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SHORT COMMUNICATION

Prolong intermittent injection of human parathyroid hormone in rats modulates the expression of several proteins in the neuropil of the cerebellar cortex.

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Abstract

INTRODUCTION: The intermittent use of recombinant human parathyroid hormone (iPTH) alters calcium metabolism and induces osteogenesis in experimental models. However, the real effects of iPTH in excitable cells and neurons that require membrane receptors to undergo membrane depolarization/repolarization (Na$^+$K$^+$ATPase) to generate ATP, voltage-gated calcium channel (calcium-IP3R-calponin) as well as GABAergic (GABAA) signaling remains unclear.
OBJECTIVES: In this study, the expression of IP3R, Na$^+$K$^+$-ATPase, GABAA and calmodulin proteins were evaluated in histological sections of the cerebellum of rats following prolonged injection of iPTH.
METHODS: Twenty Wistar rats were used in this study and randomly assigned as either or control group. The test group were subcutaneously injected with 20 µg/kg of iPTH, 3×/week for 8 weeks, while the control group received 1 ml/kg of 0.9% saline solution. The rats were euthanized on the 60th day after the first administration, and their cerebellar vermis was removed and submitted to histological and immunohistochemical evaluation for detection of IP3R, Na$^+$K$^+$-ATPase, GABAA and calmodulin proteins. The expression of proteins was evaluated in the areas corresponding to the Purkinje cells as well as in neuropil of molecular layer of cerebellum. All results were transformed into a percentage for each area analyzed to verify significance between groups.
RESULTS: Rats that received iPTH demonstrated significant reduction of IP3R, calmodulin and GABAA in Purkinje cells and neuropil of molecular layer while the expression of Na$^+$K$^+$-ATPase was similar.
CONCLUSION: It was concluded that iPTH decreased the expression of IP3R and calmodulin while it did not alter the expression of Na$^+$K$^+$-ATPase. These changes insinuate the ionic activity of calcium and sodium/potassium. Yet, the iPTH alters GABAergic signaling in Purkinje cells, suggesting neurotransmission activity changes in the cerebellum.

Abbreviations:

iPTH	- Intermittent use of recombinant human parathyroid hormone
PTH	- Parathyroid hormone
Na+K+ATPase	- Sodium potassium pump Adenosine 5'-TriPhosphatase
ATP	- Adenosine TriPhosphate
IP3R	- Receptor for inositol 1,4,5-trisphosphate
GABA	- γ-aminobutyric acid
GABAergic	- neurons that produce gamma-Aminobutyric acid (GABA)
GABAA	- γ-Aminobutyric acid type A

INTRODUCTION

Supported by the premise that the intermittent use of recombinant human parathyroid hormone (iPTH) stimulates osteogenesis, several researchers have recommended iPTH for stimulating bone formation in areas that have suffered loss due to trauma, neoplasm, cysts, or even osteoporosis. Although this development is exciting for orthopedics and craniomaxillofacial surgery, its effect on tissues and organs is still uncertain, especially those tissues that share protein expression with bone and/or are susceptible to calcium variation (Giovanini *et al.* 2018). Since protein and gene expression may be altered together with changes to systemic cellular metabolism in this therapy, there is a distinct possibility that pathological effects may result (Smajilovic *et al.* 2010).

The suggestion that the use of iPTH in high doses may induce a pathological condition in excitable neuronal cells in fact is plausible, and the intersection of some issues supports this hypothesis. Firstly, there are reports in the literature that show that PTH receptors are expressed within the brain and cerebellum neuron as well as glia cells, consequently alters the intracellular changes in Ca^{2+} concentration $[Ca^{2+}]i$ in these cells. (Harvey & Hayer 1993). Concurrently, there is also evidence that the increase in the intracellular changes in $[Ca^{2+}]i$ leads to neurotoxicity (Silver & Erecinska 1990; Bondy 1989) and thus induce degeneration of cells in the brain (Hirasawa *et al.* 2000). Thus, It should also be taken in consideration that the maintenance and regulation of Ca^{2+} homeostasis are fundamental to cell development and the functioning of excitable cells, and neurons in particular (Ryglewski *et al.* 2007).

