

Concentration-Effect Relationship of Intrapitoneal Administration of 1, 25 (OH) 2-Vitamin D in a Chronic Constriction Model of Neuropathic Pain

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ABSTRACT

Introduction: The presence of nuclear receptors of 1,25 dihydroxy vitamin D3 (1,25 vit D3), the biologically active metabolite of vitamin D, in neurons and glial cells indicates the biological effect of this vitamin in the nervous system. The present experiment was conducted to identify the effects of different doses of 1,25 vit D3 on mechanical and cold allodynia in rodent model of neuropathic pain.

Methods: A mononeuropathy was produced by chronic constrictive injury (CCI) of the sciatic nerve. 1,25 vit D3 (0.3, 0.6, 1 µg/Kg) was administered by an i.p. injection every 2 days during a month after CCI. Mechanical and cold allodynia were evaluated by Von frey filament and acetone respectively.

Results: These findings revealed the exaggerated responses in the group which received CCI. The group which was treated by 1 µg/kg of 1,25 vit D3 showed a significant reduction in pain behavior. Injection of 1,25 vit D3 did not change the response of animals to the acetone drop and von frey filament.

Discussion: Our results showed that antinoceptive effect of 1,25 vit D3 in a rodent neuropathic pain model is dose dependent and this vitamin may provide new approach for treatment of chronic pain.

1. Introduction

Neuropathic pain is defined as pain due to a lesion or dysfunction of the normal sensory pathways in either the peripheral or central nervous system (Smith et al., 2002). Metabolic disorders such as diabetes or uremia, immune deficiencies, traumatic lesions, nerve compressions, spinal cord injuries and vascular insufficiency may all cause neuropathic pain (Jensen et al., 2002). Lesions of all levels from the peripheral receptor to the highest cortical centers may all give rise to same clinical

cal pain picture, in almost all patients, allodynia (a painful response to non-painful stimulus) and hyperalgesia (an exaggerated response to normally painful stimulus) have been detected (Smith et al., 2002).

The exact mechanisms for the induction and development of allodynia and hyperalgesia in neuropathic pain are not clear, but researchers have focused on the imbalance of excitatory and inhibitory systems such as loss or down-regulation of γ -amino butyric acid (GABA) in dorsal horn (Stiller et al., 1995; Moore et al., 2002), increased release of glutamate and up-regulation of

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NMDA (N-methyl-D-aspartate) receptors located in the dorsal horn of spinal cord (Sang 2002). Accumulating evidence also implicates a reduction in neurotrophins and a role of nitric oxide (NO) in neuropathic pain conditions (Cahill et al., 2003; Levy et al., 2001; Fernyhough et al., 1999). Neurotrophins are also thought to play an important role in the regulation of development, differentiation and maintenance of the nervous system. For example, NGF (nerve growth factor) induces neural tissue regeneration and protection (Bard 1994). Some studies have shown reduction of responses to a thermal stimulus after injury, and suggest that this is due to increased numbers of NGF receptors (Wan et al., 2005). Local over production of some neurotrophins in the spinal cord has been reported to cure neuropathic pain induced by chronic constrictive injury of sciatic nerve, similarly an over production of opioid peptides and neurotrophins could be useful for a better management of chronic pain (Pohl and Meunier, 2003). NO has also been implicated in the physiopathology of a wide variety of acute and chronic neurologic disorders (Tsuda, 2008; Sparrow 1995; Celeries et al., 2006) and may be particularly important in hyperalgesic responses after peripheral or central nervous system dysfunction via sensitization of peripheral nociceptors to thermal and mechanical stimuli (Vetter et al., 2001). The nitric oxide synthase (NOS) inhibitor, N-omega-nitro-L-arginin methyl ester (L-NAME), reduces hyperalgesia (Guan et al., 2007; Li et al., 2007) and 7-nitroindazole and other NOS inhibitors delay spinal cord neurons malfunction and degeneration caused by injuries (Ikeda et al., 1998). The widespread distribution of vitamin D3 receptors in distinct portion of the sensory, motor and other parts of nervous system make this vitamin important in the nervous system (Prufer et al., 1999; Nakagawa, 2006; Eyles et al., 2005). Calcitriol (1,25-dihydroxy vitamin D3) is thought to be the most important form of vitamin D3, readily passes the blood-brain barrier, and has been implicated in a broad range of functions in the nervous system (Garcion et al., 2002; Nataf et al., 1996). Several studies have shown induction of neurotrophin synthesis by vitamin D3, specially the increased NGF in muscles and the nervous system (Brown et al., 2003; Riaz et al., 1999). Vitamin D3 appears to enhance the expression of NGF transcripts in CNS regions directly moreover it depletion decreases free NGF and GDNF (Glial cell-derived neurotrophic factor) levels in brain (Riaz et al., 1999; Feron et al., 2005). The trophic action of NGF on cholinergic neurons and GDNF on dopaminergic neurons is well identified. Vitamin D3 also regulates P75 NTR expression in neurons and is a potent regulator of neurotrophin receptors in the developing

