Effects of progesterone on T-type-Ca^{2+}-channel expression in Purkinje cells

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Graphical Abstract *Changes of T-type-Ca^{2+}-channel expression in Purkinje cells after progesterone treatment*
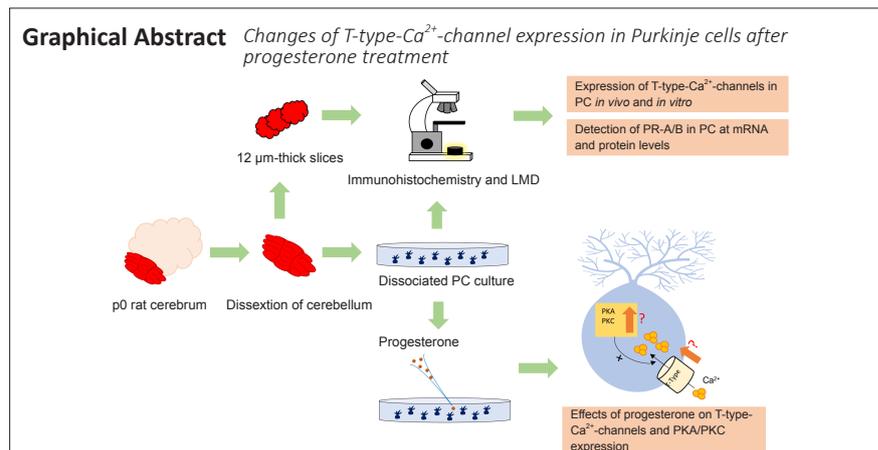
Abstract

Plasticity of cerebellar Purkinje cells (PC) is influenced by progesterone via the classical progesterone receptors PR-A and PR-B by stimulating dendritogenesis, spinogenesis, and synaptogenesis in these cells. Dissociated PC cultures were used to analyze progesterone effects at a molecular level on the voltage-gated T-type-Ca^{2+}-channels Ca$_v$3.1, Ca$_v$3.2, and Ca$_v$3.3 as they helped determine neuronal plasticity by regulating Ca^{2+}-influx in neuronal cells. The results showed direct effects of progesterone on the mRNA expression of T-type-Ca^{2+}-channels, as well as on the protein kinases A and C being involved in downstream signaling pathways that play an important role in neuronal plasticity. For the mRNA expression studies of T-type-Ca^{2+}-channels and protein kinases of the signaling cascade, laser microdissection and purified PC cultures of different maturation stages were used. Immunohistochemical staining was also performed to characterize the localization of T-type-Ca^{2+}-channels in PC. Experimental progesterone treatment was performed on the purified PC culture for 24 and 48 hours. Our results show that progesterone increases the expression of Ca$_v$3.1 and Ca$_v$3.3 and associated protein kinases A and C in PC at the mRNA level within 48 hours after treatment at latest. These effects extend the current knowledge of the function of progesterone in the central nervous system and provide an explanatory approach for its influence on neuronal plasticity.

Key Words: Ca$_v$3.1; Ca$_v$3.2; Ca$_v$3.3; neuronal plasticity; progesterone; protein kinase A; protein kinase C; Purkinje cells; rat cerebellum; T-type-Ca^{2+}-channels

Introduction

Progesterone is primarily known as a sex hormone, playing an important role in the female cycle and pregnancy. It is also synthesized *de novo* from cholesterol in neuronal and glial cells in the female and male central nervous system (Baulieu and Robel, 1990), as well as in the peripheral nervous system (Baulieu, 1997; Schumacher et al., 2012). Functionally, progesterone shows neuroprotective, antidegenerative, anti-apoptotic, and anti-inflammatory effects in the central nervous system (Schumacher et al., 2012; Singh and Su, 2013; De Nicola et al., 2018; Yilmaz et al., 2019; Tsutsui and Haraguchi, 2020). Furthermore, progesterone induces morphological changes in neurons, such as increased synaptogenesis, spinogenesis, and dendritogenesis of Purkinje cells (PC) (Sakamoto et al., 2001; Wessel et al., 2014). These structural changes in PC are mediated by the classical progesterone receptors A and B (PGR), which act via genomic pathways and are responsible for the morphological changes during the neonatal period (Wessel et al., 2014; Theis and Theiss, 2019).

To elucidate the molecular mechanisms of progesterone effects on neuronal cells like outlined above, cerebellar PC is an excellent model. Their large, highly branched, and spiny dendrites are occupied by up to 200,000 synapses per PC (Napper and Harvey, 1988; Kapfhammer, 2004), and they are capable of synthesizing progesterone (Tsutsui and Haraguchi, 2020). In the central nervous system, Ca^{2+} mediating via N-methyl-D-aspartate receptors play a key role in synaptic- and non-synaptic plasticity by activating gene expression and signal transduction of various enzymes and signaling pathways (Neveu

and Zucker, 1996; Higgins et al., 2014; Maggio and Vlachos, 2014). Less well known is that in PC, low-voltage gated T-type-Ca^{2+}-channels are also responsible for Ca^{2+}-homeostasis and neuronal plasticity (Catterall, 2000; Kitamura and Kano, 2013; Aguado et al., 2016; Leresche and Lambert, 2017). Low-voltage gated T-type-Ca^{2+}-channels contain three subtypes named Ca$_v$3.1 (encoded by *CACNA1G*), Ca$_v$3.2 (encoded by *CACNA1H*), and Ca$_v$3.3 (encoded by *CACNA1I*), which are distinguished by their terminal amino acid and depolarize close to the resting membrane potential (Talavera and Nilius, 2006; Lory et al., 2020). All three Ca$_v$3 channels have a specific subcellular distribution. Immunohistochemically, Ca$_v$3.1 and Ca$_v$3.2 were detected in the somata and dendrites of PC, the letter overall weaker. Ca$_v$3.3 had the strongest immunolabel within the cell body and dendritic arbor of PC (Molineux et al., 2006; Hildebrand et al., 2009). The effect of progesterone on T-type-Ca^{2+}-channels has not yet been fully explored, but it is known that progesterone as a neurosteroid alters neuronal and non-neuronal Ca^{2+} currents in an excitatory way, leading to Ca^{2+}-influx, probably by addressing Ca$_v$3-channels (Viero and Dayanithi, 2008; Sun and Moenter, 2010; Strünker et al., 2011; Kapur and Joshi, 2021). Like the expression and functionality of most ion channels, the expression and functionality of T-type-Ca^{2+}-channels are modulated by various signaling mechanisms, like protein kinase A (PKA) (encoded by *PRKACA*) and protein kinase C (PKC), which can be further divided into different subunits, of which the following subunits are expressed in the brain: PKCα (encoded by *PKCA*), PKCβI (encoded by *PRKCB*), PK-ε (encoded by *PRKCE*) and PKCζ (encoded by *PRKCZ*) (Pemberton et al., 2000; Metzger and Kapfhammer, 2003; Perez-Reyes, 2003; Park et al., 2006). It is known that PKC phosphorylates PGR and

