injected before the injection of ^3H 1,25 D_3 , no nuclear concentration of radioactivity is noted, and injection of excess unlabeled 25 D_3 , instead of 1,25 D_3 , does not abolish nuclear labeling (Stumpf and O'Brien 1987). Nuclear silver grains were counted in motor neurons of vitamin D-deficient mice (10-15/mouse) and uptake per nucleus was calculated. Nuclear uptake was similar for all ages 10 days or older. Thus, counts from seven mice that were evaluated were combined for assessment of a mean uptake of $5.1 \pm 1.4 \times 10^3$ molecules per nucleus.

Examples of autoradiograms of the spinal cord are shown in Figs. 1-4, and the pattern of distribution of la-

beled neurons in different segments is depicted schematically in Fig. 5.

Strongest nuclear concentration of radioactivity is found in large motor neurons in lamina IX (Figs. 1, 2) throughout the spinal cord, including the ventromedial, ventrolateral and dorsolateral subgroups of lamina IX. In the region of lamina VIII in the cervical-thoracic intumescence, on occasion a labeled neuron is found. Scattered small labeled cells are present throughout lamina II (Fig. 3). An occasional labeled cell is noted in the area of the intermediate nucleus of the lateral column and close to the spinal canal in lamina X (Fig. 4).

Sections of the sensory ganglion of the trigeminus reveal a subpopulation of neurons that show nuclear labeling, while the majority of the neurons appears unlabeled (Fig. 6).

Discussion

The results of the present studies provide further evidence for the wide-spread existence of nuclear receptors for

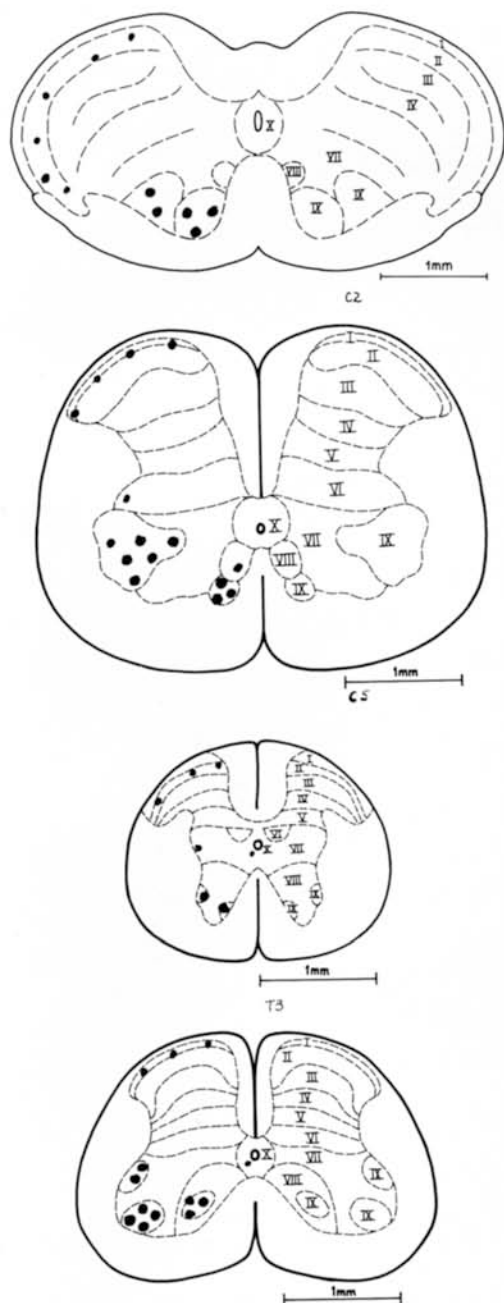


Fig. 5. Distribution of ^3H 1,25(OH) $_2$ vitamin D_3 target neurons in spinal cord upper and lower cervical, thoracic, and lumbar regions. Schematic cross sections prepared after autoradiograms. The size and density of the dots indicate the relative frequency of labeled neurons and the intensity of nuclear labeling

1,25(OH) $_2$ vitamin D_3 . The pituitary and the nervous system were probably least expected to be targets. The calcium homeostatic system was believed to be a peripherally regulated system, and considerations of a direct involvement of pituitary and brain were lacking, until the use of autoradiography provided a more precise assessment of cellular target sites (Stumpf et al. 1979, 1980b, 1982). Although there was reluctance to accept such unexpected findings, it is now apparent that nervous-endocrine system components are extensively involved as effectors for 1,25 D_3 and mediators of its action. This includes many sites in the fore-brain, midbrain, hindbrain (Stumpf and O'Brien 1987b)