brain (Eyles et al., 2003). In addition, vitamin D3 effects have also been implicated in the inhibition of NO production. 1,25 dihydroxy this type of vitamin can inhibit the synthesis of inducible NOS and increase glutathione levels, suggesting a role in brain detoxification and neuroprotective pathways (Garcion et al., 2002). Together, these data suggest that vitamin D3 may be useful in the alleviation of the chronic neuropathic pain. In the present study, we describe the reduction of tactile and cold allodynia induced after chronic constriction injury (CCI) of the sciatic nerve by intraperitoneal injection of vitamin D3.

2. Methods

A total of 70 male Wistar rats weighing 200-250g were used. All the rats in the experiment group were executed with the Guide for the care and use of laboratory animals and the protocols were approved by the local research ethics committee. The animals were kept in 12-hour light-dark cycle in clear plastic cages for at least one week before the experimental procedures. At the time of experiment, the rats were divided into seven groups of ten each. These groups comprised of:

- 1) Normal rats injected with vitamin D3 (normal + vit),
- 2) Normal rats injected with vehicle (normal+veh),
- 3) Rats subjected to CCI (CCI),
- 4) CCI rats treated with different doses (0.3, 0.6, or 1 µg/kg) of 1,25 vit D3 (CCI +vit),
- 5) CCI rats received vehicle (CCI+veh),

Anesthesia was induced with a mixture of Ketamine (100 mg/Kg) and Xylazine 2% (15mg/Kg). For induction of neuropathic pain 4 ligatures (4/0 chromic gut) were used to produce loose constriction around the sciatic nerve of left paw at spaces about 1mm apart using the Bennett & Xie model (Bennet and Xie, 1988). To technically minimize variability, the same person performed all operations for all groups. As a result of the tests, 1,25 vit D3 (Roche, product number: 0419125) was dissolved at the appropriate concentration in 80% Propylene glycol and 20% 0.05 M sodium phosphate at pH 7.4 and administrated by an i.p. injection in the volume of 0.5 ml. These amounts of vitamin D3 do not change serum calcium level (Nataf et al., 1996). Injections were initiated on the first day after the CCI and repeated every 2 days for a month after. Vehicle-treated rats received an i.p. injection of propylene glycol and

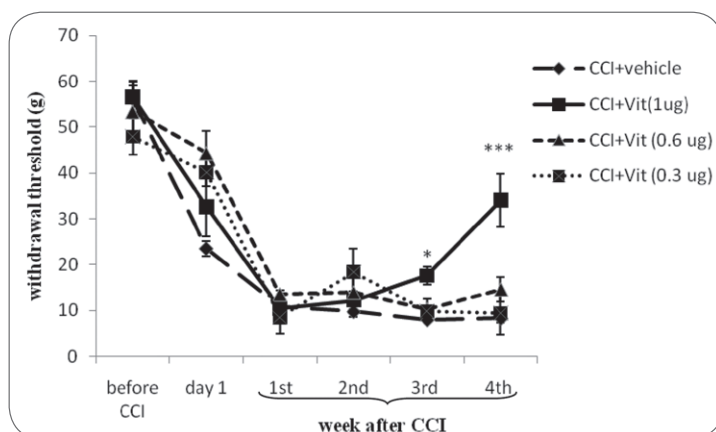
sodium diphosphate. Animals were tested before the CCI, the day after it, and every week thereafter. The effects of vitamin D3 alone, normal intact animals received higher dose of the vitamin (1 μ g/kg) (normal+vit) or vehicle (normal+veh) according to the same schedule, and were tested the day from the first injection up to the final date of the test. The vitamin or the vehicle was given to the tested rats at least 3 hours before the test in case the injection and the times of the test were the same. In order to evaluate mechanical and cold allodynia, Von Frey filaments (Stoelting Company, Wood Dale IL, and USA) and acetone were used respectively. Mechanical allodynia was determined with a series of 7 Von Frey hairs (4.56, 4.74, 4.93, 5.07, 5.18, 5.46 and 5.88). Each filament was vertically applied to the mid-plantar skin in ascending order with sufficient force to cause slight bending against the hind paw. The area tested was the mid-plantar surface of left hind paw in the area of the sciatic nerve distribution, avoiding the footpads. Each filament were applied 5 times with at least 15 second intervals between the applications. At thresholds the rats responded with a quick paw withdrawal. A bending force evoked 60% occurrences of the paw withdrawal response was set as the mechanical withdrawal threshold. To quantify cold allodynia, brisk foot withdrawal in response to acetone application was measured. The rat was placed under a transparent plastic dome on a mesh-metal floor and acetone was applied to the plantar surface of the foot. The acetone was applied 5 times (once every minute) to the left paw. The

