Biochimica et Biophysica Acta 1823 (2012) 1708–1719

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

1α,25-Dihydroxyvitamin D_3 mechanism of action: Modulation of L-type calcium channels leading to calcium uptake and intermediate filament phosphorylation in cerebral cortex of young rats

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ARTICLE INFO

Article history:
Received 14 December 2011
Received in revised form 15 June 2012
Accepted 18 June 2012
Available online 25 June 2012

Keywords:
1α,25-Dihydroxyvitamin D_3
Cerebral cortex
Calcium
Cytoskeleton
Protein kinase
VDRm

ABSTRACT

The involvement of calcium-mediated signaling pathways in the mechanism of action of 1α,25-dihydroxyvitamin D_3 (1,25D) is currently demonstrated. In this study we found that 1,25D induces nongenomic effects mediated by membrane vitamin D receptor (VDRm) by modulating intermediate filament (IF) phosphorylation and calcium uptake through L-type voltage-dependent calcium channels (L-VDCC) in cerebral cortex of 10 day-old rats. Results showed that the mechanism of action of 1,25D involves intra- and extracellular calcium levels, as well as the modulation of chloride and potassium channels. The effects of L-VDCCs on membrane voltage occur over a broad potential range and could involve depolarizing or hyperpolarizing coupling modes, supporting a cross-talk among Ca^{2+} uptake and potassium and chloride channels. Also, the Na^+/K^+-ATPase inactivation by ouabain mimicked the 1,25D action on $^{45}Ca^{2+}$ uptake. The Na^+/K^+-ATPase inhibition observed herein might lead to intracellular Na^+ accumulation with subsequent L-VDCC opening and consequently increased $^{45}Ca^{2+}$ (calcium, isotope of mass 45) uptake. Moreover, the 1,25D effect is dependent on the activation of the following protein kinases: cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase (PKCaMII), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase p38 ($p38^{MAPK}$). The modulation of calcium entry into neural cells by the 1,25D we are highlighting, might take a role in the regulation of a plethora of intracellular processes. Considering that vitamin D deficiency can lead to brain illness, 1,25D may be a possible candidate to be used, at least as an adjuvant, in the pharmacological therapy of neuropathological conditions.

1. Introduction

1α,25-Dihydroxyvitamin D_3 (1,25D), the bioactive metabolite of vitamin D_3, plays an essential role in the regulation of calcium and bone homeostasis [1]. In addition to these classic functions, vitamin D_3 has been shown to influence a variety of other systems such as mammary glands, reproductive organs, osteoblasts and the nervous system [2–9]. The effects of 1,25D are mediated by its interaction with a nuclear vitamin D receptor (VDRn), a member of the nuclear receptor superfamily of ligand-activated transcription factors [10]. This interaction leads to either upregulation or downregulation of gene expression. On the other hand, it has been reported that 1,25D acts also through a plasma membrane-associated receptor (VDRm) which modulates a complex signaling system involving rapid formation of second messengers (Ca^{2+}, cyclic AMP, inositol), activation of protein kinases (cAMP-dependent protein kinase—PKA; mitogen-activated protein kinase—MAPK) and the rapid opening of Ca^{2+} and Cl^- channels [6,7,11–14]. The VDRm has been shown to be localized in caveolae-enriched plasma membranes [15,16]. However, there is a current debate on the identity of the receptor(s) involved in the rapid nongenomic effect of vitamin D. There are two main candidates for the membrane receptor for 1,25D: the MARRS (membrane associated, rapid response, steroid-binding) protein and also new evidence indicates that the VDRn could also act as a membrane receptor [17–20].

The VDR is widely distributed in the brain, including neurons and glial cells and is associated to many different functions [21,22]. Previous studies have demonstrated that the VDR is expressed in both developing [23] and adult rat brain [24,25], as well as in human brain

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0167-4889
doi:10.1016/j.bbamcr.2012.06.023

[26,27]. Regarding its functions, several studies have postulated that 1,25D modulates the activity of brain genes and enzymes of neurotransmitter metabolism, and is also involved in neuroprotection and brain development [3,4,28,29].

The cytoskeleton of the neural cells comprises a protein network composed by microtubules, microfilaments and intermediate filaments (IFs). Neurofilaments are heteropolymers that are expressed almost exclusively in neurons. They are composed of three different polypeptides commonly referred to as heavy (NFH), medium (NFM), and light (NFL) NF subunits [30]. Under normal conditions, the three NF subunits coassemble stoichiometrically into filamentous structures playing a role in maintaining axonal caliber [31] and axonal transport [32]. Vimentin and glial fibrillary acidic protein (GFAP) are the IFs found in astrocytes [30,33]. GFAP is the IF of mature astrocytes [34], while vimentin is the IF of mesenchymal origin. The phosphorylation of IF proteins is a dynamic process controlling their physiological role in response to extracellular signals. The importance of GFAP and NF subunits on neural function is evident from the fact that perturbation of their structure accounts for several genetically determined protein misfolding/aggregation diseases [35]. Also, perikaryal accumulations/aggregations of NF proteins have been correlated with aberrantly phosphorylated NF in several neurodegenerative diseases, such as Alzheimer's disease, motor neuron diseases, and Parkinson's disease [36–38]. Also, the misregulation of the IF-associated phosphorylation system is assumed to have an important role in neurodegeneration.

Our research group has previously demonstrated that the phosphorylating system associated with the IF proteins is responsive to hormonal signals [5,39–43]. These actions can be initiated by the activation of L-type voltage-dependent Ca^{2+} channels (L-VDCC) and the signal is transduced downstream of Ca^{2+} uploading through different kinase pathways, regulating the dynamics of the cytoskeleton [5,41]. The phosphorylating system associated with the IF proteins has been previously shown to be targeted by 1,25D in the male reproductive system. However, the intracellular pathways involved in 1,25D actions targeting the neural cytoskeleton and the involvement of Ca^{2+} in such actions remain to be identified.

Calcium signaling plays a central role in neuronal cell development. Accordingly, the VDCCs are highly expressed during development and their functions are critical for developing neurons [44], as well as for synaptogenesis [45,46]. One of the ways in which Ca^{2+} channels influence neuronal activities is via signaling pathways that control gene expression and that involve transcription factors such as cyclic AMP response element-binding protein (CREB) [47,48]. Also, the critical role of VDCCs regulating the secretion of neurotransmitters must be considered [49].

It has been shown that the steroid hormone 1,25D has modulatory effects on cytoskeleton, neurotransmission, antioxidant system, as well as Ca^{2+} and Cl^- channel activities in a variety of cell types [5,50–52]. Taking into account these findings in the present study we explore the mechanisms involved in the rapid effect of 1,25D on IF phosphorylation and on $^{45}Ca^{2+}$ uptake in slices of cerebral cortex from immature male rats in an attempt to better understand the relevance of this hormone to the brain function.

2. Materials and methods

2.1. Chemicals

1α,25-Dihydroxyvitamin D_3 (1,25D), nifedipine, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89), (bisindoylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)maleimide methanesulfonate salt) Ro 31–8220, (*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl) imidazole) SB 239063,(2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) PD 98059, ouabain, theophylline, dibutyryl cAMP (di-BucAMP), 9-anthracene carboxylic acid (9-AC), flunarizine, dantrolene sodium salt, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), tolbutamide, apamine, N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl] phenyl]-N-(2-hydroxyethyl)-4 methoxybenzenesulfonamide phosphate salt, N-[2-[[[3-(4′-chlorophenyl)-2-propenyl] methylamino] methyl]phenyl]-N-(2-hydroxyethyl)-4′ methoxybenzenesulfonamide phosphate salt (KN93), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY 294002), cycloheximide, acrylamide and bis-acrylamide were purchased from Sigma Chemical Company (St. Louis, MO, USA). [^{32}P]Na_2HPO_4 that was purchased from the Brazilian Agency of Nuclear Energy (Comissão Nacional de Energia Nuclear—CNEN), São Paulo, Brazil, was from Amersham International (UK). [^{45}Ca]$CaCl_2$ (sp. act. 321 kBq/mg Ca^{2+}), and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Boston, USA). Anti-p44/42 MAP kinase (anti-ERK1/2), anti-phospho-p44/42 MAP kinase (anti-phospho ERK1/2), anti $p38^{MAPK}$ and anti-phospho $p38^{MAPK}$ antibodies were from Cell Signaling Technology, Inc. (USA). The anti-VDR (sc-1008) and anti-caveolin-1 (7C8) (sc-53564) antibodies were obtained from Santa-Cruz. The mouse anti-NeuN antibody was obtained from Chemicon International (São Paulo/SP, Brazil). The second antibody goat anti-rabbit IgG coupled to Alexa Fluor® 594, goat anti-mouse IgG coupled to Alexa Fluor® 430, ProLong® Gold Antifade Reagent and DAPI were from Molecular probes (Invitrogen). The Immobilon™ Western chemiluminescent HRP substrate was obtained from Millipore. All other chemicals were of analytical grade.

2.2. Animals

Wistar rats were bred in an animal house and maintained in an air-conditioned room (about 21 °C) with controlled lighting (12 h/12 h light/dark cycle). The suckling rats were kept with their mothers until euthanasia. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals' procedures were carried out in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00179).

2.3. Preparation of cerebral cortex slices

Ten day-old male rats were killed by decapitation, and the cerebral cortex was dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a Mcllwain chopper.

2.4. ^{32}P in vitro incorporation

2.4.1. Preincubation and ^{32}P labeling

Slices of cerebral cortex from 10 day-old male rats were initially preincubated at 30 °C for 20 min in a Krebs–HEPES medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin in the presence or absence of 10 μM H89 [40,43,53–55], 30 μM PD 98059 [43]; 10 μM nifedipine [43,56,57], 50 μM BAPTA-AM [43,58,59], 0.35 mM cycloheximide [39,43] or 1 μM Ro 31–8220 [6,60], when indicated. After preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate with or without addition of H89, BAPTA-AM, PD98059, Ro 31–8220, nifedipine or cycloheximide in the presence or absence of 1,25D when indicated. The incubation of tissue slices with 80 μCi of ^{32}P-orthophosphate supplies the label for mitochondrial production of γ^{32}P-ATP used for endogenous kinases. The excess of radioactive orthophosphate assures the maximal production of labeled ATP. This experimental approach has been developed by our

research group [61] and has been largely used in quantitative studies of the *in vitro* ^{32}P incorporation into cytoskeletal proteins in different tissues and experimental models [5,39–43,62–64]. The labeling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 ml of ice-cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris–HCl, pH 6.5), and the protease inhibitors described above. Slices were then washed twice with stop buffer to remove excess radioactivity and maintained on ice at 4 °C.

2.4.2. Preparation of the high salt-Triton insoluble cytoskeletal fraction

After the labeling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15,800×*g* for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched pellet, containing NF subunits, vimentin and GFAP, was dissolved in 1% SDS and protein concentration was determined.

2.4.3. Polyacrylamide gel electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli [65]. After drying, the gels were exposed to X-ray films (X-Omat XK1) at −70 °C with intensifying screens and finally the autoradiograph was obtained.