Neuronal calcium plays a dual role, working as both a charge carrier and as an intracellular messenger. Together with the Na^+K^+ATPase pump, calcium regulates neurotransmitter release and membrane excitability while calcium influx through calcium channels acts as a secondary messenger of electrical signals that regulate many physiological events (Prins & Michalak 2011). More specifically, intracellular calcium may trigger various signaling pathways that lead to alterations in GABAA receptors responsible for hyperpolarizing and most fast inhibitory responses (Gravielle 2021).

However, it is highlighted that each area of the central nervous system is complex and produces different responses depending on the anatomical area, stimulus and neurotransmitter (Fair *et al.* 2009). Specifically, the cerebellar cortex, possess a precise histological array with intrinsic cell types (Purkinje cells and neuron of molecular and granular layers) and well known afferent systems, that together allow for the integration system's organization makes it uniquely appropriate for neuronal understanding (Swain *et al.* 2011).

In this context, it is worth noting that calcium-dependent postsynaptic depolarization on cerebellar Purkinje cells of cerebellum is able to induce a long-lasting potentiation of GABAergic transmission at synapses formed by stellate cells. Apparently involved in motor learning, this is referred to as rebound potentiation and it is triggered by an increase in intracellular calcium resulting from calcium channel activation (Hirano & Kawaguchi 2014).

A key role in the control of Ca^{2+} demand may be attributed to the inositol 1,4,5-trisphosphate (IP_3) and IP_3 receptors (IP_3Rs) which are the main source of Ca^{2+} released from the endoplasmic reticulum that supplies intracellular calcium (Foskett & Daniel Mak 2010). Through the active IP3R mechanism, the basal levels of free cytoplasmic calcium in neurons are kept low and its concentration, as well as the action of IP3R, is orchestrated either by calcium ions or calmodulin (Prole & Taylor 2019; Woll & Van Petegem 2022).

Calmodulin is a small, acidic, heat-stable, monomeric protein that is considered to be a major Ca^{2+} -binding protein in the brain where it plays an important role in the neuronal response to changes in intracellular Ca^{2+} concentration. This protein modulates numerous Ca^{2+}-dependent enzymes, participates in cellular functions involved in neurotransmission, and acts on postsynaptic membrane modulation and synaptic vesicles (Swulius & Waxham 2008).

Since IP3R, calmodulin, Na^+K^+ATPase, and GABAA are expressed either osteoblast (Zayzafoon 2006; Takahata *et al.* 2011; Francis *et al.* 2002) or neuronal and glial cells (Sola *et al.* 2001; Gravielle 2021; Prins & Michalak 2011), the aim of this preliminary research was to evaluate the immunohistochemical expression of these proteins in Purkinje and stellate's cells of cerebellar cortex specimens treated with iPTH in a controlled study.

MATERIAL AND METHODS

Animals and Administration

Wistar rats, 5 to 6 months old, weighing 330–460 g, and with no previous disease were used in our study. All rats were maintained in a room at a controlled temperature (20-22°C) under a 12 h light–dark cycle.

The rats were arbitrarily assigned to either a control group (n = 10) or to a group that received the subcutaneous administration of 20 μg of recombinant

human PTH 1-34 (Forteo) (Indianapolis, Indiana, United States) (n = 10). The administration of iPTH was performed 3×/week (Washimi *et al.* 2010) during 8 weeks (Andreassen *et al.* 2004) until euthanasia on 60th day after the first administration. This time period included four days after the last application of iPTH, to prevent immediate effects or even rebound effects of iPTH application.

Euthanasia and Material Collection

The rats were euthanized in a chamber through exposure to CO_2 until they ceased moving. Immediately afterwards, an inverted cone bur was used to cranial osteotomy and consequently for access to brain tissue. All brain tissue was removed using a spoon-shaped curette. The cerebellum was carefully removed, and a representative sample of cerebellar vermis was collected. Each necropsied sample of vermis was fixed in 10% buffered formalin for 48 h. Afterwards, hemisectional pieces were surgically removed transversely to obtain the granule, Purkinje, and molecular layers of cerebellar cortex together with subcortical white matter fragments. The samples were dehydrated, cleared in xylene, and embedded in paraffin.