frequency of foot withdrawal was expressed as a percentage: (number of trials accompanied by brisk foot withdrawal) \times 100/ (number of trials). To minimize the differences, the same one being unaware of the animal treatments took all tests. All data are presented as the Mean \pm SEM. Differences between treatment groups were determined using repeated measures two-way ANOVAs as the between group factor. Effect of time was determined using two-way ANOVAs as the within subject factor. Student–Neuman–Keuls test was used for post hoc comparisons. Statistical significance was taken at $P < 0.05$.

3. Results

3.1. Effect of vitamin D3 on mechanical allodynia in normal and the CCI rats:

Mechanical sensitivity to innocuous tactile stimuli in the CCI rats treated with different amounts of 1, 25 vit or vehicle, are shown in Fig. 1. In the CCI animals received vehicle, mechanical allodynia-like responses were observed at one day after the CCI and continued through the fourth week. The tactile allodynia observed following CCI was attenuated in animals treated with i.p. injections of 1 μ g/kg 1,25 vit D3. From the third week onward CCI, significant differences were observed in mechanical thresholds between vitamin (1 μ g /kg) and vehicle-treated rats [$F(1,18)=62.227$, $P < 0.05$ on the 3rd week and $F(1,18)= 187.114$, $P < 0.001$ on the 4th week]. No significant differences were observed in mechanical



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Figure 1. Effect of i.p. injection of different doses of vitamin D3 and vehicle on mechanical allodynia induced by chronic constriction injury (CCI). Results compare the withdrawal threshold in the CCI rats treated with vehicle and different doses of vitamin D3 starting after CCI induction. The data reported are the Mean \pm SEM of 10 animals in each group. Asterisks (*) indicate that CCI animals treated with Vitamin D3 (1 μ g/kg) differed significantly from the CCI animals treated with vehicle (* : $P < 0.05$, ***: $P < 0.01$).

threshold of CCI rats treated with 0.3 and 0.6 $\mu\text{g}/\text{kg}$ of vitamin compared to vehicle treated CCI rats.

Withdrawal thresholds to Von Frey hairs in vehicle treated normal rats, as well as in normal ones treated with 1mg/kg of 1,25 vit D3 was about 50 g throughout the duration of the experiment and i.p. injections of 1,25 vit D3 (1 $\mu\text{g}/\text{Kg}$) did not alter sensitivity to tactile stimuli in normal uninjured animals (Fig.2). In animals receiving CCI procedures, there were noticeable decreases of withdrawal thresholds to Von Frey filaments on the injured side beginning from the very first day after CCI and mechanical allodynia-like responses were observed for at least a month after CCI, as reported by Bennett and Xie [28].

