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

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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\textsuperscript{2+}-channels Ca\textsubscript{3.1}, Ca\textsubscript{3.2}, and Ca\textsubscript{3.3} as they helped determine neuronal plasticity by regulating Ca\textsuperscript{2+}-influx in neuronal cells. The results showed direct effects of progesterone on the mRNA expression of T-type-Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{3.1} and Ca\textsubscript{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\textsubscript{3.1}; Ca\textsubscript{3.2}; Ca\textsubscript{3.3}; neuronal plasticity; progesterone; protein kinase A; protein kinase C; Purkinje cells; rat cerebellum; T-type-Ca\textsuperscript{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, anti-degenerative, anti-apoptotic, and anti-inflammatory effects in the central nervous system (Schumacher et al., 2012; Singh and Su, 2013; De Nicola et al., 2018; Ylmaz 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\textsuperscript{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\textsuperscript{2+}-channels are also responsible for Ca\textsuperscript{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\textsuperscript{2+}-channels contain three subtypes named Ca\textsubscript{3.1} (encoded by CACNA1G), Ca\textsubscript{3.2} (encoded by CACNA1H), and Ca\textsubscript{3.3} (encoded by CACNA2I), 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\textsubscript{3} channels have a specific subcellular distribution. Immunohistochemically, Ca\textsubscript{3.1} and Ca\textsubscript{3.2} were detected in the somata and dendrites of PC, the latter overall weaker. Ca\textsubscript{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\textsuperscript{2+}-channels has not yet been fully explored, but it is known that progesterone as a neurosteroid alters neuronal and non-neuronal Ca\textsuperscript{2+} currents in an excitatory way, leading to Ca\textsuperscript{2+}-influx, probably by addressing Ca\textsubscript{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\textsuperscript{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: PKCa (encoded by PKCA), PKCBI (encoded by PRKCB1), PKCe (encoded by PRKCE), and PKCG (encoded by PRKCG) (Pemberton et al., 2000; Metzger and Kapfhammer, 2003; Perez-Reyes, 2003; Park et al., 2006). It is known that PKC phosphorylates PGR and...
The aim of the present study was to verify the expression and colonization of the three T-type Ca 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 ad libitum access to food and water. At room temperature in detail (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 anesthetized with 100 µL of isoflurane solution and the skulls were exposed. A few drops of tissue-saving compound were applied to the cerebellum immediately before the removal of the 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 non-adherent 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% azur II, and 1%boc 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.

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 plus 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). The primers shown in Table 1 are all obtained from Microsynth (Balgach, Switzerland).

qRT-PCR

The qRT-PCR principle was performed using GoTaq® qPCR Master Mix (no. A6001, Promega, Waldorf, Germany). For this purpose, 10 µL of undiluted total RNA containing 500 ng of total RNA was mixed with 4 µL of cDNA. The cDNA was mixed with 1.4 µL of the primers (for LC3B, Xie et al., 2021) and 3.2 µL ddH2O. The samples were heated to 95°C for 2 minutes, followed by 40 cycles of polymerase activation and 40 cycles of 15 seconds at 95°C and 60°C for 30 seconds, then kept at 4°C for 1 minute. The next day, after thorough washing with PBS, incubation of the secondary donkey anti-rabbit antibody was followed at room temperature.
Table 1 Primers and sequences used in qRT-PCR