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induces its transcriptional activity, resulting in protein-protein interaction (Marquina-Sánchez et al., 2017; Arcos-Montoya et al., 2021; Valdés-Rives et al., 2021).

The aim of the present study was to verify the expression and colocalization of the three T-type-Ca^{2+}-channels in the cerebellum and PC at different stages of maturation using immunohistochemistry. In addition, laser microdissection (LMD) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were used to analyze the mRNA expression of these channels before investigating the direct effect of progesterone on the mRNA expression of *CACNA1G*, *CACNA1H* and *CACNA1I*, as well as on the extra-nuclear PKA and PKC in dissociated PC cultures.

Materials and Methods

Animals and surgical procedures
The rats are from the own breeding in the animal house of the Ruhr-University Bochum and were kept in a 12-hour light/dark cycle and had access to food and water *ad libitum*. As described in detail by Pieczora et al. (2017), cerebellar samples were obtained under sterile and RNase-free conditions from male and female Wistar rat pups at the day of birth (p0), the 9th postnatal day (p9), and the 30th postnatal day (p30) (Pieczora et al., 2017). The rats were decapitated and the skull was opened with sterile and RNase-free instruments. Binocular microscopy (MuellerOptronic, Erfurt, Germany) was used to isolate the cerebellum from the forebrain and the brainstem; the pia mater and superficial blood vessels were removed. When cerebella were not used directly for PC dissection, they were snap-frozen in liquid nitrogen-cooled isopentane and stored at –80°C. All procedures were carried out under established standards of the German federal state of North Rhine-Westphalia and in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes. According to current German and European legislation, the removal of organs or cells from vertebrates for scientific purposes is not considered an animal experiment if the animals have not been subject to surgical interventions or invasive treatments prior to sacrifice. Thus, decapitation of rats intended for the removal of brain/cerebellum does not need an approval or permission by local or governmental authorities.

Laser microdissection
Cryosections of p9 and p30 rat cerebella were prepared. To prevent contamination with RNases, removable parts of the cryostat were cleaned with a solution of 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M NaOH in diethyl pyrocarbonate (DEPC)-treated water. Cryosections (12 µm thickness) of rat cerebella were obtained using a cryostat (Leica microsystems CM3050 S Cryostat, Leica, Wetzlar, Germany), applied to RNase-free polyethylene naphthalate (PEN)-membrane slides (#11505189, Leica), and dried at 40°C. Five cerebellar cryosections were placed on each PEN-membrane slide. Staining was performed with 1% methylene blue, 1% azure blue II, and 1% borax in DEPC-treated water. Briefly, a few drops of the dye were applied to the cryosections and immediately removed before the samples were washed with DEPC-treated water. The sections were air-dried and stored at –80°C.

Cells were microdissected using the LMD6500 system (Leica microsystem; Pieczora et al., 2017). In each session, 1,000,000 µm^2 of lasered PC were collected in 20 µL of lysis solution (AM1931, Invitrogen, Darmstadt, Germany) and stored at –80°C. A total of 4,000,000 µm^2 (~8000 cell somata) of PC were collected from four different cerebella of p9 and p30 Wistar rats. The protocol of the RNAqueous-Micro Total RNA Isolation Kit procedure for LCM (AM1931, Ambion, Austin, Texas, USA) was used to extract total mRNA from the PC excised from the cerebellum. The lasered samples were incubated in 100 µL of lysis solution for 30 minutes at 42°C, vortexed, and then centrifuged. Then the microfilter cartridge was pre-wetted with 30 µL lysis solution for 5 minutes, 3 µL of LCM additive was added to the cell solution and vortexed. After 129 µL of 100% EtOH was mixed with the cell solution to recover large and small RNAs, the solution was filtered through the microfilter cartridge. After several washing steps, the RNA was eluted by applying 10 µL of elution solution followed by the same centrifugation steps, that are described in the following section of mRNA isolation. Finally, the samples were stored at –80°C.

Dissociated PC cultures
The method for preparation of highly purified PC cultures from p0 Wistar rat cerebella was performed as previously described (Tjaden et al., 2018). In brief, cerebella were prepared under sterile conditions, digested, triturated, and transferred to a Percoll-gradient to separate large neurons and the glial cell fraction from other cells (e.g., small neurons and fibroblasts). Subsequently, non-neuronal cells were removed by immunoadsorption onto anti-GD3-coated dishes. Then, PC were purified by immunoadsorption on mouse anti-Thy1.1- (MAB1604, Merck KGaA, Darmstadt, Germany, RRID: AB_94105) coated dishes. Afterwards, non-adherent cells were rinsed off and adherent PC were plated in 96-well plates with 100,000 cells per well and incubated at 37°C in serum-free medium containing 6.5 mL Basal Medium Eagle stock, 0.5 mL L-glutamine, 2.4 mL 10% glucose, 100 µL 100× penicillin-streptomycin, 0.5 mL serum-free supplement, and 0.5 g bovine serum albumin (BSA) (fraction V; A-9418, MilliporeSigma). The medium was replaced every other day (Tjaden et al., 2018). The stimulated group of PC was incubated for 24 and 48 hours with 10 nM progesterone (MilliporeSigma) according to the physiological progesterone levels (**Figure 1**; Hernandéz-Hernandéz et al., 2010; Wessel et al., 2014; González-Orozco and Camacho-Arroyo, 2019).
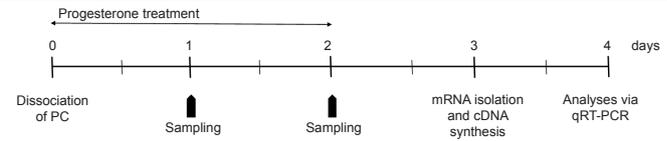