3.2. Effect of vitamin D3 on cold allodynia in normal and the CCI rats:

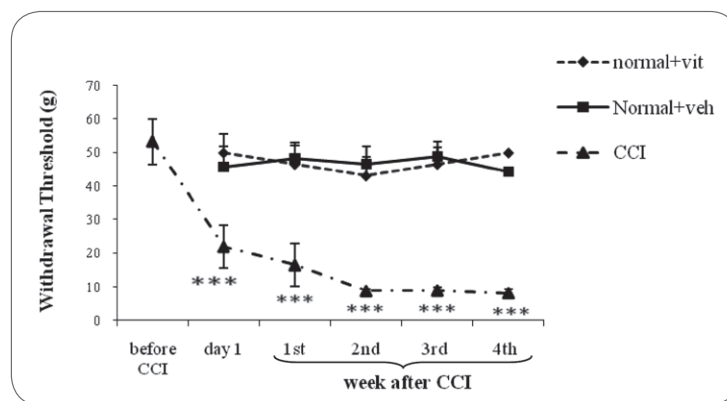
For determination of cold allodynia, a drop of acetone was applied to the plantar surface of operated (CCI) side, which resulted in brisk hindpaw withdrawal (with delays of about 0.2-0.3 sec) and subsequent hind paw shaking or licking. Fig. 3 shows the results following acetone application to the hind paw of CCI animals treated with vehicle or different amounts of 1,25 vit D3. The cold allodynia was maintained for at least four weeks following peripheral nerve injury in the CCI rats received vehicle or 0.3 $\mu\text{g}/\text{kg}$ of 1,25 vit D3 (Fig. 3). But it was reversed in the CCI animals with i.p. injection of 0.6 and 1 $\mu\text{g}/\text{kg}$ 1,25 vit D3 compared with CCI rats receiving vehicle. This beneficial effect was observed on the first day after CCI [$F(1,18)=43.408$, $P<0.05$]

and on the 3rd and 4th week after [$F(1,18)=235.552$ and $F(1,18)=405.640$ respectively $P<0.001$] in animals treated with 1 $\mu\text{g}/\text{kg}$ of vitamin D3 (compared with vehicle injection). Significant differences were observed on the 3rd and 4th week after CCI in animals injected with 0.6 $\mu\text{g}/\text{kg}$ of vitamin D3 [$F(1,18)=86.008$ on the 3rd and $F(1,18)=116.676$ on the 4th week, $P<0.05$].

Cold allodynia was induced following the CCI (Fig. 4). This was apparent as an increased percentage of hind paw withdrawal responses to acetone, beginning on the first day following CCI and repeatedly observed during the weeks following nerve injury. Uninjured rats rarely responded to acetone application. The i.p. injections of 1 $\mu\text{g}/\text{kg}$ of 1,25 vit D3 or vehicle had no significant effect on responses of normal animals during the one month evaluation period.

4. Discussion

The present study confirms that animals with chronic constriction injury of sciatic nerve show behavioral signs of mechanical and cold allodynia which are at a maximum 2 weeks after the lesion and last for a period of 4 weeks (Bennet and Xie, 1988). The main findings from this study indicate that administration of vitamin D3 can effectively reverse or reduce both cold and mechanical allodynia in rats with peripheral neuropathic pain. Similar to our finding, Lee and Chen reported the improvement of pain in the subjects with type 2 diabetic neuropathy in who the serum concentration of the vitamin D was less than 20 ng/ml. But as they stated their study was neither blinded nor randomized, resulting in



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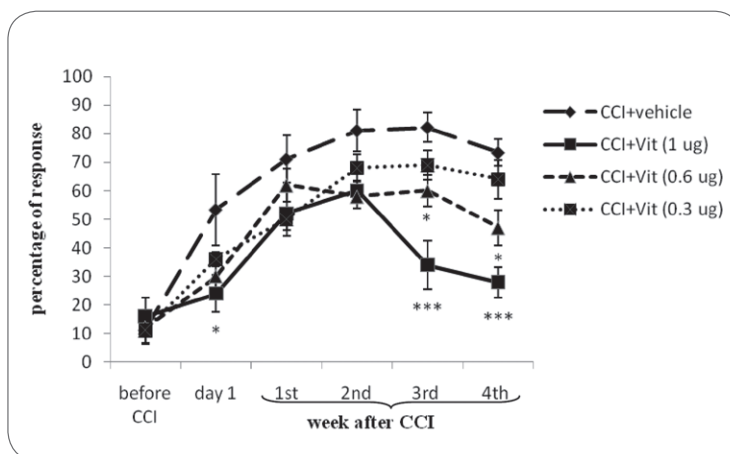
Figure 2. Mechanical allodynia in untreated CCI rats and in normal rats treated with vitamin D3 or vehicle. The data reported are the Mean \pm SEM (n=10 animals/groups). No significant difference was seen in tactile threshold in normal rat injected with vitamin D3 or vehicle. Asterisks (*) indicate the significant differences in mechanical thresholds between CCI and normal rats with vitamin D3 or vehicle. (***: $P < 0.001$)

the possibility of treatment bias. Since the treatment of diabetic neuropathic pain is generally unsatisfying and is associated with significant adverse effects and because the vitamin D supplement has no harmful effect they advocate the trial of vitamin D supplementation in vitamin D-insufficient patients with neuropathic pain (Lee and Chen, 2008).

