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Neurosteriod enhancement of glutamatergic transmission via transient receptor potential melastatin 3 chanels in the developing cerebellum

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NEUROSTEROID ENHANCEMENT OF GLUTAMATERGIC TRANSMISSION VIA TRANSIENT RECEPTOR POTENTIAL MELASTATIN 3 CHANNELS IN THE DEVELOPING CEREBELLUM

by

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DISSERTATION

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Abstract

Neurosteroids are compounds synthesized *de novo* within the central nervous system and affect brain function via genomic and non-genomic mechanisms. Sulfated neurosteroids modulate synaptic neurotransmission via interactions with various membrane receptors located at pre- and/ or postsynaptic sites, including metabotropic and ionotropic neurotransmitter receptors and ion permeable channels. The interactions of sulfated neurosteroids with these types of targets can induce rapid changes in neurotransmitter release and/or in postsynaptic responses. Since the 1940's, pregnenolone sulfate (PregS), the sulfated version of the steroid precursor, pregnenolone, has been suggested to have anti-stress, anti-aging, antidepressive, neuroprotective and memory enhancing effects in the mature brain. However, little is known on the physiological role of PregS in the development of the brain. Previous studies from our laboratory have postulated PregS as a novel enhancer of excitatory neurotransmission in the hippocampus, a brain area important for learning and memory. In the work presented here, we evaluated the effects of PregS in the developing rat cerebellum. The cerebellum

is a brain region essential for motor coordiation, balance and equilibrium, muscle tone, and cognitive and language functions. We found that PregS potently and reversibly enhanced the release of the excitatory neurotransmitter, glutamate, onto neonatal Purkinje cells (PCs), which provide the sole output from the cerebellar cortex. This effect of PregS was present at the climbing-fiber-to-PC synapse, a powerful glutamatergic synapse important for the timing of conditioned reflexes. Interestingly, the mechanism responsible for this effect of PregS is mediated by a Ca^{2+} permeable cation channel, the transient receptor potential melastatin 3 (TRPM3) channel. This channel was recently shown to be sensitive to PregS and to be involved in a number of physiological events in the periphery. However, its role in brain physiology is unknown. Thus, the studies presented here postulate TRPM3 channels as novel modulators of excitatory neurotransmission in the immature brain. In addition, we found that TRPM3 channels are highly expressed in all layers of the developing cerebellar cortex. This finding suggests that these steroid-sensitive channels play a major role in the development of this brain region. Lastly, we found that pharmacologically-induced increases in endogenous PregS-like neurosteroids potentiate glutamatergic transmission at PCs in organotypic hindbrain cultures. This finding sets up the stage for future experiments that will investigate the effect of TRPM3 channels and endogenous PregS in the development of the cerebellar cortex.

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1. INTRODUCTION

1.1 Overview of the cerebellum

The cerebellum is an area of the hindbrain known for its important role in motor movement coordination, balance and equilibrium, and muscle tone. Interestingly, the cerebellum has undergone a significant size enlargement through evolution, which occurred in parallel with the acquisition of higher human cognitive skills, such as language (Leiner, 2010). Over the last three decades, it has been demonstrated that the cerebellum does not only communicate with motor and sensory regions in the cerebral cortex, but it is also heavily interconnected with areas involved in cognitive and language functions, and even with brain regions associated with affective states (Schmahmann and Sherman, 1997, Strick et al., 2009). These cerebro-cerebellar connections are arranged in a closed-loop fashion where a specific cerebellar output channel within the cerebellar nuclei provides the main synaptic input to a specific cerebellar cortical area and vice versa (Strick et al., 2009). Parallel to anatomical evidence for the role of the cerebellum in higher and executive brain functions, a plethora of functional evidence has been presented (Strick et al., 2009, Leiner, 2010, Stoodley and Schmahmann, 2010). These studies have shown that patients with cerebellar damage present not only motor syndromes such as dysmetria, dysarthria, and ataxia but also executive, visual spatial, and linguistic impairments, as well as affective dysregulation (reviewed in (Stoodley and Schmahmann, 2010)). It is then apparent that dysregulation of cerebellar circuitry functions can have widespread adverse effects in overall brain function. Clear examples for these are the cerebellar dysfunctions caused by acute and chronic ethanol intoxication, which include dysarthria, nystagmus, ataxia, intention tremor, and executive function

alterations. The latter are likely caused by damage of the connections between the cerebellar hemispheres and the frontal cortex (reviewed in (Valenzuela et al., 2010)). Furthermore, alterations in cerebellar cortex development and synapse formation have been found to be implicated in the pathophysiology of autism, schizophrenia and ataxia , which are accompanied by cognitive impairments and emotional symptoms (Ito-Ishida et al., 2008, Stoodley and Schmahmann, 2010).

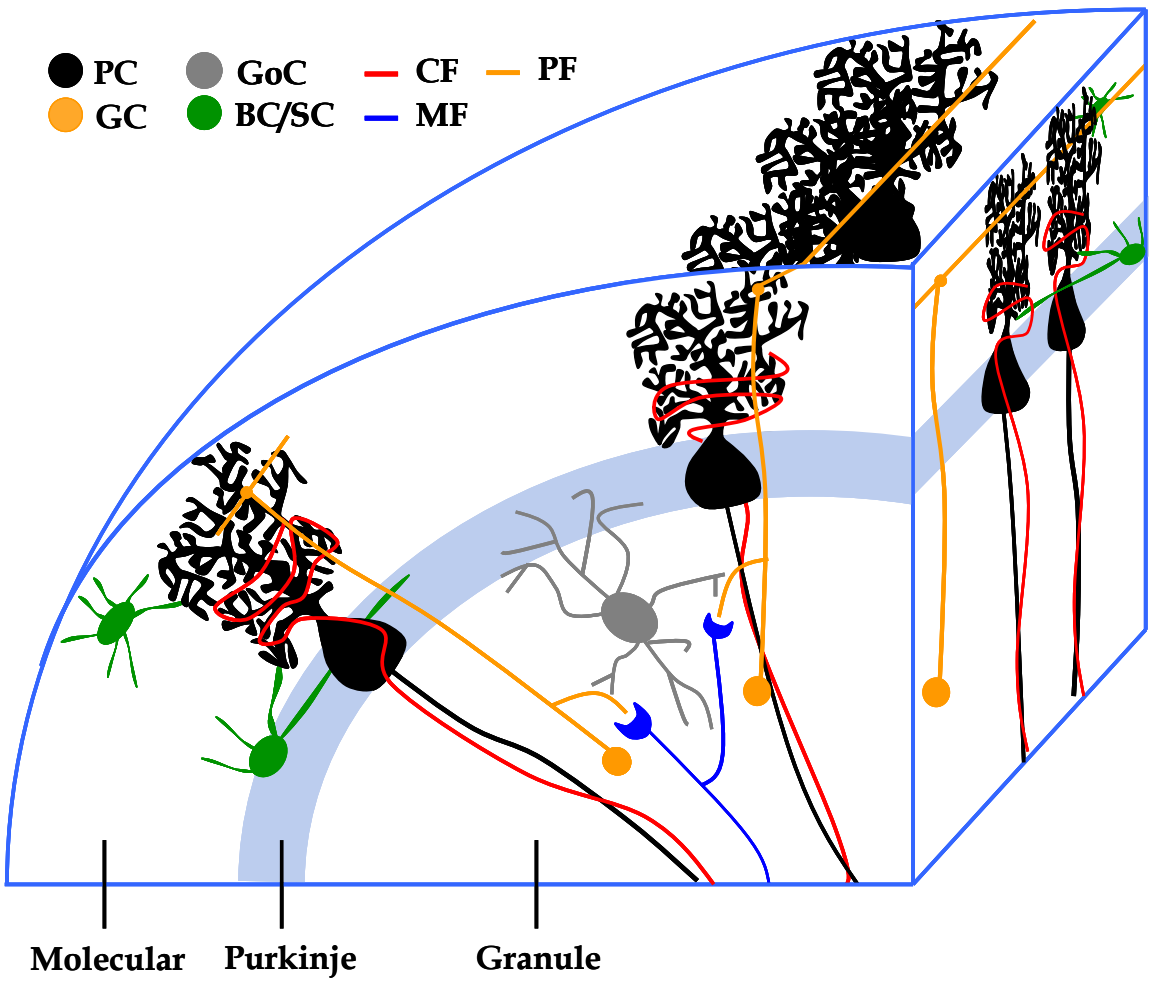

Figure 1.1. Schematic representation of the cerebellar cortical layers and their cellular components. PC: Purkinje cell; GoC: Golgi cell, GC: granule cell, BC: basket cell (located more basally), SC: stellate cell (located more apically), MF: mossy fiber, CF: climbing fiber, PF: parallel fiber. The molecular, Purkinje, and granule cell layers are indicated at the bottom (From Valenzuela et al 2010).

The cerebellum contains the largest number of neurons of any region of the brain and its structure is comparable to that of computing machines. Basic components are assembled into modular packages that contain similar circuitry and large numbers of such similar modules are organized into parallel processing networks, each of which sends a bundle of fibers to specific parts of the cerebral cortex (Leiner, 2010). The cerebellar cortex contains the circuitry modules that compute the information from the cerebral cortex. This cortex receives external inputs form the inferior olive, thought to encode the timing of sensory input (reviewed in (Liu et al., 2008a)), and from various areas of the cerebral cortex, the vestibular system, the spinal cord, and the reticular formation via the mossy fibers (reviewed in (Dean et al., 2010)).

The only output from this cerebellar region are the Purkinje cells (PCs), which send the processed information to the deep cerebellar nuclei for final integration (Jaeger, 2011). PCs are GABAergic neurons arranged as a monolayer located in between the granule cell layer and the most superficial layer, the molecular layer (Figure 1.1). PCs receive inhibitory input from GABAergic interneurons in the molecular layer, namely the stellate (SCs) and basket cells (BCs). The cell bodies of the SCs populate the most external two thirds of the molecular layer, whereas the cell bodies of BCs are located in the one third closest to the PCs bodies. BCs synapse onto the initial segment of the PC axon, forming the pinceaux synapse, where interbasket fiber septate-like junctions also establish unique axoaxonic synapses (Sotelo, 2008). On the other hand, SCs synapse on the planar and extensive dendritic tree of the PCs, which traverses the molecular layer. PCs receive excitatory, glutamatergic input from the parallel and the climbing fibers, PFs and CFs, respectively (Figure 1.1). The former arise from the bifurcated axonal

projections of granule cells in the granule cell layer, and the latter correspond to the axons of inferior olivary neurons. PFs synapse onto the distal dendrites while the CF synapses onto the proximal dendrites. These two types of axonal projections also communicate to SCs and BCs, but while PFs directly synapse onto these interneurons, CFs communicate via glutamate spillover (Szapiro and Barbour, 2007, Cesa and Strata, 2009). Additionally, while several PFs form more than 200,000 synapses onto PCs, only one CF arbor, containing about 300 varicosites, contacts a single PC (Cesa and Strata, 2009). PFs and CFs inputs to PCs also differ in their strength, synaptic plasticity, and baseline release probability. PFs produce brief excitatory postsynaptic potentials generating single action potentials, or simple spikes. Activation of PFs exhibits a stimulus strength-dependent response, which exhibits prominent paired-pulse facilitation (PPF) of presynaptic origin (Etzion et al., 2009). The CF induces a large (all-or- none) depolarization of the soma and dendrites of PCs with a burst of action potentials, or complex spike (Schmolesky et al., 2002, Cesa and Strata, 2009). Also, the CF-to-PC synapse has been shown to operate at a baseline of high release probability and, consistent with this, it exhibits paired pulse depression (PPD) of presynaptic origin (Hashimoto and Kano, 1998).

1.2 Development of the cerebellar cortex.

Cerebellar neuronal precursors, in rodents, start to migrate and differentiate from the ventricular epithelium and the rhombic lip at around embryonic day (E) 9 (Bosman and Konnerth, 2009). Despite the early start of cerebellar development, the most active period of cerebellar maturation occurs from birth to approximately the end of the second postnatal week and parallels motor development (Swinny et al., 2005). In humans, this

period is roughly equivalent to the last trimester of pregnancy (Dobbing and Sands, 1973). Studies have shown that from 24 weeks to 40 weeks of gestation, the volume of the human cerebellum undergoes a 5-fold increase (Volpe, 2009).

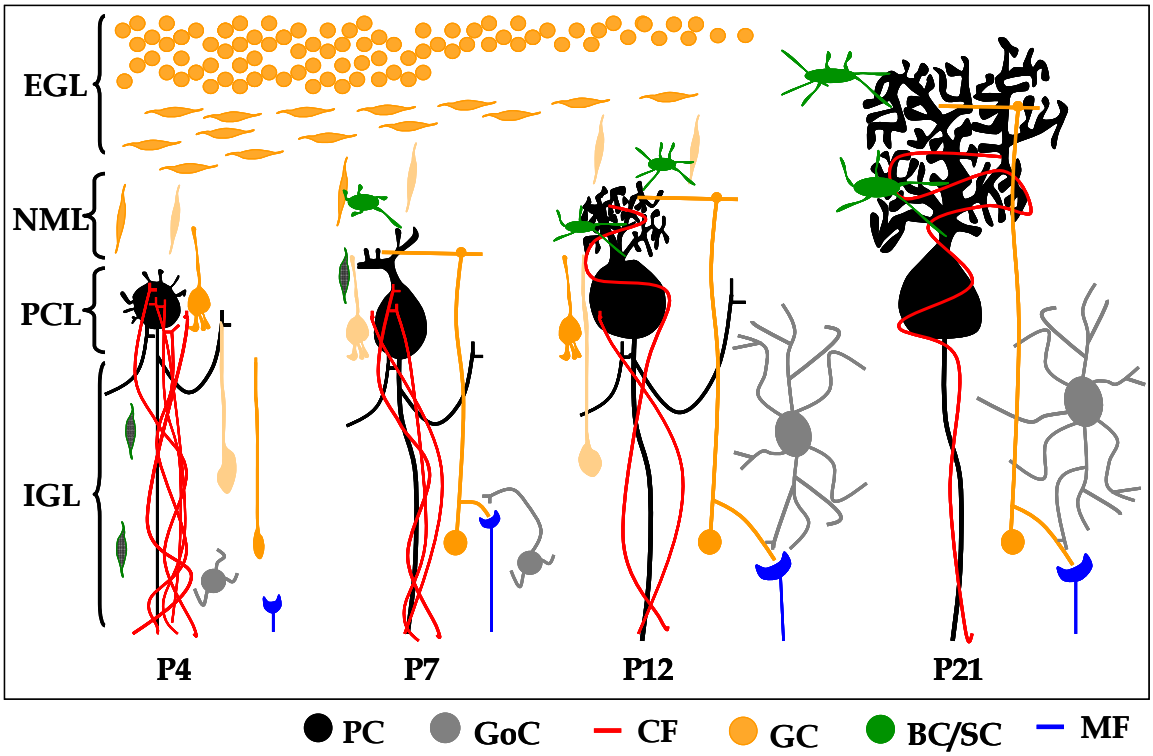

Figure 1.2. Schematic representation of the cerebellar cortex development. At P4, GC are migrating from the external granule layer (EGL) to the internal granule layer (IGL), where GoC and MF are already present, - GCs migrate tangentially within the EGL and along the Bergmann glia in the nascent molecular layer -, undifferentiated BC and SC are migrating toward the nascent molecular layer (NML), and PCs are innervated by around 5 CFs. By P7, immature GCs as well as BC/SCs continue to migrate; while their more mature counterparts start to innervate PCs. Additionally, GCs start to receive GoC and MF inputs. At this stage the CF elimination process has started. At P12, already differentiated BCs innervate the proximal dendrites of PCs whereas SCs innervate the distal ones, while immature GC migration and CF elimination are still in place. By the end of the third postnatal week, P21, the external granule layer disappears completely and CF monoinnervation is achieved. Also note that by this age the axon collaterals of PCs have disappeared. Adapted from Valenzuela et al, 2010.

At birth, the rodent cerebellar cortex contains four layers, namely, the external granule layer, the nascent molecular layer, the PC layer, and the internal granule layer

(Figure 1.2). The external granule layer contains proliferating granule cells, which migrate to their final destination in the internal granule layer in a saltatory fashion, alternating phases of forward movement with pauses, along the radial/Bergmann glia (Chedotal, 2010). The nascent molecular layer houses newly formed stellate, and basket cells, and immature PCs dendrites. In the PC layer, the somas of PCs, (organized in a multilayered fashion and innervated by multiple climbing fibers) and immature Bergmann glia are found (Douyard et al., 2007). The internal granule layer contains sparse granule cells, the GABAergic Golgi cells, and the mossy fibers. Mossy fibers arrive at the inner granule cell layer around postnatal day 3; the Golgi cell is present at the time of birth and starts forming synapses onto granule cells after postnatal day 7 (Takayama and Inoue, 2006). It has been shown that granule cell migration is completed by postnatal day (P) 21, when the external granule layer disappears (Sotelo, 2008). Comparably, in humans, granule cell proliferation and migration starts during the third gestational trimester and continues into the first year of life (Dobbing and Sands, 1973, Swinny et al., 2005).