Figure 1 | **Experimental timeline.**

mRNA isolation
To obtain total RNA of cultured PC, we used the NucleoSpin® miRNA isolation kit (No. 740304, Macherey-Nagel, Düren, Germany) and followed the manufacturer's protocol for animal tissue and cultured cells. Briefly, our samples containing 100,000 PC were resuspended in 300 µL of lysis buffer (ML) and incubated for 5 minutes at room temperature. After incubation, 150 µL of 100% EtOH was added and immediately vortexed for 5 seconds. Samples were loaded onto the NucleoSpin RNA column and centrifuged at 11,000 × *g* at room temperature for 1 minute. The flow-through was kept for later steps. Membrane desalting buffer (MDB, 350 µL) was added to the column and after another centrifugation (see above), 100 µL of rDNase was added directly to the membrane of the column and incubated for 15 minutes at room temperature. Protein precipitation (MP, 300 µL) buffer was added to the saved flow-through, vortexed for 5 seconds, and centrifuged at 11,000 × *g* for 3 minutes. The supernatant was carefully poured onto a NucleoSpin protein removal column and centrifuged at 11,000 × *g* at room temperature for 1 minute. To the flow-through, 800 µL of binding buffer (MX) was added to set the binding conditions for small RNA. Now samples were loaded onto the column incubated with rDNase and briefly centrifuged. Finally, the RNA column was washed in several steps with wash buffers (MW1 and MW2) and the total RNA was eluted in 30 µL nuclease-free water to achieve a high RNA concentration and stored at –80°C.

cDNA synthesis
The cDNA synthesis was performed with qScript cDNA SuperMix (95048-100, Quantabio, Beverly, MA, USA). For this purpose, 10 µL of undiluted total RNA containing 500 ng of total RNA was mixed with 4 µL qScript cDNA SuperMix and 6 µL DEPC water. These 20 µL samples were incubated according to the protocol for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C and cooled to 4°C in the C1000^{TM} Thermal cycler (Bio-Rad, Hercules, CA, USA) and stored at –20°C.

qRT-PCR
All analyses were done in triplicates using GoTaq® qPCR Master Mix (no. A6001, Promega, Walldorf, Germany). To achieve an amount of 200 ng/µL of cDNA per well, 4 µL of the cDNA was mixed with 3.2 µL ddH$_2$O, 1.4 µL upstream and 1.4 µL downstream primer and 10 µL of the Promega Master Mix and pipette into a Hard-Shell®PCR 96-well plate (96, skirted well plate, 4titude). After pipetting, the plate was sealed with Microseal® "B" seal foil (no. MSB1001, Bio-Rad, Hercules, CA, USA). The primers shown in **Table 1** are all obtained from Microsynth (Balgach, Switzerland).

qRT-PCR was performed using a CFX Connect^{TM} Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Samples were heated to 95°C for 2 minutes for polymerase activation and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C were set for amplification. In the end, samples were heated to 65°C for 300 seconds for dissociation. The 2$^{-\Delta\Delta Ct}$ method was used to analyze the relative mRNA expression (Zhang et al., 2015), with all C*t*-values normalized against the C*t*-values of the reference gene *GAPDH*.

Immunohistochemistry
Immunohistochemistry was performed using purified rat PC (48 hours *in vitro*) and cryosections of p0 and p9 rat cerebella. After fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes and thorough washing with PBS (2 × 2 minutes and 2 × 10 minutes), cells were permeabilized with 0.25% Triton (MilliporeSigma, T8532) in PBS for 10 minutes. Immunostaining of 12-µm-thick cryosections was performed on SuperFrost Plus Adhesion slides (Thermo Fisher Scientific, Waltham, MA, USA, J1800AMNZ). The cerebella were fixed overnight with 4% paraformaldehyde and for 2 days with 30% saccharose before being frozen and embedded in tissue-freezing medium (No. 14020108926, Leica). As the first step of immunohistochemical labeling, samples were permeabilized with 1% Triton in PBS for 15 minutes. To block non-specific binding sites, the samples were then treated with 10% goat serum (1:50 in PBS, MilliporeSigma; G9023) for 20 minutes. After intensive washing of the samples with PBS (2 × 2 minutes plus 2 × 10 minutes), the primary mouse anti-calbindin (marker for PC) antibody (1:600 in PBS, MilliporeSigma; C9848, RRID: AB_476894) was applied at 4°C overnight. The next day, after thorough washing with PBS (2 × 2 minutes plus 2 × 10 minutes), the samples were incubated with goat anti-mouse tetramethylrhodamine (TRITC) antibody (1:1000 in PBS T5393, MilliporeSigma; RRID: AB_261699) at room temperature for 2 hours. After washing with PBS (2 × 2 minutes plus 2 × 10 minutes), samples were additionally incubated with the following antibodies against T-type-Ca^{2+}-channels at 4°C overnight: rabbit anti-Cav3.1 (1:1500 in PBS, Alomone Labs, Jerusalem, Israel; ACC21, RRID: AB_2039779); rabbit anti-Cav3.2 (1:1500 in PBS, Alomone Labs, ACC25, RRID: AB_2039781) or rabbit anti-Cav3.3 (1:1000 in PBS, Alomone Labs; ACC009, RRID: AB_2039783). On the 3rd day, after thorough washing with PBS, incubation of the secondary donkey anti-rabbit antibody was followed at room