Several lines of evidence indicate that the CNS constitutes a target for vitamin D (Garcion et al., 2002) and genes encoding enzymes involved in the metabolism of this hormone are present in the CNS (Neveu et al., 1994; Taniura et al., 2006). Vitamin D3 receptors have also been reported in hippocampus (Taniura et al., 2006; Langub et al., 2001) although nociceptive regions (e.g. spinal cord) have not yet been evaluated. It has been shown that active form of vitamin D3 is synthesized from its precursor, 25-hydroxy vitamin D3, by activated microglial cells (Neveu et al., 1994), and chronic peripheral treatment of rats with vitamin D3 can retard the age-related decrease in neuronal density in hippocampus (Landfield, 1996). It is possible that similar mechanisms underlie the beneficial effects of vitamin D3 treatment reduces neuropathic pain symptoms.

It has been reported that deficiency causes muscle weakness and pain in children and adult (Michael and Holic, 2003), and replacement vitamin D3 therapy promptly decrease the symptoms of muscle weakness and pain in these patients (Grober, 2010). Hyperesthesia, considered a symptom of chronic pain, reportedly

resolved with vitamin D3 therapy and reoccurred when vitamin D3 levels declined (Meyers, 2003). So reducing vitamin D3 deficiency may help preventing many chronic diseases and reducing pain intensity (Meyers, 2003; Beer et al., 2005; Harari et al., 2011). Vitamin D is a neurotrophic substance and modulates neuromuscular function and neuronal growth and differentiation. Also it is one of the more potent molecules which can increase NGF mRNA expression in the brain. In recent years several 1,25 dihydroxy vitamin D3 analogues have been discovered acting as efficient inducers of neurotrophic factors such as NGF, GDNF and NT-3, with reduced hypercalcemia producing side effects (Riaz et al., 1999; Eyles et al., 2003; Beer et al., 2005). Fukuoda et al showed that the active form of vitamin D3 reduces the symptoms of peripheral neuropathy through production of NGF (Fukuoka et al., 2001). NGF reverses thermal hyperalgesia after chronic constriction injury of sciatic nerve, but this appears to be time dependent. Immediate injection of NGF following CCI blocks behavioral hyperalgesia (possibly by reducing secondary cell death in the spinal cord, e.g. vulnerable inhibitory GABAergic interneuron), but when delayed for 4 days following CCI, infusion of NGF produces no effect on thermal hyperalgesia (Ro et al., 1999). Thus one possible mechanism underlying the beneficial effects of vitamin D3 treatment is by neuroprotection via increasing NGF levels in spinal cord during the early post-CCI stages.



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Figure 3. Effect of i.p. injection of different doses of vitamin D3 and vehicle on cold allodynia induced by chronic constriction injury (CCI). Results compare the withdrawal threshold in the CCI rats receiving different doses of vitamin D3 or vehicle starting after CCI induction. The data reported are the Mean \pm SEM of 10 animals in each group. Significant difference is seen between CCI animals with Vehicle and CCI animals received 1 and 0.6 μ g/kg of vitamin D3 after CCI. *: $P < 0.05$, ***: $P < 0.001$