1.2.1 PC development and synapse formation.

PCs are born at E13 and after their final mitosis they migrate along radial glial fibers over the already formed deep cerebellar nuclei into the cerebellar anlage. Importantly PCs, undergo marked morphological and functional changes during the first 2 postnatal weeks. They merge from two to three irregular rows into a single layer, develop an extensive, flattened, dendritic tree, and form synaptic connections (Kurihara et al., 1997, Eilers et al., 2001, Sotelo and Dusart, 2009). At around P2, PCs grow provisional somatic synaptic protrusions, onto which the first synaptic glutamatergic

connections will form (Kurihara et al., 1997, Eilers et al., 2001, Kawa, 2003). By E17, CFs start to contact PCs. At first, PCs are innervated by multiple CFs; by the end of the third postnatal week, all but the strongest of these fibers are eliminated through a process of synapse competition and elimination (Bosman and Konnerth, 2009, Hashimoto et al., 2009)

The process of CF synapse elimination is divided into three different phases. It starts with the functional differentiation of CF inputs and it is followed by the activity-dependent early and late phases of synapse elimination. The number of CFs innervating a PC can be determined using electrophysiology techniques. In these types of experiments, immature PCs are patch clamped and CF inputs are stimulated at different intensities. CF-evoked excitatory postsynaptic current responses (CF-eEPSCs) in PCs are then analyzed (Figure 1.3A and B). Given the characteristic all-or-none response of CF-to-PC synapses, the amplitude of the evoked responses will remain constant until an additional CF starts contributing to this response. Every time a CF input is recruited into the CF-eEPSC response, its amplitude will increase in a step-like manner (Figure 1.3B). The number of steps is indicative of the number of CF inputs and the amplitude of each CF-eEPSC step reflects the strength of an individual CF input (Bosman and Konnerth, 2009). Using this experimental approach, it has been shown that, previous to the start of synapse elimination, the relative synaptic strengths of multiple CFs innervating the same PC differ in an age-dependent manner (Figure 1.3C). The amplitudes of CF-eEPSC steps are comparable at P3, while those at P12 are very different between the largest CF and the smallest CF (Hashimoto and Kano, 2005). At the end of the first postnatal week, multiple CF inputs have been functionally differentiated into one strong and a few weak ones

(Figure 1.3D). Morphological studies have shown that at P4-7, many CFs crawl around the PC layer; these CFs are denominated the "creeper type" and their swellings do not aggregate at a particular PC soma, but instead scatter randomly and contact multiple PCs at the soma and the dendrites. Also at this age, other CFs surround specific PC somata and form aggregated terminals on them, these synapses are called the 'nest type' (Sugihara, 2005, Hashimoto and Kano, 2008, Kano and Hashimoto, 2009). It is possible that weak CF terminals inputs correspond to creeper type CFs and strong ones arise from nest type CFs.

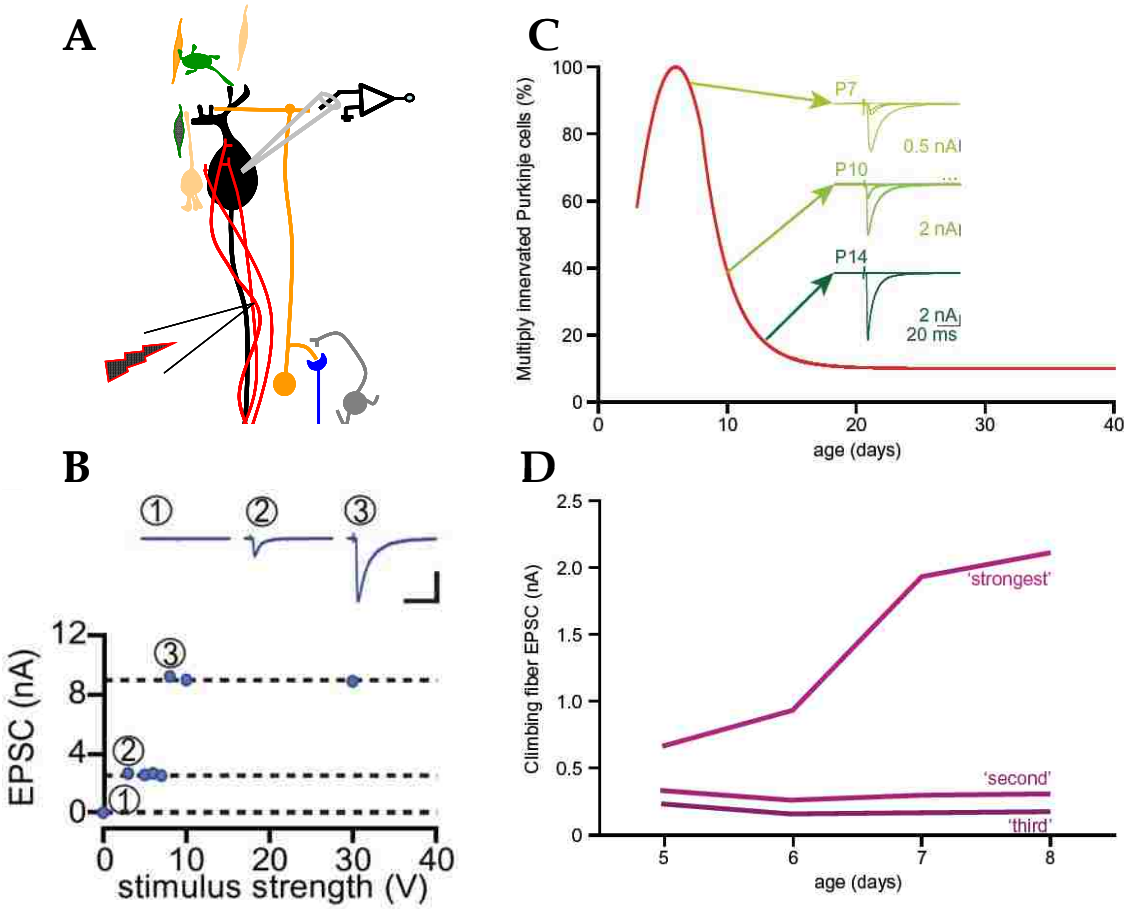

Figure 1.3. Neonatal PCs are innervated by multiple CFs. (A) Schematic representation of the electrophysiological paradigm used to record CF-evoked EPSCs. (B) Example of the CF-eEPSC steps in a P10 mouse PC showing sequential recruitment of two climbing fibers by increasing stimulation strength. Scale bars 4 nA, 20 ms. (C) The number of PCs innervated by more than one

CF reaches almost 100% around P7 and then rapidly declines. (D) The strongest CF synapse increases rapidly in synaptic strength, while the weaker synapses remain fairly stable. (Adapted from Bosman and Konnerth 2009 and Valenzuela et al 2010).

The mechanisms responsible for this functional differentiation are not well understood. Nonetheless, it has been observed that in P5-9 PCs, upon PC depolarization and/or high frequency stimulation of CFs, strong CF inputs undergo long-term potentiation (LTP) whereas weak ones undergo long-term depression (LTD) (Bosman et al., 2008, Ohtsuki and Hirano, 2008). This suggests that plasticity mechanisms may play a role in the functional differentiation of multinumerary CF inputs. An early phase of surplus CF input elimination occurs between P7 and P11. This early phase is independent of PF inputs. The late phase of CF synapse elimination takes place between P12 and P16 and has been shown to be dependent on PF synapse formation, which starts at the end of the first postnatal week. In GluRδ2 knockout mice, where PF-to-PC synapse formation is impaired, the regression of CFs is normal from P5 to P11, whereas the average number of CFs innervating individual PCs is significantly larger than that of control mice from P12 afterward (Hashimoto and Kano, 2008).

The formation of GABAergic synapses onto PCs also occurs early in postnatal life and ends by P21. The first GABAergic interneurons to contact PCs are the BCs (Takayama and Inoue, 2004a). The formation of BC-to-PC synapses (the pinceaux synapse) starts at around P7 (Ango et al., 2004, Sotelo, 2008). However, functional GABAergic synapses have been detected as early as P2 (Kawa, 2002). These early GABAergic synapses could be axonal collaterals from neighboring PCs, which mediate $GABA_A$ receptor–dependent traveling waves of activity in neonatal P4-P14 PCs, but not

in mature PCs. These GABAergic traveling waves may play a role in the maturation of cerebellar circuitry (Watt et al., 2009).

During the period prior to the formation of GABAergic synapses onto PCs, $GABA_A$ receptors can depolarize the membrane potential and activate voltage-gated Ca^{2+} channels. These excitatory actions of $GABA_A$ receptors have been suggested to contribute to the activity-dependent maturation of cerebellar cortical circuitry components, including climbing fibers (Eilers et al., 2001, Watt et al., 2009).

1.2.2 Modulators of brain maturation

During the rat neonatal period, the proper development of the PC dendrites and its synaptic connections, are facilitated by a number of endogenous factors acting as anterograde and/or retrograde messengers. These modulating factors include: brain derived neurotrophic factor, polysialic acid, neural cell adhesion molecule, glutamate and GABA, and neurosteroids (Levenes et al., 2001, Tsutsui et al., 2003, Duguid and Smart, 2004, Duguid et al., 2007, Huang et al., 2007b). In the development of brain cortical structures, some factors influence synapse formation by acting as modulators of synaptic transmission. It is known that glutamate signaling regulates nearly all aspects of synapse formation and maturation. Likewise, GABA transmission has been shown to have trophic effects on neurite growth during the embryonic and perinatal periods, which is largely explained by the GABA depolarizing action in immature neurons that triggers Ca^{2+} influx and intracellular signaling cascades (Huang et al., 2007b). Therefore, factors that modulate neurotransmitter release as well as the activation of neurotransmitter receptors

and Ca^{2+} channels are potential players in the process of synapse formation and maturation.

During development, spontaneous as well as experience-generated synaptic transmission sculpt neuronal circuits, and provide the initial configuration of connections necessary for function and survival. It is well established that neurosteroids regulate synaptic transmission by altering the function of pre and/or postsynaptic neurotransmitter receptors.

1.2.3 Steroids and Development

Steroids are cholesterol metabolites with a core structure formed by four cycloalkane rings (Figure 1.4). The synthesis of these compounds starts with the translocation of cholesterol from the outer to the inner mitochondria membrane, by the steroidogenic acute regulatory protein (StAR). Then, cholesterol is transformed into pregnenolone by virtue of the cytochrome P450 side-chain cleavage enzyme (P450scc). Pregnenolone is the precursor to other steroids (Figure 1.4). Steroids have been long recognized to modulate development and, in fact, have been shown to be essential. Disruption of steroid production in P450scc knockout mice leads to death at P1-2. However, steroid administration can rescue these mice (Hu et al., 2002).

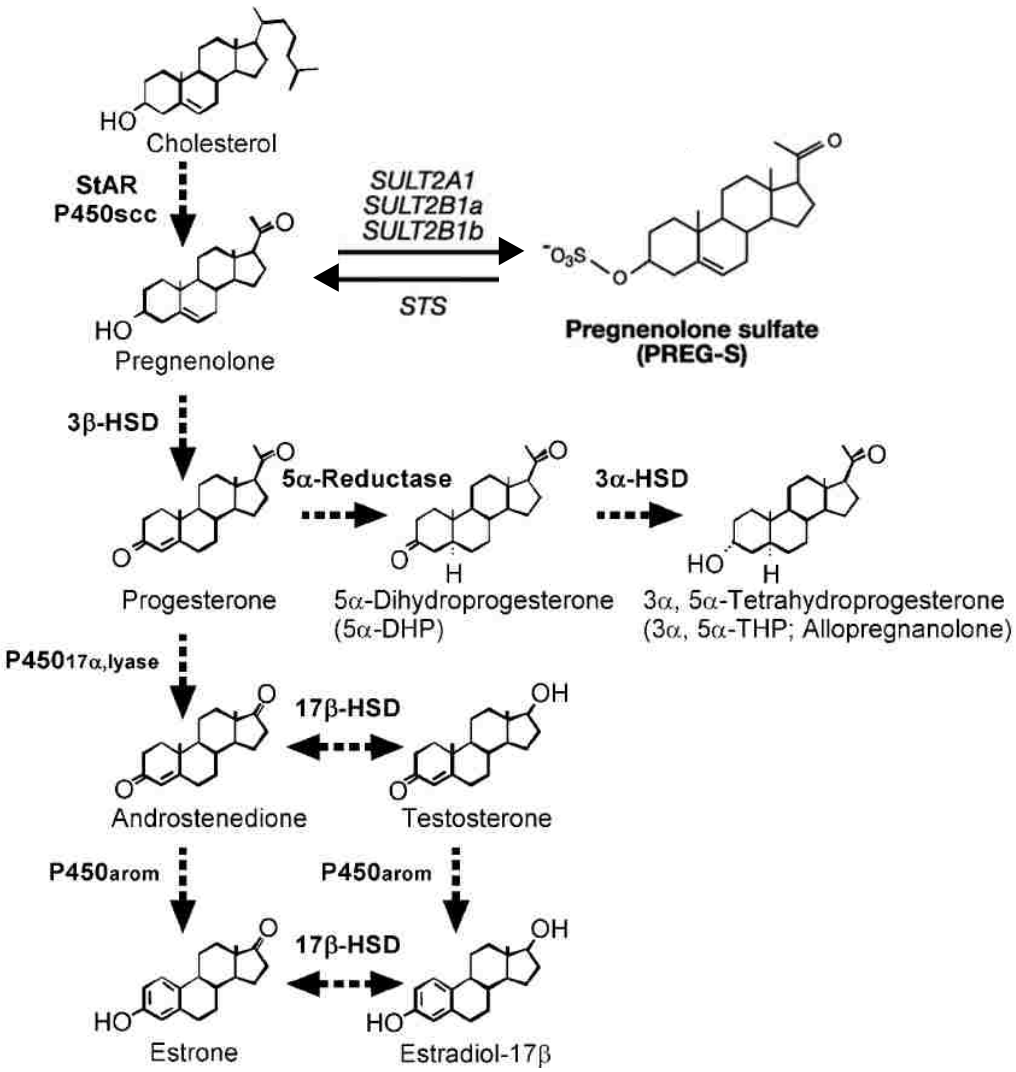

Figure 1.4. Synthesis of steroids. Adapted from Schumacher 2007 and Tsutsui 2008

Hormonal steroids organize the developing brain during a perinatal sensitive period and have enduring consequences for adult behavior. In male rodents testicular androgens are aromatized in neurons to estrogens and initiate distinct cellular processes that ultimately determine the masculine phenotype. In brain regions such as the preoptic area, the ventro-medial nucleus and the mediobasal hypothalamus, steroids modulate mainly cell number and dendritic morphology. The mechanisms responsible for these

effects include estradiol-induced modulation of prostaglandin synthesis, presynaptic release of glutamate, postsynaptic changes in glutamate receptors, and changes in cell adhesion molecules (reviewed in (Wright et al., 2010)).

Importantly, steroids synthesized *de novo* in the brain have also been shown to play important roles during development. These steroids, locally synthesized in the brain, are collectively called neurosteroids. Among these developmental regulators are pregnenolone and its sulfated ester, progesterone, allopregnenolone, and DHEA and its sulfate. These steroids have been shown to be involved in myelination, oligodendrocyte differentiation, neurogenesis, neuroprotection, and neurite outgrowth (reviewed in (Mellon, 2007)).

1.2.4 Neurosteroids influence PC development

The PC was the first neuronal cell type in the brain identified as neurosteroidogenic. These cells express several kinds of neurosteroidogenic enzymes such as StAR, P450scc, and 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD), starting in the neonatal period. Interestingly, the expression of P450scc appears immediately after differentiation. PCs have been proposed to synthesize pregnenolone and its sulfated ester, progesterone, and progesterone metabolites (Tsutsui et al., 2003). Neuroactive steroids such as estradiol, allopregnanolone, and progesterone have been shown to play a role in PC survival, dendritic growth, spine formation and synaptogenesis (Sakamoto et al., 2001, 2002, Tsutsui et al., 2003, Griffin et al., 2004, Sasahara et al., 2007).

A morphological study showed that estrogen administration during neonatal life resulted in dendritic growth, spinogenesis, and synaptogenesis in mouse PCs. Conversely, decreases in endogenous estrogen, produced by knocking down the P450 aromatase enzyme, led to the inhibition of dendritic growth, spinogenesis, and synaptogenesis. These developmental effects were shown to be mediated by an estrogen-induced increase in the expression of brain-derived neurotrophic factor (Sasahara et al., 2007).

Allopregnanolone has neuroprotective and neurogenesis-inducing effects (Mellon, 2007). In the cerebellum, allopregnanolone increases PC survival in a mouse model of Niemann-Pick type C disease, an autosomal recessive, childhood neurodegenerative disease characterized by defective intracellular cholesterol trafficking. In this model, there is an age-dependent decrease in the ability to synthesize allopregnanolone. Importantly, administration of allopregnanolone at P7-10 delays the onset of neurological symptoms, such as ataxia and tremor, and increases PC survival (Griffin et al., 2004).

In cerebellar slice cultures from neonatal rats, progesterone increases the density of PC dendritic spines, in a dose-dependent manner, via modulation of the nuclear progesterone receptors A and B (Sakamoto et al., 2002). Similarly, an *in-vivo* study showed that administration of progesterone to neonatal (P3-5) rats resulted in an increase in the density of PC spine synapses (Sakamoto et al., 2001).

Although, the synthesis of other neurosteroids, such as pregnenolone and its sulfate ester, in the developing PC, has been suggested, it is not clear whether these participate in the maturation of synapses onto PCs. Work from our laboratory has implicated a pregnenolone sulfate (PregS)-like neurosteroid as a modulator of synaptic

transmission in the developing hippocampus. These studies showed that a retrogradely released PregS-like neurosteroid enhances glutamate release onto CA1 pyramidal cells via activation of presynaptic N-Methyl-D-aspartate (NMDA) receptors (Mameli et al., 2005). Interestingly, the deleterious effects of ethanol on hippocampal development are mediated in part by alterations in the production of this sulfated neurosteroid, which may result in premature stabilization of excitatory synapses. Ethanol strengthens AMPA receptor-mediated transmission in the CA1 region by reducing the failure rate of low-efficacy synapses. A PregS antibody scavenger and blockade of neurosteroid synthesis prevents this effect of ethanol. A pharmacologically-induced increase in PregS production, using a sulfatase inhibitor, mimicked alcohol's effects (Mameli and Valenzuela, 2006).

