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short report

Upregulation of the 1,25-Dihydroxyvitamin D_3 Receptor in Neonatal Brain Injury

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Abstract

The Vitamin D_3 receptor (VDR) has been found to play a role in brain development through its identification in brain and spinal cord neurons of developing fetal rats. Also, studies have shown that Vitamin D_3 increases the amount of transcripts coding for its own receptor, VDR, and also for neurotrophins, further indicating a role in neurogenesis and protection.

Objectives:

To investigate the neuroprotective role of 1, 25-Dihydroxyvitamin D_3 and its receptor (VDR) in neonatal brain injury.

Methods:

Neural Stem Cells (NSC'S) were cultured and treated with various concentrations of glutamate. Samples were collected and analyzed through a protein assay and Western Blots. The unilateral carotid ligation model and the use of a hypoxia chamber were used to induce brain injury. In immunohistochemistry, brains previously extracted from 5-and 12-day-old mice kept in room air, hypoxic, and hypoxic-ligated conditions were stained for VDR and Cleaved Caspase-3 proteins. The stained slices were observed using a fluorescent light microscope.

Results and Conclusions:

In neural stem cells, the amount of VDR protein increased by 3.6-fold under 0.50 mM glutamate conditions, 4.2-fold under 0.75 mM glutamate, and up to 6.9-fold under 3.0 mM glutamate conditions. In hypoxia-ligation mouse brain tissue, the VDR receptor was found in areas of apoptosis, indicating that damaged or stressed cells produced more VDR than cells in normal conditions. These results suggest a role of Vitamin D_3 Receptor in brain injury; however, further studies must be completed to understand the role of Vitamin D_3 in such injury.

Introduction

Premature neonates are vulnerable to many conditions including neonatal brain injury. Neonatal brain injury affects thousands of children yearly leading to conditions such as cerebral palsy. In 2006, it was estimated that the preterm birth rate was 12.8% of the roughly 4 million live births in that year[1]. MRI data of these infants showed that about 50% exhibited some form of cerebral white matter injury[2]. Many researchers are investigating the potential role of many candidate mechanisms to confer neuroprotection. One of the difficulties in studying this condition is the multifactorial nature of this condition. As we learn more about the disease process we may one day be able to decrease the amount of destruction to the premature brain.

The main causes that play a role in the preterm brain's susceptibility to injury include hypoxia-ischaemia and infection/inflammation. Due to their inability to breathe effectively and maintain cerebral blood flow, preterm infants commonly have reduced oxygen levels in vital organs such as the brain[3]. This leads to cell death, particularly in the premyelinated oligodendrocytes (pre-OLs), an important type of glial cell which functions in neuronal insulation and conduction of action potentials throughout the central nervous system. Additionally, maternal or fetal infection/inflammation also contribute to perinatal brain injury through downstream mechanisms such as increased glutamate levels. These can lead to excitotoxicity, or neuronal death, due to the over activation of receptors for the excitatory neurotransmitter glutamate. It has been found that elevated levels of glutamate correspond directly with the amount of white matter injury present in the brain[3].

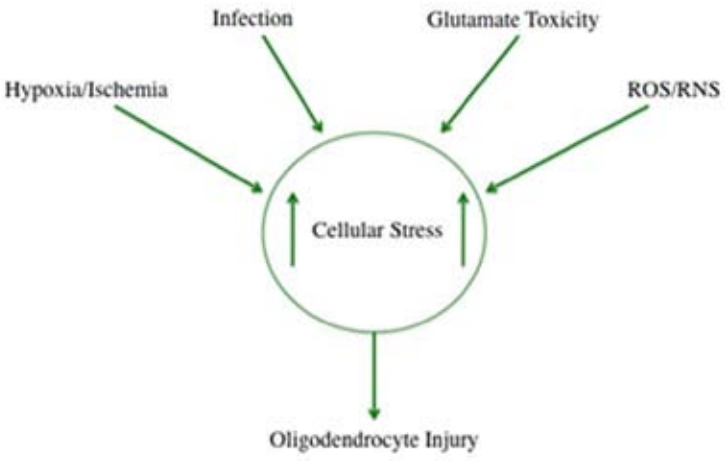


Figure 1.Known mechanisms of oligodendrocyte injury

In the developing brain, neurogenesis occurs through the differentiation of neural stem cells (NSCs). NSCs possess the capacity to differentiate into many cell types including pre-OLs, the targets of white matter injury in preterm infants. Due to their multipotent nature, NSCs are an ideal candidate for research in perinatal brain injury, and specifically in its prevention at an early stage.