| Name         | Sequence (5’–3’) |
|--------------|-----------------|
| GAPDH*       | F: ACT CCC ATT CTT CCA CCT TTG |
|              | R: CCG TGG TGT AGG CAT ATT |
| CACNA1G      | F: GTC ATT TGG TGT GGC TTC TCC |
|              | R: TGG TAG TGA TGT TCC TGG TGT C |
| CACNA1H      | F: CTT CAT CTT CGG CAT TGT TGG |
|              | R: CCT CCG TCT GCT AGT AT |
| CACNA1J      | F: AGG CTG TCA CTC ACA TCT CT |
|              | R: TAC TCG TGA ACT TCC TGG CT |
| PKRACA       | F: CCA TTA CGG GGG ATT GGG AG |
|              | R: GCC TAG ATG AGG TCC |
| PKRCB        | F: ATG ACC AAA CAC CCA GGA |
|              | R: TGG CGG ATT CTC CTT GGG AT |
| PKRCE        | F: TCT CCT TGT GAC CAG GAA CT |
|              | R: GGG ACG CTA GAG GGA TGG |
| PKRCKZ       | F: GAA AGG ATA TGA GGC TTC GC |
|              | R: GAG GAC GTG GCA GCG TTT AT |
| PGR          | F: AGC ATG TCA GTG GAC AGA TG |
|              | R: TAA GGC ACA AGG AGT ATG |
| Calbindin    | F: GAA GAA GAG CAG CTT CAG AA |
|              | R: GCC CTT GAT CAT GCA CAA A |
| GFAP         | F: GAG TGG TAT CTT AAC AAG TT |
|              | R: TTC GTG CAG GGT GGA TTT CT |
| Neur 9       | F: GAG AAG CAG TAT AAC ATG GAG |
|              | R: CAT ATG GGC GTC TTA AAT AT |
| Vglu1        | F: CCA TGG GAG GCC TAA TAT TT |
|              | R: GCC CTT GGA GTG GTA GTC AT |
| Alpha-GABAR  | F: CGG ATG AAG CTT GTC AAC TT |
|              | R: TCT TCT GGG ACC TCT AAT GAA T |

* GAPDH was used as a reference gene. F: Forward; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GAP: glial fibrillary acidic protein; Neur 9: neuronal nuclear; PGR: classical progesterone receptors A and B; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; R: reverse.

Table 2 mRNA expression of T-type-Ca2+-channels and single PC on mRNA level.

| Gene          | CACNA1G | CACNA1H | CACNA1J | CACNA2D | CACNA2E | CACNA2B | CACNA2A | CACNB1 | CACNB2A | CACNB2B | CACNB3 | CACNB4 | CACNB5 | CACNB6 | CACNB7 | CACNB8 | CACNB9 | CACNB10 |
|---------------|---------|---------|---------|---------|---------|---------|---------|--------|---------|---------|--------|--------|--------|--------|--------|--------|--------|---------|
| Value         | p9      | p9      | p9      | p30     | p30     | p30     | p30     | p9     | p9      | p9      | p9     | p9     | p9     | p30     | p30     | p30     | p30     | p9      |
| Expression    | 1.00 ± 0.04 | 1.00 ± 0.04 | 1.00 ± 0.04 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 | 1.40 ± 0.02 |

**Results**

**T-type-Ca2+-channel expression in dissociated and cerebellar PC**

In a first step, the colocalization of T-type-Ca2+-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-Ca2+-channels were present in PC in the p9 and p30 samples (CACNA1G: p9 = 1.00 ± 0.04, p30 = 1.40 ± 0.02; CACNA1H: p9 = 1.01 ± 0.01, p30 = 1.12 ± 0.03). The mRNA expression showed a slight decrease at p30 compared with p9, while CACNA1G and CACNA1J showed an increase in expression between p9 and p30.

**Progesterone influences the mRNA expression of T-type-Ca2+-channels in PC**

Before taking a closer look at the effect of progesterone on T-type-Ca2+-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 could be detected around the soma and in 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 T-type-Ca2+-channel expression in purified and cultured PC 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 (Tadden et al., 2018).