The development of PCs could be influenced by PregS via a mechanism of retrograde release similar to the one found in the neonatal hippocampus (Mameli et al., 2005). In addition, these cells could be exposed to systemically circulating PregS. Studies have shown that maternal and fetal PregS levels in plasma are increased during pregnancy and are highest during delivery. During midpregnancy, it has been shown that PregS levels in the fetal blood serum is over 5 times higher than in serum from the mother and in the amniotic fluid (Bicikova et al., 2002). Although, sulfated steroids are believed not to be able to cross the blood brain barrier, it is possible that sulfated steroids are transported inside cells and taken up from the blood through the blood brain barrier via active transport processes. It has been shown that intravenously administered PregS may cross this barrier without being hydrolyzed to the more lipophilic pregnenolone. Also, sulfo-conjugated steroids have been shown to cross the sheep fetal blood brain

barrier (Schumacher et al., 2008). Therefore, it is of interest to investigate the sensitivity of the neonatal cerebellar cortex to PregS, as well as the possible roles of this neurosteroid in the development of the cerebellar cortical circuitry.

1.3 Molecular targets of PregS.

Sulfated steroids and in particular, PregS, act on a wide range of synaptic membrane receptors and/or ion channels, thereby rapidly modulating synaptic transmission. They can act on presynaptic sites to modulate neurotrasmitter release and/or on postsynaptic sites to modulate ion channels and neurotransmitter receptors, leading to significant changes in neuronal excitability.

It has been shown that PregS can enhance currents through NMDA receptors (Wu et al., 1991). However, NR2D-containing NMDA receptors expressed in Xenopus oocytes are inhibited by this neurosteroid (Malayev et al., 2002, Jang et al., 2004). PregS also act at $GABA_A$ and glycine receptors were it exerts an inhibitory effect (Wu et al., 1990, Wu et al., 1997). PregS-mediated potentiation of α7 nicotinic acetylcholine receptors, α1 receptors, voltage-gated Ca^{2+} channels, metabotropic σ1 receptors, and the inwardly rectifying potassium (Kir) channel 2.3 has also been suggested (Meyer et al., 2002, Chen and Sokabe, 2005, Dong et al., 2005, Hige et al., 2006, Monnet and Maurice, 2006, Kobayashi et al., 2009). In addition, PregS inhibits kainate and AMPA receptors (Wu et al., 1991).

Recently, it has been shown that certain members of the superfamily of transient receptor potential (TRP) channels are sensitive to PregS. The capsaicin receptor (the TRPV1 vanilloid channel) was shown to be inhibited by PregS in dissociated dorsal root

ganglion neurons. These results postulated PregS as a pharmacological agent for the reduction of reducing capsaicin receptor-mediated nociception (Chen and Wu, 2004). PregS-induced activation of TRPM3 melastatin receptors has been demonstrated in TRPM3-expressing human embryonic kidney and pancreatic β cells (Wagner et al., 2008). Interestingly, TRPM2, TRPM7, TRPM8, TRPV4, and TRPV6 were found to be insensitive to PregS (Wagner et al., 2008). Additionally, TRPC channels have been suggested to mediate the PregS-induce increase in glutamate release at dentate gyrus hilar neurons and perhaps also PCs; however, the involvement of a specific TRPC channel in this effect and direct activation by PregS has not been reported (Lee et al., 2010, Zamudio-Bulcock and Valenzuela, 2011).

1.3.1 Transient receptor potential channels as steroid receptors

The members of the superfamily of TRP channels share the common features of six transmembrane segments, varying degrees of sequence homology, and permeability to cations. However, they display a remarkable diversity of cation selectivities and specific activation mechanisms. This diverse family of cation channels responds to various stimuli such as temperature, mechanical stress, light, sound, osmolarity, and several endogenous and exogenous chemical signals. It is believed that they act mainly by changing the membrane potential and increasing intracellular Ca^{2+} (Venkatachalam and Montell, 2007). The TRP superfamily of cation channels contains approximately 50 members expressed from yeast to human. It is divided into 7 subfamilies on the basis of structural homology as follows, the TRPC ('Canonical') group, the TRPV ('Vanilloid') group, the TRPM ('Melastatin') group, the TRPP ('Polycystin'), the TRPML ('Mucolipin'), the TRPA ('Ankyrin'), and the TRPN ('NOMP') family (Nilius and Voets, 2005).

1.3.2 The steroid sensitive TRPM3 channel.

As mentioned above, TRPM3 channels have been recently found to be potently enhanced by PregS (Wagner et al., 2008, Majeed et al., 2010, Klose et al., 2011). The TRPM3 gene displays a highly conserved organization in mouse, rat, and human. In mouse, this gene is located in chromosome 19b, contains 28 exons, and its size is about 850 kb (Oberwinkler et al., 2005b). The TRPM3 gene encodes the largest number of variants within the TRP gene family. In mouse, five variants have been identified (mTRPM3α1-5) and in human seven of them have been identified (hTRPM3 a-f and hTRPM31325) (Oberwinkler and Phillipp, 2007). Interestingly, in intron 8 the TRPM3 gene hosts the gene for the microRNA, miR-204. Given that microRNAs induce downregulation of genes very different from their host gene, miR-204 is not likely to control the expression of TRPM3 in a direct autoregulatory manner (reviewed in (Oberwinkler and Phillipp, 2007)); however, it could regulate other genes related to TRPM3 function.

The TRPM3 protein features six transmembrane domains, a conserved TRP motif and a coiled region in its C-terminus (Fig. 1.5). Additionally, it contains the characteristic 700 amino acid-long TRPM homology region in the N-terminal. Within the amino terminus the TRPM3 protein contains four putative calmodulin-binding sites, suggesting a Ca^{2+}/calmodulin dependent regulation of these channels. However, such regulation is yet to be tested (Oberwinkler and Phillipp, 2007). TRPM3 proteins assemble an ion conducting channel. The permeability of this channel depends on the splice event in the linker region between the transmembrane helices five and six. Thus, different splice

variants are permeable to different ions (Grimm et al., 2003). In general the selectivity of TRPM3 channels for Ca^{2+} is high (PCa:PNa = 1.6).

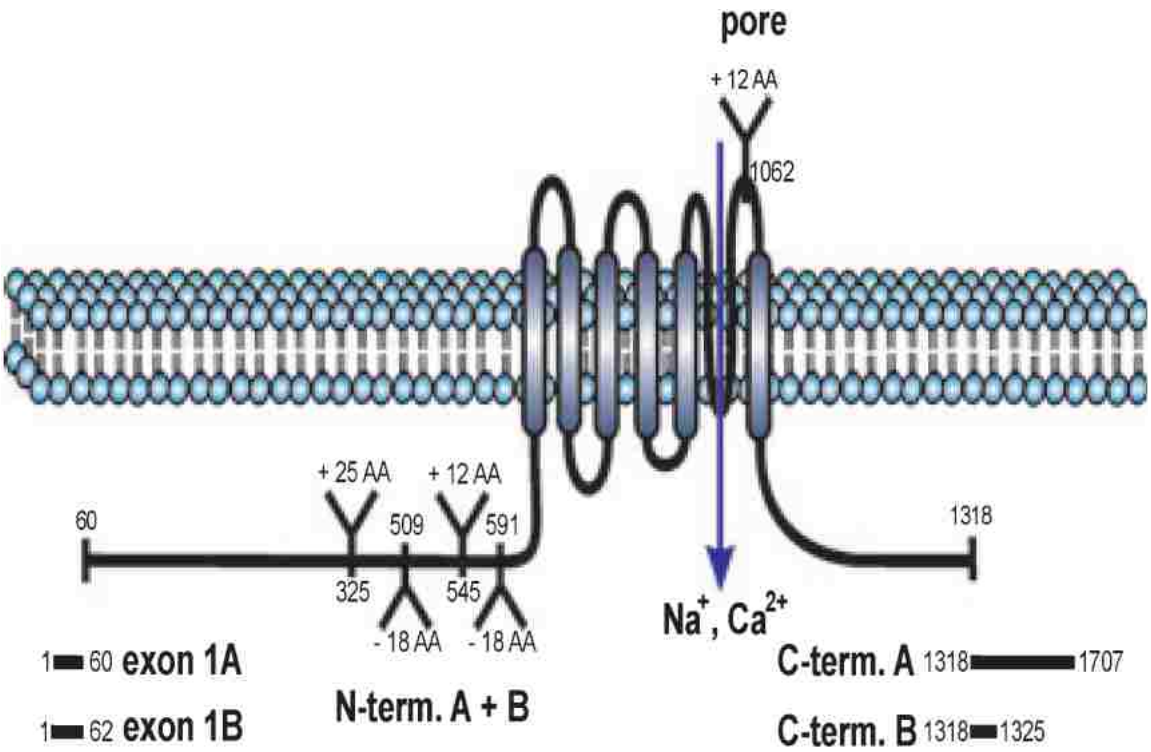

Figure 1.5. Transmembrane topology of TRPM3 and known sequences variations of TRPM3 resulting from alternative splice and transcription initiation processes (Hoffmann et al., 2010)

In TRPM3-expressing human embryonic kidney cells, TRPM3 forms a constitutively active Ca^{2+} and Mn^{2+} permeable channel (Grimm et al., 2003). The spontaneous TRPM3 currents are enhanced by hypotonic extracellular solutions and cell swelling. TRPM3 can also be activated by Ca^{2+} store depletion or sphingolipids (Venkatachalam and Montell, 2007). In addition TRPM3 channels carry outwardly rectifying and voltage-dependent currents. However, the degree of rectification varies depending on the splice variant (Grimm et al., 2003, Oberwinkler et al., 2005b). In humans, the highest expression of TRPM3 has been found in kidney, brain, pituitary, ovaria, and pancreas. In the mouse,

the brain is the main source of TRPM3 expression. The mouse brain regions where TRPM3 has been detected include the dentate gyrus, lateral septal nuclei, indisium griseum, tenia tecta, choroid plexus, corpus callosum, cerebellum, and brain stem (Grimm et al., 2003, Hoffmann et al., 2010). However, the functional significance of TRPM3 in the brain remains largely unknown.

1.4 Summary.

In the cerebellar cortex the first three postnatal weeks represent an active period of synapse formation and stabilization. PCs are highly neurosteroidogenic sites throughout development and the steroids progesterone, allopregnanolone, and estradiol have been shown to modulate the morphological maturation of PCs and of developing synapses (Tsutsui et al., 2003). PregS is a neuro-active steroid capable of rapidly affecting synaptic transmission via modulation of membrane ion channels. This neurosteroid is known to strengthen glutamatergic transmission in the neonatal hippocampus (Mameli et al., 2008). However, despite its proposed presence in the developing PC, the role of PregS in PC synapse development had not been explored.

The studies described in this dissertation characterize the effects of PregS in neurotransmission in neonatal PCs and identify the steroid-sensitive TRPM3 channel as a novel modulator of glutamatergic transmission in the developing brain.

2. Goals of this study

Specific Aim 1. Do PregS-like neurosteroids modulate neurotransmission in developing Purkinje cells?

Neuroactive steroids have been shown to play an important role in processes such as learning and memory and maturation of brain circuits (Grobin and Morrow, 2001). They modulate synaptic transmission by acting on a wide range of synaptic membrane ion channels and regulate the excitatory/inhibitory balance in the brain. The neuroactive steroid pregnenolone sulfate (PregS), in addition to modulating synaptic transmission in the adult brain (Chen and Sokabe, 2005, Schiess and Partridge, 2005, Monnet and Maurice, 2006) has also been shown to potentiate glutamatergic transmission in the immature hippocampus (Mameli et al., 2005). Another brain region that could be targeted by PregS during development is the cerebellum. The cerebellar Purkinje cell (PC) is an active neurosteroidogenic cell, which expresses the cholesterol side chain cleavage enzyme, P450scc, in adulthood and neonatal life. P450scc synthesizes pregnenolone from cholesterol (Ukena et al., 1998). Therefore, we investigated whether PregS affected GABAergic and/or glutamatergic transmission in developing PCs.

Specific Aim 2. What is the mechanism underlying the PregS-induced increase in glutamate release onto neonatal Purkinje cells?

PregS has been shown to modulate glutamate release in various brain regions via modulation of several targets such as σ1, α7 nicotinic acetylcholine, dopamine 1,

adrenergic α 1 receptors, and transient receptor potential C channels (Baulieu et al., 2001, Dong et al., 2005, Schiess and Partridge, 2005, Lee et al., 2010). In the immature hippocampus, PregS acts via NMDA receptor activation. In these studies we systematically tested the involvement of known PregS targets as well as modulators of glutamatergic transmission in the developing cerebellum, on the PregS-induced increase in glutamate release at immature PCs.

Specific Aim 3. Does the PregS-induced increase in glutamatergic transmission modulate the development of climbing fiber - and/or parallel fiber-to-Purkinje cell synapses?

Using acute slice preparations we were able to characterize and elucidate the mechanism by which PregS exerts its potentiating effects on glutamate release at developing PCs. However, it is of interest to investigate whether endogenous PregS regulates the development of glutamatergic synapses onto PCs. In these studies we used organotypic cultures to investigate whether increasing the levels of endogenous PregS-like neurosteroids during development could modulate glutamatergic transmission at cerebellar PCs

3. Pregnenolone Sulfate Increases Glutamate Release at Neonatal Climbing Fiber-to-Purkinje Cell Synapses

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3.1 Abstract

Development of cerebellar Purkinje cells (PCs) is modulated by neuroactive steroids. Developing hippocampal pyramidal neurons retrogradely release a pregnenolone sulfate (PregS)-like neurosteroid that may contribute to glutamatergic synapse stabilization. We hypothesized that PregS could exert a similar effect on developing PCs. To test this hypothesis, we performed whole-cell patch-clamp recordings from PCs in acute cerebellar vermis slices from neonatal rats. PregS induced a robust (~3,000 %) and reversible increase in AMPA receptor-mediated miniature excitatory postsynaptic current (AMPA-mEPSC) frequency without affecting the amplitude, time-to-rise, or half-width of these events. PregS also increased the frequency of $GABA_A$ receptor-mediated miniature postsynaptic currents, but to a significantly lesser extent (<100%). The PregS-induced increase of AMPA-mEPSC frequency was not significantly decreased by antagonists of receptors (NMDA, glycine, α7 nicotinic acetylcholine, and σ1) that have been shown to modulate glutamatergic transmission at PCs and/or mediate the actions of PregS on neurotransmitter release. Ca^{2+} chelation experiments suggested that PregS acts by increasing presynaptic terminal $[Ca^{2+}]_i$, an effect that is independent of voltage-gated Ca^{2+} channels, but is blocked by the antagonist of transient receptor potential (TRP) channels, La^{3+}. PregS also increased the amplitude of EPSCs evoked by climbing fiber (CF) stimulation and decreased the paired-pulse ratio of these events. Neither CF- nor parallel fiber- evoked EPSCs were affected by PregS in slices from juvenile rats. These results suggest that glutamate release at CF-to-PC synapses is an important target of PregS in the neonatal cerebellar cortex, an effect that may play a role in the refinement of these synapses.

Keywords: neurosteroid, cerebellar cortex, synaptic transmission, development, Purkinje cell, climbing fiber

3.2 Introduction

The cerebellum is essential for motor coordination, motor learning, timing of conditioned reflexes, and also for a number of higher cognitive functions (Ito, 2002). Alterations in the function of neurons of the cerebellar cortex and deep cerebellar nuclei have been implicated in the pathophysiology of several neurological and psychiatric disorders, including autism, schizophrenia, alcoholism, fetal alcohol syndrome, and ataxia (Schmahmann, 2004, Ito-Ishida et al., 2008, Shi et al., 2009). The mature cerebellar cortex consists of three layers—the granule layer is the innermost layer, the Purkinje layer is the middle layer and the molecular layer is the outermost layer. The granule cell layer contains the cerebellar granule cells and Golgi cells. Cerebellar granule cells receive excitatory input from the brain stem and spinal cord via mossy fibers and inhibitory input from Golgi cells. The axons of granule cells form the parallel fibers (PFs) that provide glutamatergic input to distal dendrites of Purkinje cells (PCs). PCs also receive glutamatergic input from the inferior olive via the climbing fibers (CFs), which synapse onto more proximal dendrites. γ-aminobutyric acid (GABA)- releasing interneurons (i.e. basket and stellate cells) located in the molecular layer provide inhibitory input to PCs; these cells are collectively known as molecular layer interneurons (MLIs). PCs are GABAergic neurons that project to deep cerebellar nuclei and their axons constitute the only output of the cerebellar cortex.

PCs undergo marked morphological and functional changes during the first 2 postnatal weeks in rodents; the first 10-12 days of life in rats is equivalent to the 3rd trimester of pregnancy in humans and is a critical period for PC maturation. During this period, PCs merge into a single layer, develop an extensive dendritic tree, and form

synaptic connections (Kurihara et al., 1997, Eilers et al., 2001). The development of CF-to-PC synapses precedes that of PF-to-PC synapses. CF-to-PC synapses have been detected as early as embryonic day (E) 19 (Morara et al., 2001). From the onset of CF-to-PC synaptogenesis until postnatal day (P) 4-7, the number of CF inputs per PC reaches a maximum of approximately 5. These immature CF-to-PC inputs differ in synaptic strength and decrease in number through the 3rd postnatal week by a process of synapse elimination of all but the strongest CF input (reviewed in (Bosman and Konnerth, 2009).