Although the relationships between Vitamin D_3 and calcium absorption are understood, recent research suggests that 1,25-dihydroxyvitamin D_3 plays a role in brain development. The Vitamin D_3 receptor (VDR) has been identified in the brain and spinal cord neurons of developing fetal rats[4], indicating that vitamin D_3 must have some role in neurogenesis. Furthermore, Vitamin D_3 has been found to have a neuroprotective role, as it regulates the production of nerve growth factor (NGF) and the expression of other neurotrophins, or neuroprotective compounds. Studies have also shown that VDR transcripts existed in rat oligodendrocytes and VDR-positive cells were present in brain white matter. Vitamin D_3 increased the amount of transcripts coding for its own receptor, VDR and also for NGF, further con-

firming a role in neurogenesis and protection[4].

Methods and Materials

Cell Culture and Sample Collection

Experimental cell types (Neural Stem Cells) were cultured in sterile conditions at 37°C. NSC cells were treated with increasing millimolar concentrations (0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM) of glutamate. Samples were collected, Ripa Buffer was added, and stored at -20°C.

Protein Analysis

A Bradford Protein Assay was completed for all samples to determine their respective protein concentrations. Samples were then prepared and loaded into a 10% Tris-HCl gel for Gel Electrophoresis. The gels were allowed to run and equilibrate in a 1X Transfer Buffer. They were then transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus. After transfer was complete, blots were probed for various proteins (VDR, Cleaved Caspase-3, and Actin) using the SNAP i.d.™ Protein Detection System. Probed blots were then developed in a dark room using western blotting detection reagents.

Unilateral Carotid Ligation

Mice were anesthetized using 2% isofluorane per IACUC protocol. Temperature regulation occurred using a warming blanket. Using sterile technique, a midline tracheal incision was made and the left common carotid was exposed and then cauterized. The incision was closed using Dermabond. Animals were placed back with mother after 15 minutes. The animals were then placed in a plexiglass hypoxia chamber at 12% overnight. The brains were collected after 24 hours.

Immunohistochemistry

Five- and twelve- day- old mice kept in various conditions (Room Air, Hypoxia, and Hypoxia-Ligation) were euthanized. Their brains were extracted, fixed with 4% Paraformaldehyde (PFA), and cryosectioned at 16 microns. Brain slices were fluorescently stained for the VDR protein and Cleaved Caspase-3. Analysis was completed using a Zeiss fluorescent light microscope.

Results

The role of the Vitamin D_3 Receptor, VDR, in injury or protection is not fully understood. To examine the role of VDR in injury we used undifferentiated neural stem cells in the presence of increasing concentrations of glutamate, to model glutamate toxicity, a known mechanism of oligodendrocyte injury. Glutamate toxicity concentrations used were from 0-3mM. It was found that as glutamate increases, there is a concomitant increase in VDR protein level (Figure 2A). To assess the amount of cell death, we probed the same blot for Cleaved Caspase-3 and probed for Actin to normalize the results (Figure 2A). Cleaved Caspase-3 levels were normalized to Actin and represented in Figure 2B. There is a statistical increase in cell death at 0.25 and 0.50mM, $p < 0.05$, compared to control. VDR levels were also normalized using Actin as the"housekeeping" protein and Figure 1C demonstrates a general increase in VDR levels with a statistical increase of protein concentration seen at 0.75, 1 and 3 mM glutamate concentrations, $p < 0.01$. The increase seen in VDR almost reaches 7 fold as seen at 3mM.