2.5. Western blot analysis

In some experiments tissue slices were treated with 1,25D during 5, 10, 15 and/or 30 min, then total tissue extracts were processed to Western blot analysis. The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4 °C in blocking solution containing the following monoclonal antibodies designated to study the MAPK cascade: anti-ERK1/2, anti-phospho ERK1/2, anti $p38^{MAPK}$ and anti-phospho $p38^{MAPK}$ antibodies (diluted 1:2000). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase conjugated anti-rabbit IgG 1:1000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using Immobilon™ Western chemiluminescent HRP substrate.

2.6. Quantitation of Western blot and ^{32}P incorporation into IF proteins

The radioactivity incorporated into cytoskeletal proteins as well as the immunoblots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and by determining optical densities for the band corresponding to each protein. Control values were then normalized to 100% and the percentage changes in the test samples were calculated. Protein loading was controlled by Coomassie blue staining the gels.

2.7. $^{45}Ca^{2+}$ uptake

Slices of cerebral cortex from 10 day-old male rats were preincubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 37 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). After that, the medium was changed by fresh KRb with 0.1 μCi/ml $^{45}Ca^{2+}$ during 60 min. Finally, 1,25D was included in this KRb containing $^{45}Ca^{2+}$ and the tissues were incubated either in the absence (control) or presence of 1,25D (10^{-13}, 10^{-12}, 10^{-10}, 10^{-9}, and 10^{-7} M) for 5 min. In some experiments channel blockers or kinase inhibitors were added during the last 20 min before hormone addition and maintained during all the incubation period (see figures). The following drugs were used: nifedipine (10 μM), flunarizine (1 μM), 9-AC (1 μM), BAPTA-AM (50 μM), dantrolene (50 μM), tolbutamide (100 μM), ouabain (1 μM), H89 (10 μM), theophylline (50 μM), di-BucAMP (500 μM), Ro 31–8220 (20 μM), LY 294002 (10 μM), SB 239063 (10 μM), PD 98059 (10 μM), and KN93 (10 μM) [43,52,66–69].

Extracellular $^{45}Ca^{2+}$ from the cortical slices was thoroughly washed off in a washing solution containing 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, 10 mM $LaCl_3$, pH 7.3 (30 min in washing solution). The presence of La^{3+} during the washing stage was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$ [70]. After washing, tissue slices were homogenized with 0.5 M NaOH solution, 100 μl aliquots were placed in scintillation fluid and counted in a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA) [52,66,69], and 5 μl aliquots were used for protein quantification as described by Lowry and colleagues [71].

2.8. VDR and caveolin-1 immunohistochemistry

Control animals were anesthetized with xylazine (0.1 ml/100 g body weight, i.m.) and ketamine (0.1 ml/100 g body weight, i.m.) and perfused with phosphate buffered saline (PBS) with heparin (50 ml in the 15-day-old rats) followed by 4% paraformaldehyde diluted in 0.1 M phosphate buffer (pH 7.4) at 4 °C of the same flow rate and total amount. The perfusion rate was approximately 100 ml. After perfusion, the brain was extracted from the skull, weighed, and placed in the same fixing solution for 72 h. After fixation, the brain was washed for 1 h in running water, dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 95% and absolute ethanol) and cleared with xylol. Brains were then embedded in paraplastic resin (Histosec; Merk). Coronal serial sections (3 μm thickness) were obtained with a microtome and serially collected on glued slides. Brain slides were deparaffinized in toluene, rehydrated in ethanol and rinsed in tap water. After this step, sections were placed in 0.1 M sodium citrate and citric acid buffer, heated for 20 min in a 350 W microwave oven, allowed to cool for 60 min, and rinsed in tap water and in PBS three times. Sections then were contoured with Dakopen and nonspecific binding sites were blocked with 3% PBS-BSA for 60 min at room temperature. Thereafter sections were incubated overnight with rabbit anti-VDR polyclonal antibody, mouse anti-caveolin-1 (1:500 in 3% PBS-BSA) and/or mouse anti-NeuN (1:1000 in 3% PBS-BSA) at 4 °C. After thorough rinsing, all sections were incubated with goat anti-mouse IgG conjugated to Alexa Fluor® 430 (1:500) and/or goat anti-rabbit IgG conjugated Alexa Fluor® 594 (1:500) for 45 min at room temperature and washed with PBS. Nucleus were stained with DAPI (0.25 μg/ml) and covered with ProLong® Gold Antifade Reagent and coverslips. Negative controls were performed without primary antibody. Images were captured with a fluorescence microscope (Olympus, BX-41) equipped with a digital camera (3.3 Mpixel QCOLOR3C, Qimaging™) and image acquisition software (Qcapture Pro 5.1, Qimaging™).

2.9. Statistical analysis

The results are means ± S.E.M. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by a Bonferroni multiple comparison test. Differences were considered to be significant when $p<0.05$.

3. Results

3.1. Glial and neuronal IF proteins as a target of membrane-initiated actions of 1,25D

Slices of cerebral cortex were exposed to different concentrations of 1,25D and the *in vitro* phosphorylation of IF-enriched cytoskeletal fraction was investigated. Results showed that 1,25D exposure for 30 min increased the IF phosphorylation at doses ranging from 10^{-10} to 10^{-7} M (Fig. 1). The following experiments were carried out by using 10^{-10} M 1,25D. The hormone-induced IF phosphorylation was totally independent on protein synthesis, as attested by using 0.35 mM cycloheximide, a potent inhibitor of protein synthesis (Fig. 2), therefore, supporting a nongenomic action of 1,25D directed to the IF-associated phosphorylating system.

3.2. Involvement of PKA, PKC, PKCaMII and MAPK on 1,25D-induced IF phosphorylation

The contribution of different protein kinases known to target the IF proteins [5] was investigated. Interestingly, the effect of 1,25D (100 pM) was unaffected by the presence of either protein kinase C (PKC) or MAP kinase kinase (MEK) inhibitors (Ro 31–8220 and PD 98059, respectively) (Fig. 3A). However, 10 μM H89 (PKA inhibitor) or 10 μM KN93 (PKCaMII inhibitor) totally prevented the 1,25D-induced hyperphosphorylation (Fig. 3B). Altogether, these findings demonstrate a role for PKA and PKCaMII in the mechanism of the hormonal action on the homeostasis of the phosphorylating system targeting the cytoskeleton.

3.3. Involvement of calcium on 1,25D-induced IF phosphorylation

The contribution of calcium signaling to the 1,25D-triggered events targeting the cytoskeleton was verified by using 10 μM nifedipine (L-VDCC blocker) and 50 μM BAPTA-AM (cell permeable calcium quelator). Results showed that co-incubation of 1,25D with each one of these drugs was able to prevent the hormone-stimulated IF-phosphorylation, evidencing a calcium-dependent mechanism (Fig. 4). In order to clarify these findings, the mechanisms involved in 1,25D-mediated Ca^{2+} entrance were investigated.

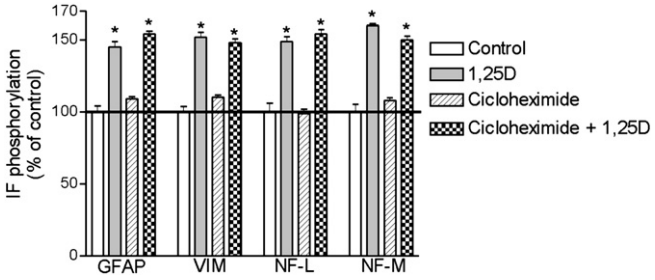

Fig. 2. IF phosphorylation induced by 1,25D is independent of protein synthesis. Slices of cerebral cortex were pre-incubated in the presence or absence of 0.35 mM cycloheximide for 45 min and incubated with ^{32}P-orthophosphate with or without (100 pM) 1,25D during 30 min, as described in Material and methods. The cytoskeletal fraction was extracted, and analyzed by SDS-PAGE and the radioactivity that was incorporated into GFAP, vimentin, NF-L and NF-M was measured by determining the optical density values for the band corresponding to each protein. Data are reported as means ± SEM of 10 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: $^{*}P<0.01$.

3.4. Involvement of VDCC and intracellular calcium in the stimulatory effect of 1,25D on $^{45}Ca^{2+}$ uptake

As depicted in Fig. 5, exposure to the secosteroid hormone at 10^{-10} M for 5 min was able to increase the $^{45}Ca^{2+}$ uptake.

In order to verify the involvement of L- and T-type VDCCs on the stimulatory effect of 1,25D (10^{-10} M) on $^{45}Ca^{2+}$ uptake, nifedipine

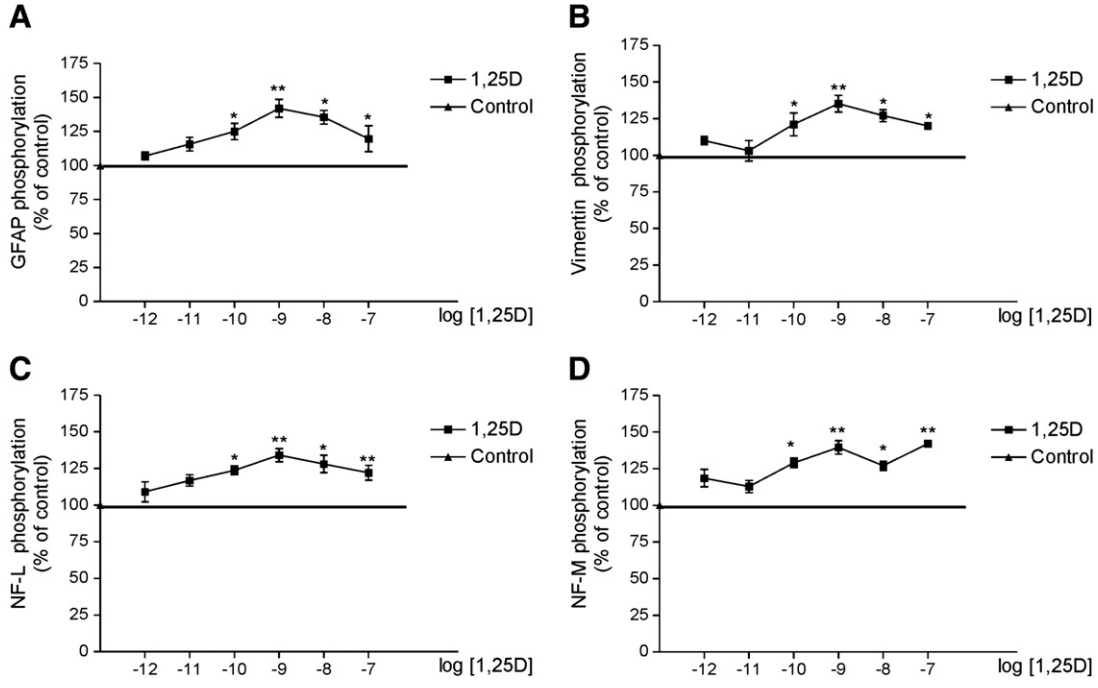

Fig. 1. Effect of different concentrations of 1,25D on IF phosphorylation. Slices of cerebral cortex were incubated with ^{32}P-orthophosphate in the absence or presence of 1,25D at doses ranging from 10^{-12} to 10^{-7} M. The cytoskeletal fraction was extracted, and analyzed by SDS-PAGE and the radioactivity that was incorporated into GFAP, vimentin, NF-L and NF-M was measured by determining the optical density values for the band corresponding to each protein. Data are reported as mean ± SEM of 12 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: $^{*}P<0.01$; $^{**}P<0.001$.