In immature neurons, glutamatergic and GABAergic transmission have been shown to regulate the maturation of dendrites and axons, as well as synaptogenesis and synaptic refinement. Glutamate controls dendritic arbor growth via N-methyl-D-aspartic acid (NMDA) receptor-dependent Ca^{2+} transients and insertion of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors (Cline and Haas, 2008). GABA acts mainly by binding to $GABA_A$ receptors, which conduct Cl- ions out of developing neurons, leading to membrane potential depolarization and activation of voltage-gated Ca^{2+} channels. This is in contrast to $GABA_A$ receptors in mature neurons where Cl- flows into the cell, causing membrane potential hyperpolarization (Huang et al., 2007b). Therefore, factors that modulate the glutamatergic and GABAergic neurotransmitter systems in developing neurons can have an impact on synapse formation and maturation. Among these factors are neuroactive steroids produced in the brain either from peripheral precursors or independently of these precursors (i.e. neurosteroids) (Mellon, 2007). Neurosteroids and neuroactive steroids such as allopregnanolone, progesterone, and estradiol have been shown to play a role in PC survival, dendritic growth, spine formation, and synaptogenesis (Tsutsui et al., 2003, Sasahara et al., 2007). However, it is

unknown whether other neurosteroids participate in the maturation of synapses onto PCs. Work from our laboratory demonstrated that an endogenous pregnenolone sulfate (PregS)-like neurosteroid produces a long-lasting strengthening of glutamatergic transmission in developing CA1 hippocampal pyramidal neurons (Mameli et al., 2005). Based on these results, we hypothesized that PregS also enhances glutamatergic synaptic transmission at developing PC synapses. To test this hypothesis we used whole-cell patch-clamp electrophysiological techniques in acute cerebellar slices from rats and measured changes in glutamatergic transmission upon bath application of PregS. For comparison, we also tested the effect of PregS on GABAergic transmission in immature PCs.

3.3 Experimental procedures

Unless specified, all chemicals were from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO). All experiments were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee and conformed with National Institutes of Health guidelines. Cerebellar vermis parasagittal slices (250 μm-thick) were prepared from neonatal (P4-10) or juvenile (P15-21) Sprague-Dawley rats and recordings from PCs were performed, as previously described (Mameli et al., 2008). The artificial cerebrospinal fluid (ACSF) was equilibrated with 95%O2/5%CO2 and contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 10 glucose, and 10 μM SR 95531 or 1 μM GYKI 53655 for AMPA-mEPSC and $GABA_A$-mPSC recordings, respectively. For these recordings the membrane potential was held at -70 mV. For Ca^{2+} free recordings, the $MgSO_4$ concentration was increased to 3 mM and 1 mM ethylene glycol tetraacetic acid (EGTA) was added to reduce contaminant Ca^{2+} levels (Perez-Velazquez et al., 1994). Patch pipette resistances were between 2 and 5.5 MΩ. AMPA-mEPSCs, $GABA_A$-mPSCs and CF stimulation- evoked EPSCs (CF-eEPSCs) were recorded from neonatal rat slices using an internal solution containing (in mM): 150 CsCl, 1.5 $MgCl_2$, 10 HEPES, 0.1 BAPTA (Calbiochem, La Jolla, CA), 2 Na_2-ATP, 0.4 Na-GTP, at pH 7.3. For CF-eEPSC and PF-eEPSC recordings from juvenile rat slices, the internal solution contained (in mM): 120 CsOH, 120 D-gluconic acid, 3 tetraethylammonium-Cl, 10 HEPES, 0.1 BAPTA, 2 Na_2-ATP, 2 Na-GTP and 0.2 Mg-ATP. The access resistance was between 10 and 30 MΩ. If the access resistance changed > 30%, the recording was discarded.

Recordings of mPSCs were obtained in presence of 0.5 μM tetrodotoxin (TTX; Calbiochem, La Jolla, CA).

For CF-eEPSC recordings, an ACSF-filled glass unipolar stimulating electrode was used and was placed on the granule cell layer, ~ 150 μm from the PC. A pair of current pulses, (duration of 100 μs; intensity 20-100 μA) was delivered every 15 s with an inter-event-interval of 50 or 60 ms. The holding potential was -70 mV in neonatal rat slices. In juvenile rat slices, the amplitude of the CF-eEPSC dramatically increased with respect to that of neonatal rat slices. To improve voltage-clamp stability in juvenile rat slices, the holding potential was therefore changed to -10 mV and the Ca^{2+} concentration in the ACSF was lowered to 0.5 mM (Mg^{2+} concentration was increased to 2.5 mM). All responses evoked were typical of CF-to-PC synapses, as they were all-or-none, and showed paired-pulse depression. PF-eEPSCs were recorded from juvenile rat slices using a holding potential of -50 mV or -70 mV. For these recordings, the stimulation electrode was placed in the molecular layer (duration of 100 μs; intensity 20-100 μA). For all stimulation experiments, 4 mM QX-314 bromide was added to the internal solution. The paired-pulse ratio (PPR) was calculated as eEPSC2/eEPSC1.

Data were acquired with pClamp 9.2 or 10 (Molecular Devices, Sunnyvale, CA) at a filtering frequency of 2 kHz and an acquisition rate of 10 kHz. Miniature events were analyzed using Mini Analysis (Synaptosoft, Decatur, GA); only events that had an amplitude at least eight times greater than the noise (average 0.84 ± 0.04 pA; n = 11) were selected for analysis. Individual recordings were analyzed using the Kolmogorov-Smirnov (K-S) test using a conservative value for significance of $p < 0.01$. Evoked EPSCs were analyzed with Clampex 9.2 (Molecular Devices, Sunnyvale, CA). Statistical

analyses of pooled data were performed with GraphPad Prizm 4 (GraphPad Software, San Diego, CA). Data were initially analyzed with the D'Agostino and Pearson omnibus normality test. If data followed a normal distribution, these were analyzed using parametric tests. If this was not the case, then non-parametric tests were used. The level of significance was $p < 0.05$. Data were normalized to baseline and expressed as percent of baseline, or as change in Hz ± SEM.

3.4 Results

3.4.1 PregS enhances the frequency but not the amplitude of AMPA-mEPSCs in neonatal PCs

We tested the effect of PregS on AMPA-mEPSCs in neonatal rat (P4-10) slices. These events were recorded in presence of the $GABA_A$ receptor antagonist, SR 95531 (10 μM) and, under these conditions, were completely blocked by the AMPA receptor antagonist, GYKI 53655 (50 μM; n = 9; not shown). Fig 3.1A-C shows that bath application of PregS (25 μM) for 5 min, reversibly enhanced AMPA-mEPSC frequency without having an effect on amplitude. The effect of PregS on AMPA-mEPSC frequency was robust (3,580 ± 1,073 % of baseline; $p < 0.0001$, n=21) (Fig 3.1C). K-S test analysis revealed that the AMPA-mEPSC inter-event interval was significantly decreased in 21 out of 21 neurons, whereas the amplitude was increased in 5 out of 21 neurons, decreased in 6 out of 21 and was not affected in 10 out of 21. The frequency of AMPA-mEPSCs (in Hz) in the absence and presence of PregS is shown in Fig 3.1C and Table 3.1; PregS increased the frequency of these events approximately 13-fold. The percent increase in the frequency of these events was inversely correlated with the baseline frequency, while the increase in Hz showed no correlation with the baseline frequency (Fig 3.1D). PregS did not affect the AMPA-mEPSC time-to-rise (107 ± 4.6 % of baseline) or half-width (102 ± 2.8 % of baseline) (not significant by Wilcoxon Signed Rank Test; n = 21) (Table 3.1). We also tested the effect of a lower concentration of PregS (5 μM) on the frequency of AMPA-mEPSCs and found that it significantly enhanced AMPA-mEPSC frequency (401.1 ± 114.7 % of baseline; raw change from baseline 0.6 ± 0.26 Hz; n=4; the K-S test showed a significant reduction in the inter-event interval in 3 out of 4 cells) without

affecting amplitude, time-to-rise or half-width (data not shown). To determine whether PregS had an effect on AMPA-mEPSC frequency in slices from older animals, we measured its effect at P12. Using a Cs-gluconate-based solution, we found that 25 μM PregS significantly increased AMPA-mEPSC frequency, but to a lesser extent than in PCs from P4-10 rats (184.3 ± 13.83 % of baseline; raw frequency change 0.97 ± 0.46 Hz; n=4; KS test <0.01 in all 4 cells).

3.4.2 PregS enhances AMPA-mEPSC frequency independently of neurotransmitter receptors.

PregS has been shown to modulate glutamate release onto immature as well as mature neurons via modulation of a number of neurotransmitter receptors; namely, NMDA, σ1, and α7 nicotinic acetylcholine receptors (α7nACh) (Meyer et al., 2002, Chen and Sokabe, 2005, Mameli et al., 2005, Schiess and Partridge, 2005). In addition, activation of NMDA and glycine receptors has been shown to facilitate transmitter release onto developing PCs (Kawa, 2003, Bidoret et al., 2009). Previous findings in the developing hippocampus suggested that PregS enhances glutamate release onto CA1 pyramidal neurons from neonatal rats via activation of presynaptic NMDA receptors (Mameli et al., 2005). In cultured hippocampal neurons PregS enhances mEPSC frequency via modulation σ1 receptors, and at perforant path-to-dentate granule cell synapses, it produces a similar effect via modulation of α7nACh receptors (Kawa, 2002, Meyer et al., 2002, Chen and Sokabe, 2005). Therefore, we measured the effect of PregS on AMPA-mEPSCs in the presence of DL-2-Amino-5-phosphonopentanoic acid (DL-APV) (100 μM), strychnine (1 μM), BD-1063 (1 μM), and methyllycaconitine (MLA; 200 nM) to block NMDA, glycine, σ1, and α7nACh receptors, respectively. In the

presence of these agents, PregS increased AMPA-mEPSC frequency to a similar extent as under control conditions (Fig 3.2). The K-S test showed that PregS significantly decreased the inter-event interval of AMPA-mEPSCs in all cells regardless of the pharmacological blocker used. Basal AMPA-mEPSC properties were not significantly affected by any of these agents (Table 3.1).

3.4.3 PregS enhances AMPA-mEPSC frequency by increasing presynaptic $[Ca^{2+}]_i$ via activation of a TRP channel

We next used the membrane-permeable Ca^{2+} chelator, BAPTA-AM (50 μM), to test the role of Ca^{2+} in the mechanism of action of PregS. A 10 minute incubation with BAPTA-AM, followed by a 5 minute washout, resulted in no significant change in basal AMPA-mEPSC frequency (Table 3.1) and a significant reduction of the PregS-induced increase of AMPA-mEPSC frequency (565.2 ± 379 % of baseline, $^{**}p < 0.05$; raw frequency change of 0.65 ± 0.3 Hz from baseline, $^{***}p < 0.05$, Fig 3.3) when compared to controls (2,824 ± 1,014 % of baseline; raw frequency change 11.41 ± 1.82 Hz from baseline, Fig 3.3B). It is noteworthy that, with this treatment, the K-S test showed that, 5 out of 9 cells showed no significant change in the AMPA-mEPSC inter-event interval with PregS bath application, and the remaining 4 showed a significant decrease. BAPTA-AM is expected to non-selectively chelate Ca^{2+} both in the pre- and post-synaptic compartments. To determine whether PregS acts by selectively increasing Ca^{2+} in one of these compartments, we tested the effect of postsynaptic Ca^{2+} chelation by dialyzing BAPTA (10 mM) into PCs through the patch pipette. The K-S test showed that in 3 out of 3 cells, PregS significantly enhanced the frequency of AMPA-mEPSCs (2,541 ± 864.4 %

of baseline; raw frequency increase of 13.69 ± 7.15 Hz from baseline, n=3, data not shown), suggesting that PregS acts by increasing $[Ca^{2+}]_i$ in presynaptic terminals.

To further assess if Ca^{2+} entrance into the presynaptic terminal was required for this effect of PregS, we performed experiments in Ca^{2+} free ACSF in the presence of the Ca^{2+} chelator, EGTA (1mM); under these conditions, the baseline AMPA-mEPSC frequency (0.25 ± 0.11 Hz) was not significantly different from control ($p > 0.05$ by unpaired t-test, n=4; see Table 3.1 for control values) and the K-S test showed that in 4 out of 4 cells PregS did not significantly change the AMPA-mEPSC inter-event interval (132.6 ± 28.01 % of baseline, not significant by Wilcoxon Signed Rank Test; raw frequency change of 0.027 ± 0.043 Hz from baseline, n=4, data not shown).

At calyx of Held developing synapses, PregS was shown to increase glutamate release via facilitation of voltage-gated Ca^{2+} channels (VGCCs) (Hige et al., 2006). To test the involvement of these channels in the effect of PregS on neonatal PCs, we performed recordings in the presence of Cd^{2+} (100 μM), a broad spectrum VGCC antagonist. As shown in Table 3.1, Cd^{2+} did not significantly change basal AMPA-mEPSC parameters. Moreover, in the presence of Cd^{2+}, PregS potently increased the frequency of AMPA-mEPSCs (2,064 ± 458.30 % of baseline with a raw frequency increase of 17.18 ± 4.53 Hz, n= 11 , Fig 3.3), suggesting that VGCC are not involved in this effect.

Recently, transient receptor potential (TRP) cation channels have been shown to be modulated by PregS, which results in increases in $[Ca^{2+}]_i$ (Wagner et al., 2008, Lee et al., 2010). Therefore, we tested the effect of La^{3+}, a blocker of a number of TRP channel

subtypes (Albert et al., 2006). A 30 min incubation with La^{3+} (100 μM) did not significantly decrease baseline AMPA-mEPSC frequency (Table 3.1). Taken together with the lack of an effect of BAPTA-AM and Ca^{2+}-free ACSF on basal mEPSC frequency, this result suggests that if tonic levels of PregS are present at CF-to-PC synapses, these are not sufficiently high to support quantal glutamate release, in agreement with our previous finding that a significant level of neuronal activity is required for retrograde release of endogenous PregS-like neurosteroids (Mameli et al., 2005). However, after incubation with La^{3+}, PregS did not significantly increase AMPA-mEPSC frequency (152.2 ± 48.07 % of baseline; raw frequency change of -0.0021 ± 0.13 Hz, n= 11, Fig 3.3), suggesting that PregS increases AMPA-mEPSC frequency via modulation of a TRP channel.

3.4.4 PregS increases PPR at CF-to-PC synapses in neonatal rat slices but does not affect PPR in CF- and PF-to-PC synapses in juvenile rat slices.

During the neonatal period in rats, CFs are the major glutamatergic input to PCs, with PF connections beginning to arrive after the first postnatal week (Bosman and Konnerth, 2009, Cesa and Strata, 2009). To determine whether PregS enhanced AMPA-mEPSC frequency by potentiating glutamate release at CF-to-PC synapses, we measured paired-pulse plasticity of CF-eEPSCs in neonatal PCs. These evoked responses were abolished in the presence of the AMPA receptor blocker, GYKI 53655 (50 μM). We found that CF-eEPSC1 amplitude was significantly increased to 135.5 ± 2.43 % of control, from a baseline value of 1,262 ± 258.8 pA ($p < 0.001$ vs 100%; n=10) in the presence of PregS (25 μM) and this effect was partially reversible (Fig 3.4). Increases in the probability of transmitter release are typically associated with decreases in the PPR.

We found that PregS induced a significant and reversible decrease in the PPR to 75.87 ± 2.31 % of control, from a baseline value of 0.39 ± 0.03 ($p < 0.001$ vs 100%; $n = 10$). This finding suggests that PregS increases glutamate release probability at CF-to-PC synapses.

CF mono-innervation is achieved during the first three postnatal weeks, during which CF-to-PC synapses undergo three distinct developmental phases. During the first postnatal week, multinumerary CF synapses onto one PC, which are composed of a strong CF synapse and several weaker ones, become functionally differentiated. This process is thought to be achieved via competition among CF inputs and is followed by an early phase and a late phase of synapse elimination of redundant inputs, the last one being dependent on PF-to-PC synapse formation (Kano and Hashimoto, 2009). We next investigated whether PregS also enhanced glutamate release from CFs beyond the functional differentiation phase (i.e. during the first week) and the early phase of synapse elimination (i.e. during P7-12). To this end, we performed paired-pulse stimulation recordings of CF-eEPSCs in juvenile rat slices (P15-21) and found that PregS (25 μM) did not significantly affect the PPR of these events (baseline PPR was 0.42 ± 0.04; Fig 3.5). The amplitude of CF-eEPSC1 also remained unchanged in the presence of PregS. These results suggest that PregS increases glutamate release probability at the CF-to-PC synapse only during the phases of functional differentiation and early synapse elimination.

We also tested whether PregS affected glutamate release at PF-to-PC synapses, which start forming at the end of the first postnatal week, continuing their development through the end of the third postnatal week when the migration of granule cells is completed and the outer granule cell layer disappears (Kurihara et al., 1997, Scelfo and

Strata, 2005). To this end, we recorded paired-pulse plasticity of PF-eEPSCs in juvenile rat slices. We found that PregS did not change the amplitude or the PPR of PF-eEPSCs (Fig 3.6), suggesting that the effect of PregS on glutamate release is specific to developing CF inputs.