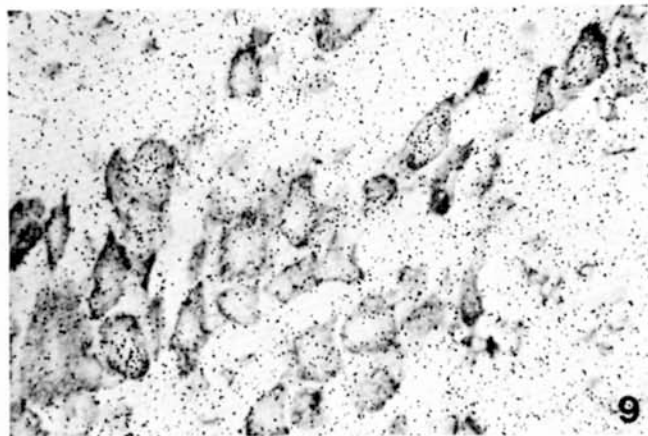


Fig. 6. Autoradiogram of ganglion trigeminale after injection of ^3H 1,25(OH) $_2$ vitamin D_3 , showing concentration of radioactivity in nuclei of certain neurons. Exposure 390 days, $\times 800$

and spinal cord, as well as pituitary thyrotropes (Stumpf et al. 1979; Sar et al. 1980), thyroid follicle epithelial cells (Stumpf and O'Brien 1987a), thymus reticular cells (Stumpf and Downs 1987), pancreas B cells (Clark et al. 1980, 1987), adrenal medullary cells (Clark et al. 1986), Sertoli cells (Stumpf et al. 1987a), gastrin producing cells (Stumpf et al. 1980, 1987b), kidney macula densa and podocytes (Stumpf et al. 1980), and others. The situation with 1,25 D_3 in the 80's is reminiscent of the situation with estradiol in the 60's. When our autoradiographic techniques were applied, the newly discovered targets for estradiol (e.g., heart, Leydig cells, thymic reticular cells, skin dermal papilla of hair, brain cortex, medulla oblongata and spinal cord) were unexpected and overwhelming and not readily accepted as significant findings.

Our data indicate that the selective uptake of 1,25 D_3 by spinal motor neurons is similar to nuclear uptake in other target sites. Based on the observed average nuclear uptake of 5100 molecules per nucleus we calculate that motor neurons bind on average 1300 fmol/mg DNA, a value which is greater than we have observed for pancreatic islets, intestine, kidney, or parathyroid glands (Clark et al. 1987). The existence of these nuclear receptors for 1,25 D_3 directly affects the neuromuscular unit in both adult and neonatal mice. Dihydrotestosterone (Sar and Stumpf 1977), corticosterone (Duncan and Stumpf 1984) and aldosterone (Stumpf and Sar 1979) are also concentrated in spinal motor neurons and, therefore, appear to be also of importance to motor neuron function.

An effect of vitamin D on neuromuscular function has been implicated in clinical studies. Clinical myopathy associated with deficiency of or resistance to 1,25 D_3 is reversible by treatment with 1,25 D_3 (Heyburn et al. 1983; Peacock 1977; Schott and Wills 1976). The observed neural abnormalities associated with the myopathy (Mallette et al. 1975) is consistent with proposed effects of 1,25 D_3 on motor neurons.

In light of our present findings and the evidence for low receptor levels in skeletal muscle (Simpson et al. 1985; Boland et al. 1985) it would appear that 1,25 D_3 affects the neuromuscular unit at three levels. At the organismal level 1,25 D_3 maintains appropriate plasma calcium for excitation coupling. At the neural level 1,25 D_3 may modulate moto-

neuron electrophysiology and neurotrophic function. At the contractile level, 1,25D₃ may directly act on muscle cells to alter muscle function. Based on our autoradiographic evidence, while this latter possibility may exist and can not be excluded, uptake by nuclei of skeletal muscle has not been observed (Kim et al. 1985) under conditions, when other tissues display high levels of nuclear concentration of radioactivity after injection of ³H 1,25D₃. While experimental evidence suggests that maintenance of normal plasma calcium levels is the primary mechanism whereby 1,25D₃ affects neuromuscular function (Wassner et al. 1983), the present data suggest a strong role for vitamin D on skeletal muscle development, maintenance and function through neurotrophic and neurophysiologic influences.

The localization of ³H 1,25D₃ in neurons in the substantia gelatinosa and in the trigeminal ganglion indicates involvement of 1,25D₃ in sensory tactile or pain perception, or both. Nuclear neuronal labeling, although sparse in number, in the region of the lateral column intermediate nucleus and in lamina X, further suggests a role of 1,25D₃ in these autonomic neurons.

Effects of 1,25D₃ on the manufacture of 28k calcium-binding protein is a possible action to be considered. Calcium-binding protein has been found in neurons of the substantia gelatinosa (Garcia-Segura et al. 1984). Combined steroid autoradiography-immunohistochemistry (Sar and Stumpf 1980) could resolve, whether there is colocalization of ³H 1,25D₃ and calcium-binding protein or most likely of ³H 1,25D₃ and specific peptide messengers in identical neurons, which would provide further clues for the mechanisms of action of 1,25D₃.

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