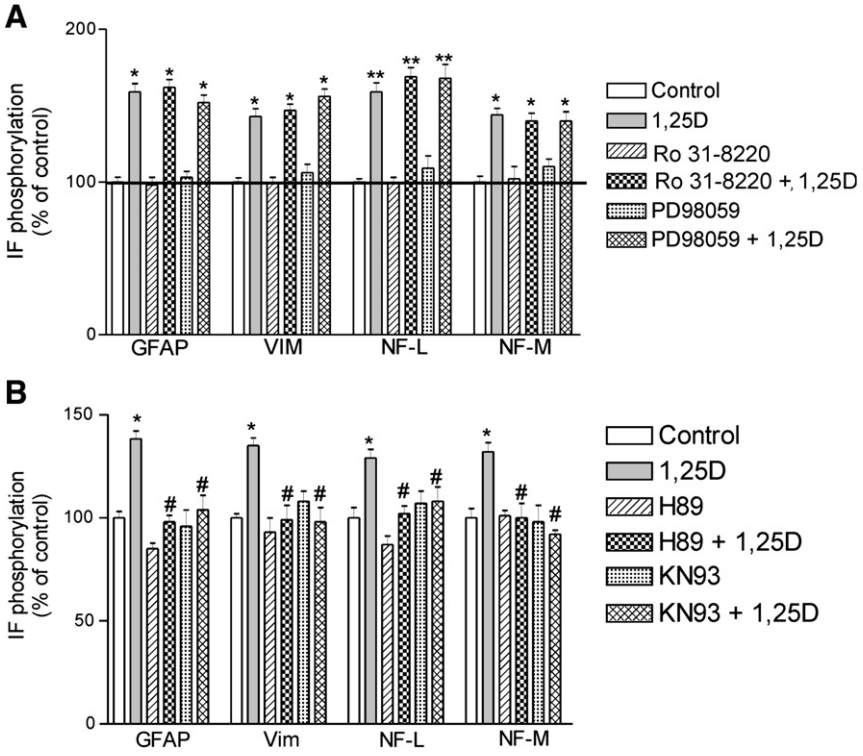

Fig. 3. Involvement of (A) PKC, MAPK, (B) PKA and PKCaMII signaling pathways on 1,25D-induced IF phosphorylation. Slices of cerebral cortex were pre-incubated in the presence or absence of 1 μM Ro 31–8220 (PKC inhibitor), 30 μM PD 98059 (MEK inhibitor), 10 μM H89 or 10 μM KN93 and incubated with ^{32}P-orthophosphate with or without addition of drugs and/or 1,25D (100 pM). The cytoskeletal fraction was extracted, and analyzed by SDS-PAGE and the radioactivity that was incorporated into GFAP, vimentin, NF-L and NF-M was measured by determining the optical density values for the band corresponding to each protein. Data are reported as means ± SEM of 10 animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: *P<0.01; **P<0.01 compared with control group. $^{\#}$P<0.05 compared with 1,25D group.

and flunarizine, L- and T-VDCC blockers, respectively, were used. Results showed that nifedipine (10 μM) prevented the 1,25D action while flunarizine (1 μM) did not alter the hormone effect, indicating the participation of L-VDCC in the mechanism of hormone action (Fig. 6A). Once the participation of L-VDCC on $^{45}Ca^{2+}$ uptake in cerebral cortex was established, we also sought to determine if the intracellular Ca^{2+} levels could play a role in regulating the VDCC activity by using BAPTA-AM to chelate the intracellular Ca^{2+}. Therefore we found that in the presence of BAPTA-AM the stimulatory effect of 1,25D on the L-VDCC was abrogated. In addition, the use of dantrolene (50 μM) allowed us to set the implication of ryanodin receptors in the mechanism of action of 1,25D (Fig. 6B).

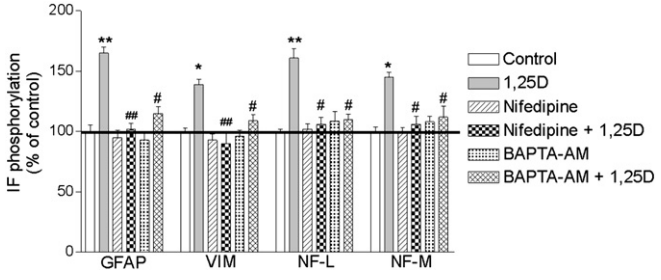

Fig. 4. Involvement of calcium on 1,25D-induced IF phosphorylation. Slices of cerebral cortex were pre-incubated in the presence or absence of 10 μM nifedipine or 50 μM BAPTA-AM for 20 min and incubated with ^{32}P-orthophosphate with or without (100 pM) 1,25D and/or inhibitors. The cytoskeletal fraction was extracted, and analyzed by SDS-PAGE and the radioactivity that was incorporated into IFs was measured by determining the optical density values for the band corresponding to each protein. Data are reported as means ± SEM of 10 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: **P<0.001, *P<0.01 compared to control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared to 1,25D group.

3.5. Involvement of K^+ and Cl^- channels in the stimulatory effect of 1,25D on $^{45}Ca^{2+}$ uptake

The VDCCs can open in response to changes on resting membrane potential; therefore, in order to investigate if K^+ efflux or Cl^- influx could lead to $^{45}Ca^{2+}$ uptake through VDCCs we used specific blockers for ATP-dependent K^+ channels (tolbutamide), as well as for calcium-dependent Cl^- channels (9-AC). Results showed that both tolbutamide (100 μM) and 9-AC (1 μM) prevented the hormone stimulated $^{45}Ca^{2+}$ uptake (Fig. 6C). These data suggest the involvement of K^+ and Cl^- channels in the mechanism of action of 1,25D in cerebral cortex.

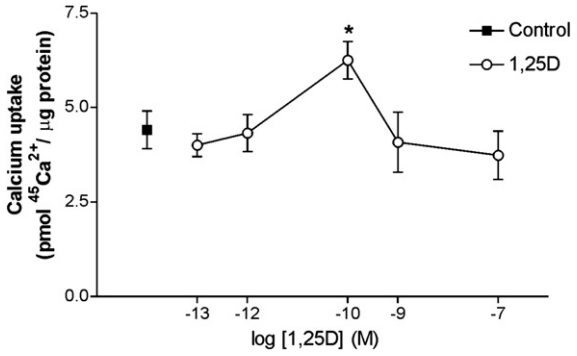

Fig. 5. Dose–response curve of 1,25D on $^{45}Ca^{2+}$ uptake on cerebral cortex. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.1 μCi/ml of $^{45}Ca^{2+}$. The slices were incubated with or without 1,25D at different concentrations (10^{-13}, 10^{-12}, 10^{-10}, 10^{-9} and 10^{-7} M) for 5 min. Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: *P<0.05 compared with control group.

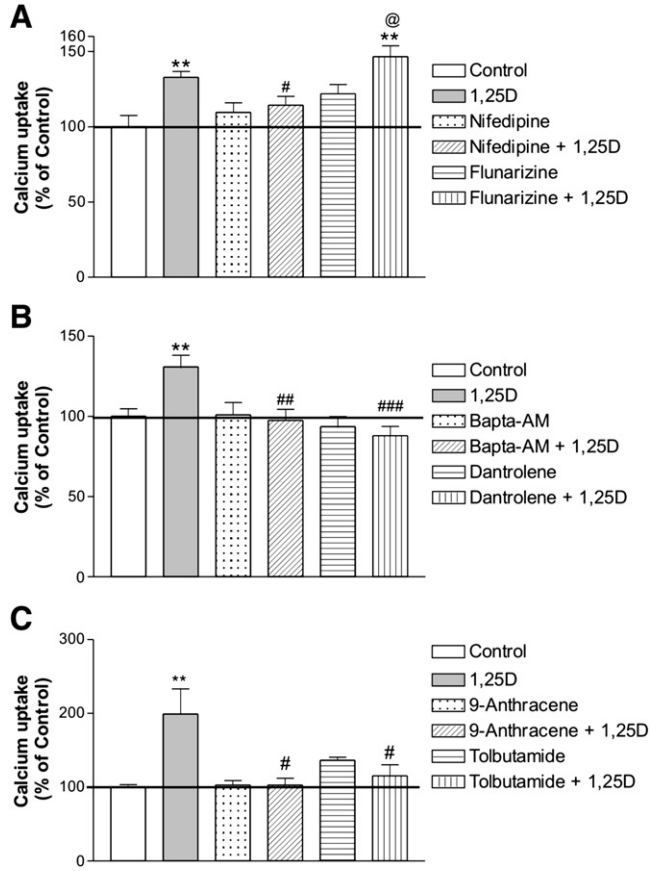

Fig. 6. Involvement of (A) voltage-dependent calcium channels (VDCCs) and (B) intracellular Ca^{2+} levels, as well as (C) K^+ and Cl^- channels on $^{45}Ca^{2+}$ uptake induced by 1,25D (100 pM) in cerebral cortex of rats. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.1 μCi/ml of $^{45}Ca^{2+}$. In the last 20 min preincubation 10 μM nifedipine (L-VDCC blocker) or 1 μM flunarizine (T-VDCC blocker) (A); 50 μM BAPTA-AM (intracellular calcium chelator) or 50 μM dantrolene (ryanodine calcium channel blocker) (B); 100 μM tolbutamide (ATP-dependent potassium channel blocker) or 1 μM 9-AC (calcium-dependent chloride channel blocker) (C), was added to the incubation medium. After that, the slices were treated with or without 1,25D for 5 min (incubation). Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: $^{**}P<0.01$ compared with control group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with 1,25D group. $^{@}P<0.05$ compared with flunarizine group.

3.6. Involvement of Na^+/K^+-ATPase in the stimulatory effect of 1,25D on $^{45}Ca^{2+}$ uptake

To evaluate the consequences of Na^+/K^+-ATPase inactivation on $^{45}Ca^{2+}$ uptake we incubated the slices of cerebral cortex in the presence of ouabain (1 μM), a digitalic glicoside. The pump inhibition mimicked the 1,25D action on $^{45}Ca^{2+}$ uptake (Fig. 7), without induced cell death evaluated by lactate dehydrogenase (LDH) release (results not shown).

3.7. Involvement of the PKA signaling pathway in the stimulatory effect of 1,25D on $^{45}Ca^{2+}$ uptake

We also investigated whether the PKA signaling pathway could be involved in the 1,25D effect on $^{45}Ca^{2+}$ uptake. Results demonstrated that H89 (PKA inhibitor) prevented the $^{45}Ca^{2+}$ uptake stimulated by 1,25D. Furthermore, an elevation of cAMP levels in response to 500 μM di-BucAMP (a cAMP synthetic analog), was able to increase $^{45}Ca^{2+}$ uptake. In addition, we did not observe an additional stimulatory effect when the tissue was co-incubated with the hormone, suggesting that cAMP on its own participates in the mechanism of action of 1,25D

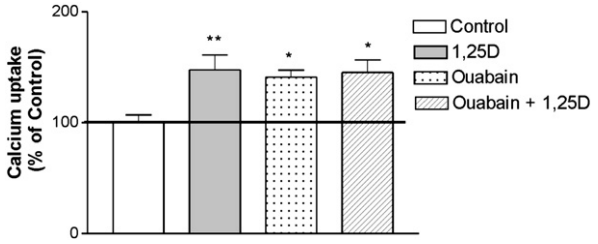

Fig. 7. Involvement of Na^+/K^+-ATPase on $^{45}Ca^{2+}$ uptake in cerebral cortex of rats. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.1 μCi/ml of $^{45}Ca^{2+}$. In the last 20 min preincubation 1 μM ouabain (Na^+/K^+-ATPase inhibitor) was added to the incubation medium. After that, the slices were treated with or without 1,25D (100 pM) for 5 min (incubation). Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: $^{*}P<0.05$ and $^{**}P<0.01$ compared with control group.

on $^{45}Ca^{2+}$ uptake. Also, the effect of 1,25D was sustained in the presence of a phosphodiesterase inhibitor (50 μM theophylline) (Fig. 8A), reinforcing the involvement of the cAMP/PKA signaling pathway in the 1,25D-induced $^{45}Ca^{2+}$ uptake.