3.4.5 PregS also increases GABA release in developing PCs but to a lesser extent than glutamate release.

For comparison, we investigated the effect of PregS on GABAergic transmission at PCs. PregS has been shown to decrease the frequency of spontaneous inhibitory PSCs (IPSCs) as well as mIPSCs in cultured pyramidal hippocampal neurons (Teschemacher et al., 1997, Mtchedlishvili and Kapur, 2003), but it has been suggested to increase spontaneous IPSCs in PCs (Tsutsui and Ukena, 1999). We found that PregS (25 μM) also enhanced the frequency of $GABA_A$-mPSCs in neonatal rat slices (183.9 ± 28.37 % of baseline, with a raw frequency increase of 0.84 ± 0.33 Hz from baseline, n=10; $p < 0.001$ by one sample t-test; the K-S test revealed that 6 out of 10 cells showed an statistically significant decrease in $GABA_A$-mPSC inter-event interval) (Fig 3.7; Table 3.2). In addition, PregS significantly decreased the amplitude of these events (80.88 ± 5.35 % of baseline, $p < 0.05$ by one sample t test; raw amplitude decrease 18.52 ± 6.17 pA from baseline) (Table 3.2). Time-to-rise (99.86 ± 2.98 % of baseline), and half-width (97.66 ± 2.69 % of baseline) were not significantly affected by PregS application (Table 3.2).

3.5 Discussion

3.5.1 PregS enhances quantal glutamate release onto neonatal cerebellar PCs

A miniature postsynaptic current is produced by the opening of postsynaptic neurotransmitter receptors in response to the presynaptic release of individual quanta of neurotransmitter. Therefore, increases in the frequency of these events, are indicative of an increase in presynaptic neurotransmitter release. On the other hand, changes in the amplitude, time-to-rise, or half-width of miniature postsynaptic currents suggest an alteration in the current through postsynaptic neurotransmitter receptors and are, therefore, indicative of a postsynaptic change. We report here that, in neonatal PCs, PregS increases the frequency of AMPA-mEPSCs, but does not affect the amplitude, time–to-rise, or half-width of these events, indicating that it acts presynaptically by increasing the release of glutamate onto PCs. This effect was observed at concentrations of PregS (5 and 25 μM) that have been previously shown to increase quantal glutamate release and are near the estimated concentration of an endogenous PregS-like neurosteroid in glutamatergic synapses of developing CA1 pyramidal neurons (i.e., 17 μM); (Meyer et al., 2002, Dong et al., 2005, Mameli et al., 2005, Hige et al., 2006, Lee et al., 2010). To the best of our knowledge, the PregS effect at PC glutamatergic transmission shown here is the most robust action of this agent on quantal transmitter release reported to-date. Previous studies have shown that 25 μM PregS increases mEPSC frequency in developing hippocampal synapses by about 400% (Mameli et al., 2005), and in the mature prelimbic cortex, a similar concentration of PregS (20 μM) enhanced mEPSC frequency by about 200% (Dong et al., 2005). Moreover, the effect of PregS on glutamatergic transmission in PCs was reversible, in contrast to its effect on

immature CA1 pyramidal neurons, suggesting that the late postsynaptic phase of the PregS-induced plasticity—that involves NMDA receptor-dependent recruitment of AMPA receptors into silent syapses in CA1 pyramidal neurons— does not take place in PCs (Mameli et al., 2005). A possible explanation for this finding is that postsynaptic NMDA receptors in developing PCs have different characteristics, which make them unable to recruit AMPA receptors into the membrane in response to changes in glutamate release.

3.5.2 The PregS-induced increase of quantal glutamate release onto PCs is independent of activation of neurotransmitter receptors

Another unique property of the effect of PregS at PCs is that it was insensitive to blockade of a number of receptors (NMDA, glycine, α7nACh, and σ1 receptors), which have been previously shown to mediate PregS effects and/or modulate neurotransmitter release onto neonatal PCs. Although activation of NMDA receptors mediates the PregS-induced increase in glutamate release in neonatal hippocampal neurons (Mameli et al., 2005), this is not the case in the neonatal cerebellum. Postsynaptic NMDA receptors have been shown to contribute to CF-EPSCs before P21 (Piochon et al., 2007); however, presynaptic NMDA receptors may not be present at neonatal CF-to-PC synapses and this could be an explanation for their lack of contribution to the mechanism of action of PregS. An alternative target of PregS that we considered was the glycine receptor, which has been shown to enhance the frequency of spontaneous EPSCs and IPSCs in neonatal PCs (Kawa, 2003). Nonetheless, in the presence of strychnine, the PregS-induced increase in AMPA-mEPSC frequency was not affected. These results are not surprising given that the glycine-induced increase in sEPSC frequency was almost completely

abolished by TTX (Kawa, 2003), and that PregS has been shown to inhibit recombinant glycine receptors (reviewed in (Gibbs et al., 2006). Another plausible candidate for the effect of PregS on PCs was the Ca^{2+} permeable α7nACh receptor. This receptor has been shown to modulate the PregS-induced facilitation of synaptic transmission in the hippocampus (Chen and Sokabe, 2005). In addition, acetylcholine increases spontaneous EPSC and IPSC frequency in developing PCs (Kawa, 2002). We found that blockade of α7nACh receptors did not affect the PregS-induced increase in AMPA-mEPSC frequency. PregS has also been shown to modulate glutamate release via activation of metabotropic σ1 receptors. PregS-induced modulation of σ1 receptors enhances facilitated glutamate release in the mature hippocampus (Schiess and Partridge, 2005), and increases spontaneous glutamate release in cultured hippocampal neurons (Meyer et al., 2002). We found that blockade of σ1 receptors did not abolish the effect of PregS in PCs. Moreover, the σ1 receptor agonist, pre-084 did not affect AMPA-mEPSC frequency in P9-12 PCs (n=4, data not shown), suggesting that these receptors do not play a role in the spontaneous release of glutamate onto neonatal PCs. Taken together, these findings indicate that NMDA, glycine, α7nACh, and σ1 receptors are not involved in the mechanism of action PregS at neonatal PCs.

3.5.3 The PregS-induced increase of quantal glutamate release requires an elevation in presynaptic $[Ca^{2+}]_i$ and is dependent on activation of TRP channels

Previously, PregS-induced increases in glutamate release have been shown to be dependent on elevations in $[Ca^{2+}]_i$; incubation with the membrane permeable Ca^{2+} chelator, BAPTA-AM, blocked the PregS-induced increase of AMPA-mEPSC frequency in developing hippocampal neurons (Meyer et al., 2002, Mameli et al., 2005). Consistent

with these studies, we found that pre-incubation with BAPTA-AM (50 μM), followed by a 5 minute washout of BAPTA-AM before PregS bath application, dramatically decreased the PregS-induced increase of AMPA-mEPSC frequency. However, this treatment did not completely abolish the effect of PregS on neonatal PCs, which could be explained by incomplete Ca^{2+} chelation. Absence of extracellular Ca^{2+} abolished the PregS-induced increase in AMPA-mEPSC frequency in neonatal PCs. This result, combined with the fact that dyalizing BAPTA into the PCs through the patch pipette did not block the increase in mEPSC frequency, supports the conclusion that elevation in Ca^{2+} entrance into the presynaptic terminal is required for this effect of PregS.

We found that the PregS-induced increase in AMPA-mEPSC frequency is blocked by La^{3+}. This finding is in agreement with the results of a recent study showing that PregS increases sEPSC frequency in dentate gyrus hilar neurons, an effect that was blocked by non-selective TRP channel antagonists, including La^{3+} (Lee et al., 2010). La^{3+} antagonizes several members of the canonical (C) family of TRP channels (TRPC3, 5, 6, and 7) (Albert et al., 2006), suggesting that PregS could act by potentiating these channels. TRPC channels have been found to be expressed in presynaptic nerve terminals (Goel et al., 2002, Nichols et al., 2007) and their activation has been implicated in neurotransmitter release modulation (Selvaraj et al.). Interestingly, in the cerebellum, the expression of TRPC channels has been shown to be developmentally regulated; during the first 6 postnatal weeks, TRPC3 is abundantly expressed in comparison to TRPC4 and TRPC6 channels (Huang et al., 2007a). Additionally, TRPC3 channels mediate mGluR1-dependent slow EPSCs in PCs from young mice whereas TRPC1 channels mediate these currents in PCs from older animals (Kim et al., 2003, Hartmann et

al., 2008). Given that the effect of PregS on glutamate release at PCs is restricted to the neonatal period, it is possible that the loss of PregS sensitivity in more mature neurons is a consequence of a change in the subunit composition of cerebellar TRPC channels.

An alternative TRP channel that could mediate the effects of PregS is TRPM3 (Kraft and Harteneck, 2005). PregS was shown to activate both recombinant TRPM3 channels expressed in HEK 293 cells and also native TRPM3 channels expressed in pancreatic β cells and vascular smooth muscle, where PregS stimulates insulin secretion and contraction, respectively (Wagner et al., 2008, Naylor et al., 2010). La^{3+} has been shown to antagonize recombinant TRPM3 channels expressed in HEK 293 cells (Grimm et al., 2003). Importantly, TRPM3 channels were recently found to be expressed in developing neurons of the cerebellum and brain stem of Wistar rats. Interestingly, as development progresses, expression of these channels becomes restricted to oligodendrocytes (Hoffmann et al., 2010) and this could explain the lack of PregS sensitivity of PCs in slices from juveniles rats. Clearly, the role of TRPM3 channels in the mechanism of action of PregS in the developing cerebellum should be further investigated.

3.5.4 PregS enhances glutamate release evoked by electrical stimulation of CFs in slices from neonatal rats.

The all-or-none responses produced by CFs on mature PCs are mediated by a massive release of glutamate that nearly saturates the postsynaptic AMPA receptors (Konnerth et al., 1990). Therefore, at these synapses, an increase in glutamate release probability would not be expected to dramatically increase CF AMPA-eEPSC amplitude

(Ohtsuki and Hirano, 2008). Accordingly, the effect of PregS on AMPA-eEPSCs was substantially smaller than on AMPA-mEPSCs. Interestingly, we found that the effect of PregS on the probability of glutamate release at PCs is specific to CF synapses during the neonatal period (i.e. P4-10), where CF-to-PC synapses undergo differentiation and the first stages of elimination; importantly, PregS did not significantly affect glutamatergic transmission at CF-to-PC synapses (and also PF-to-PC synapses) beyond these developmental period (i.e. in slices from P15-21 rats). Thus, during this restricted developmental period, PregS could act on CF-to-PC synapses to strengthen glutamatergic transmission. Studies from our laboratory have shown that a PregS-like neurosteroid can be retrogradely released upon depolarization of hippocampal CA1 pyramidal cells, which subsequently increases glutamate release onto these cells (Mameli et al., 2005). In a similar fashion, CF-induced depolarization of neonatal PCs could stimulate the retrograde diffusion of a PregS-like neurosteroid, which in turn would increase the probability of glutamate release at CF terminals via activation of TRP channels, thereby contributing to the maturation of these synapses. It is important to note that the CF-eEPSC evaluated in this study were the largest ones found in each cell, representing the responses of both weak and strong CF inputs. Previous studies have shown that strong and weak CF synapses differ in their response to stimulation; while strong CF synapses undergo long term potentiation, under the same stimulation paradigm, weak CF synapses undergo long term depression (Bosman et al., 2008, Ohtsuki and Hirano, 2008). Therefore, future studies should investigate whether PregS differentially modulates the probability of glutamate release and plasticity in strong versus weak CF-to-PC synapses and how this

modulation affects CF synaptic competition and the subsequent achievement of monoinnervation.

3.5.5 PregS increases GABAergic transmission onto neonatal PCs

We found that PregS significantly enhanced the frequency and decreased the amplitude of $GABA_A$-mPSCs. Our findings are in contrast with previous results showing that PregS decreases $GABA_A$-mPSC frequency in hippocampal neurons, suggesting a decrease in GABA release upon PregS application (Teschemacher et al., 1997, Mtchedlishvili and Kapur, 2003). Both of these studies were performed in cultured hippocampal neurons (3-9 week *in vitro*) that were obtained from late embryonic or neonatal rats, where it was found that PregS reduced the frequency of mIPSCs by 20 to 40% at concentrations between 30 nM and 50 μM (Teschemacher et al., 1997, Mtchedlishvili and Kapur, 2003). The discrepancy between the previous studies and our current findings could be due to differences in presynaptic release mechanisms, and their sensitivity to PregS, in hippocampal neurons vs. PCs. During the first postnatal week, $GABA_A$ receptors are excitatory and have been suggested to contribute to the activity-dependent maturation of cerebellar synapses, including those of CFs (Eilers et al., 2001, Watt et al., 2009). Therefore, the PregS-induced enhancement of GABAergic transmission during this developmental period could play a role in the maturation of GABAergic as well as glutamatergic synapses onto PCs. However, the specific source for the PregS-sensitive GABA release sites remains undetermined, as molecular layer interneurons to PC synapses start developing at the end of the first postnatal week (Ango et al., 2004). A plausible candidate is the PC axonal collaterals to neighboring PCs, which have been detected as early as P4 (Watt et al., 2009).

The PregS-induced decrease in the amplitude of $GABA_A$-mPSCs is in agreement with previous reports showing that PregS inhibits $GABA_A$ receptor-mediated currents (Akk et al., 2001). However, Mtchedlishvili and Kapur, (2003) found no effect of PregS on the amplitude of $GABA_A$ mediated events and Teschemacher et al., (1997) reported a non-reversible 15% decrease in the amplitude of mIPSCs upon PregS application. This effect, however, was not attributable to a PregS- induced decrease in $GABA_A$ receptor - mediated currents, because of its late onset and lack of concentration dependency, and was better explained by current run-down. Our finding that the effect of PregS on $GABA_A$-mPSC amplitude is reversible (see Fig. 3.7B) suggests that PregS could directly inhibit postsynaptic $GABA_A$ receptors.

3.6 Conclusion

Cerebellar PCs are highly neurosteroidogenic cells. The cytochrome P450scc, which catalyzes the conversion of cholesterol to the neurosteroid precursor pregnenolone, has been found in the soma and dendrites of PCs in rodents, avians, amphibians and other lower vertebrates. This enzyme appears in rat PCs immediately after their differentiation (reviewed in (Tsutsui and Ukena, 1999). Therefore, it is possible that PCs synthesize an endogenous counterpart of PregS, which could play a role in the maturation of CF-to-PC synapses (for a detailed discussion on whether PregS per se is a veritable neurosteroid, see (Dong et al., 2005, Schumacher et al., 2008, Valenzuela et al., 2008). Importantly, PCs are highly sensitive to developmental ethanol exposure (Servais et al., 2007) and studies from our laboratory have shown that chronic prenatal ethanol exposure increases the levels of PregS-like neurosteroids in the developing brain and that ethanol increases the efficacy of immature hippocampal synapses in a PregS-like neurosteroid-dependent

manner (Caldeira et al., 2004, Mameli et al., 2005, Mameli and Valenzuela, 2006, Valenzuela et al., 2008). Therefore, alterations in the developmental actions of PregS at PCs may play a role in the pathophysiology of the cerebellar alterations that characterize fetal alcohol spectrum disorders.

Acknowledgement

We thank Dr. Don Partridge for critically reading the manuscript.

3.7 Figure Legends

Figure 3.1. PregS (25 μM) robustly increases the frequency of AMPA-mEPSCs in PCs from neonatal rats. (A) Sample traces for a P5 rat, calibration: 20 pA, 0.2 s. Low panel average events, calibration: 4.1 pA, 3 ms. (B) Corresponding cumulative probability plots; PregS decreased the inter-event interval ($p < 0.01$ by K–S test; 86 events/3 min before PregS and 2050 events/3 min during PregS); the inset further illustrates the robust increase that PregS induces on the inter-event interval at an expanded scale. The bottom panel shows that amplitude of these events is not significantly affected by PregS. (C) Average time courses of AMPA-mEPSC frequency and amplitude expressed as a percent of baseline (top panel) and as the raw change (bottom panel) (n=21). (D) The percent increase in frequency induced by PregS is inversely correlated to the baseline frequency ($r = -0.7390$; $p < 0.05$ by Spearman correlation test, n=21) (top panel). However, the PregS-induced increase in frequency in Hz is not correlated to the baseline frequency ($r=-0.09775$; $p=0.6734$ by Pearson correlation test) (bottom panel).

Figure 3.2. The PregS (25 μM)-induced increase in AMPA-mEPSC frequency is independent of NMDA, glycine, σ1, or α7nACh receptors. (A) Sample traces illustrating the PregS effect on AMPA-mEPSC frequency in PCs from P4-5 rats in the presence of the NMDA and glycine receptors blockers, DL-AP5 (100 μM) and strychnine (1 μM); the σ1 receptor blocker, BD-1063 (1 μM); and the α7nACh receptor blocker,

MLA (200nM); calibration: 50 pA and 0.2 s. (B) Summary graphs of the percent frequency (left panel) and the raw frequency increases (right panel) induced by PregS in the presence of NMDA, glycine, σ1 or α7nACh receptor antagonists. For both, percent and raw frequency increases, one-way ANOVA followed by Dunn's multiple comparison test showed no significant difference between controls (n=11) and DL-APV/strychnine (n=11), BD-1063 (n=9) or MLA (n=10). The effect of PregS on AMPA-mEPSC frequency from Fig 1 is shown again for comparison.

Figure 3.3. PregS (25 µM) increases AMPA-mEPSC frequency by elevating $[Ca^{2+}]_i$ possibly via modulation of TRP channels. (A) Sample traces illustrating the effect of PregS on AMPA-mEPSC frequency in PCs from P4-5 rats in the presence of BAPTA-AM, Cd^{2+} and La^{3+}, calibration: 50 pA and 0.2 s. (B) Summary graphs of the percent frequency (left panel) and the raw frequency (right panel) changes induced by PregS in the presence of these agents. For both, percent and raw frequency increases, one-way ANOVA followed by Dunn's multiple comparison test showed a significant difference between controls (n=11), BAPTA-AM (n=9; * $p < 0.05$) and La^{3+} (n=11; *** $p < 0.001$). The effect of PregS on AMPA-mEPSC frequency from Fig 1 is shown again for comparison.