Table 1 | Primers and sequences used in qRT-PCR

Name	Sequence (5'–3')
GAPDH*	F: ACT CCC ATT CTT CCA CCT TTG
	R: CCC TGT TGC TGT AGC CAT ATT
CACNA1G	F: GTC ATT TGC TGT GCC TTC TTC
	R: TGT TAG TGA TGT TCC TGG TGT C
CACNA1H	F: CTT CAT CTT CGG CAT TGT TGG
	R: CCT CCT CCG TCT GGT AGT AT
CACNA1I	F: AGC CTG TCA CTC ACA TCT CT
	R: TAC TGC TGA ACT TCC TGG CT
PRKACA	F: CAC TTA CGG CGG ATT GGG AG
	R: TCC CGG TAG ATG AGG TCC AG
PRKCB	F: ATG ACC AAA CAC CCA GGC AA
	R: TGG CTG AAT CTC CTT GCG TT
PKCA	F: AAT CTG GGA CTG GGA TCG GA
	R: ATC CAC TGG CTG GCA TCT TC
PRKCE	F: TCC CCT TGT GAC CAG GAA CT
	R: GCC ATC AGT AGA CGA CGA GG
PRKCZ	F: GAA AGG ATA TGG GGC CTC GC
	R: GAG GAC GTG GCA GCG TTT AT
PGR	F: AGC ATG TCA GTG GAC AGA TG
	R: TAA GGC ACA GCG AGT AGA ATG
Calbindin	F: GAA GGA AAG GAG CTG CAG AA
	R: TCT GCC CAT ATT GAT CCA CAA A
GFAP	F: GAG TGG TAT CGG TCC AAG TTT
	R: TTG GCG GCG ATA GTC ATT AG
NeuN	F: GAG AAG CTG AAT GGG ACG AT
	R: CAT ATG GGT TCC CAG GCT
Vglut1	F: CCT GCG CAG TCG TCA TAT AAT
	R: GCC CTT GGA GTG TGA GTA TC
Alpha GABAR-6	F: CCG ATG AGA CTG GTT AAC TTC C
	R: TCT TCT GGG ACC TCT ACT GAA T

* GAPDH was used as a reference gene. F: Forward; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; NeuN: neuronal nuclei; PGR: classical progesterone receptors A and B; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; R: reverse.

temperature for 2 hours (1:1000 in PBS, Thermo Fisher Scientific; AF488, RRID: AB_2535792). Finally, staining with Hoechst 33342 trihydrochloride (1:1000 in PBS, Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany, B2261) was performed for 20 minutes before the samples were rinsed with PBS and covered with Fluorshield™ (MilliporeSigma, F6937) on microscope slides (76 mm × 26 mm, Thermo Fisher Scientific).

Statistical analysis
Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) were used for statistical analysis. An unpaired two-tailed t-test was performed to prove statistical significance between the relative expression levels of mRNAs. $P < 0.05$ was accepted as statistically significant.

Results

T-type-Ca^{2+}-channel expression in dissociated and cerebellar PC
In a first step, the colocalization of T-type-Ca^{2+}-channels and PC was characterized by immunohistochemistry in p0 (**Figure 2A**) and p9 (**Figure 2B**) cerebellar slice cultures and dissociated PC cultures (**Figure 2C**). All three T-type-Ca^{2+}-channels were expressed in calbindin-positive PC. In the neonatal stage p0, PC in the cerebellum appeared as tightly grouped cell clusters surrounded by an internal and external granular cell layer (**Figure 2A**). Here, T-type-Ca^{2+}-channels could mainly be detected in the PC layer (PCL) at the somata of the PC and the internal granular cell layer. In p9, the PC dendrites had already spread into the molecular layer (MCL), so the expression of the T-type-Ca^{2+}-channels was visible in the somata of the PC in the PCL and along the dendrites of the PC in the MCL (**Figure 2B**). After dissociated PC were purified and cultured for 2 days (p0 + 2div) (**Figure 2C**), Ca$_v$3.1, Ca$_v$3.2, and Ca$_v$3.3 were detectable both in the soma and along the dendrites, whereby Ca$_v$3.3 was strongest expressed in the soma (**Figure 2Cc**).

Expression of *CACNA1G*, *CACNA1H*, and *CACNA1I* in microdissected PC
In a second step, T-type-Ca^{2+}-channel mRNA expression during maturation was studied in laser microdissected p9 and p30 PC (**Figure 3**). The PC in tissue younger than that could not be laser dissected due to the unclear allocation of the PC in this tissue. At p9, four distinct layers could be localized in the cerebellar cortex, external granular cell layer, MCL, PCL, and the internal granular cell layer (**Figure 3A**). At p30, the cells of the outer granular layer have migrated through the MCL and PCL into the internal granular cell layer, so that a three-layered morphology with MCL, PCL, and GCL on the inner side was visible (**Figure 3A**). For both maturation stages, qRT-PCR analysis showed that PC could be isolated precisely using LMD, to further analyze the

colocalization of T-type-Ca^{2+}-channels and single PC on mRNA level. This is underlined by the strong expression of PC-specific marker calbindin compared with markers for glial and other neuronal cells as analyzed via qRT-PCR (**Figure 3B**). At p9 78.21% and at p30 64.90% of the cells were attributable to PC, while other cell markers for glial cells and neurons other than PC were only detectable at low levels in the LMD samples (p9: glial fibrillary acidic protein (GFAP; marker for astrocytes) = 14.44%, neuronal nuclei (NeuN; marker for neuronal cells other than PC (Mullen et al., 1992)) = 3.18%, Vglut1 (marker for glutamatergic neurons (Lin et al., 2017)) = 2.78%, alpha-GABAR-6 (marker for cerebellar granule cells (Wisden, 1995)) = 0.97%; p30: GFAP = 3.36%, NeuN = 4.51%, Vglut1 = 14.82%, alpha-GABAR-6 = 12.42%; **Figure 3B**). To verify the mRNA expression pattern of T-type-Ca^{2+}-channels in PC, qRT-PCR analysis of the LMD samples was performed (**Figure 3C**). The results showed that *CACNA1G*, *CACNA1H*, and *CACNA1I* were present in PC in the p9 and p30 samples (*CACNA1G*: p9 = 1.00 ± 0.04, p30 = 1.40 ± 0.02; *CACNA1H*: p9 = 1.00 ± 0.04, p30 = 0.54 ± 0.02; *CACNA1I*: p9 = 1.01 ± 0.13, p30 = 1.28 ± 0.03). *CACNA1H* mRNA expression showed a slight decrease at p30 compared with p9, while *CACNA1G* and *CACNA1I* showed an increase in expression between p9 and p30.