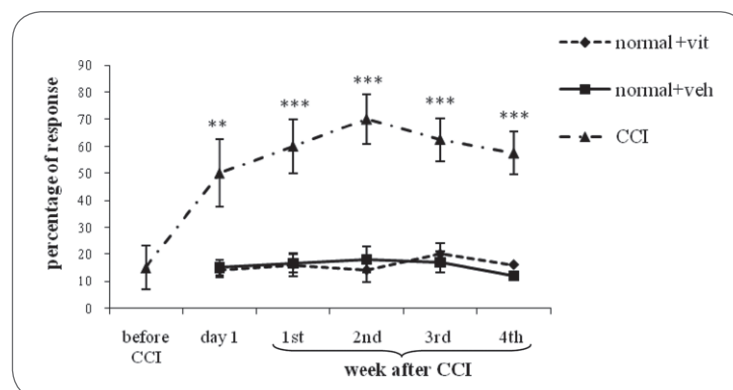
There are many controversial evidences about the effect of neurotrophins when the pain is a prominent feature. In contrast to experiments indicating the antinociceptive effect of neurotrophins (Pohl and Meunier, 2003; Wilson-Gerwing and Verge, 2006) some studies express that neurotrophins specially NGF is involved in pain transduction mechanisms and play a key role in many persistent pain states, notably those associated with inflammation. Wu et.al showed that NGF mRNA is increased and a large-molecular-weight form of NGF protein is expressed in the region adjacent to the incision and high concentration of NGF in incised tissue contributes to hyperalgesia in incisional pain (Wu et al., 2007).

Administration of monoclonal anti-NGF antibody in animal models of neuropathic pain reversed tactile allodynia when administered 2 weeks after injury and repeated injecting this antibody to CCI mice for 3 weeks produced a sustained reversal of tactile allodynia with no development of tolerance (Wild et al., 2007). Neutralizing antibodies directed against the TrkA receptor may display potent analgesic effects in inflammatory and chronic pain (Ugolinin et al., 2007). In an experiment using different strains of rats, it was shown that different mechanisms exist in different strains and these have been partially attributed to differences in the endogenous opioid and noradrenergic systems. Since neurotrophins modulates both the endogenous opioid and noradrenergic system, pain sensitivity may be differentially influenced by neurotrophins (Herradon et al., 2007). As different mechanisms involved neuropathic pain resulting from nerve injury is therapeutically chal-

lenging to treat due to the complexity of signaling pathways and molecules involved.

Another potential mechanism believed to play a role in neuropathic pain state is the increased production of nitric oxide in the spinal cord and nitric oxide second messenger pathways in the periphery following peripheral nerve injury (Sparrow, 1995; Celeries et al., 2006). NO initiated events appear to be involved in the induction of hyperalgesia and allodynia in chronic pain models (Sparrow, 1995; Okudoco and Onal, 2005) and the inhibition of NO synthesis by L-NAME reduces neuropathic pain syndrome (Sparrow, 1995; Celeries et al., 2006). Higher NO production is also thought to be a consequence of and at the same time a contributor to increased CNS damage in other conditions, such as multiple sclerosis, where glial cell-derived NO may cause the death of oligodendrocytes. Human NO synthase mRNA is markedly elevated in demyelinated regions of the brain of multiple sclerosis patients compared to control brain (Bagasra et al., 1995). Steroid-like hormones have been shown in vitro to antagonize NOS synthesis in different cell types. In an animal model of multiple sclerosis, treatment with vitamin D3 following the appearance of clinical symptoms, which are accompanied by enhancement of NOS expression, resulted in significant improvement (Bo et al., 1994). Thus, another possible explanation for the attenuation of neuropathic pain by i.p. injections of vitamin D3 could be the peripheral and/or central inhibition of NO production.

It seems that the effect of vitamin D3 in cold and mechanical allodynia occurs at different initiation times,



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Figure 4. Cold allodynia in untreated CCI rats and normal rats treated with vitamin D3 or vehicle. The data reported are the Mean \pm SEM (n=10/group). No significant changes in cold allodynia were seen in the normal rats after Vitamin D3 injections comparing to vehicle treated animals. In CCI rats, the percentage of withdrawal response to acetone application was increased at all time points following the surgery. Asterisks (*) indicate significant differences compared to normal rats. (**: $P < 0.01$, ***: $P < 0.001$)

and that i.p. injections of vitamin D3 diminish cold allodynia more effectively than mechanical allodynia. These results go with the data reported by other investigators showing distinct patterns of initiation and time courses for mechanical and thermal allodynia seen after nerve injury and may indicate distinct mechanisms underlying different types of allodynia (Mamlber et al., 1998)

In summary, the present work demonstrates that intraperitoneal injection of an active form of vitamin D3 can alleviate allodynic symptoms of neuropathic pain, and could be a valuable tool in the management of chronic pain associated with nerve injury. Further investigation is needed for determination the exact mechanisms involved in its analgesic action.

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