Figure 3.4. PregS (25 µM) increases CF-eEPSC amplitude and decreases the PPR in PCs from neonatal rats. (A) Sample traces from a P6 rat, calibration: 500 pA, 50 ms.

(B) Average time course of the effect of PregS (25 μM) on the amplitude on CF-eEPSC1 and PPR (n=10).

Figure 3.5. PregS (25 μM) does not affect CF-eEPSC amplitude or the PPR in PCs from juvenile rats. (A) Sample traces from a P15 rat, calibration: 100 pA, 50 ms. (B) Average time course of CF-eEPSC1 amplitude and PPR (n=7).

Figure 3.6. PregS (25μM) does not affect PF-eEPSC amplitude or the PPR in PCs from juvenile rats. (A) Sample traces from a P18 rat, calibration: 200 pA, 20 ms. (B) Average time course of PF-eEPSC1 amplitude and PPR (n=6).

Figure 3.7. PregS (25μM) increases the frequency of $GABA_A$-mPSCs in PCs from neonatal rats (A) representative traces from a P6 rat. Calibration: 50 pA, 1 s. Right panel average events, calibration: 20.5 pA, 4 ms. (B) Time courses of the PregS- induced changes of $GABA_A$-mPSC frequency and amplitude.

3.8 Tables

Table 3.1. AMPA-mEPSC parameters in PCs from neonatal rats in the absence and presence of PregS or pharmacological receptor blockers.

*** $p<0.01$ vs. control by one-way ANOVA followed by Dunn's multiple comparison test.

Experimental condition (n)	Frequency (Hz)	Amplitude (pA)	Time-to-rise (ms)	Half-Width (ms)
Control (11)	0.80 ± 0.22	23.20 ± 1.55	2.27 ± 0.12	1.98 ± 0.12
APV + Strychnine (11)	0.81 ± 0.16	24.71 ± 1.68	2.93 ± 0.39	2.23 ± 0.14
BD1063 (9)	0.49 ± 0.14	20.16 ± 1.19	2.54 ± 0.25	2.18 ± 0.13
MLA (10)	0.31 ± 0.10	23.44 ± 2.72	2.69 ± 0.28	2.13 ± 0.24
BAPTA-AM (9)	0.66 ± 0.31	21.36 ± 1.46	2.79 ± 0.42	2.56 ± 0.3
PregS (11)	12.2 ± 1.98 ***	22.62 ± 1.50	2.59 ± 0.20	2.07 ± 0.15
Cd^{2+} (11)	1.79 ± 0.58	20.75 ± 1.52	2.08 ± 0.20	2.08 ± 0.16
La^{3+} (11)	0.44 ± 0.17	23.89 ± 2.69	2.05 ± 0.12	1.97 ± 0.15

Table 3.2. $GABA_A$-mPSC parameters in PCs from neonatal rats in the absence and presence of PregS (25 µM).

* $p < 0.05$ by paired t-test,** $p < 0.05$ by Wilcoxon signed rank test.

Experimental condition (n=10)	Frequency (Hz)	Amplitude (pA)	Time-to-rise (ms)	Half-Width (ms)
Control	1.88 ± 0.90	85.77 ± 10.35	2.79 ± 0.08	4.64 ± 0.23
PregS	2.72 ± 1.14 **	67.25 ± 7.54 *	2.78 ± 0.10	4.47 ± 0.20

Figure 3.1. PregS (25 µM) robustly increases the frequency of AMPA-mEPSCs in PCs from neonatal rats.

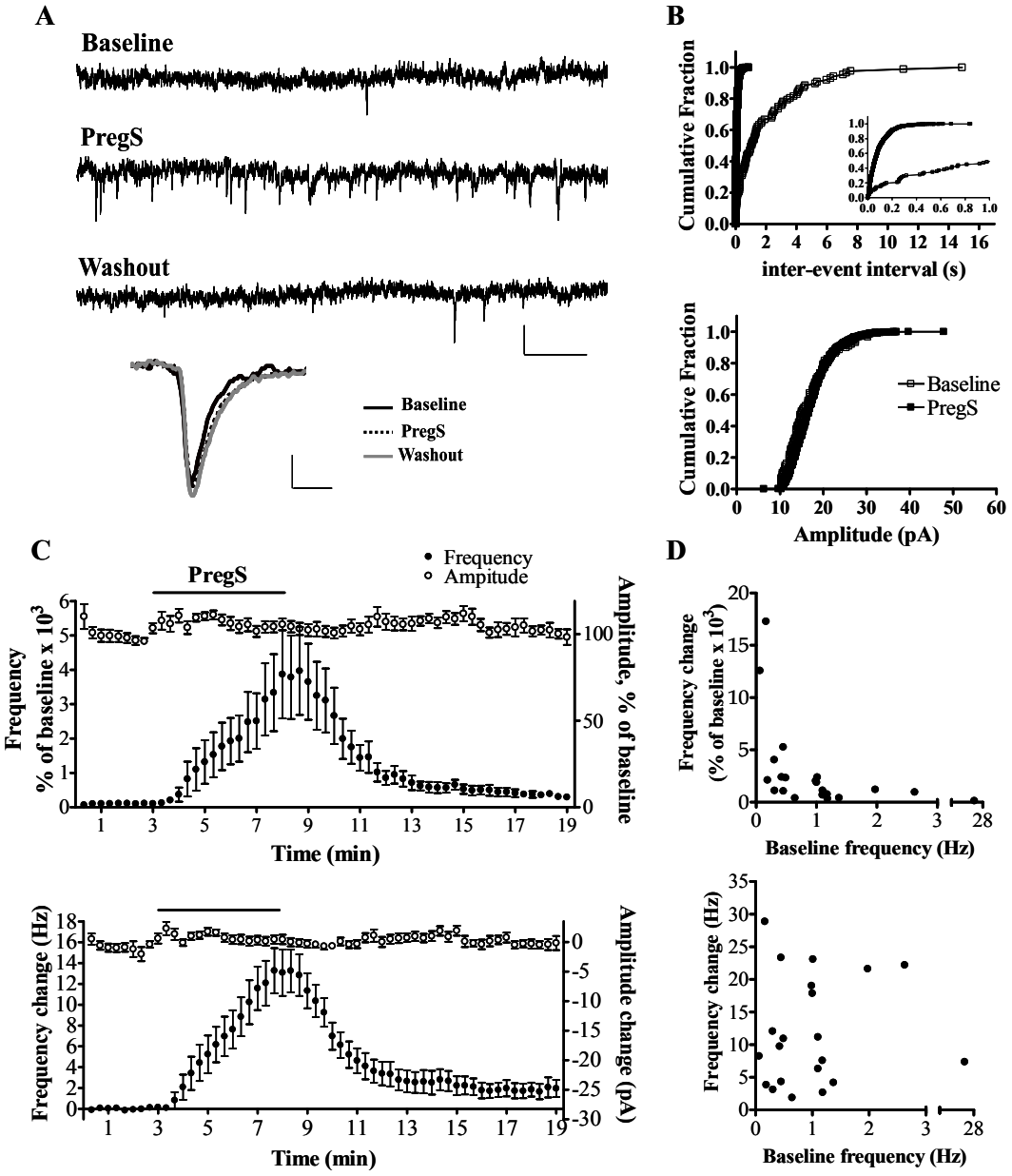


Figure 3.2. The PregS (25 µM)-induced increase in AMPA-mEPSC frequency is independent of NMDA, glycine, σ1, or α7nACh receptors.

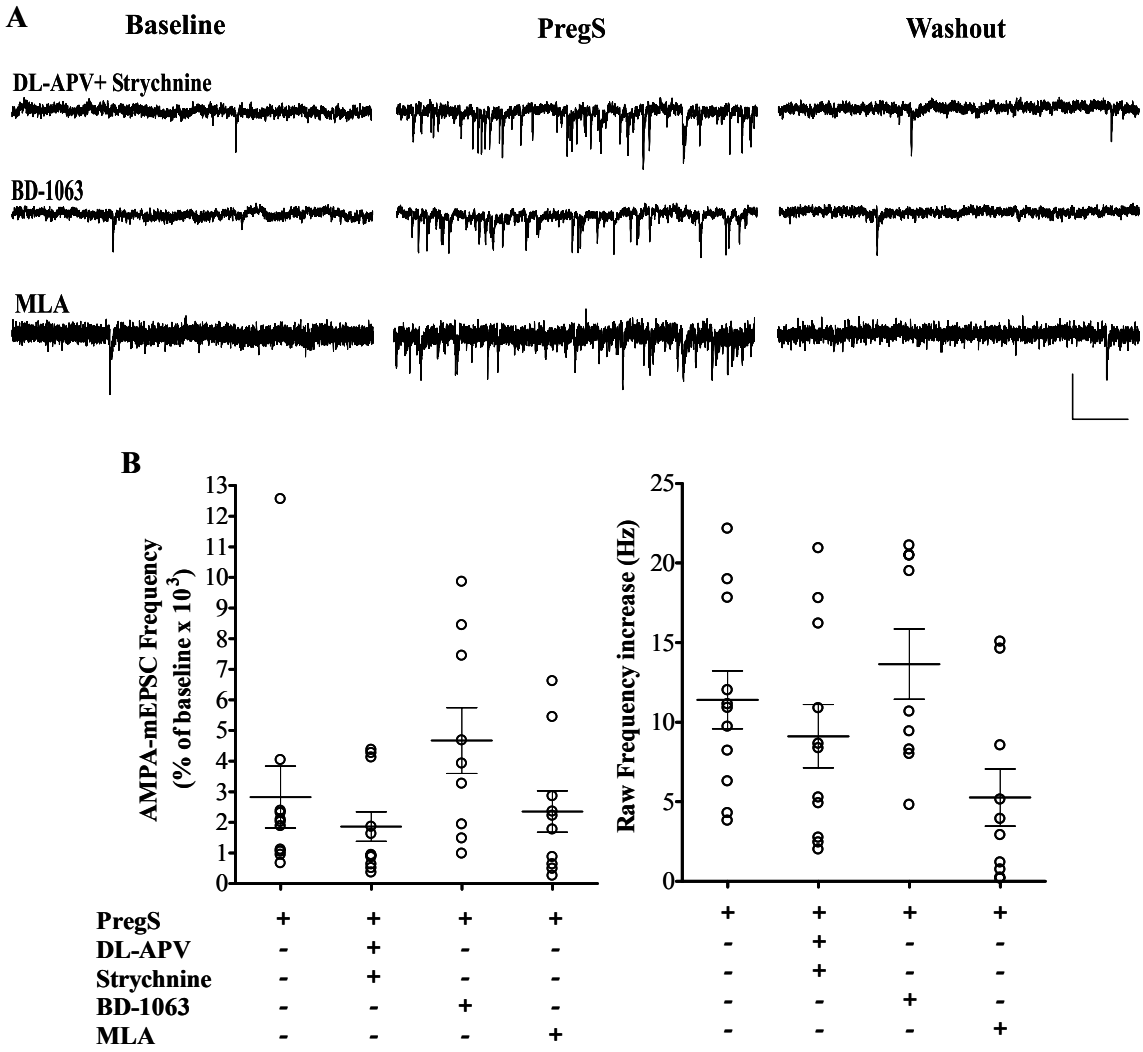


Figure 3.3. PregS (25 µM) increases AMPA-mEPSC frequency by elevating [Ca2+]i possibly via modulation of TRP channels.

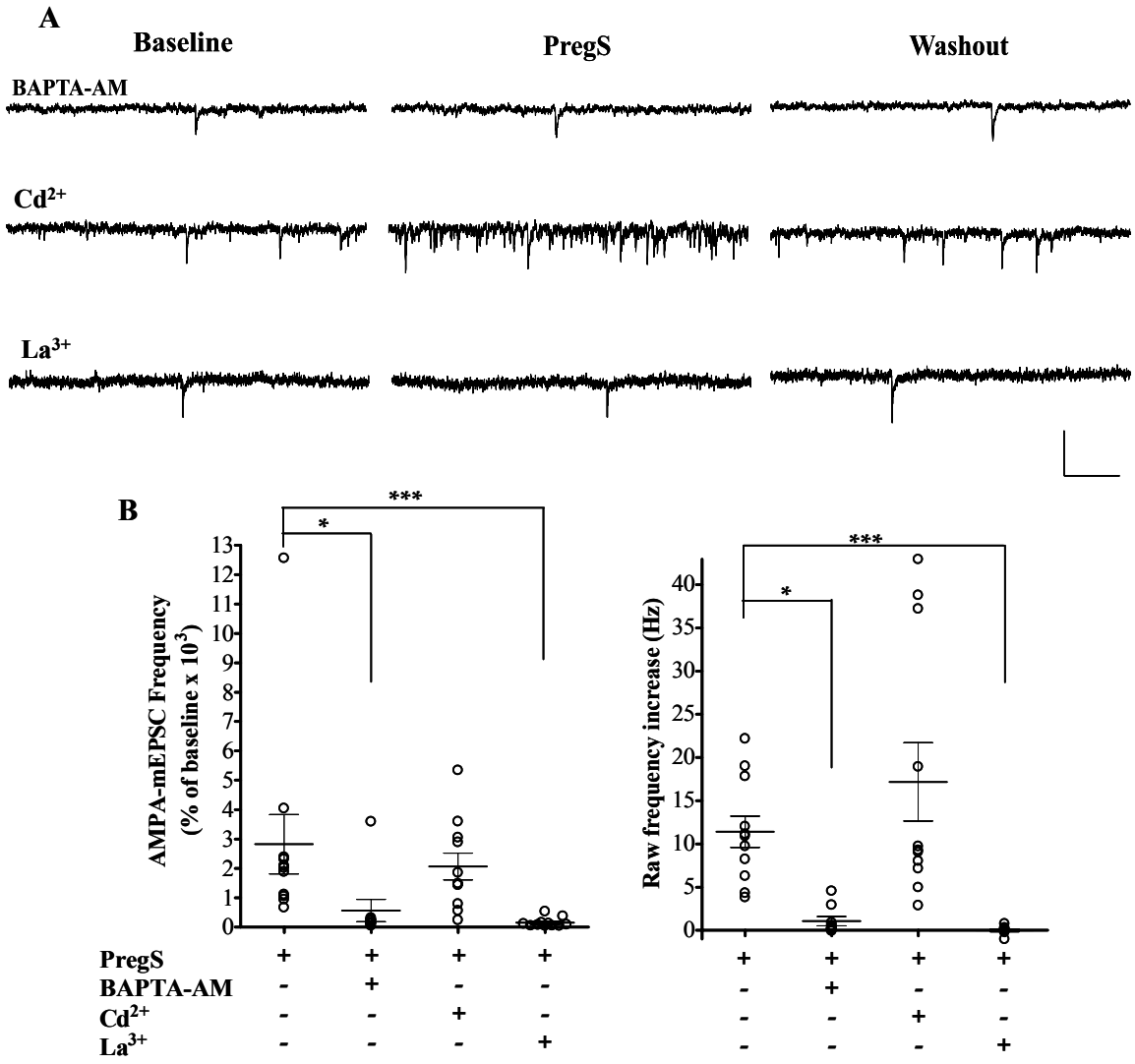


Figure 3.4. PregS (25 μM) increases CF-eEPSC amplitude and decreases the PPR in PCs from neonatal rats.

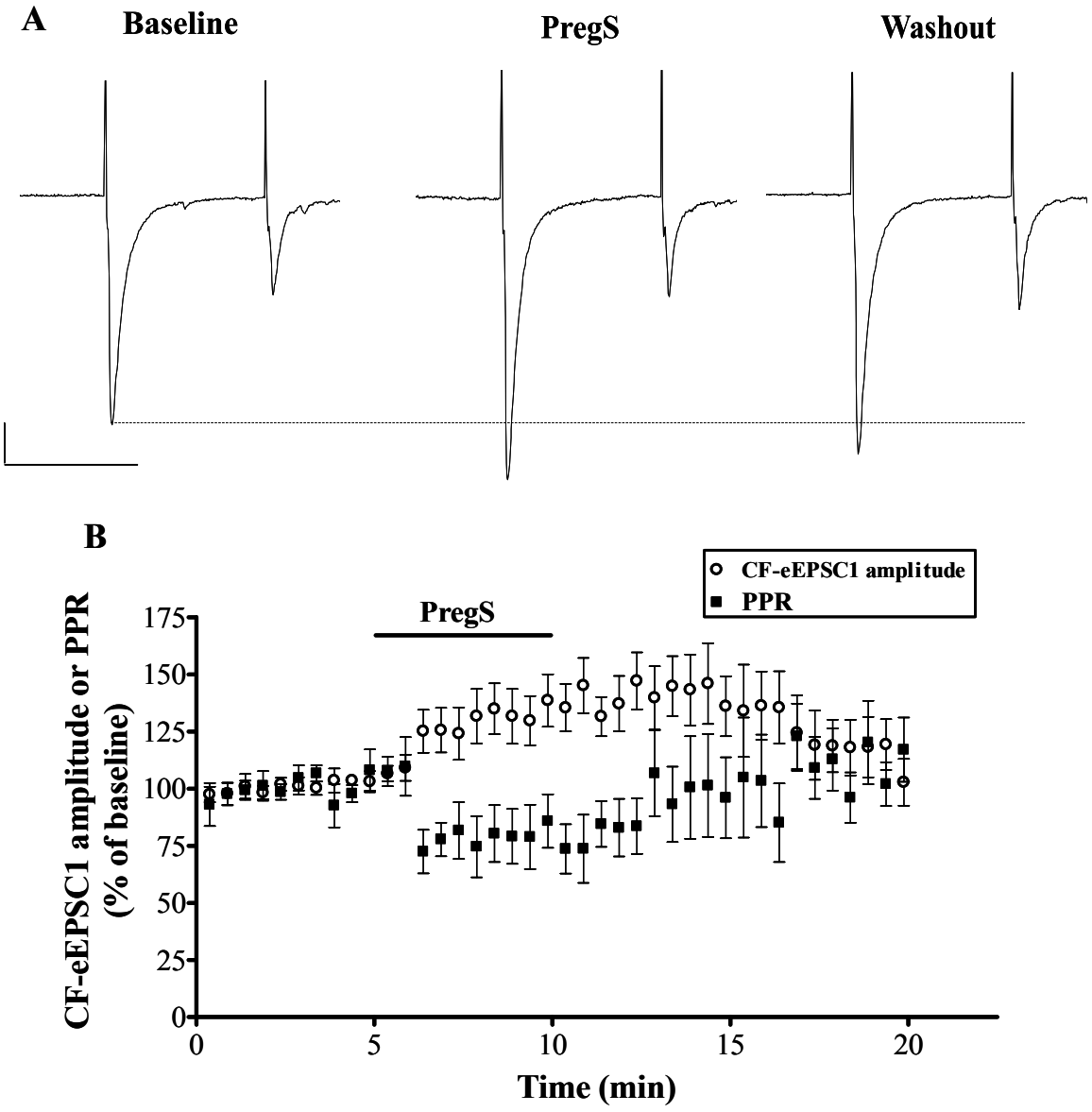


Figure 3.5. PregS (25 μM) does not affect CF-eEPSC amplitude or the PPR in PCs from juvenile rats.