Immunohistochemical Procedure

The serial sections of 3-μm samples were prepared and mounted on slides and the paraffin was removed in xylene. The sections were re-hydrated in consecutive ethanol washes (100%, 95%, 70% GL). Antigenic retrieval was performed in phosphate-citrate buffer (pH 6.0, Sigma-Aldrich, MO, USA) for 15 min (3× cycle of 5 min each in a microwave at high power), and non-specific binding was blocked via incubation with a 4% non-fat milk solution for 30 min. Mounted on slides, the samples were incubated for 18 h at 4°C with primary antibodies anti-IP3R antibody (1.0 mg/ml, Abcam, Cambridge, UK, ab-5804, with a dilution factor of 1:1000), anti-calmodulin-1,2,3 antibody (1.0 mg/ml, Abcam, Cambridge, UK, ab-45689, with a dilution factor of 1:1100), anti-Na^+K^+ATPase antibody (Santa Cruz, USA, sc-16043, with a dilution factor of 1:200), and anti-GABAA Rα1-6 antibody (Santa Cruz, USA, sc-376282, with a dilution factor of 1:50). Afterwards, sections were incubated in a second antibody kit containing an alkaline phosphatase substrate for 40 min at room temperature and then revealed with a Fast Red chromogenic substrate for 15 min. Each incubation step was preceded by a 5 min wash with PBS. The sections were counterstained with Harris's hematoxylin. Each immunohistochemical procedure was performed three times. A negative control was produced using the same method, but with 0.01 M Phosphate-buffered saline pH 7.4 as the primary antibody.

Image Analysis

The images of immunohistochemistry sections were captured by a digital camera (Samsung, South Korea) at a light microscope with an original magnification of ×200. The area established was localized in transitional area between the molecular and granular layer, where the Purkinje cells and cell bodies of stellated cells on the molecular layer were evident. Each captured micrograph was transferred to an imaging program Adobe Photoshop (Apple Mac. Inc.). From the original images, an area of 4 mm^2 was carefully traced and cut out. An image of 1-mm slide micrometer was used to calibrate all measurements. The areas of interest were established from a region where there were a higher number of positive cells for both Purkinje cells and for neuron bodies of the molecular layer (stellate cells). All immunohistochemical measurements were performed using the software ImageJ (https://imagej.nih.gov/ij). The immunoexpression for calmodulin, IP3R, anti-GABAA Rα1-6, and Na^+K^+ATPase of each protein in Purkinje cells was considered positive independent of its expression in membrane or cytoplasm. All cells (positive and negative) obtained through micrograph were manually counted and tagged. The percentage of Purkinje cells was obtained by simple ratio between positive cells and the total number of cells counted in the established area.

In the molecular layer, the presence of proteins in the stellated cells and neuropil were accounted together. A perimeter of positive neuropil as well as stellate cells identified as a red color was carefully delineated, and positive area were computed together. After data tabulation, all data were transformed into a percentage (positive area/ total area of 4 mm^2) in order to homogenize interpretation of the results.

Statistical Analysis

Data was expressed as median and maximum/ minimum values. The nonparametric Mann-Whitney test was performed to compare the differences between groups followed by Student–Newman–Keuls test. A value of $p \leq 0.05$ was considered statistically significant. All analyses were performed using the statistical software Statistical Package for Social Science (SPSS; version 20.0; SPSS Inc. Chicago, IL, USA).

RESULTS

The present study demonstrated that prolonged administration of iPTH significantly decreased the expression of IP3R, calmodulin, and GABAA while it did not alter the expression of Na^+K^+ATPase. A brief description of the histological features found among groups was done, while the histomorphometric data were summarized in Fig.1.