If VDR levels change during stress, they should increase during certain pathological states. One such pathological state is in hypoxia-ischemia, which we were able to mimic by using the unilateral carotid ligation method. Full details of the procedure are described in methods section, but animals were anesthetized, common carotid ligated, and then placed in hypoxia. The VDR levels in control hemispheres (right) were compared to VDR levels in ligated hemispheres (left). In Figure 3, A (nuclear), C (Cleaved-Caspase 3), E (VDR), G (merged) of control hippocampus are compared to the ligated hemisphere in Figure 3, B (nuclear), D (Cleaved-Caspase 3), F (VDR) and H (merged), respectively. There is a dramatic increase seen in VDR shown in

Figure 2.(A)

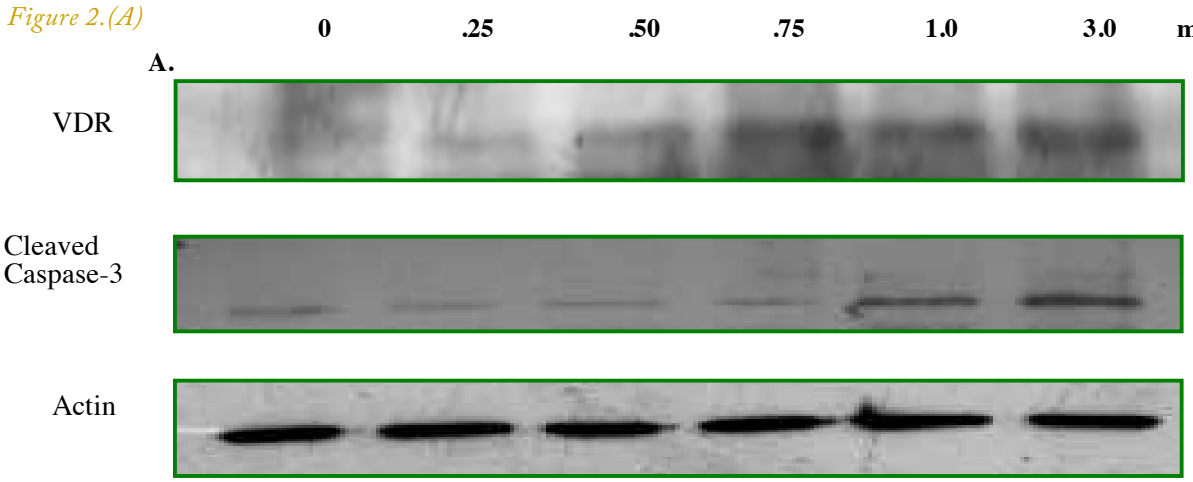
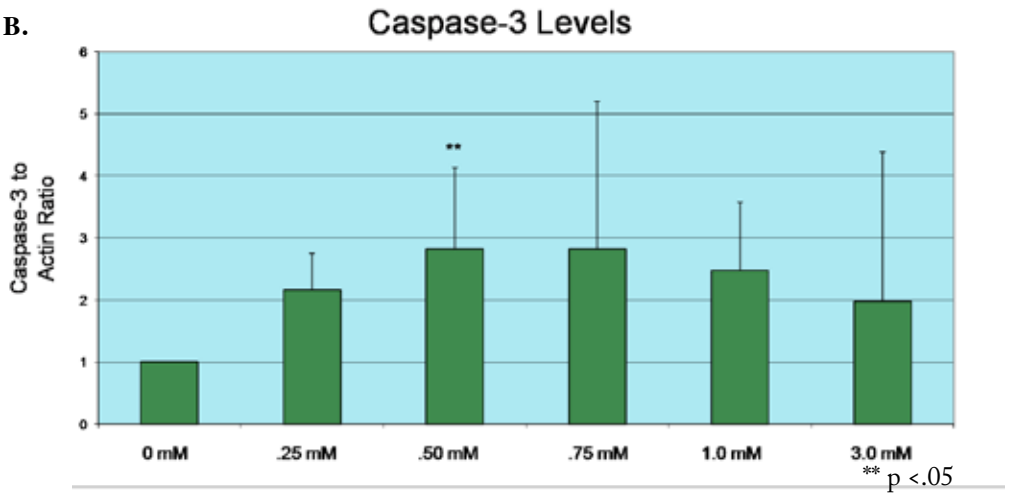


Figure 2 (A-C). Protein analysis of neural stem cells under increasing glutamate concentrations

(A) Representative immunoblot probed for VDR, Cleaved Caspase-3, and Actin proteins

(B-C on page 34)

B.