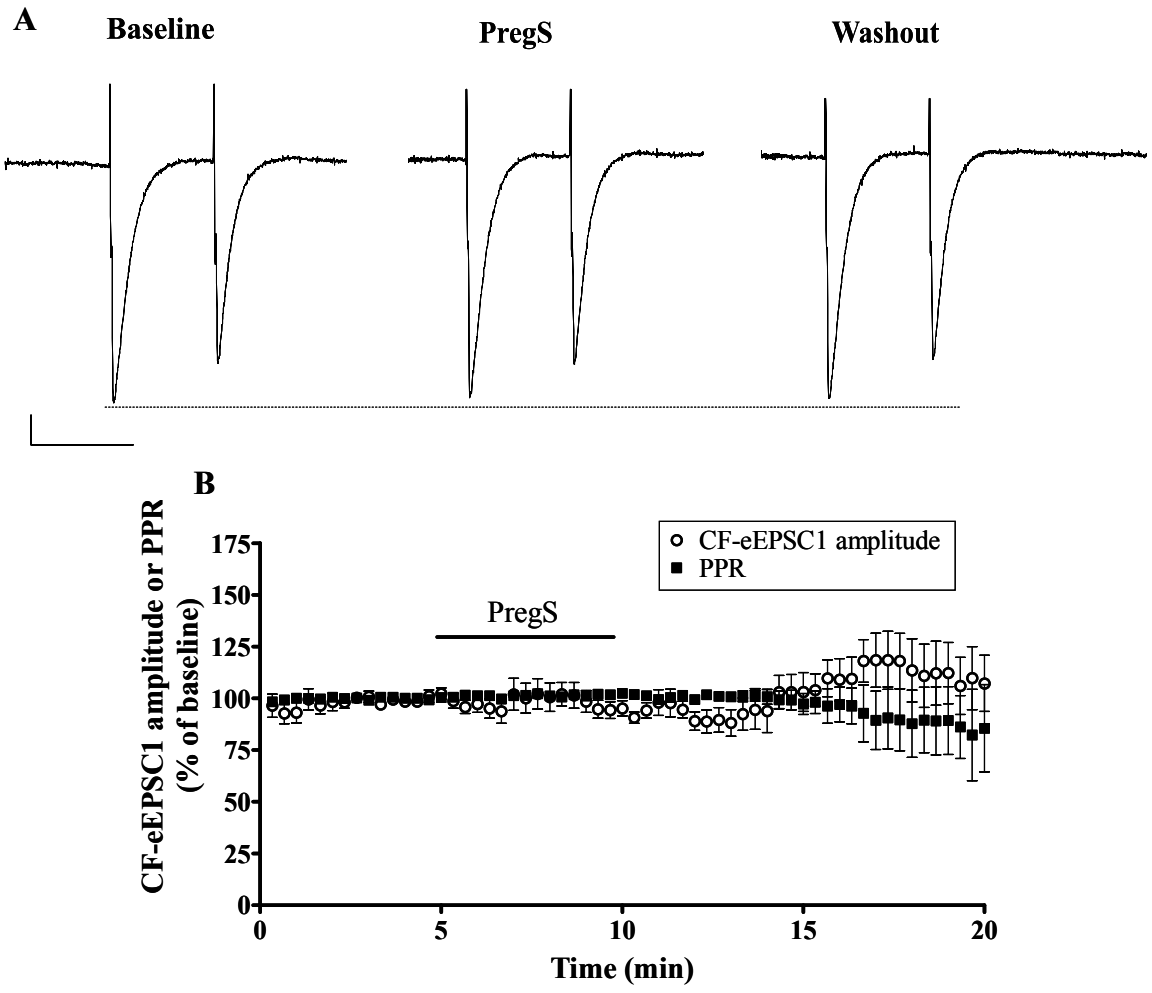


Figure 3.6. PregS (25μM) does not affect PF-eEPSC amplitude or the PPR in PCs from juvenile rats.

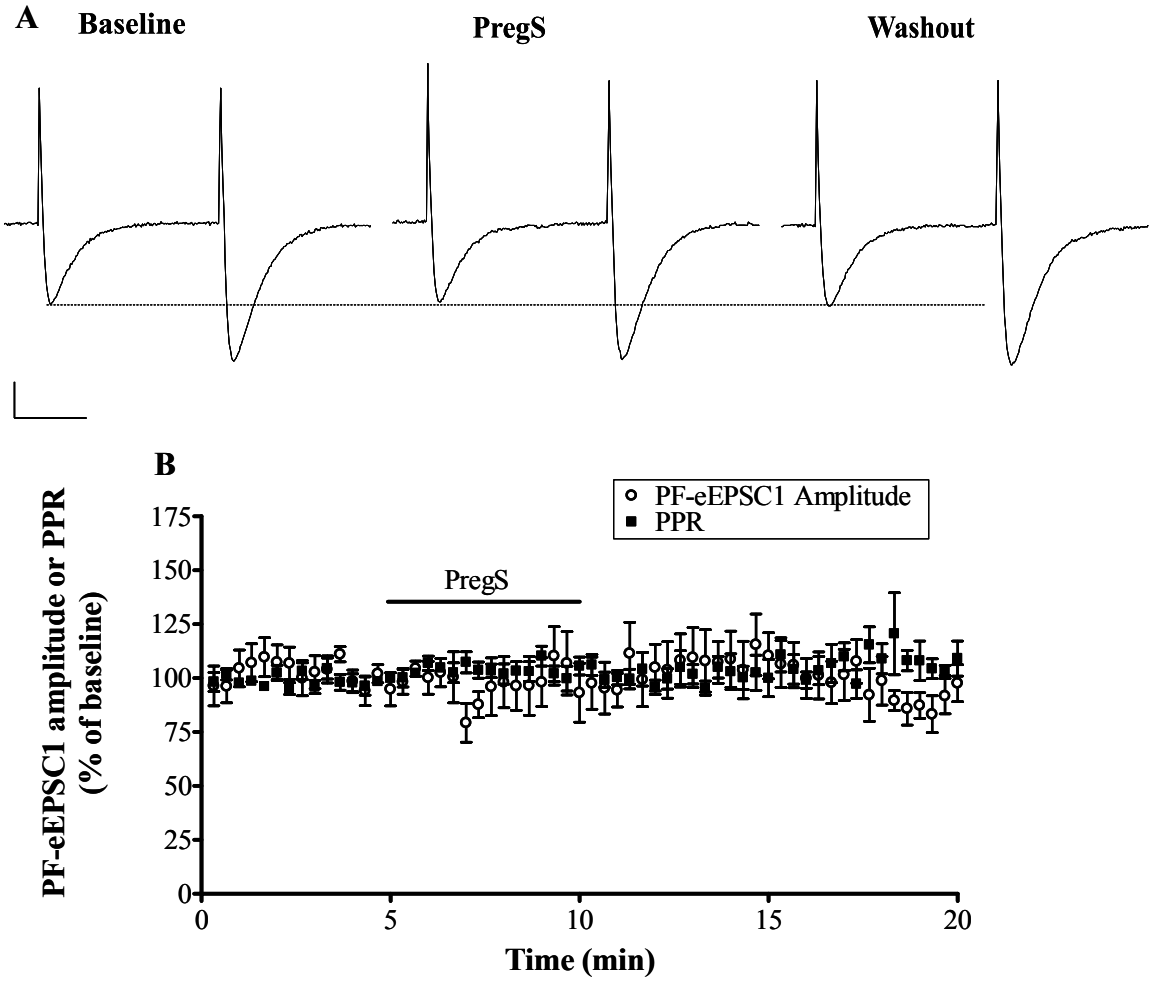
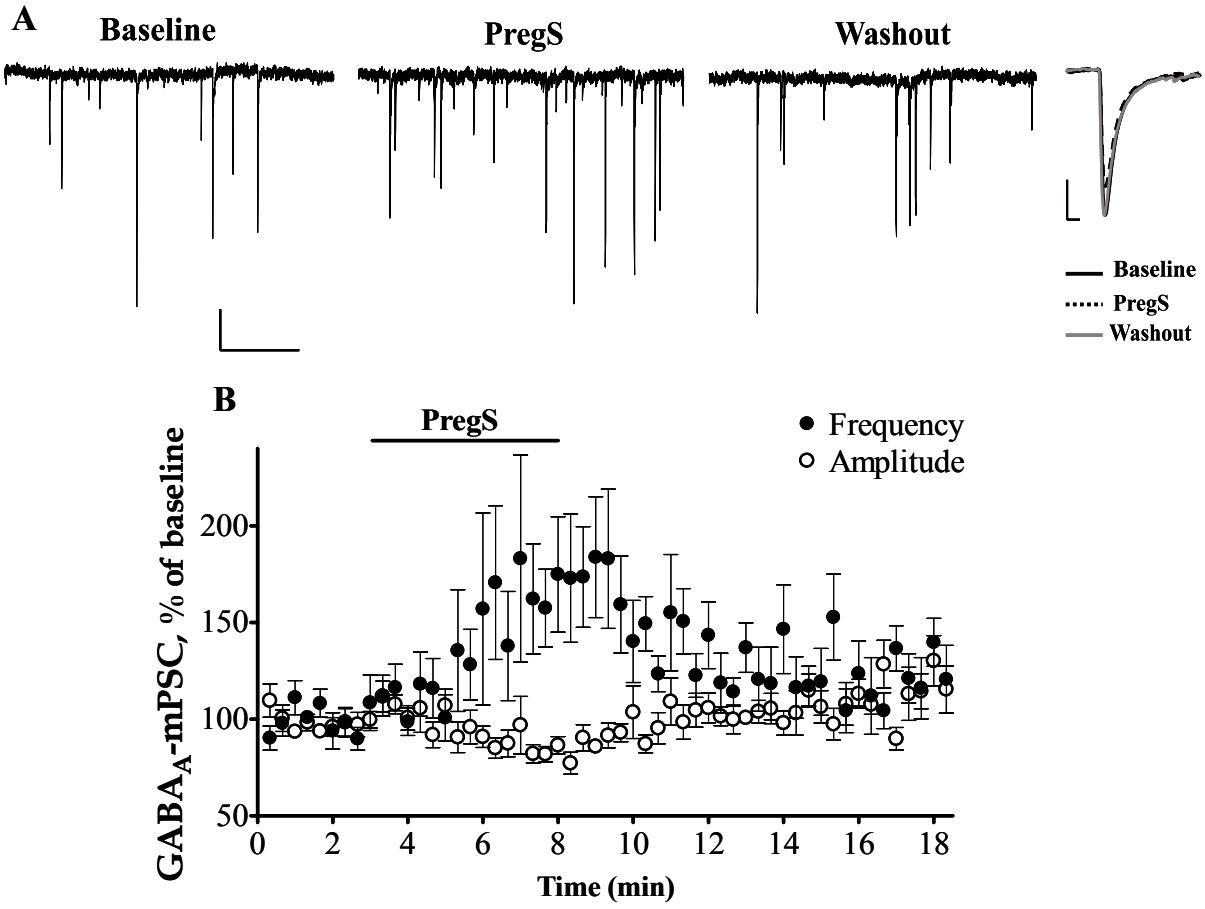


Figure 3.7. PregS (25μM) increases the frequency of $GABA_A$-mPSCs in PCs from neonatal rats



4. Activation of steroid-sensitive transient receptor potential M3 channels potentiates glutamatergic transmission at cerebellar Purkinje neurons from developing rats

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(Sumitted, Jornal of Neurochemistry)

4.1 Abstract

The functional implications of transient receptor potential melastatin 3 (TRPM3) activation, the most recently described member of the melastatin subfamily of cation permeable TRP channels, have begun to be elucidated in recent years. The discovery of TRPM3 activation by the steroid pregnenolone sulfate (PregS) has shed new light on the physiological role of TRPM3. For example, TRPM3 activation enhances insulin secretion from β pancreatic cells, induces contraction of vascular smooth muscle cells, and is also involved in the detection of noxious heat. Although TRPM3 expression has been detected in several regions of the developing and mature brain, little is known about the roles of TRPM3 in brain physiology. Here, we demonstrate the abundant expression of TRPM3 steroid-sensitive channels in the developing cerebellar cortex. We also show that TRPM3-like channels are expressed at glutamatergic synapses in neonatal PCs. We recently showed that PregS potentiates spontaneous glutamate release onto neonatal Purkinje cells (PCs) during a period of active glutamatergic synapse formation; we now show that this effect of PregS is mediated by TRPM3-like channels. Mefenamic acid, a recently discovered TRPM3 antagonist, blocked the effect of PregS on glutamate release. The PregS effect on glutamate release was mimicked by other TRPM3 agonists (nifedipine and the epipregnanolone sulfate) but not by a TRMP3-inactive steroid (progesterone). Our findings identify TRPM3 channels as novel modulators of glutamatergic transmission in the developing brain.

4.2 Introduction

The mammalian transient receptor potential melastatin (TRPM) channel subfamily includes 8 members based on their homology to melastatin, a putative tumor suppressor that may be involved in the pathophysiology of melanoma. The members of the TRPM subfamily are characterized by sequence similarities along the 700 amino-acid long N-terminus and transmembrane domains, whereas the C-terminal domains show diverse structures (reviewed in (Venkatachalam and Montell, 2007, Wu et al., 2011). TRPM proteins assemble into ion conducting channels that respond to a variety of stimuli including temperature, osmolarity, various chemical signals, changes in membrane voltage, oxidative stress and intracellular Ca^{2+} (Harteneck, 2005). While most of the TRPM channels are activated in a unique manner, TRPM3 behaves as a polymodal regulated ion channel (Harteneck and Schultz, 2007, Oberwinkler and Phillipp, 2007). Beside the recently described activation by temperature, TRPM3 is activated by the neuroactive steroids pregnenolone sulfate (PregS) and epipregnanolone sulfate, as well as by the L-type voltage-gated Ca^{2+} channel antagonist, nifedipine, and hypotonicity (Grimm et al., 2003, Lee et al., 2003, Grimm et al., 2005, Wagner et al., 2008, Majeed et al., 2010, Vriens et al., 2011). Electrophysiological experiments in heterologous expression systems demonstrated that TRPM3 forms an ion channel permeable to Ca^{2+}, Na^{+}, Mg^{2+}, and Mn^{2+} (Grimm et al., 2005, Oberwinkler et al., 2005a). The polymodal activation mode of TRPM3 corresponds to the diversity of cells expressing TRPM3; these channels have been linked to vascular smooth muscle contraction, modulation of glucose-induced insulin release from pancreatic islets, detection of noxious heat in dorsal

root ganglia and in development of oligodendrocytes (Wagner et al., 2008, Hoffmann et al., 2010, Naylor et al., 2010, Vriens et al., 2011).

Since the first functional description of TRPM3, northern blot analyses argue for the expression of TRPM3 in the brain (Grimm et al., 2003, Lee et al., 2003, Oberwinkler et al., 2005a). These studies revealed the presence of several distinct transcription products in the mouse brain. The detection of sequence discrepancies due to alternative splicing and exon usage suggests the expression of multiple TRPM3 mRNA transcripts (Lee et al., 2003, Oberwinkler et al., 2005). *In situ hybridization* and immunohistochemistry approaches revealed expression of TRPM3 in the epithelial cells of the choriod plexus, oligodendrocytes and neurons (Oberwinkler et al., 2005, Hoffmann et al., 2010). The functional consequences of TRPM3 activation in the brain have just begun to be elucidated and one physiological role for TRPM3 in the brain has been recently suggested. At the onset of myelination and into adulthood, TRPM3 expression was found in oligodendrocytes, where TRPM3 activation induced increases in intracellular Ca^{2+}. These findings suggest that TRPM3 channels are important players in oligodendrocyte differentiation and CNS myelination (Hoffmann et al., 2010). In the immature brain, the presence of TRPM3 was also detected (Hoffmann et al., 2010); however, the physiological role of these channels in the developing brain has not been explored. Studies from our laboratory suggest that the TRPM3 activator, PregS, is an important regulator of the formation and refinement of glutamatergic synapses in the developing brain (Mameli et al., 2005, Valenzuela et al., 2008). In the developing cerebellum, PregS potently and reversibly enhances glutamate release onto Purkinje cells (PCs) of neonatal rats, an effect that is independent of the known PregS-sensitive targets

N-Methyl-D-aspartate (NMDA), glycine, α7 nicotinic acetylcholine, and σ1 receptors, as well as voltage-gated Ca^{2+} channels (Zamudio-Bulcock and Valenzuela, 2011). PregS increases glutamate release by enhancing Ca^{2+} entrance into presynaptic terminals via a target that is sensitive to La^{3+}, a blocker of TRP channels (Zamudio-Bulcock and Valenzuela, 2011). Based on this finding, we hypothesized that TRPM3 channels are expressed at developing glutamatergic synapses on PCs and that they mediate the PregS-induced enhancement in glutamatergic transmission. We tested this hypothesis using immunohistochemistry and slice electrophysiology.