IP3R

A lower expression of IP3R was found in iPTH group when compared with the control group. The expression of IP3R was detected in Purkinje cells (Fig.1A), as well as cells that compounded either molecular layer or granular layer. Yet, it was notorious the exuberant expression of IP3R on neuropil of cerebellum cortex (Fig.1B). In

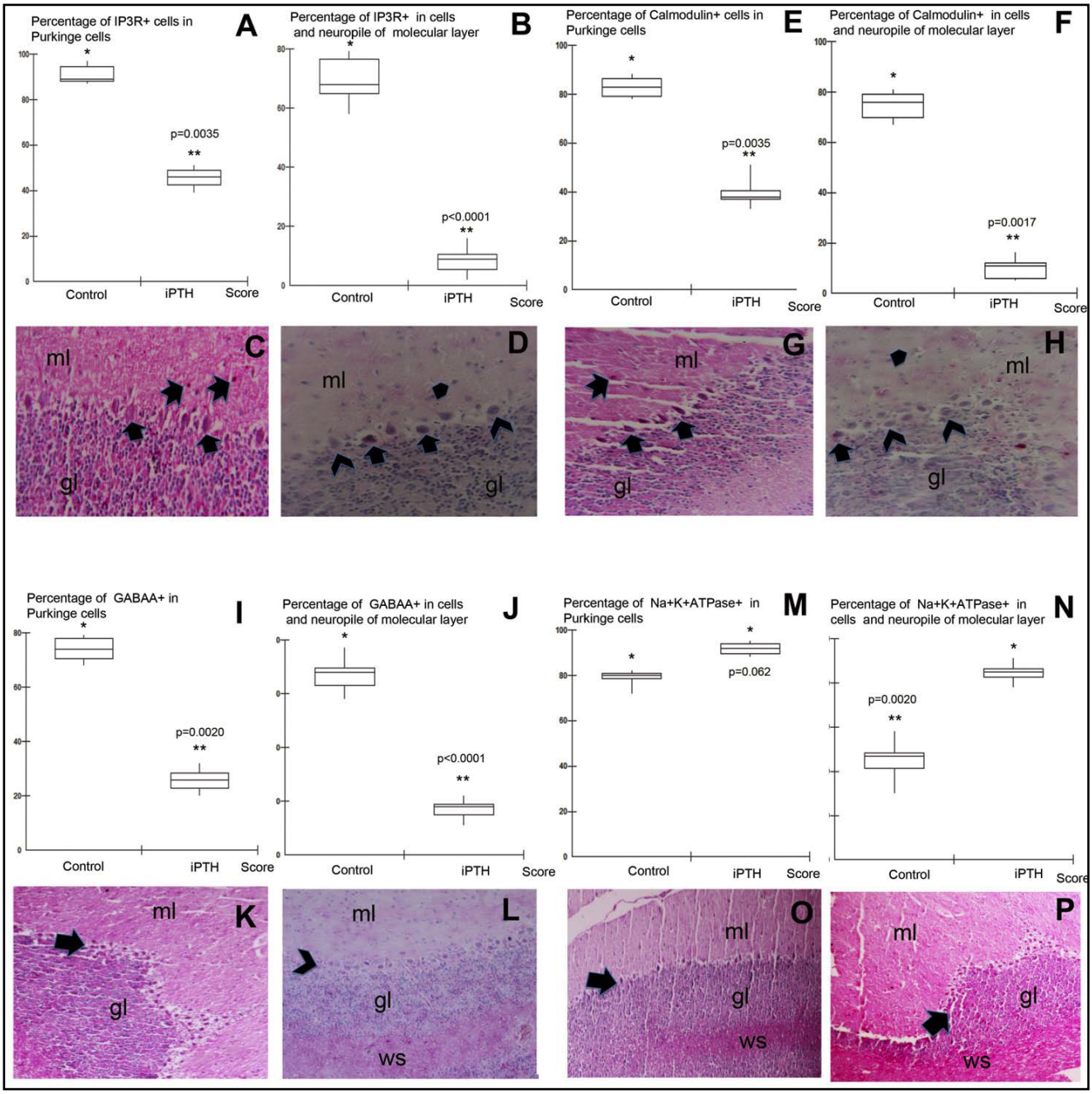

Fig. 1. Figure A and B demonstrate Box plot diagram revealing the median, maximum, and minimum values of IP3R expression on Purkinje cells and molecular layer, respectively. Micrograph C shows the histological frame demonstrating expression of IP3R in control group. Verify the intense expression of immunopositivity both in Purkinje cells (arrows) and stellate cells of molecular layer (notched arrow), while micrograph D shows loss of immunopositivity for Purkinje cells (Chevron arrow) and stellate cells (pentagon). Figure E and F reveal Box plot diagram exhibiting the median, maximum, and minimum values for calmodulin expression respectively on Purkinje cells and molecular layer. Micrograph G demonstrates the expression of calmodulin in control showing expression of immunopositivity for Purkinje cells (arrows) and stellate cells of molecular layer (notched arrow). Micrograph H shows evident lack of expression of calmodulin for Purkinje cells (Chevron arrow) and stellate cells (pentagon). Figure I and J show Box plot diagram revealing the median, maximum, and minimum values of GABAA expression respectively on Purkinje cells and molecular layer of cerebellar cortex. Micrograph K shows the expression of GABAA in control group. Verify the expression of GABAA in Purkinje cells (arrows) and on cells and neuropil of molecular layer. Differently micrograph shows suppression of immunopositivity for GABAA in Purkinje cells (Chevron arrow) and molecular layer. Similarly, micrographs M and N reveal Na^+K^+ATPase respectively for Purkinje cells and molecular layer. Differently, there lower expression of protein in control group (Micrograph O) specially in white mater of cerebellum, and molecular layer when compared to specimens that received iPTH, while in Purkinje cells the expression of Na^+K^+ATPase was similar (arrows). (Micrographs C, D, G, and H original magnification 200×, fast red immunohistochemical technique; Micrographs K, L, O and P original magnification 100×, fast red immunohistochemical technique; **iPTH** = intermittent use of recombinant human parathyroid hormone; **IP3R** = Receptor for inositol 1,4,5-trisphosphate, **GABAA** = γ-Aminobutyric acid type A, Na^+K^+ATPase = sodium potassium pump Adenosine 5'-TriPhosphatase; ml= molecular layer; gl= granular layer; wm = white mater).