3.8. Involvement of PKCaMII, PI3K, PKC and $p38^{MAPK}$ in the stimulatory effect of 1,25D on $^{45}Ca^{2+}$ uptake

The participation of other protein kinases such as PKCaMII, PI3K, PKC, and MAPK signaling pathways in 1,25D actions was investigated by using the specific inhibitors: (10 μM) KN93, (10 μM) LY294002, (20 μM) Ro 31–8220, (10 μM) PD 98059 and (10 μM) SB239063, respectively. The outcomes showed that KN93, LY 294002, as well as

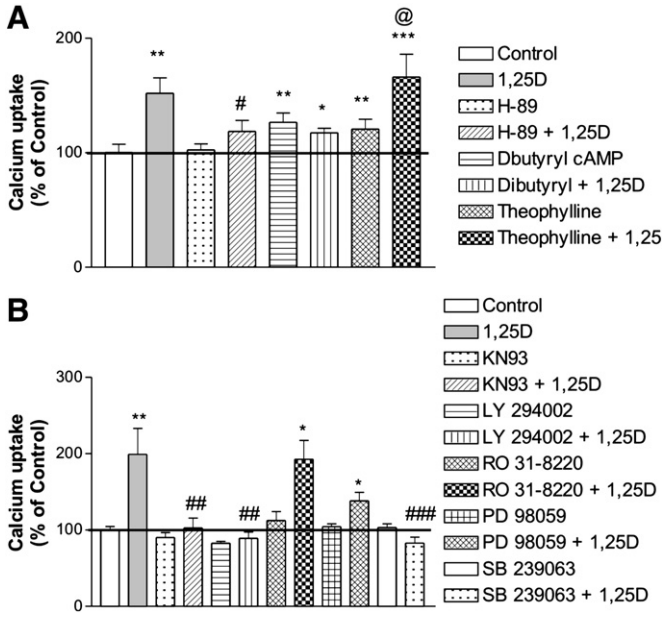

Fig. 8. Involvement of (A) the PKA signaling pathway, (B) Ca^{2+}/calmodulin-dependent protein kinase, phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), mitogen activated protein kinase (MAPK), and $p38^{MAPK}$ on $^{45}Ca^{2+}$ uptake in cerebral cortex of rats. Slices of cerebral cortex were pre-incubated for 60 min in the presence of 0.1 μCi/ml of $^{45}Ca^{2+}$. In the last 20 min preincubation 10 μM H89 (PKA inhibitor) or 500 μM dibutyryl (cAMP analog), or 100 μM theophylline (phosphodiesterase inhibitor) (A); 10 μM KN93 (PKCamII inhibitor) or 10 μM LY 294002 (PI3K inhibitor), or 20 μM RO 31–8220 (PKC inhibitor), or 10 μM PD 98059 (MAPK inhibitor), or 10 μM SB 239063 ($p38^{MAPK}$ inhibitor) was added to the incubation medium. After that, the slices were treated with or without 10^{-10} M 1,25D for 5 min (incubation). Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by a Bonferroni multiple comparison test are indicated: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with 1,25D group. $^{@}P<0.05$ compared with theophylline group.

SB239063 totally prevented the stimulatory effect of the hormone while Ro 31–8220 and PD98059 did not change the 1,25D stimulation on $^{45}Ca^{2+}$ uptake (Fig. 8B), suggesting that the ability of 1,25D to increase $^{45}Ca^{2+}$ uptake is associated to activation of PKCaMII, PI3K and $p38^{MAPK}$.

3.9. MAPK modulation by 1,25D

To attest the modulation of MAPKs by 1,25D, we investigated the effect of different times (5, 10, 15 and 30 min) of exposure to the hormone on the activation profile of the enzymes, measuring the total and phosphorylated levels of ERK1/2 and $p38^{MAPK}$ in cerebral cortex from immature rats. Results showed that the total levels of ERK1/2 and $p38^{MAPK}$ were unaltered from 1 to 30 min (Fig. 9A and C), while the phospho-ERK1/2 and phospho-$p38^{MAPK}$ levels significantly increased the first 5 min incubation returning to control levels at 30 min exposure to the hormone (Fig. 9B and D).

3.10. VDR/caveolin-1 and VDR/NeuN immunolocalization in cerebral cortex from young rats

Fig. 10 shows the VDR and caveolin-1 immunolocalization in the cerebral cortex cells from young rats. The nuclear staining with DAPI (blue) was demonstrated in Fig. 10A. The analysis revealed a strong cellular peripheral VDR immunoreactivity (red), but also a weak staining is shown in the nucleus of neural cells from cerebral cortex (Fig. 10B). In addition, immunoreactive caveolin-1 (green) was largely distributed in the cytosol and cell membrane but was largely absent in the nucleus (Fig. 10C). The colocalization of VDR and caveolin-1 is depicted in Fig. 10D. Apparently, VDR and caveolin-1 are immunolocalized in neurons (distinct nucleolus within an ovoid nucleus and

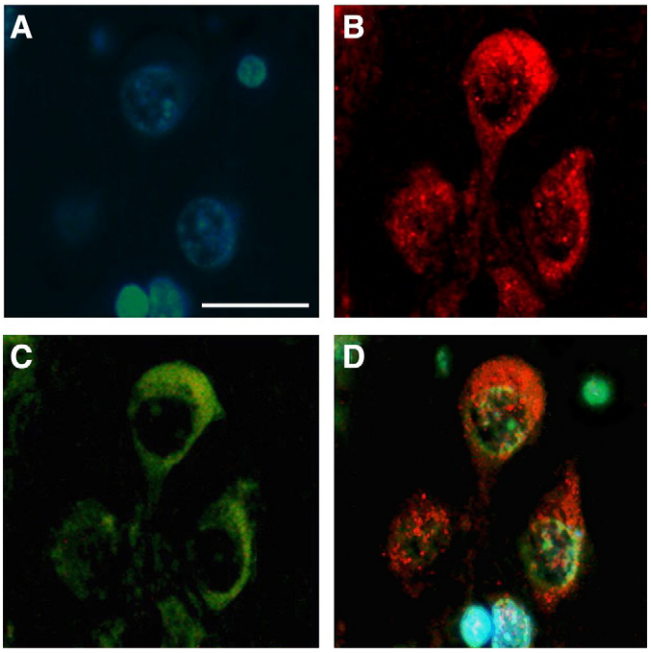

Fig. 10. Immunohistochemical double staining shows the immunolocalization of VDR and caveolin-1 in neurons of cerebral cortex of rats. Immunofluorescence images show the cell nuclei stained by 40,6-diamidino-2-phenylindole (DAPI) (blue, A), the subcellular localization of VDR (red, B) and caveolin-1 (green, C). Merged image of A, B and C illustrating co-localization of both VDR and caveolin-1 proteins (orange, D). Bars = 50 μm.

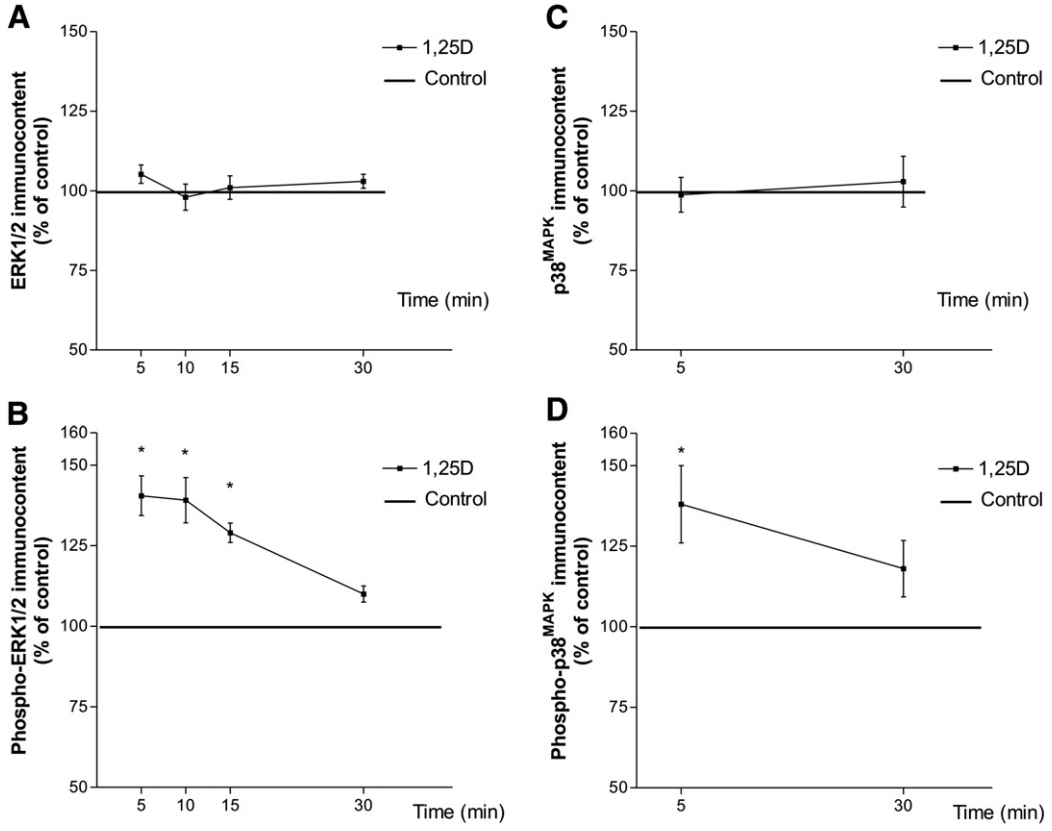

Fig. 9. Effect of 1,25D on ERK1/2 (A and B) and $p38^{MAPK}$ (C and D) activation in cerebral cortex of rats. Time course activation of ERK1/2 and $p38^{MAPK}$. After 20 min pre-incubation tissue slices were incubated for 1, 5, 15 or 30 min with 1,25D (100 pM). Tissue was lysed and the total and phospho levels of ERK1/2 and $p38^{MAPK}$ were measured by Western blot. Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by a Tukey–Kramer multiple comparison test are indicated: $^{*}P<0.05$ compared with control group.