Progesterone influences the mRNA expression of T-type-Ca^{2+}-channels in PC
Before taking a closer look at the effect of progesterone on T-type-Ca^{2+}-channels in PC, the expression of *PGR* in the purified and cultured p0 PC was analyzed via relative mRNA expression analysis with the help of qRT-PCR (**Figure 4A**) and immunohistochemistry (**Figure 4B**). *PGR* was expressed in p0 (0 hours) PC and after 1 div (24 hours) and with a decrease in mRNA expression after 2 div (48 hours) (**Figure 4A**). Immunohistochemically, PGR, shown in green can be detected around the soma and the dendrites of dissociated PC (p0 + 2 div), marked with anti-calbindin in red (**Figure 4B**).

Subsequently, the effects of 24- and 48-hour progesterone treatment (10 nM) on the expression of T-type-Ca^{2+}-channels in purified and cultured PC (p0 + 2 div) were investigated (**Figure 4C**). Possible effects of progesterone could unfortunately not be performed on older PC because they did not survive the processes of isolation, purification, and cultivation (Tjaden et al., 2018).

However, the experiments performed with p0 PC showed that progesterone affects T-type Ca^{2+} channels in different ways, such that both downregulation and upregulation are present at both observed time points. *CACNA1G* mRNA was initially down-regulated after 24 hours of progesterone treatment but showed a significant increase after 48 hours of stimulation with 10 nM progesterone ($P < 0.05$). In contrast, *CACNA1H* mRNA levels were significantly down-regulated after 24 and 48 hours of progesterone treatment ($P < 0.01$). *CACNA1I* mRNA levels were significantly up-regulated after 24 and 48 hours of progesterone treatment ($P < 0.01$).

Progesterone increases the mRNA expression of various protein kinases
Next, we investigated whether progesterone additionally affects the expression of the protein kinase A (PKA), which is encoded by the *PRKACA* gene and α-(PKCA), β-(PRKCB), ε-(PRKCE), ζ- subunits (PRKCZ) of PKC in PC. Again, the isolated, purified, and cultured PC were incubated with 10 nM progesterone for 24 and 48 hours. qRT-PCR analysis showed that all above-mentioned protein kinases increased significantly after progesterone treatment (**Figure 5**). Specifically, *PRKACA* mRNA showed a significant increase after progesterone incubation, notably within 24 hours ($P < 0.05$). The mRNA level of *PKCA* and *PRKCB* was also significantly up-regulated after 24 and 48 hours of progesterone treatment ($P < 0.05$ or $P < 0.001$). A highly significant up-regulation of *PRKCE* and *PRKCZ* mRNA expression was also detected after 24 and 48 hours of treatment with 10 nM progesterone ($P < 0.01$ or $P < 0.001$; **Figure 5**).