group that received iPTH, scarce Purkinje cells exhibited expression for IP3R, while distribution for this protein in neuropil always occurred randomly and in scarce form. The expression of IP3R in cells that compounded either molecular layer or granular layer was suppressed.

CALMODULIN

Similarly to IP3R, the expression of calmodulin in iPTH group was significantly lower than the control group (Fig. 1E and 1F). The expression of calmodulin was exuberant in Purkinje cells, molecular layer, granular layer as well neuropil of cerebellum cortex. In contrast, the expressions of calmodulin in the cortex and the white matter were scarce in group that received iPTH.

GABAA

The expression of GABAA also was significantly lower in iPTH group than the control group (Fig. 1 I and J). The protein was exuberant in Purkinje cells, and molecular layer, while granular layer and its neuropil the expression of GABAA was scarce. In contrast, the expression of calmoduin was occasional in Purkinje cells and molecular layer, while in granular layer the expression of GABAA was inhibited.

Na+K+ATPase

Although the expression of Na^+K^+ATPase looked to be more intense in the Purkinje cells of iPTH group, the difference was not statistically significant than the control group (Fig. 1M). However, the expression Na^+K^+ATPase in the neuropil of molecular layer of cerebellar cortex in the iPTH group was higher than the control group (Fig. 1N).

DISCUSSION

Granule cells are the most abundant neuronal type in the central nervous system, and its axons ascend toward the pial surface. It has a T-shaped bifurcation where each Purkinje cell receive input from a single climbing fiber, generating postsynaptic depolarization and giving rise to a brief train of action potentials which together constitute the "complex spike" that is associated with a surge in Ca^{2+} concentration (Hoxha *et al.* 2016).

It is noteworthy that both Na^+K^+ATPase and IP3R are known to play an important role in regulating intracellular calcium. While Na^+K^+ATPase regulates calcium entry through the Na^+/Ca^{++} exchanger by altering intracellular Na^+ concentration (Tian & Xie 2008), IP3R is responsible for the movement of calcium either from the extracellular to the intracellular microenvironment, or for the intracellular influx through intramembranous calcium reserves, especially from endoplasmic reticulum calcium stock (Foskett 2010).