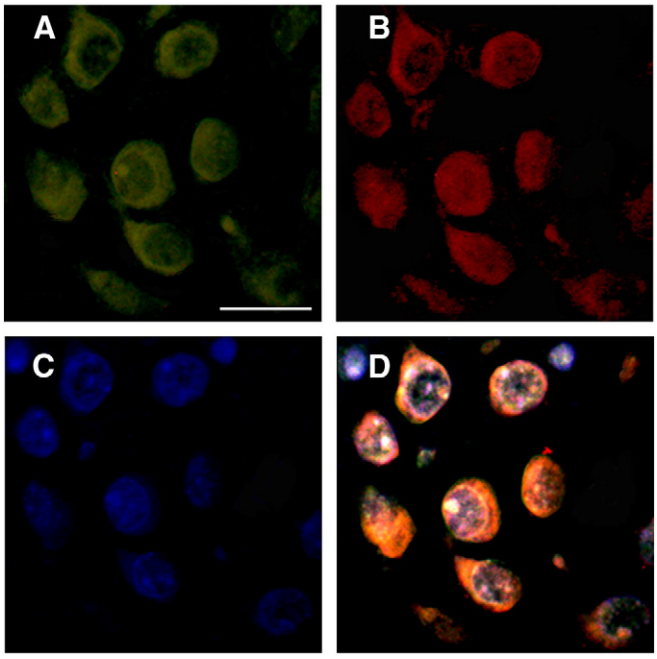

Fig. 11. Immunohistochemical double staining showing the immunolocalization of VDR and NeuN in neurons of cerebral cortex of immature rats. Immunofluorescence images show NeuN staining (green, A), VDR localization (red, B), cell nuclei stained by DAPI (blue, C), and merged image of A, B and C illustrating immunolocalization of both VDR and NeuN proteins (orange, D) in neuronal cells. Bars = 50 μm.

elongated perikarya) and are not present in glial cells (characterized by their much smaller size when compared with the neighboring neurons).

Fig. 11 shows the VDR and NeuN immunolocalization in the cerebral cortex cells from 15-day-old rats. Results demonstrated the NeuN immunoreactivity (green; Fig. 11A) and VDR immunoreactivity (red; Fig. 11B). The nuclear staining with DAPI (blue) was shown in Fig. 11C. The outcomes clearly demonstrated that VDR and NeuN are co-localized, indicating the expression of the VDR in neurons.

4. Discussion

In this study we found that 1,25D induced nongenomic effects in cerebral cortex of immature rats by modulating Ca^{2+}-mediated signal transduction, the homeostasis of the cytoskeleton, and neurochemical parameters. Results showed that the mechanism of hormonal action involves the participation of protein kinases, intra- and extracellular Ca^{2+}, as well as the modulation of Cl^- and K^+ channels.

The evidence that vitamin D plays critical roles in brain physiology is becoming recognized from findings linking vitamin D deficiency and the development of neuropsychiatric disorders [72,73]. Vitamin D and its metabolites were found in human cerebrospinal fluid several years ago [74], however, the evidence that this hormone plays important roles in brain was only provided after demonstration of the presence of the VDR in the central nervous system [75–77] and the involvement of 1,25D in brain function and development [73,78–80]. Nonetheless, several questions are still open concerning the 1,25D mechanism of action in brain. Interestingly, a strong VDR immunostaining in close proximity to the neuronal cell membrane is clearly visible, which is colocalized with the plasma membrane protein caveolin-1. It is classically described that 1,25D regulates gene transcription through modulation of VDRn [1]. Moreover, initiation of rapid cellular responses is attributed to a putative plasma membrane-associated receptor (VDRm) which is present in a caveolae-enriched membrane fraction [12,16]. In this context MARRS binding protein [17,18] and the classical VDR [15] have been suggested as possible candidates for the VDRm. Taking into account these findings, our data support the conclusion that the classical VDR, which is normally found in the nucleus, is also a neuronal plasma membrane-associated receptor, as demonstrated by the colocalization of VDR and caveolin-1 in cerebral cortex of immature rats. However, we could not exclude the participation of MARRS protein in the mechanism of action of 1,25D in cerebral cortex. Further experiments will be necessary to clarify this point.

Considering the relevance of the cytoskeleton to the physiology of neural cells, we were interested to assess the molecular mechanisms underlying the actions of 1,25D on the phosphorylation of GFAP, vimentin and neurofilament subunits in the cerebral cortex of immature rats. In this context, we have previously demonstrated the vimentin phosphorylation and polymerization/aggregation as a target of 1,25D in immature rat testis [5]. Therefore, in the present report we have demonstrated that 1,25D is upstream of complex membrane-initiated signaling pathways leading to phosphorylation of the IF proteins from astrocytes (GFAP and vimentin) and neurons (NF-L and NF-M). However, although the immunohistochemical analyses indicated that VDR is only expressed in neurons, 1,25D induced the phosphorylation of GFAP and vimentin, which are proteins expressed in an astrocyte. The mechanisms underlying such effects probably involve complex signaling events and interactions between cells. In this context, the neurons may provide additional opportunities for cross-talk between them and the neighboring cells, such as the astrocytes. Also, other proteins, such as MARRS, might be participating in the mechanism of action of 1,25D in glial cells, as demonstrated in other tissues [19,20].

Considering the relevance of Ca^{2+} overload on the modulation of the phosphorylating system associated with the cytoskeleton [5,42,81,82], the blockade of L-VDCC and the intracellular Ca^{2+} chelation totally prevented the effect of 1,25D on IF phosphorylation. Increased cytosolic Ca^{2+} levels lead to astrocyte and neuronal IF phosphorylation by second messenger-dependent protein kinases, PKA and PKCaMII, supporting the evidence that the neural isoforms of adenylyl cyclase are activated by calcium [83]. Our results show that intracellular Ca^{2+} levels are required for the 1,25D actions, as demonstrated by treating cerebral cortex slices with BAPTA-AM, an intracellular Ca^{2+} chelator, which prevented both hormone-induced IF phosphorylation and $^{45}Ca^{2+}$ uptake. Ca^{2+} itself is usually the physiological ligand for the ryanodine receptor in the endoplasmic reticulum (ER) [84]. In this context, we evidenced a role for this receptor on the 1,25D-induced $^{45}Ca^{2+}$ uptake by using dantrolene, a ryanodine receptor blocker. Taken together, our results suggest that the increase in intracellular Ca^{2+} levels could activate ryanodine receptors leading to L-VDCC opening.

Calcium acts as an intracellular messenger controlling cell development and function and the involvement of Ca^{2+}-mediated signaling mechanisms in vitamin D effects is currently demonstrated [5,6,9,52,85]. Also, our results provide evidence that $^{45}Ca^{2+}$ uptake induced by 1,25D is associated with the modulation of L-VDCC but is unaffected by T-VDCC. These findings highlight the critical role of L-VDCC as a mediator of the actions of 1,25D targeting phosphorylation sites on IF proteins, although we cannot exclude the participation of other VDCCs in the modulation of intracellular Ca^{2+} levels.

The Ca^{2+} influx via VDCCs frequently occurs after plasma membrane depolarization. These mechanisms then set off a cascade of events including activation of protein kinases, which phosphorylate head domain sites on GFAP and NFL [64]. To clarify the involvement of ionic fluxes in the mechanism of action of 1,25D, we investigated the participation of Cl^- and K^+ channels and also the Na^+/K^+-ATPase activity in the hormone-induced $^{45}Ca^{2+}$ uptake via L-VDCC. Our results showed that both ouabain (Na^+/K^+-ATPase inhibitor) and 1,25D increased $^{45}Ca^{2+}$ uptake in cerebral cortex slices. It is important to emphasize that coincubation with ouabain and 1,25D did not result in an additive effect, suggesting that they might have a similar mechanism

on Na^+/K^+-ATPase activity. However, the mechanisms underlying such effects need further investigation. Taking into account these findings, the Na^+/K^+-ATPase inhibition might lead to intracellular Na^+ accumulation with subsequent L-VDCC opening and consequent $^{45}Ca^{2+}$ uptake. In this context, Nemoto et al. [86] showed that intracellular levels of Na^+ are upstream of Ca^{2+} influx through L-VDCC in adrenal chromaffin cells. Interestingly, the available results presented here clearly indicate that Cl^- and K^+ fluxes are important components involved in the $^{45}Ca^{2+}$ uptake activated by 1,25D in cerebral cortex, although the precise mechanisms underlying such participation are unknown. It is important to consider that the modulation of neuronal excitability might be under the control of L-VDCC activity, and also that these channels may act in opposing manners, depending on the prevalence of coupling to K^+ channels. Geier et al. [87] have demonstrated that the effects of L-VDCCs on membrane voltage occur over a broad potential range and could involve depolarizing and hyperpolarizing coupling modes, supporting the cross-talk among $^{45}Ca^{2+}$ uptake and K^+ and/or Cl^- channels we are evidencing.

In this context, $^{45}Ca^{2+}$ uptake through VDCCs could be modulated by complex mechanisms involving the activities of different protein kinases [63,88,89] through direct phosphorylation of the channel. Therefore, the possible involvement of different serine/threonine kinases such as PKA, PKC, PI3K, PKCaMII and MAPKs in the stimulatory effect of 1,25D-induced $^{45}Ca^{2+}$ uptake in cerebral cortex of young rats was examined. These data suggest that 1,25D-induced $^{45}Ca^{2+}$ uptake is triggered by the influx of this ion through L-VDCC and that PKA plays an important role in mediating this effect which are in line with previous studies [6,9,85] and with the evidence that PKC and PKA play central roles in signal transduction in many cell types [66,88,89]. Accordingly, it has been demonstrated that L-VDCC phosphorylation by PKA in skeletal muscle resulted in an activation and opening of the channels [90–92]. Moreover, Vela et al. [93] showed that PKA and PKC activation leads to depolarization-induced Ca^{2+} entry through L-VDCC in GH3 cells. These data reinforce our results demonstrating the involvement of PKA and PKC in inducing channel opening and Ca^{2+} entry leading to IF phosphorylation in cerebral cortex of immature rats. The participation of the cAMP/PKA pathway in the 1,25D mechanism of action was also reinforced by experiments showing that dibutyryl cAMP as well as theophylline might induce $^{45}Ca^{2+}$ uptake. Otherwise, in contrast with previous studies [6,9], the nongenomic effects of 1,25D on IF protein phosphorylation and $^{45}Ca^{2+}$ influx are independent on PKC and ERK1/2 activation in developing cerebral cortex. The reasons leading to these findings remain to be clarified.

Interestingly, the activation of PKCaMII, $p38^{MAPK}$ and PI3K is important to support the hormonal action of 1,25D on Ca^{2+} homeostasis. Recently, Nemoto et al. [86] demonstrated that inhibition of $p38^{MAPK}$ prevented the $^{45}Ca^{2+}$ uptake via VDCCs. Moreover, PI3K activation was found to be an upstream effector modulating L-type Ca^{2+} currents in cardiomyocytes [94]. Also, the phosphorylation of the β subunit of L-VDCC by PKB/Akt, a key effector of the PI3K signaling pathway, was demonstrated to modulate the gating of these channels [95,96], further supporting the involvement of PI3K on Ca^{2+} influx induced by 1,25D as observed by us in immature rat brain.

The early stages of brain development are recognized as the periods of intense synaptogenesis associated with enhanced neuronal activity and augmented synaptic plasticity [97]. Interestingly, the PI3K activation was associated with induction of functional synaptogenesis in the hippocampus [98]. Taken together, this evidence supports the idea that 1,25D also might induce synapse formation through PI3K-mediated mechanisms in immature rat brain.