## Discussion

Co-localization of Ca$_v$3 channels in PC was demonstrated both *in vivo* and *in vitro*
LMD is a very elegant method for analyzing microdissected PC. However, a technical limitation was that only single-cell somata could be lasered out of the fixed tissue, with no possibility for further studies on dendrites of the PC, where the T-type-Ca^{2+}-channels are also located. Nevertheless, since immunohistochemistry of the studied T-type-Ca^{2+}-channels in the *in vivo* models did not differ from *in vitro* models, as co-localization was shown in both cerebellar slice cultures and LMD dissected PC, as well as in cultured PC, then the dissociated PC model was used. In the next step, the mRNA expression of T-type-Ca^{2+}-channels and their regulating protein kinases and the influence of progesterone were studied. T-type-Ca^{2+}-channels and their regulating protein kinases PKA and PKC are of high interest for Ca^{2+}-homeostasis and neuronal plasticity. It is known that neuronal cells benefit from interactions and cell-cell contacts in neuronal networks. Granule and glia cells have a major influence on the regulation of PC development via synaptic contacts and neurotrophic signaling (Seil, 2001; Yamada and Watanabe, 2002; Willett et al., 2019). The Ca^{2+}-influx into PC, regulated by synaptic activity with the subsequent regulation of gene expression, is a major player in neuronal and synaptic plasticity (Han et al., 2007; Yang and Lisberger, 2014; Lin et al., 2017; Mateos-Aparicio and Rodríguez-Moreno, 2020). In summary, a disadvantage is that dissociated and purified PC somehow shows deprivation symptoms. Nevertheless, this model offers the possibility to investigate the influence of progesterone with no other neurons or glial cells influencing the metabolic processes (Tjaden et al., 2018).
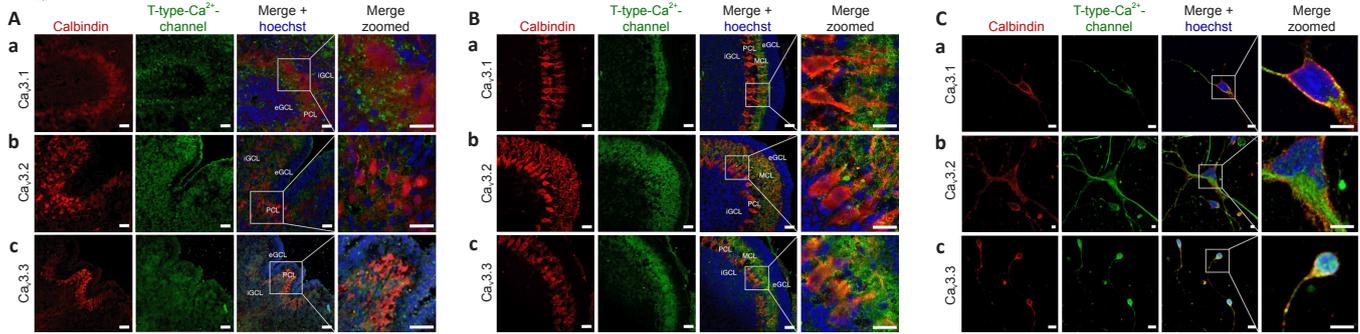
Figure 2 | Expression of T-type-Ca²⁺-channels in p0 (A) and p9 (B) PC of the cerebellum and dissociated PC (C).
(A, B) Immunostaining of cryosections (12 μm in thickness) of rat cerebellum with antibodies against calbindin (red) and Caᵥ3.1 (a), Caᵥ3.2 (b), and Caᵥ3.3 (c) in green. Cell nuclei are counterstained with Hoechst 33342 in blue. Scale bars: 20 μm. (C) Immunostaining of cultured PC (p0 + 2div) with antibodies against calbindin in red and Caᵥ3.1 (a), Caᵥ3.2 (b), and Caᵥ3.3 (c) in green. Cell nuclei are counterstained with Hoechst in blue. Scale bars: 5 μm. eGCL/iGCL: External/internal granular cell layer; MCL: molecular cell layer; p0: The day of birth; p9: the 9ᵗʰ postnatal day; PC: Purkinje cell; PCL: Purkinje cell layer.
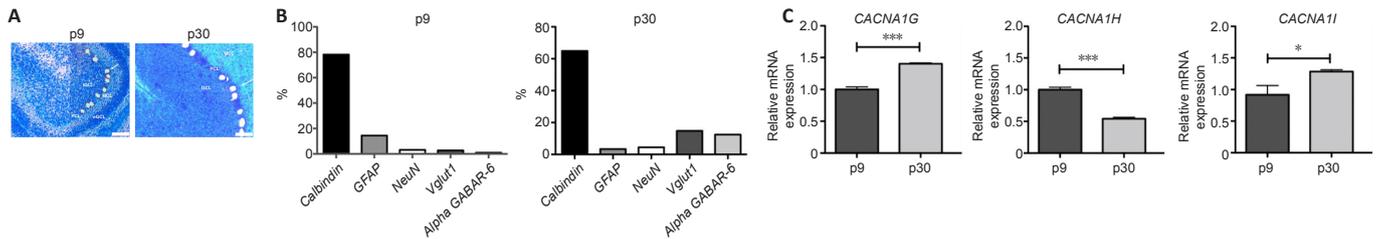
Figure 3 | Expression of T-type-Ca²⁺-channels in laser microdissected PC.
(A) Methylene blue-stained cryosections (12 μm in thickness) of p9 and p30 rat cerebella. White arrows mark microdissected PC. Scale bars: 100 μm. (B) Analysis of purity of microdissected PC via qRT-PCR based on mRNA expression of PC-specific markers (*Calbindin*), other neural cells (*NeuN*, *alpha GABAR-6*, and *Vglut1*) and astrocytes (*GFAP*) at the 9ᵗʰ postnatal day (p9) and the 30ᵗʰ postnatal day (p30) with GAPDH as a reference gene. All values were added and the percentage of each marker was calculated by estimating the portion of the total; *n* = 3. (C) Relative mRNA expression of *CACNA1G*, *CACNA1H*, and *CACNA1I* in microdissected PC at p9 and p30 normalized to GAPDH. The analysis was performed by qRT-PCR. Data are shown as the mean ± SEM; *n* = 4; statistical analyses with unpaired two-tailed *t*-test; significance level: *P*(*CACNA1G*) = 0.0006, *P*(*CACNA1H*) = 0.0004, *P*(*CACNA1I*) = 0.0481. **P* < 0.05, ****P* < 0.001. *n* = numbers of repetition of the experiment. eGCL/iGCL: External/internal granular cell layer; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GCL: granular cell layer; GFAP: glial fibrillary acidic protein; MCL: molecular cell layer; NeuN: neuronal nuclei; PC: Purkinje cell; PCL: Purkinje cell layer; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.
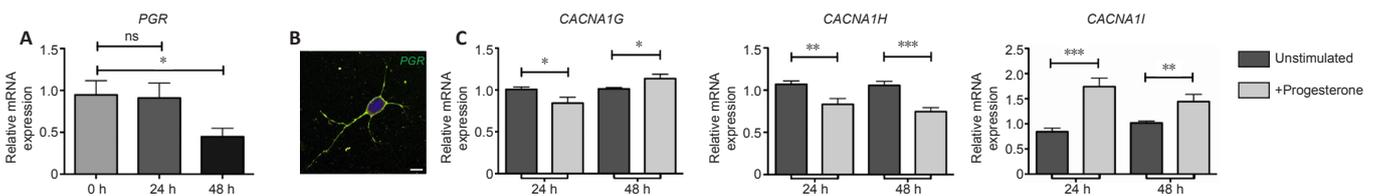
Figure 4 | Expression of PGR in PC (A, B) and the effect of progesterone on T-type-Ca²⁺-channel mRNA expression in dissociated PC cultures (C).
(A) Analysis of *PGR* mRNA expression in dissociated PC culture p0 (0 hours), 1 div (24 hours) and 2 div (48 hours) by quantitative reverse transcription-polymerase chain reaction with *GAPDH* as reference genes. Data are provided as the mean ± SEM; statistical analyses with unpaired two-tailed *t*-test. Significance level, p0–1div: not significant, 1–2div: *P* = 0.0301; *n* = 3. (B) Immunohistochemistry of dissociated PC p0 + 2div, anti-PGR is shown in green, anti-calbindin in red, nuclear staining was done with Hoechst 33342 in blue. Scale bar: 10 μm. (C) After incubation of dissociated p0 PC with 10 nM progesterone for 24 and 48 hours, the relative mRNA expression pattern of *CACNA1G*, *CACNA1H*, and *CACNA1I* normalized to GAPDH was evaluated; *n* = 4. Data are provided as the mean ± SEM; unpaired two-tailed *t*-test was performed for statistical analyses. Significance level: *P*(*CACNA1G* 24 hours) = 0.0246, *P*(CACNA1G 48 hours) = 0.0115, *P*(*CACNA1H* 24 hours) = 0.0063, *P*(*CACNA1H* 48 hours) = 0.0001, *P*(*CACNA1I* 24 hours) = 0.0006, *P*(*CACNA1I* 48 hours) = 0.0089. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *n* = numbers of repetition of the experiment. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PGR: classical progesterone receptors A and B; PC: Purkinje cell.
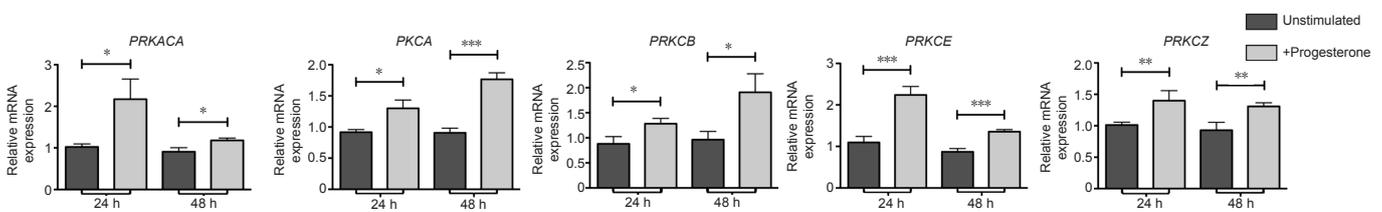
Figure 5 | Effects of progesterone on the mRNA expression of protein kinases.
Relative mRNA expression pattern of the protein kinases *PRKACA*, *PKCA*, *PRKCB*, *PRKCE*, and *PRKCZ* normalized against *GAPDH* in dissociated p0 PC-cultures after treatment with 10 nM progesterone for 24 and 48 hours compared with unstimulated controls (quantitative reverse transcription-polymerase chain reaction); *n* = 4. Data is shown as the mean ± SEM; statistical analyses with unpaired two-tailed *t*-test. Significance level: *P*(PRKACA 24 hours) = 0.0487, *P*(PRKACA 48 hours) = 0.021, *P*(PKCA 24 hours) = 0.0151, *P*(PKCA 48 hours) = 0.0001, *P*(PRKCB 24 hours) = 0.0343, *P*(PRKCB 48 hours) = 0.0294, *P*(PRKCE 24 hours) = 0.0004, *P*(PRKCE 48 hours) = 0.0006, *P*(PRKCZ 24 hours) = 0.0053, *P*(PRKCZ 48 hours) = 0.0036. *n* = numbers of repetition of the experiment. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PC: Purkinje cell; PGR: classical progesterone receptors A and B.