However, the experiments performed with p0 PC showed that progesterone affects T-type Ca2+ channels in different ways, such that both downregulation and upregulation are present at both observed time points. CACNA1G mRNA was not significantly 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). CACNA1J 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- (PKCα), β- (PKCβ), ε- (PKCε), γ-, δ-, and θ-subunits (PKCγ, PKCδ, PKCζ) in PC. Again, the isolated, purified, and cultured PC were incubated with 10 nM progesterone for 24 and 48 hours. qRT-PCR analysis showed a significant increase in expression of 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 levels of PKCβ and PKCε were significantly up-regulated after 24 and 48 hours of progesterone treatment (P < 0.05 or P < 0.001). A highly significantly up-regulation of PRKCE and PKRCK2 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 Ca3 channels in PC was demonstrated both in vivo and in vitro**

LMO is a very elegant method for analyzing microdissected PC. However, a technical limitation was that single-cell somata could be isolated precisely using LMD, to further analyze the colocalization of T-type-Ca2+-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 the PC was not 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 = 3.38%, Vglut1 (marker for neuronal glutaminergic neurons = 15.75%). In summary, a technical limitation of LMD is 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-Ca2+-channels are also located. Nevertheless, since immunohistochemistry of the studied T-type-Ca2+-channels in the in vivo model did not differ from the in vitro models, as PC degeneration was via synaptic contacts and neurotrophic signaling (Seil, 2001; Yamada and Watanabe, 2002; Willett et al., 2019), the Ca2+-influx into PC, regulated by synaptic activity with the subsequent regulation of gene expression, is a major player in neuronal inactivation and synaptic plasticity (Han et al., 2007; Yang and Lisberger, 2014; Lin et al., 2017; 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 (Tadden et al., 2018).
Effects of progesterone on the mRNA expression of protein kinases.

Relative mRNA expression pattern of the protein kinases PRKACA, PKA, PKCB, PKACE, and PRKZ 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(PRKCB 48 hours) = 0.0001, P(PRKCB 24 hours) = 0.0343, P(PKACE 48 hours) = 0.0394, P(PRKCE 24 hours) = 0.0004, P(PRKCE 48 hours) = 0.0006, P(PRKZ 24 hours) = 0.0053, P(PRKZ 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-Ca\(^{2+}\)-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 regulation of hormones and neurotransmitters (Strünker et al., 2011; Zamboni 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\(^{2+}\)-currents are primarily regulated by changes in membrane potential, other factors such as hormones and neurotransmitters acting through signaling intermediaries like PKA and PKC additionally regulate channel activity (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 CACNA1I and CACNA1G. 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 PKA-ζ and PKC 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.

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; Arcos-Montoya et al., 2021). PKA and PKC are two members 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 isoforms differing in their sensitivity (Newton, 2010). The four conventional isoforms include PKCa, PKCb, PKCd, and PKCy, they are sensitive to the second messenger diacylglycerol or Ca\(^{2+}\). The four novel isoforms include PKCe, -ε, -η, and -θ, being sensitive only to diacylglycerol. While PKCe and -ε are classical 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), PKCa (encoded by PKCA), PKCb (encoded by PKCB1), PKCd (encoded by PKCD), PKCe (encoded by PKCE), PKε (encoded by PKCEQ), PKδ (encoded by PRKCA2), as they are known to be expressed in neuronal tissue in rat brain (Metzger and Kaphammer, 2003). The present study showed that progesterone positively influences the mRNA expression of all protein kinases examined. Furthermore, the resulting increased activity of the kinases would be desirable. Any ways with increased PKA/PKCe-level, a higher reserve pool that could be phosphorylated and activated is available here.

Conclusion

The 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 PKA-ζ and PKC 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-Ca\(^{2+}\)-channels in Purkinje cells.

Progesterone diffuses into PC and influences gene expression in the nucleus via the classical PRG. It causes an increased expression of PKA and PKC, which leads to an increased Ca\(^{2+}\)-influx. Due to increased expression of Ca\(^{2+}\) and Ca\(^{2+}\) induced by progesterone, Ca\(^{2+}\)-influx is increased. More functional T-type-Ca\(^{2+}\)-channels and an increased Ca\(^{2+}\)-influx promote dendrogenesis, 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.

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