It is now clear that there are interfaces between 1,25D nongenomic and genomic actions [15,99]. The involvement of $p38^{MAPK}$ and PI3K in the mechanism of action of 1,25D suggests that probably a nongenomic-initiated action might lead to late activation of genomic

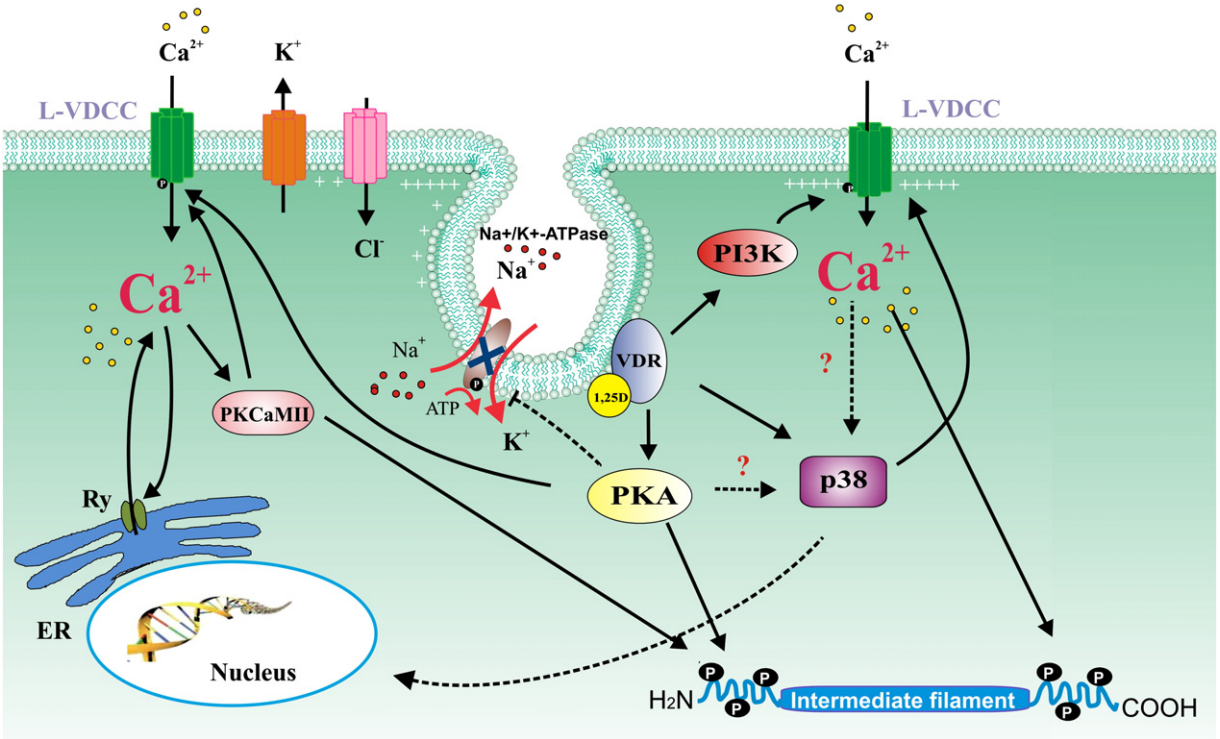

Fig. 12. Schematic representation of 1,25D-induced signaling pathways mediating cerebral cortex Ca^{2+} uptake and IF phosphorylation. 1,25D binds to the membrane-associated VDR and stimulates a variety of signaling pathways such as PKA, PI3K, PKCaMII and p38. 1,25D might inhibit Na^+/K^+ ATPase activity with subsequent Na^+ accumulation which can lead to membrane depolarization and K^+, Cl^- and VDCC opening. The activated form of PKA might phosphorylate L-VDCC which could promote the entry of extracellular Ca^{2+} into the cells. PKA can also phosphorylate the ryanodine (Ry) receptor and promote the Ca^{2+} release from stocks into endoplasmic reticulum (ER). Intracellular Ca^{2+} activates Ca^{2+} messenger systems, such as PKCaMII that also could phosphorylate L-VDCC. These events might be also involved in the protein synthesis independent astrocyte and neuronal IF phosphorylation and consequently lead to cytoskeleton remodeling.

pathways, considering that both of these kinases can activate transcriptional factors and modulate gene transcription [15]. Moreover, the modulation of MAPK signaling pathways may transduce the 1,25D signal into complex cellular events, such as aromatase expression [8], that clearly require gene transcription, although not necessarily the participation of VDRn. However, further experiments are necessary to clarify this point. Recently, Sun et al. [99] demonstrated that the $p38^{MAPK}$ signaling pathway might regulate VDR mRNA expression and mediate Ca^{2+} signaling in preadipocytes. These findings present an evident connection among $p38^{MAPK}$, transcriptional activity and a variety of possibilities in vitamin D actions.

The participation of PKCaMII modulating Ca^{2+} channels and IF phosphorylation demonstrated in this study is in agreement with previous reports showing that this kinase induces L-VDCC to enter a gating mode. The authors showed that PKCaM-mediated phosphorylation is an essential signaling event triggering L-VDCC facilitation [100]. The PKCaMII is one of the most prominent protein kinases present in the brain. The activity of PKCaMII regulates important neuronal functions, such as neurotransmitter synthesis and release, modulation of ion channel activity, cellular transport, cytoskeleton phosphorylation, cell morphology and synaptic plasticity. Also, it is involved in learning and memory, as well as in modulation of gene expression [40,43,100,101]. It has also been reported that PKCaMII can phosphorylate and activate the glutamatergic receptor AMPA resulting in an increase of Ca^{2+} entry into postsynaptic neurons and that this event could be related to long-term potentiation [101], and consequently could be involved with learning and memory. The involvement of different protein kinases in the mechanism of action of 1,25D in the brain provides an evidence of the hormonal action and intracellular targets of the hormone during brain development.

In summary, the present study strongly suggests that 1,25D induces IF phosphorylation and Ca^{2+} influx by modulating L-VDCC opening through different kinase activities, as well as by regulating ionic fluxes in cerebral cortex of young rats. An overview of the proposed mechanism of action for 1,25D in the cerebral cortex of young rats is depicted in Fig. 12. The modulation of Ca^{2+} entry into neural cells by the hormone might participate in the regulation of a plethora of intracellular processes, such as IF phosphorylation, modulation of neurotransmission, enzymatic activity, protein synthesis and cell proliferation, as well as synapse formation, reinforcing 1,25D as a neuroactive substance that could participate in brain pathology and physiology. Considering that vitamin D deficiency can lead to several types of brain illness, it may be a possible candidate to be used, at least as an adjuvant, in the pharmacological therapy of neuropsychiatric conditions.

Acknowledgements

This work was supported by grants from CAPES, CNPq-Brazil, PPG-BQA-UFSC, FUNPESQUISA-UFSC and FAPESC. Special thanks to Biology, Embryology and Genetic Department/Biological Sciences Centre (BEG-CCB/UFSC) for microscope facilities (FluorBEG) and to the biologists Chirle Ferreira and Demétrio Gomes for technical assistance. LZ was registered on Pharmacy Postgraduate Program/UFSC-Brazil and on Ecole Doctorale Normande de Biologie Intégrative, Santé, Environnement/Université de Caen-France (Project CAPES/COFECUB 554/07).