C.

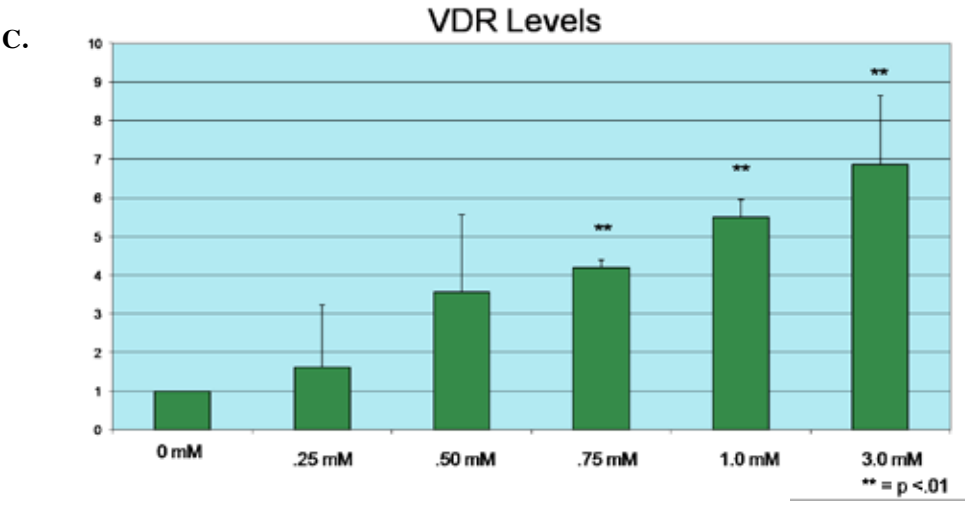


Figure 2. (B-C)

*(B) Caspase-3 levels in neural stem cells increase, p<0.05 in ** experiments, in increasing concentrations of glutamate: 0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM*

*(C) VDR levels in neural stem cells increase, p<0.01 in ** experiments, in increasing concentrations of glutamate: 0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM*

Figure 3F compared to Figure 3E, which additionally seems to correlate with cells that are Cleaved-Caspase 3 positive. Controls are not shown in this figure but no primary antibody controls were used to normalize the fluorescence.

In the experiment in Figure 3 these brains were not assessed for the specific cell type that responds to stress. The distribution of VDR in the brain is not well understood. Therefore, this experiment was designed to demonstrate specific cell types that express VDR. In Figure 4 (B and D), we demonstrate that VDR is expressed highly in NeuN positive cells.

Discussion

Currently, there is little understanding of brain injury and even less understanding of neonatal brain injury. There is a growing need in the vulnerable population of premature neonates to gain more insight into the pathology of this condition. It is well known that certain conditions such as infection and inflammation are linked to neonatal brain injury[3]. The current animal models to study neonatal brain injury use animals such as mice at different stages of development. The current model we use at 5 days will assess potential white matter injury but older animals are better models for grey matter injury as assessed in our current experiments. There is no evidence that links brain injury to the vitamin D receptor.

In our current study, we have linked VDR to excitotoxicity in neural stem cells. Neural stem cells are the most vulnerable cell population in neonatal brain injury. We have made a first step in understanding the role of VDR in neonatal brain injury by demonstrating that this receptor responds to stress. It can be argued that increases in VDR may be harmful to the cell but we have not done experiments to address these issues. The cells could be exhausting all potentially protective pathways before cell death. We can address both of these scenarios through genetic manipulation of the VDR in vitro and in vivo by experiments that involve RNA silencing.

The response of VDR in neuropathology is under investigated. Therefore, our study suggests that there may be a potential role in VDR in brain injury. Our preliminary findings suggest there is an increase in VDR in our model of hypoxia-ischemia. Using the unilateral carotid ligation we can tease out the role of hypoxia alone on the VDR and also do time-course experiments in these animals to further elucidate the role of VDR to see if it is an early or late responder.