It should be highlighted that when stoichiometric levels of intracellular calcium ions are high, excess calcium is transferred to cell organelles (mitochondria and endoplasmic reticula) through IP3R channels, maintaining optimal low calcium levels (Foskett & Daniel Mak 2010). This biological effect is coordinated by the calcium-dependent protein calomdulin that orchestrates this physiological signal, and is also involved in neurotransmission where, in Purkinje cells, it stimulates the GABBA channel which induces inhibitory neurotransmission for the stellate cells in the molecular layer (Swulius & Waxham 2008). In our study it was verified on control group that the IP3R, CaM, Na^+K^+ATPase and GABAA are strongly expressed in Purkinje cells, while Na^+K^+ATPase is weakly to moderately present in the neuropil of the molecular layer of the cerebellar cortex (Fig. 1).

It is noteworthy that suppression or dysfunctional alterations of GABAA or IP3R occur in numerous disorders such as epilepsy, anxiety, depression, autism, schizophrenia, as well as hyperexcitability (Gravielle 2021). Herein, we demonstrated that the iPTH induced lack of expression of GABAA, IP3R, and calmodulin on Purkinje cells. At the same time the specimens that received iPTH revealed intense expression of Na^+K^+ATPase, although it does not demonstrate statistical significance, it was slightly higher in cerebellar cortex when compared to control group. Thus, it may be inferred herein that iPTH may induces cerebellar hyperexcitability and consequently a neuropathological phenomenon (Sola *et al.* 2001).

Either the loss of expression of GABAA or of IP3R channels seems to be associated with the action of iPTH. Similar to our observation, Hong *et al.* described a close relationship between suppression of the GABA receptor while PTH is high in parathyroid adenomas (Hong *et al.* 2016). Analogously, Khudaverdyan and Ter-Markosyan (Khudaverdyan & Ter-Markosyan 2000) also described a decrease in binding sites and GABAA expression in neuronal ganglia when subjected to the action of parathyroid hormone or even due hypercalcemia caused by this hormonal action. According to the authors, these data suggest that not only the PTH may modulate the action and function of neurons, but also may induce a neuroprotective effects, minimizing and protecting neuronal tissues from inhibition.

However, it is noteworthy that Purkinje cells are rich in GABA and have a natural inhibitory effect in cerebellum. Thus, this peculiar biological situation seems to induce an influence divergent of the cerebral cortex, promoting a cerebellar hyperexcitation. This biological event may be inferred in the present since we detected an intense expression of Na^+K^+ATPase either in white mater or on neuropil that comprise the molecular layer of the cerebellar cortex.

Simultaneously, there is important evidence that iPTH increases intracellular calcium levels as well as cAMP signaling, and that together these effects induce a phosphatidylinositol hydrolysis, condition that suppresses IP3R and inhibits its immunohistochemical expression (Wheeler *et al.* 2008), explaining the effect of the decrease in IP3R in the present study.

Interestingly, there was little expression of calmodulin in specimens that received iPTH. Unlike what occurs with receptor channel protein, the suppression of calmodulin does not necessarily denote loss of protein, however, this seems to be a data point that supports the hypothesis of hypercalcemia as associated with neuronal hyperexcitability while also contributing to loss of IP3R.

In order to explain this inference, Clapham (Clapham 2007) notes that in hypercalcemia a large amount of Ca^{2+} binds to calmodulin and modifies it structurally by forming a Ca^{++}/calmodulin complex, a condition which mimics reduction in protein expression. According to the author, this amplifies the signal generated by Ca^{2+} which activates phosphorylation pathways and induces a protein kinase dependent on Ca^{2+}/calmodulin that may in turn inhibit the basal and calmodulin-stimulated activities of the Ca^{2+} pump through phosphorylation in Ser^{45} in the N-terminal regulatory domain as well as reduce IP3R expression.

Despite this study´s small sample group and the omission of measuring the quantities of each protein, it was concluded that iPTH reduced the expression of IP3R and calmodulin while it did not alter the expression of $Na^{+}K^{+}$-ATPase. Such changes insinuate the ionic activity of calcium and sodium/potassium. Yet, the iPTH altered GABAergic signaling in Purkinje cells, suggesting neurotransmission activity changes in the cerebellum.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no competing conflict of interest interests.

Ethical approval from the institutional Animal Care Committee was obtained (protocol #296-2016). All applicable international and institutional Animal Care Committee guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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