References

[1] R. Bouillon, W.H. Okamura, A.W. Norman, Structure–function relationships in the vitamin D endocrine system, Endocr. Rev. 16 (1995) 200–257.
[2] L.A. Plum, H.F. DeLuca, Vitamin D, disease and therapeutic opportunities, Nat. Rev. Drug Discov. 9 (2010) 941–955.
[3] R. Bouillon, G. Carmeliet, L. Verlinden, E. van Etten, A. Verstuyf, H.F. Luderer, L. Lieben, C. Mathieu, M. Demay, Vitamin D and human health: lessons from vitamin D receptor null mice, Endocr. Rev. 29 (2008) 726–776.
[4] E. Garcion, N. Wion-Barbot, C.N. Montero-Menei, F. Berger, D. Wion, New clues about vitamin D functions in the nervous system, Trends Endocrinol. Metab. 13 (2002) 100–105.
[5] A. Zamoner, P. Pierozan, L.F. Vidal, B.A. Lacerda, N.G. Dos Santos, C.S. Vanzin, R. Pessoa-Pureur, Vimentin phosphorylation as a target of cell signaling mechanisms induced by 1alpha,25-dihydroxyvitamin D_3 in immature rat testes, Steroids 73 (2008) 1400–1408.
[6] L. Zanatta, A. Zamoner, R. Gonçalves, A.P. Zanatta, H. Bouraïma-Lelong, S. Carreau, F. Silva, 1α,25-dihydroxyvitamin D(3) signaling pathways on calcium uptake in 30-day-old rat Sertoli cells, Biochemistry 50 (2011) 10284–10292.
[7] L. Zanatta, A. Zamoner, A.P. Zanatta, H. Bouraïma-Lelong, C. Delalande, B. Bois, S. Carreau, F.R. Silva, Nongenomic and genomic effects of 1α,25(OH)(2) vitamin D(3) in rat testis, Life Sci. 89 (2011) 515–523.
[8] L. Zanatta, H. Bouraïma-Lelong, C. Delalande, F.R. Silva, S. Carreau, Regulation of aromatase expression by 1α,25$(OH)_2$ vitamin D_3 in rat testicular cells, Reprod. Fertil. Dev. 23 (2001) 725–735.
[9] L. Zanatta, A. Zamoner, R. Gonçalves, A.P. Zanatta, H. Bouraïma-Lelong, Bois, S. Carreau, F.R. Silva, Effect of 1α,25-dihydroxyvitamin D3 in plasma membrane targets in immature rat testis: ionic channels and gamma-glutamyl transpeptidase activity, Arch. Biochem. Biophys. 515 (2011) 46–53.
[10] C. Carlberg, Ligand-mediated conformational changes of the VDR are required for gene transactivation, J. Steroid Biochem. Mol. Biol. 89–90 (2004) 227–232.
[11] P. Lips, Vitamin D physiology, Prog. Biophys. Mol. Biol. 92 (2006) 4–8.
[12] A.W. Norman, M.T. Mizwicki, P.G. Derek, Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model, Nat. Rev. Drug Discov. 3 (2004) 27–41.
[13] L.P. Zanello, A.W. Norman, Rapid modulation of osteoblast ion channel responses by 1alpha,25$(OH)_2$-vitamin D_3 requires the presence of a functional vitamin D nuclear receptor, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 1589–1594.
[14] J.C. Fleet, Rapid, membrane-initiated actions of 1,25 dihydroxyvitamin D: what are they and what do they mean? J. Nutr. 134 (2004) 3215–3218.
[15] M.R. Haussler, P.W. Jurutka, M. Mizwieki, A.W. Norman, Vitamin D receptor (VDR)-mediated actions of 1α,25$(OH)_2$vitamin D_3: genomic and non-genomic mechanisms, Best Pract. Res. Clin. Endocrinol. Metab. 25 (2011) 543–559.
[16] J.A. Huhtakangas, C.J. Olivera, J.E. Bishop, L.P. Zanello, A.W. Norman, The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25$(OH)_2$-vitamin D_3 *in vivo* and *in vitro*, Mol. Endocrinol. 18 (2004) 2660–2671.
[17] I. Nemere, M.C. Dormanen, M.W. Hammond, W.H. Okamura, A.W. Norman, Identification of a specific binding protein for 1α,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia, J. Biol. Chem. 269 (1994) 23750–23756.
[18] I. Nemere, R. Ray, W. McManus, Immunochemical studies on the putative plasmalemmal receptor for 1,25$(OH)_2D_3$ in chick intestine, Am. J. Physiol. Endocrinol. Metab. 278 (2000) E1104–E1114.
[19] I. Nemere, The 1,25D3-MARRS protein: contribution to steroid stimulated calcium uptake in chicks and rats, Steroids 70 (2005) 455–457.
[20] W. Wu, G. Beilhartz, Y. Roy, C.L. Richard, M. Curtin, L. Brown, D. Cadieux, M. Coppolino, M.C. Farach-Carson, I. Nemere, K.A. Meckling, Nuclear translocation of the 1,25D3-MARRS (membrane associated rapid response to steroids) receptor protein and NFkappaB in differentiating NB4 leukemia cells, Exp. Cell Res. (2010) 1101–1108.
[21] C. Annweiler, G. Allali, P. Allain, S. Bridenbaugh, R.A.-M. Schott, W. Kressig, O. Beauchet, Vitamin D and cognitive performance in adults: a systematic review, Eur. J. Neurol. 16 (2009) 1083–1089.
[22] W.E. Stumpf, M. Sar, S.A. Clark, H.F. DeLuca, Brain target sites for 1,25-dihydroxyvitamin D_3, Science 215 (1982) 1403–1405.
[23] T.D. Veenstra, K. Prufer, C. Koenigsberger, S.W. Brimijoin, J.P. Grande, R. Kumar, 1,25-Dihydroxyvitamin D_3 receptors in the central nervous system of the rat embryo, Brain Res. 804 (1998) 193–205.
[24] W.E. Stumpf, L.P. O'Brien, 1,25 $(OH)_2$ vitamin D_3 sites of action in the brain: an autoradiographic study, Histochemistry 87 (1987) 393–406.
[25] K. Prufer, T.D. Veenstra, G.F. Jirikowski, R. Kumar, Distribution of 1,25-dihydroxyvitamin D_3 receptor immunoreactivity in the rat brain and spinal cord, J. Chem. Neuroanat. 16 (1999) 135–145.
[26] M.K. Sutherland, M.J. Somerville, L.K. Yoong, C. Bergeron, M.R. Haussler, D.R. McLachlan, Reduction of vitamin D hormone receptor mRNA levels in Alzheimer as compared to Huntington hippocampus: correlation with calbindin-28k mRNA levels, Brain Res. Mol. Brain Res. 13 (1992) 239–250.
[27] D.W. Eyles, S. Smith, R. Kinobe, M. Hewisond, J.J. McGrath, Distribution of the vitamin D receptor and 1a-hydroxylase in human brain, J. Chem. Neuroanat. 29 (2005) 21–30.
[28] J.J. McGrath, F.P. Féron, T.H.J. Burne, A. Mackay-Simc, D.W. Eyles, Vitamin D_3 implications for brain development, J. Steroid Biochem. Mol. Biol. 89–90 (2004) 557–560.
[29] D. Eyles, J. Brown, A. Mackay-Sim, J. McGrath, F. Feron, Vitamin and brain development, Neuroscience 118 (2003) 641–653.
[30] S. Ackerley, P. Thornhill, A.J. Grierson, J. Brownlees, B.H. Anderton, P.N. Leigh, C.E. Shaw, C.C. Miller, Neurofilament heavy chain side arm phosphorylation regulates axonal transport of neurofilaments, J. Cell Biol. 161 (2003) 489–495.
[31] R. Perrot, R. Berges, A. Bocquet, J. Eyer, Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration, Mol. Neurobiol. 38 (2008) 27–65.
[32] S.M. de Waegh, V.M.Y. Lee, S.T. Brady, Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal-transport by myelinating Schwann-cells, Cell 68 (1992) 451–463.
[33] B. Albert, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Molecular Biology of the Cell, Garland Science, fifth ed., Taylor and Francis Group, New York, 2008.