Neural stem cells can also be labeled in brain slices similar to how we determined the presence of VDR on NeuN positive cells. Our study has opened up many doors of investigation that will

Immunohistochemistry

Non-Ligated (Right Hemisphere) Ligated (Left Hemisphere)

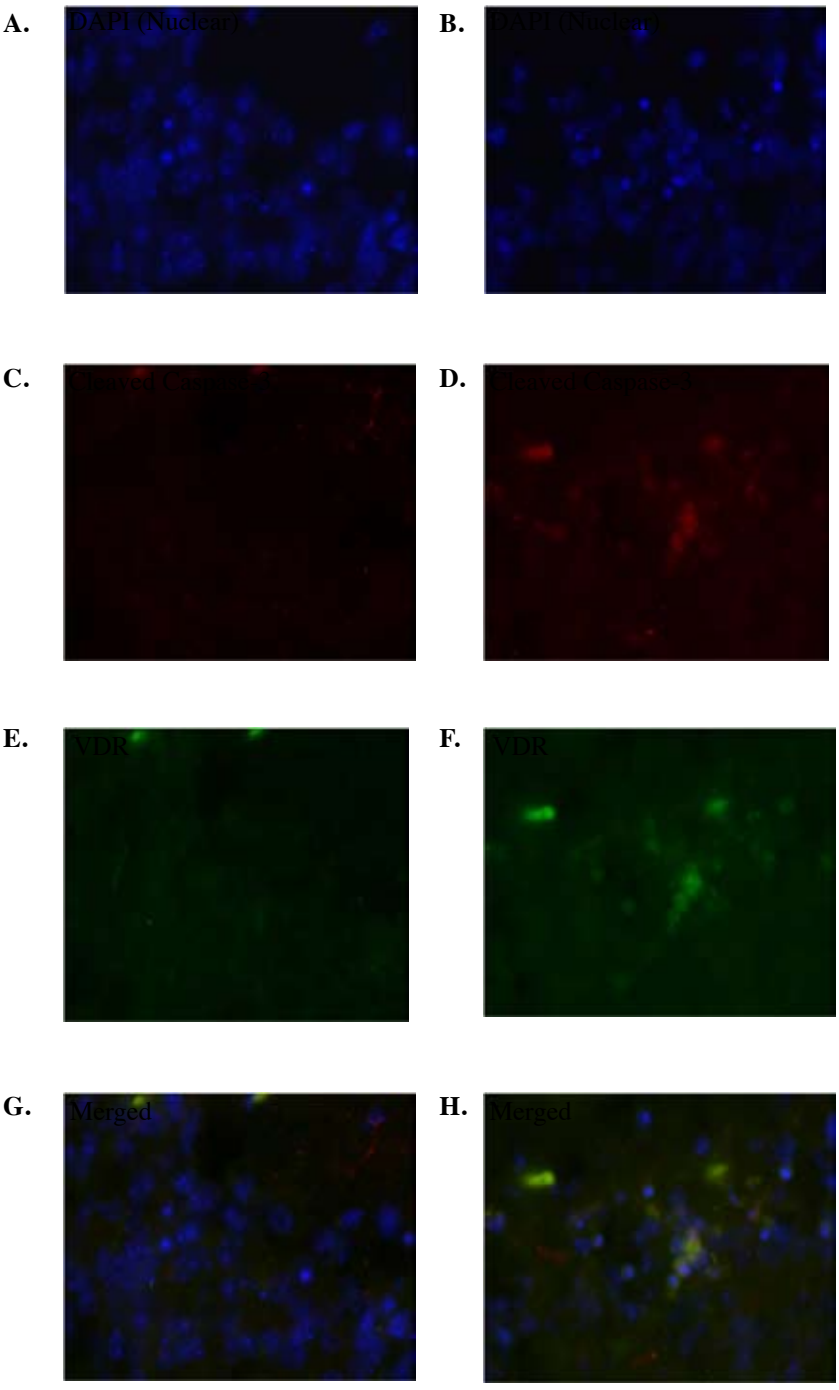


Figure 3. Immunohistochemistry analysis of neonatal mouse brain tissue in hypoxia-ischemia with effects from a unilateral carotid ligation (right column images) and no ligation effects (left column images)
(A) and (B) Nuclear-Dapi. (C) and (D) Cleaved Caspase-3-Cy3. (F) VDR-FTIC increases in ligated (left) hemisphere as compared to non-ligated (right) hemisphere of brain (E). Also, VDR co-localizes with Cleaved Caspase-3 in areas of brain injury (H).