Influence of progesterone on mRNA level of T-type-Ca²⁺-channels in PC

Voltage-gated Ca^{2+}-channels play a key role in regulating intracellular Ca^{2+}-levels and triggering various processes such as gene expression, synaptic plasticity, and the secretion of hormones and neurotransmitters (Perez-Reyes, 2003; Wheeler et al., 2012; Bannister and Beam, 2013; Catterall et al., 2013; Zamponi et al., 2015). T-type-Ca^{2+}-channels are activated at low membrane depolarization and the different subtypes differ in their voltage-dependence and kinetic properties. While $Ca_v3.1$ and $Ca_v3.2$ produce large and transient Ca^{2+}-currents with short burst firing, which corresponds to the typical signature of native neuronal T-currents, the fast deactivation kinetics of $Ca_v3.3$ generates more transient currents during one action potential and indicates small and fast inactivating Ca^{2+} currents (Lory et al., 2020). These isotype-specific gating properties lead to different channel behaviors during neuronal activity, which guarantees constant signal processing and transmission in PC (Klöckner et al., 1999; McRory et al., 2001; Chemin et al., 2002; Engbers et al., 2013). T-type-Ca^{2+}-channels are primarily regulated by changes in membrane potential, but other factors such as hormones and neurotransmitters acting through signaling intermediaries like PKA and PKC additionally regulate channel activity and expression (Pemberton et al., 2000; Kim et al., 2006; Chemin et al., 2007; Hildebrand et al., 2009; Hu et al., 2009). The present study showed for the first time that progesterone affects the mRNA expression of T-type-Ca^{2+}-channels in PC by increasing the mRNA level of *CACNA1G* and *CACNA1I*, while the mRNA level of *CACNA1H* is decreased after treatment with progesterone. Whether this also has an impact on channel expression *in vivo* and whether the different kinetics of the channels have an impact on PC can only be speculated and should be investigated in future studies. So far only studies in non-neuronal cells could show an interaction of progesterone with Ca_v3-channels (Strünker et al., 2011).

Impact of progesterone on protein kinases

Protein kinases act as signal transducers and are part of multiple regulatory processes concerning cell development and are also involved in the processes of learning and maintenance of memory (Shobe, 2002; Snyder et al., 2005; Borodinova et al., 2017; Bennison et al., 2020). PKA and PKC are both parts of the serine/threonine kinase family (Turnham and Scott, 2016). While PKA is a cAMP-dependent protein kinase encoded by the *PRKACA* gene (Taylor et al., 2012), PKC is divided into a conventional, novel and atypical isoenzymes differing in their sensitivity (Newton, 2010). The four conventional isoenzymes include PKCα, PKCβI, PKCβII, and PKCγ, they are sensitive to the second messenger diacylglycerol or Ca^{2+}. The four novel isoenzymes include PKCδ, -ε, -η, and -θ, being sensitive only to diacylglycerol. While PKCζ and -ι are considered atypical protein kinases, which are controlled by protein-protein interactions via a specific ligand-binding site (Newton, 2018). Our study refers to the protein kinases PKA (encoded by *PRKACA*), PKCα (encoded by *PKCA*), PKCβI (encoded by *PRKCB*), PK-ε (encoded by *PRKCE*) and PKCζ (encoded by *PRKCZ*), as they are known to be expressed in neuronal tissue in rat brain (Metzger and Kapfhammer, 2003). The present study showed that progesterone positively influences the mRNA expression of all protein kinases examined. Further investigations to confirm the resulting increased activity of the kinases would be desirable. Any ways with increased PKA-/PKC-level, a higher reserve pool that could be phosphorylated and activated is available here.

The effects of various protein kinases on T-type-Ca^{2+}-channels have been studied intensively but some of them remain controversial. Older studies showed no effect or inhibition of PKC on T-type-Ca^{2+}-channels (Schroeder et al., 1990; Tseng and Boyden, 1991). In contrast, more recent studies show an increased Ca^{2+}-influx in the three T-type-Ca^{2+}-channels mediated by PKC and PKA (Pemberton et al., 2000; Kim et al., 2006; Park et al., 2006).