[34] L.F. Eng, R.S. Ghirnikar, Y.L. Lee, Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000), Neurochem. Res. 25 (2000) 1439–1451.
[35] S.L. Green, J.M. Westendorf, H. Jaffe, H.C. Pant, L.C. Cork, E.A. Ostrander, F. Vignaux, J.R. Ferrell, Allelic variants of the canine heavy neurofilament (NFH) subunit and extensive phosphorylation in dogs with motor neuron disease, J. Comp. Pathol. 132 (2005) 33–50.
[36] P. Grant, H.C. Pant, Neurofilament protein synthesis and phosphorylation, J. Neurocytol. 29 (2000) 843–872 (Review).
[37] R.C. Lariviere, J.P. Julien, Functions of intermediate filaments in neuronal development and disease, J. Neurobiol. 58 (2004) 131–148 (Review).
[38] R.A. Nixon, R.K. Sihag, Neurofilament phosphorylation: a new look at regulation and function, Trends Neurosci. 14 (1991) 501–506 (Review).
[39] A. Zamoner, P.F. Corbelini, C. Funchal, D. Menegaz, F.R. Silva, R. Pessoa-Pureur, Involvement of calcium-dependent mechanisms in T3-induced phosphorylation of vimentin of immature rat testis, Life Sci. 77 (2005) 3321–3335.
[40] A. Zamoner, C. Funchal, L. Heimfarth, F.R. Silva, R. Pessoa-Pureur, Short-term effects of thyroid hormones on cytoskeletal proteins are mediated by GABAergic mechanisms in slices of cerebral cortex from young rats, Cell. Mol. Neurobiol. 26 (2006) 209–224.
[41] A. Zamoner, C. Funchal, M.C. Jacques-Silva, C. Gottfried, F.R. Barreto Silva, R. Pessoa-Pureur, Thyroid hormones reorganize the cytoskeleton of glial cells through Gfap phosphorylation and Rhoa-dependent mechanisms, Cell. Mol. Neurobiol. 27 (2007) 845–865.
[42] A. Zamoner, L. Heimfarth, R. Pessoa-Pureur, Congenital hypothyroidism is associated with intermediate filament misregulation, glutamate transporters down-regulation and MAPK activation in developing rat brain, Neurotoxicology 29 (2008) 1092–1099.
[43] A. Zamoner, L. Heimfarth, S. Oliveira Loureiro, C. Royer, F.R. Mena Barreto Silva, R. Pessoa-Pureur, Nongenomic actions of thyroxine modulate intermediate filament phosphorylation in cerebral cortex of rats, Neuroscience 156 (2008) 640–652.
[44] C.P. Turner, R. Miller, C. Smith, L. Brown, K. Blackstone, S.R. Dunham, R. Strehlow, M. Manfredi, P. Slocum, K. Iverson, M. West, S.L. Ringler, Z.C. Berry, Widespread neonatal brain damage following calcium channel blockade, Dev. Neurosci. 29 (2007) 213–231.
[45] T.A. Basarsky, V. Parpura, P.G. Haydon, Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution, J. Neurosci. 14 (1994) 6402–6411.
[46] K.P. Scholz, R.J. Miller, Developmental changes in presynaptic calcium channels coupled to glutamate release in cultured rat hippocampal neurons, J. Neurosci. 15 (1995) 4612–4617.
[47] A.E. West, W.G. Chen, M.B. Dalva, R.E. Dolmetsch, J.M. Kornhauser, A.J. Shaywitz, M.A. Takasu, X. Tao, M.E. Greenberg, Calcium regulation of neuronal gene expression, PNAS 98 (2001) 11024–11031.
[48] A. Mantelas, A. Stamatakis, I. Kazanis, H. Philippidis, F. Stylianopoulou, Control of neuronal nitric oxide synthase and brain-derived neurotrophic factor levels by GABA-A receptors in the developing rat cortex, Brain Res. Dev. Brain Res. 145 (2003) 185–195.
[49] D. Atlas, O. Wiser, M. Trus, The voltage-gated Ca^{2+} channel is the Ca^{2+} sensor of fast neurotransmitter release, Cell. Mol. Neurobiol. 21 (2001) 717–731.
[50] P.T. Kumar, S. Antony, M.S. Nandhu, J. Sadanandan, G. Naijil, C.S. Paulose, Vitamin D_3 restores altered cholinergic and insulin receptor expression in the cerebral cortex and muscarinic M3 receptor expression in pancreatic islets of streptozotocin induced diabetic rats, J. Nutr. Biochem. 22 (2011) 418–425.
[51] L.P. Zanello, A.W. Norman, Stimulation by 1α,25(OH)2-vitamin D_3 of whole cell chloride currents in osteoblastic ROS 17/2. 8 cells: a structure–function study, J. Biol. Chem. 272 (1997) 22617–22622.
[52] D. Menegaz, A. Barrientos-Duran, A. Kline, F.R.M.B. Silva, A.W. Norman, M.T. Mizwicki, L.P. Zanello, 1α,25$(OH)_2$-vitamin D_3 stimulation of secretion via chloride channel activation in Sertoli cells, J. Steroid Biochem. Mol. Biol. 11 (2010) 127–134.
[53] C. Funchal, A. Zamoner, A.Q. dos Santos, S.O. Loureiro, M. Wajner, R. Pessoa-Pureur, Alpha ketoisocaproic acid increases phosphorylation of intermediate filament proteins from rat cerebral cortex by mechanisms involving Ca^{2+} and cAMP, Neurochem. Res. 30 (2005) 1139–1146.
[54] S. Ciarmatori, D. Kiepe, A. Haarmann, U. Huegel, B. Tönshoff, Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I, J. Mol. Endocrinol. 38 (2007) 493–508.
[55] C.A. Barlow, K. Kitiphongspattana, N. Siddiqui, M.W. Roe, B.T. Mossman, K.M. Lounsbury, Protein kinase A-mediated CREB phosphorylation is an oxidant-induced survival pathway in alveolar type II cells, Apoptosis 13 (2008) 681–692.
[56] H. El Beheiry, A. Ouanounoua, P.L. Carlen, L-type calcium channel blockade modifies anesthetic actions on aged hippocampal neurons, Neuroscience 147 (2007) 117–126.
[57] S. Kim, S-Y. Nah, H. Rhim, Neuroprotective effects of ginseng saponins against L-type Ca^{2+} channel-mediated cell death in rat cortical neurons, Biochem. Biophys. Res. Commun. 365 (2008) 399–405.
[58] P. Benquet, J. Le Guen, Y. Pichon, F. Tiaho, Differential involvement of Ca(2+) channels in survival and neurite outgrowth of cultured embryonic cockroach brain neurons, J. Neurophysiol. 88 (2002) 1475–1490.
[59] J. Kolikova, R. Afzalov, A. Giniatullina, A. Surin, R. Giniatullin, L. Khiroug, Calcium-dependent trapping of mitochondria near plasma membrane in stimulated astrocytes, Brain Cell Biol. 35 (2006) 75–86.
[60] A. Rosso, M. Pansera, A. Zamoner, L. Zanatta, H. Bouraïma-Lelong, S. Carreau, F.R. Silva, 1α,25(OH)(2)-vitamin D(3) stimulates rapid plasma membrane calcium influx via MAPK activation in immature rat Sertoli cells, Biochimie 94 (2012) 146–154.
[61] C. Funchal, L.M.W. de Almeida, S.O. Loureiro, L. Vivian, P.L. Pelaez, F.D.B. Pessutto, A.M. Rosa, M. Wajner, R. Pessoa-Pureur, *In vitro* phosphorylation of cytoskeletal proteins from cerebral cortex of rats, Brain Res. Protoc. 11 (2003) 111–118.
[62] L. Heimfarth, S.O. Loureiro, A. Zamoner, P. de L. Pelaez, C.W. Nogueira, J.B. Rocha, R. Pessoa-Pureur, Effects of *in vivo* treatment with diphenyl ditelluride on the phosphorylation of cytoskeletal proteins in cerebral cortex and hippocampus of rats, Neurotoxicology 29 (2008) 40–47.
[63] S.O. Loureiro, L. Heimfarth, P. de L. Pelae, C.S. Vanzin, L. Viana, A.T. Wyse, R. Pessoa-Pureur, Homocysteine activates calcium mediated cell signaling mechanisms targeting the cytoskeleton in rat hippocampus, Int. J. Dev. Neurosci. 26 (2008) 447–455.
[64] P. Pierozan, A. Zamoner, A.K. Soska, B.O. de Lima, K.P. Reis, F. Zamboni, M. Wajne, R. Pessoa-Pureur, Signaling mechanisms downstream of quinolinic acid targeting the cytoskeleton of rat striatal neurons and astrocytes, Exp. Neurol. 233 (2012) 391–399.
[65] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[66] A. Zamoner, C. Royer, K.P. Barreto, R. Pessoa-Pureur, F.R. Silva, Ionic involvement and kinase activity on the mechanism of nongenomic action of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex from young rats, Neurosci. Res. 57 (2007) 98–103.
[67] A. Katz, Y. Lifshitz, E. Bab-Dinitz, E. Kapri-Pardes, R. Goldshleger, D.M. Tal, S.J.D. Karlish, Selectivity of digitalis glycosides for isoforms of human Na, K-ATPase, J. Biol. Chem. 285 (2010) 19582–19592.
[68] D. Menegaz, A. Rosso, C. Royer, L.D. Leite, A.R.S. Santos, F.R.M.B. Silva, Role of 1,25$(OH)_2$ vitamin D_3 on alpha-[1-^{14}C]MeAIB accumulation in immature rat testis, Steroids 74 (2009) 264–269.
[69] D. Menegaz, C. Royer, A. Rosso, A.Z.P. De Souza, A.R.S. Santos, F.R.M.B. Silva, Rapid stimulatory effect of thyroxine on plasma membrane transport systems: calcium uptake and neutral amino acid accumulation in immature rat testis, Int. J. Biochem. Cell Biol. 42 (2010) 1046–1051.
[70] S. Batra, C. Sjögren, Effect of estrogen treatment on calcium uptake by the rat uterine smooth muscle, Life Sci. 32 (1983) 315–319.
[71] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[72] A. Ubbenhorst, S. Striebich, F. Lang, U.E. Lang, Exploring the relationship between vitamin D and basic personality traits, Psychopharmacology (Berl.) 215 (2011) 733–737.
[73] Y. Miyake, K. Tanaka, W. Fukushima, S. Sasaki, C. Kiyohara, Y. Tsuboi, T. Yamada, T. Oeda, T. Miki, N. Kawamura, N. Sakae, H. Fukuyama, Y. Hirota, M. Nagai, Fukuoka Kinki Parkinson's Disease Study Group, Lack of association of dairy food, calcium, and vitamin D intake with the risk of Parkinson's disease: a case–control study in Japan, Parkinsonism Relat. Disord. 17 (2011) 112–116.
[74] S. Balabanova, H.P. Richter, G. Antoniadis, J. Homoki, N. Kremmer, J. Hanle, W.M. Teller, 25-Hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in human cerebrospinal fluid, Klin. Wochenschr. 62 (1984) 1086–1090.
[75] H.J. Bidmon, W.E. Stumpf, Distribution of target cells for 1,25-dihydroxyvitamin D_3 in the brain of the yellow bellied turtle *Trachemys scripta*, Brain Res. 640 (1994) 277–285.
[76] H.J. Bidmon, W.E. Stumpf, Vitamin D target systems in the brain of the green lizard *Anolis carolinensis*, Anat. Embryol. (Berl.) 193 (1996) 145–160.
[77] M. Musiol, W.E. Stumpf, H.J. Bidmon, C. Heiss, A. Mayerhofer, A. Bartke, Vitamin D nuclear binding to neurons of the septal, substriatal and amygdaloid area in the Siberian hamster (*Phodopus sungorus*) brain, Neuroscience 48 (1992) 841–848.
[78] L.D. Brewer, N.M. Porter, D.S. Kerr, P.W. Landfield, O. Thibault, Chronic 1alpha,25-$(OH)_2$ vitamin D_3 treatment reduces Ca^{2+}-mediated hippocampal biomarkers of aging, Cell Calcium 40 (2006) 277–286.
[79] D.W. Eyles, F. Feron, X. Cui, J.P. Kesby, L.H. Harms, P. Ko, J.J. McGrath, T.H. Burne, Developmental vitamin D deficiency causes abnormal brain development, Psychoneuroendocrinology 34 (2009) S247–S257.
[80] F. Marini, E. Bartoccini, G. Cascianelli, V. Voccoli, M.G. Baviglia, M.V. Magni, M. Garcia-Gil, E. Albi, Effect of 1alpha,25-dihydroxyvitamin D_3 in embryonic hippocampal cells, Hippocampus 20 (2010) 696–705.
[81] S.O. Loureiro, P. de Lima Pelaez, L. Heimfarth, D.O. Souza, M. Wajner, R. Pessoa-Pureur, Propionic and methylmalonic acids increase cAMP levels in slices of cerebral cortex of young rats via adrenergic and glutamatergic mechanisms, Biochim. Biophys. Acta 1740 (2005) 460–466.
[82] S.O. Loureiro, L. Heimfarth, B.A. Lacerda, L.F. Vidal, A. Soska, N.G. dos Santos, A.T. de Souza Wyse, R. Pessoa-Pureur, Homocysteine induces hypophosphorylation of intermediate filaments and reorganization of actin cytoskeleton in C6 glioma cells, Cell. Mol. Neurobiol. 30 (2010) 557–568.
[83] D. Steiner, D. Saya, E. Schallmach, W.F. Simonds, Z. Vogel, Adenylyl cyclase type-VIII activity is regulated by G (betagamma) subunits, Cell. Signal. 18 (2006) 62–68.
[84] G.S. Bird, J.W. Putney Jr., Calcium, In: in: G.J. Siegel, R.W. Albers, S.T. Brady, D.L. Price (Eds.), Basic Neurochemistry: Molecular, Cellular and Medical Aspects, Elsevier Academic Press, San Diego, 2006, pp. 379–390.
[85] A.R. de Boland, A. Norman, Evidence for involvement of protein kinase C and cyclic adenosine 3′,5′ monophosphate-dependent protein kinase in the 1,25-dihydroxy-vitamin D_3-mediated rapid stimulation of intestinal calcium transport, (transcaltachia), Endocrinology 127 (1990) 39–45.
[86] T. Nemoto, S. Miyazaki, T. Kanai, T. Maruta, S. Satoh, N. Yoshikawa, T. Yanagita, A. Wada, Nav1.7-Ca^{2+} influx-induced increased phosphorylations of extracellular signal-regulated kinase (ERK) and p38 attenuate tau phosphorylation via

glycogen synthase kinase-3 beta: priming of Nav1.7 gating by ERK and p38, Eur. J. Pharmacol. 640 (2010) 20–28.
[87] P. Geier, M. Lagler, S. Boehm, H. Kubista, Dynamic interplay of excitatory and inhibitory coupling modes of neuronal L-type calcium channels, Am. J. Physiol. Cell Physiol. 300 (2011) C937–C949.
[88] T. Gao, T.S. Puri, B.L. Gerhardstein, A.J. Chien, R.D. Green, M.M. Hosey, Identification and subcellular localization of the subunits of L-type calcium channels and adenylyl cyclase in cardiac myocytes, J. Biol. Chem. 272 (1997) 19401–19407.
[89] J.H. Ko, W.S. Park, Y.E. Earm, The protein kinase inhibitor, staurosporine, inhibits L-type Ca^{2+} current in rabbit atrial myocytes, Biochem. Biophys. Res. Commun. 329 (2005) 531–537.
[90] C.F. Chang, L.M. Gutierrez, C. Mundina-Weilenmann, M.M. Hosey, Dihydropyridine-sensitive calcium channels from skeletal muscle. II. Functional effects of differential phosphorylation of channel subunit, J. Biol. Chem. 266 (1991) 16395–16400.
[91] O. Delbono, M. Renganathan, M.L. Messi, Regulation of mouse skeletal muscle L-type Ca^{2+} channel by activation of the insulin-like growth factor-1 receptor, J. Neurosci. 17 (1997) 6918–6928.
[92] B.D. Johnson, T. Scheuer, W.A. Catterall, Convergent regulation of skeletal muscle Ca^{2+} channels by dystrophin, the actin cytoskeleton, and cAMP-dependent protein kinase, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 4191–4196.
[93] J. Vela, M.I. Pérez-Millán, D. Becu-Villalobos, G. Díaz-Torga, Different kinases regulate activation of voltage-dependent calcium channels by depolarization in GH3 cells, Am. J. Physiol. Cell Physiol. 293 (2007) C951–C959.
[94] Z. Alvin, G.G. Laurence, B.R. Coleman, A. Zhao, M. Hajj-Moussa, G.E. Haddad, Regulation of L-type inward calcium channel activity by captopril and angiotensin II via the phosphatidyl inositol 3-kinase pathway in cardiomyocytes from volume-overload hypertrophied rat hearts, Can. J. Physiol. Pharmacol. 89 (2011) 206–215.
[95] A.C. Dolphin, G protein modulation of voltage-gated calcium channels, Pharmacol. Rev. 55 (2003) 607–627.
[96] P. Viard, A.J. Butcher, G. Halet, A. Davies, B. Nürnberg, F. Heblich, A.C. Dolphin, PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane, Nat. Neurosci. 7 (2004) 939–946.
[97] B. Sutor, H.J. Luhmann, Development of excitatory and inhibitory postsynaptic potentials in the rat neocortex, Perspect. Dev. Neurobiol. 2 (1995) 409–419.
[98] G. Cuesto, L. Enriquez-Barreto, C. Caramés, M. Cantarero, X. Gasull, C. Sandi, A. Ferrús, A. Acebes, M. Morales, Phosphoinositide-3-kinase activation controls synaptogenesis and spinogenesis in hippocampal neurons, J. Neurosci. 31 (2011) 2721–2733.
[99] C. Sun, R. Qi, L. Wang, J. Yan, Y. Wang, p38 MAPK regulates calcium signal-mediated lipid accumulation through changing VDR expression in primary pre-adipocytes of mice, Mol. Biol. Rep. 39 (2012) 3179–3184.
[100] I. Dzhura, Y. Wu, R.J. Colbran, J.R. Balser, M.E. Anderson, Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels, Nat. Cell Biol. 2 (2000) 173–177.
[101] E. Miyamoto, Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus, J. Pharmacol. Sci. 100 (2006) 433–442.