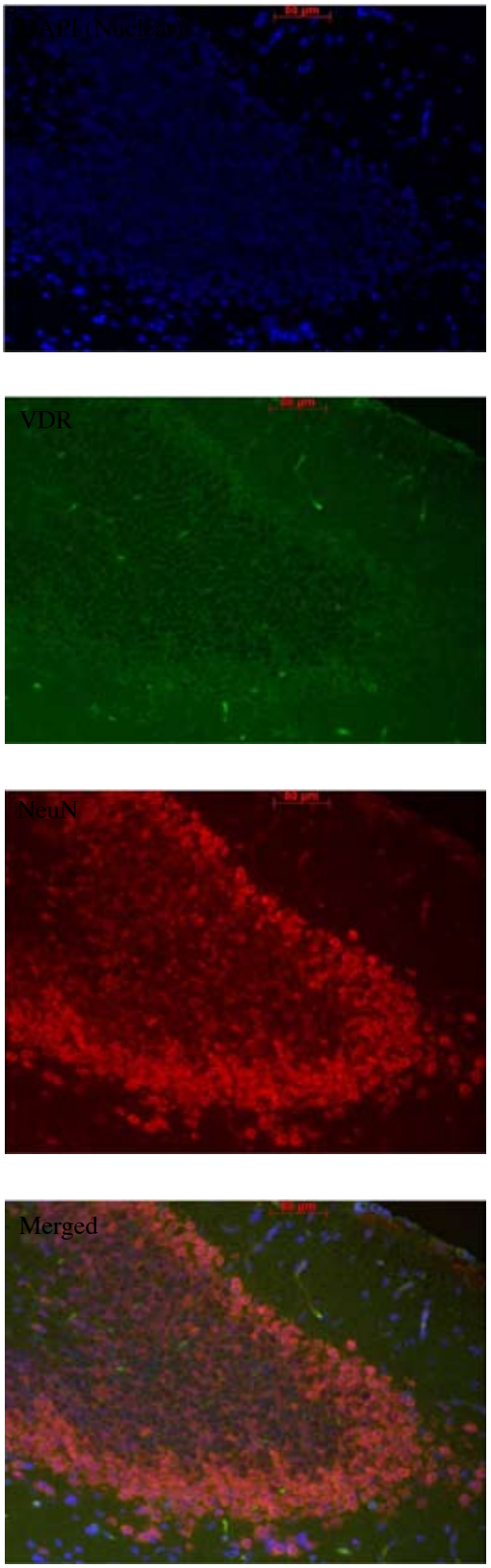


Figure 4. Immunohistochemistry of left hippocampus (ligated left common carotid) and Cell-specific VDR localization.(A)Nuclear-Dapi. VDR is expressed in neurons of the hippocampus, as indicated by NeuN- Cy3 (C), VDR- FITC (B), and merged (D).

require multiple experiments to further characterize VDR in a cell-specific fashion. We will propose to sort neural stem cells from control and injured brains to determine if there is a difference in viability. It will be important to determine if VDR has a detrimental or protective effect in these cells.

Conclusions

Based on the novel results of this investigation, we conclude that VDR may be directly involved in cell stress by responding to the conditions of excitotoxicity and hypoxia-ligation. Future studies must be conducted in order to understand cell and tissue response to these conditions in the presence of Vitamin D_3. Also, it is necessary to understand Vitamin D_3 mechanisms in damaged cell and tissue samples. Ultimately, after understanding the full neuroprotective potential of Vitamin D_3, drug therapy to animals and later to preterm infants may be completed, thus allowing for a preventative measure against brain injury in the neonate.

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