Based on our results and in the context of literature, we assume that progesterone is capable to influence neuronal plasticity via increasing the expression of different T-type-Ca^{2+}-channels and their regulating enzymes. This cross-talk between PGR and PKC in glioblastomas and an interaction between PKA and PKC and progesterone levels in extra-neuronal cells have already been described previously (Marquina-Sánchez et al., 2017; Nemer et al., 2018; Arcos-Montoya et al., 2021; Valdés-Rives et al., 2021). We hypothesize that progesterone increases the expression of $Ca_v3.3$ and $Ca_v3.1$ and additionally alters the activation of all three T-type-Ca^{2+}-channels by higher levels of PKA and PKC presumably associated with increased activity as well as the increased expression of the Ca_v3-channels on protein level, which should be confirmed in further experiments. Park et al. (2006) have shown that increased channel activation by PKC is not necessarily accompanied by an increased density of T-type-Ca^{2+}-channels on the surface. Progesterone can thus lead to increased functionality of the T-type-Ca^{2+}-channels via up-regulation of PKA and PKC, assuming it is accompanied by increased activity of the kinases, which would lead to higher Ca^{2+}-influx without additional increase in the mRNA levels of the Ca_v3 channels.

The current work focused on a subset of possible T-type-Ca^{2+}-channels regulating protein kinases. Further studies could include the effect of progesterone on tyrosine kinases or calmodulin-dependent protein kinase II and their phosphorylation behavior, factors also discussed in the context of activating T-type-Ca^{2+}-channels (Wolfe et al., 2002, 2003; Welsby et al., 2003; Kim et al., 2006; Park et al., 2006; Asmara et al., 2017).

Progesterone as a medical treatment in neuronal diseases

Progesterone is known to have various neuroprotective, neuroregenerative, and antidegenerative effects in the peripheral and central nervous system (Kim et al., 2012; Kipp et al., 2012; Guennoun, 2020). Increased myelination, restored skin innervation density and other biochemical changes such as normalization of Na^+-K^+-ATPase are responsible for the neuroregenerative effects of progesterone (De Nicola et al., 2006; Leonelli et al., 2007; Roglio et al., 2008; Ghoumari et al., 2020). It also exerts neuroprotective effects by reducing edema, oxidative stress, apoptosis, and inflammation (Schumacher et al., 2007; Stein et al., 2008; De Nicola et al., 2018). This study brings up new considerations to the way progesterone works neuroregenerative as channelopathies and inappropriate Ca^{2+}-entry via T-type-Ca^{2+}-channels have been associated with neuronal diseases such as epilepsy and neuropathic pain (Nelson et al., 2006; Heron et al., 2007; Jagodic et al., 2007; Belardetti and Zamponi, 2008; Lory et al., 2020). Here a better understanding of signaling pathways and regulatory mechanism of T-type-Ca^{2+}-channels will help to understand the pathophysiology of various neuronal diseases to possibly develop new therapeutic approaches. This study showed that progesterone is probably involved in the regulation of Ca^{2+}-homeostasis of neuronal cells as it has an impact on the mRNA expression of T-type-Ca^{2+}-channels and the expression of their regulating protein kinases. This is new therapeutic approaches to discuss the roles of progesterone. In this regard, protein kinases are not only relevant by regulating T-type-Ca^{2+}-channels, but also because they are integral components of the regulation of neuronal growth and synaptic transmission. It has been shown that PKA and PKC as "master regulators of neuritogenesis and therapeutic targets for axon regeneration" are crucial for neurite formation and increased activity of PKA leads to a tripled percentage of neurites (Shea et al., 1995; Xu et al., 2012; Bennison et al., 2020). Nevertheless, the exact effects of progesterone on neuronal Ca2+-homeostasis and on the activity of PKA and PKC need further research, especially with a focus on the electrophysiological level.

Conclusion

This study can provide an explanatory approach for the effects of progesterone on the plasticity of PC by analyzing the mRNA expression of molecules known for their important roles in neuronal plasticity. Here, progesterone regulates the gene expression of T-type-Ca^{2+}-channels. It affects the mRNA expression of *CACNA1I* within 24 hours and of *CACNA1G* within 48 hours of progesterone treatment. Also, progesterone probably regulates the function of T-type-Ca^{2+}-channels via up-regulation of the expression of protein kinases. In addition, mRNA expression of PKA and PKC is significantly increased by progesterone, leading to the hypothesis that this increase results in enhanced Ca^{2+} entry through the three T-type-Ca^{2+}-channels (**Figure 6**). The exact molecular mechanisms of the impact of progesterone need further study. Unfortunately, our study is limited to the mRNA level, studies on protein level and electrophysiological studies could help verify the exact effects of progesterone on the calcium channels.
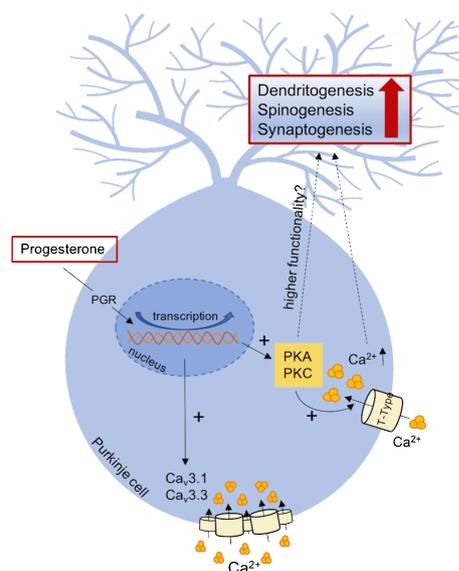
Figure 6 | Schematic presentation of the hypothesis on the effects of progesterone on T-type-Ca²⁺-channels in Purkinje cells.
Progesterone diffuses into PC where it influences gene expression in the nucleus via the classical PGR. It causes an increased expression of PKA and PKC, which leads to an increased Ca^{2+}-influx. Due to increased expression of $Ca_v3.1$ and $Ca_v3.3$ induced by progesterone, Ca^{2+}-influx is increased. More functional T-type-Ca^{2+}-channels and an increased Ca^{2+}-influx promote dendritogenesis, spinogenesis, and synaptogenesis, leading to neuronal plasticity. Ca^{2+}: Calcium ions; PGR: classical progesterone receptors A and B; PKA: protein kinase A; PKC: protein kinase B.

Author contributions: *Study concept: CT; definition of intellectual content: CT, VM; literature search: AE, JT, VT; experimental studies: AE, JT; data acquisition:*

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