4.3 Materials and Methods

Unless specified, all chemicals were from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO). All experiments were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

4.3.1 Immunohistochemistry

TRPM3 expression in the cerebellar cortex was studied using immunohistochemistry. For these experiments, parasagittal cerebellar cortex sections were prepared from 5 postnatal day (P) 6-8 Sprague Dawley rats from three different litters. Tissue preparation was performed as follows: brains were removed after deep anesthesia with 250 mg/kg ketamine and immersion-fixed in 4% paraformaldehyde overnight. Subsequently, brains were cryo-protected in 30% sucrose in phosphate buffered saline (PBS) at 4°C until they sank to the bottom. Brains were then imbedded in O.C.T compound (Tissue Tek, the Netherlands) and flash-frozen by submersion in

isopentane cooled in a dry ice/methanol bath. Brains were then cryo-sectioned in the sagittal plane and placed on superfrost plus micro slides (VWR international, West Chester, PA), stored at -20^{o}C for a period no longer than two weeks. Sections were washed 3 times in PBS and permeabilized with 0.5% Triton X-100. Simultaneously, nonspecific binding was blocked with 10% normal donkey serum, 10% normal goat serum, and 10% bovine serum albumin. Primary antibodies (polyclonal rabbit anti-TRPM3; polyclonal guinea pig anti-VGlut2, AB5907 Millipore, Billerica, MA; and monoclonal mouse anti-calbindin, sc-70478, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), were incubated overnight at 4^{o}C. The TRPM3 antibody was raised in rabbits and affinity purified as previously described (Hoffmann et al., 2010), using the following synthetic peptide NH2-CDMPYMSQAQEIHLQEKEAEEPEKPTKE-CONH2 corresponding to positions 795 to 821 of TRPM3 splice variants A and B. All secondary antibodies were purchased from Millipore. Secondary antibodies (goat anti-rabbit alexa fluor 555 for TRPM3 detection, donkey anti-guinea pig CY5 conjugate for VGlut2 detection and donkey anti-mouse alexa 488 for calbindin detection) were incubated for 1 h at room temperature. Sections were rinsed and mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined with a Zeiss LSM510 confocal microscope (Carl Zeiss, Germany). Z-stack images were taken using a 40x immersion objective and an optical section thickness of 1 μm, which was the optimal pinhole diameter for best resolution. Detector gain and laser percent output parameters were optimized for each channel in one section and maintained throughout all the studies. Two z-stack image files from different cerebellar cortical regions were taken per each animal.

Co-localization was determined using Slidebook 5 image analysis software (Intelligent Imaging Innovations, Inc., Denver, CO). After background subtraction for each individual channel, one mask per channel was created. Subsequently, the area over the PC layer region was selected and extrapolated to each image in the z-stack. Then, a colocalization mask across two channels within that area was created. Colocalization masks were created using the math function "AND", which recognizes the voxels that are positive for both proteins. Percent colocalization was calculated using the total number of voxels in an individual channel mask compared to the number of colocalized voxels in the respective colocalization mask. The triple colocalization analysis was done by creating a new colocalization mask using the colocalization mask for two given channels and the mask of the individual channel that was not considered in the first colocalization mask (Vo et al., 2004).

4.3.2 Slice electrophysiology

Cerebellar vermis parasagittal slices (250 μm-thick) were prepared from neonatal (P4–P10) Sprague–Dawley rats. Alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid (AMPA) channel-mediated miniature excitatory postsynaptic currents (AMPA-mEPSCs) were recorded from PCs. The artificial cerebrospinal fluid (ACSF) was equilibrated with 95% O_2/5% CO_2 and contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 10 glucose, 10 μM 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid (SR 95531), and 0.5 μM tetrodotoxin (TTX; Calbiochem, La Jolla, CA). The internal solution contained (in mM): 150 CsCl, 1.5 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 0.1 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Calbiochem, La Jolla,

CA), 2 Na_2-ATP, 0.4 Na-GTP, at pH 7.3. Using these recording conditions, we previously demonstrated that the mEPSC are blocked by 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 53655; 50 μM), confirming that these events are AMPA receptor mediated (Zamudio-Bulcock and Valenzuela, 2011). If the access resistance changed >30%, the recording was discarded. The membrane potential was held at -70 mV.

Data were acquired with pClamp 10 (Molecular Devices, Sunnyvale, CA) at a filtering frequency of 2 kHz and an acquisition rate of 10 kHz. Miniature events were analyzed using Mini Analysis (Synaptosoft, Decatur, GA). In all cases, changes in mEPSC frequency were quantified by measuring changes in inter-event interval. Individual recordings were analyzed using the Kolmogorov–Smirnov (K–S) test using a conservative value for significance of $p < 0.01$. Statistical analyses of pooled data were performed with GraphPad Prizm 4 (GraphPad Software, San Diego, CA). Data were initially analyzed with the D'Agostino and Pearson omnibus normality tests. If data followed a normal distribution, these were analyzed using parametric tests. If this was not the case, then non-parametric tests were used. The level of significance was $p < 0.05$. Data were normalized to baseline and expressed as percent of baseline ± standard error of the mean.

4.4 Results

4.4.1 TRPM3 expression in the neonatal cerebellar cortex

We performed immunohistochemistry experiments to assess the expression of TRPM3 in the neonatal cerebellar cortex in parasagittal sections from P6-8 rats. During the neonatal period, the cerebellar cortex is a four layered structure consisting of the external granule cell, the nascent molecular, the PC and the internal granule cell layers (Fig 4.1A). Glutamatergic axons form temporary somatic synaptic protrusions on PC bodies, which are arranged in a pseudomonolayer (Kurihara et al., 1997, Eilers et al., 2001, Kawa, 2003). During the first 14 postnatal days, all glutamatergic terminals onto PCs express the vesicular glutamate transporter VGlut2. Subsequently, VGlut2 is expressed selectively in climbing fiber (CF) terminals and VGlut1 becomes the main vesicular glutamate transporter in parallel fiber (PF) terminals (Miyazaki et al., 2003). Therefore, we used VGlut2 as a marker for glutamatergic terminals in the neonatal cerebellar cortex. To detect PCs, we used the Ca^{2+}-binding protein calbindin, which is highly expressed in PCs. We found a strong TRPM3 expression in PCs (Fig. 4.1A). Other cells within the PC layer were labeled by the anti-TRPM3 antibody; based on their localization, these cells likely correspond to developing Bergman glia, which enwrap CF -to-PC synapses during postnatal development (Douyard et al., 2007). Interestingly, TRPM3 was also found in the inner granule cell layer, the nascent molecular layer, and the external granule cell layer (Fig. 4.1A). Preincubation of the anti-TRPM3 antisera with the synthetic peptide used for immunization, completely blunted the staining reaction, demonstrating the specificity of the immunohistochemical assay (Fig. 4.1B).

4.4.2 TRPM3 is expressed at glutamatergic synapses in neonatal PCs

TRPM3 expression in the PC layer and its localization with glutamatergic synapses in the PC layer at P6-8 was further analyzed by quantifying signals from double- and triple-stained sections. We found TRPM3 abundantly expressed in PC cell bodies; the quantification revealed that 37.32 ± 11.1 % (Fig. 4.2A; n = 5) of calbindin colocalized with TRPM3 and 22.64 ± 3.56 % (not shown; n = 5) of TRPM3 staining overlapped with calbindin staining. In addition, 43.37 ± 4.84 % of glutamatergic terminals on PCs, visualized by VGlut2 and calbindin staining, were also positive for TRPM3 (Fig. 4.2B, n = 5). Consistent with the presence of immature CF terminals not associated with PCs at this age (Hashimoto et al., 2009), not all VGlut2 staining colocalized with calbindin (32.06 ± 3.13 %, not shown; n = 5).

4.4.3 Characterization of increases in glutamate release induced by TRPM3-like channels.

Our previous finding that PregS increases glutamate release at PCs, taken together present results indicating that TRPM3 channels are expressed at glutamatergic synapses in these cells, prompted us to assess the role of these channels in the mechanism of action of PregS. To this end, we used recently characterized TRMP3 pharmacological tools. Within the group of fenamates, a class of small compounds derived from N-phenyl-substituted anthranilic acid, mefenamic acid has been described to selectively block TRPM3 with a half-maximal inhibitory concentration of 6.6 ± 1.8 μM (IC_{50} of > 300 μM on other TRP channels) (Klose et al., 2011). Therefore, we tested whether the PregS-induced increase of quantal glutamate release was affected by this potent TRPM3 channel

blocker. To measure quantal glutamate release, we recorded AMPA-mEPSC from PCs using whole-cell patch-clamp techniques. Using this approach, we previously demonstrated that PregS reversibly increases AMPA-mEPSC frequency but not amplitude, indicating that this neuroactive steroid acts by increases glutamate release rather than via modulation of postsynaptic AMPA receptors (Zamudio-Bulcock and Valenzuela, 2011). Mefenamic acid (25 μM) significantly attenuated the PregS-induced increase in AMPA-mEPSC frequency. The K-S test showed that, in the presence of mefenamic acid, PregS did not significantly change the inter-event interval in 2 out of 5 cells, significantly increased it in 1 cell and significantly decreased it in 2 cells. In controls, PregS strongly increased AMPA-mEPSC frequency (4,716 ± 1,755 % of baseline, n = 5) (Fig.4.3A, B, E and F). However, in the presence of mefenamic acid, this PregS dependent increase was significantly reduced (138.7 ± 39.58 % of baseline, n = 5) (Fig. 4.3C, D, E and F).

Epipregnanolone sulfate, a pregnanolone isomer derived from progesterone, has been shown to activate recombinant TRPM3 channels expressed in HEK cells (Majeed et al., 2010). Therefore, we tested whether bath application of epipregnanolone sulfate affected glutamatergic transmission onto neonatal cerebellar PCs. Bath application of epipregnanolone sulfate (25 μM) significantly increased AMPA-mEPSC frequency (454.3 ± 95.3 % of baseline, n = 11) (Fig. 4.4A and C). The K-S test showed that 9 out of 11 cells had a significant decrease in the inter-event interval. The amplitude of these events was not significantly affected by epipregnanolone sulfate (106.2 ± 9.95 % of baseline). The K-S test showed that in 8 out of 11 cells, epipregnanolone sulfate did not significantly affect AMPA-mEPSC amplitude; in 3 out of 11 cells, it induced a

significant decrease in the amplitude of these events. We have previously reported that the TRP channel blocker, La^{3+}, blocks the PregS-induced increase in AMPA-mEPSC frequency (Zamudio-Bulcock and Valenzuela, 2011). In the presence of La^{3+} (100 μM), epipregnanolone sulfate did not significantly increase AMPA-mEPSC frequency (114.9 ± 16.22 % of baseline, n = 8) (Fig. 4.4C, D, E and F). The K-S test showed that in the presence of La^{3+}, epipregnanolone sulfate did not significantly affect the inter-event interval in 5 out of 8 cells and that there was a significant decrease in 2 out 8 cells, and a significant increase in 1 cell.

As a control, we tested progesterone, a steroid that has been shown to lack at stimulating actions on TRPM3 (Wagner et al., 2008, Majeed et al., 2010). The application of progesterone (25 μM) affected neither the frequency (107.1 ± 15.57 % of baseline, n = 6) nor the amplitude (108 ± 10.15 % of baseline) of AMPA-mEPSCs (Fig. 4.5). In 5 out of 6 cells, the K-S test showed no significant change in the inter-event interval of these events. The K-S test showed no significant change in AMPA-mEPSC amplitude in 4 out of 6 cells; there was a significant increase in 1 cell and a significant decrease in 1 cell.

The increase in AMPA-mEPSC frequency induced by steroid sulfates and the blockade of this effect by both mefenamic acid and La^{3+} provide strong evidence for the participation of TRPM3-like channels in this process. To exclude effects based on the steroid structure of the activating compounds, we chose nifedipine, a non-steroideal L-type Ca^{2+} channel blocker with TRPM3-stimulating properties (Wagner et al., 2008). Bath application of nifedipine (20 μM) significantly increased AMPA-mEPSC frequency (2,680 ± % of baseline, n = 8) (Fig. 4.6A,B,E and F). The K-S test showed that in 8 out of

8 cells there was a significant decrease in the inter-event interval. The average amplitude of AMPA-mEPSCs was not significantly changed by nifedipine (90.92 ± 2.25% of baseline) (Fig. 4.6A and B). The K-S test showed that, in 5 out of 8 cells, nifedipine did not change the amplitude of these events. In the other 3 cells, nifedipine significantly decreased the amplitude of these events. La^{3+} (100 μM) significantly reduced the nifedipine-induced increase of AMPA-mEPSC frequency (169.1 ± 44.67 % of baseline, n = 9) (Fig. 4.6C and D). In the presence of La^{3+}, the K-S test showed that nifedipine did not change the inter-event interval of these events in 5 out of 9 cells, and that it significantly decreased it in 3 out of 9 cells and increased it in 1 cell.

4.5 Discussion

This study provides evidence supporting the hypothesis that TRPM3 channels mediate the effect of PregS on glutamate release in the neonatal cerebellar cortex and constitutes the first demonstration of a physiological effect of TRPM3 channels in neurotransmission. The findings can be summarized as follows. Immunohistochemistry experiments indicate that TRPM3 splice variants A and/or B are abundantly expressed in the developing cerebellar cortex and that these colocalize with glutamatergic synapses in PCs. Furthermore, the previously reported effect of PregS on the frequency of AMPA-mEPSCs is inhibited by the TRPM3 blocker, mefenamic acid. In addition, the TRPM3 agonists, epipregnanolone sulfate and nifedipine, mimicked the PregS-induced increase of AMPA-mEPSC frequency; however, the TRPM3 inactive agent progesterone was not able to mimic the PregS effect.

4.5.1 TRPM3 channels are expressed in the developing cerebellar cortex

The expression of TRPM3 channels in the neonatal cerebellar cortex was found to be abundant and present in all 4 layers of the developing cerebellar cortex. This expression profile suggests that these channels may play a role in the maturation of cerebellar cortex circuitry. During the neonatal period there are ongoing cell maturation and migration processes that depend heavily on Ca^{2+} signaling and, therefore, might be influenced by TRPM3 channel activation. Certainly, future studies should focus on investigating the role of these channels on the overall development of the cerebellar cortex.

Importantly, our results suggest that a considerable number of VGlut2-containing glutamatergic synapses in PCs contain TRPM3 channels, although electron microscopic studies are needed to elucidate the precise synaptic compartment containing these channels. TRPM3 and calbindin showed colocalization, but most of the TRPM3 staining in the PC layer was not localized on PCs. Collectively, these results indicate that in the PC layer, TRPM3 is expressed in three locations. First, TRPM3 is localized in VGlut2 positive glutamatergic synapses onto PCs, which, at least in part, correspond to CF-to-PC synapses, as suggested by our finding that PregS alters paired-pulse plasticity of CF-evoked EPSCs (Zamudio-Bulcock and Valenzuela, 2011). Second, TRPM3 is present in PC somata in locations other than glutamatergic synapses, perhaps in developing GABAergic pinceaux synapses and/or axonal collaterals from neighboring PCs (Watt et al., 2009); supporting this possibility are previous findings that PregS, although less strongly, also increases GABAergic transmission onto neonatal PCs (Zamudio-Bulcock and Valenzuela, 2011). Third, TRPM3 is expressed in other cell types present in the developing PC layer, with the most likely candidates being Bergman glia and migrating granule cells. In the neonatal cerebellar cortex, Bergmann glial fibers associate with granule cells migrating from the external granule cell layer to their final destination in the internal granule cell layer. Therefore, it will be important to assess whether these channels are located on Bergmann glia and play a role in this process (Yamada et al., 2000).

4.5.2 Steroid-sensitive TRPM3 channels increase glutamate release

We have previously shown that the effect of PregS on glutamatergic transmission in the neonatal cerebellar cortex is blocked by the non-selective TRP channel blocker, La^{3+}. Mefenamic acid, a clinically used non-steroidal anti-inflammatory agent, was recently shown to be a selective and potent TRPM3 blocker (Klose et al., 2011). This finding enabled us to more selectively test the involvement of TRPM3 channels in the mechanism of action of PregS. Mefenamic acid blocking of the PregS-induced increase of AMPA-mEPSC frequency provided the first line of evidence of the involvement of this channel in the PregS effect.

PregS and epipregnanolone sulfate are structurally related compounds. Not surprisingly, similar to PregS, epipregnanolone sulfate has been shown to inhibit $GABA_A$ receptors (Park-Chung et al., 1999). PregS is a positive modulator of NMDA receptors whereas epipregnanolone sulfate inhibits these receptors by acting at a distinct site from that of PregS (Park-Chung et al., 1997). In the case of TRPM3 stimulation, both PregS and epipregnanolone sulfate act at the same site; however, these agents stimulate TRPM3 with a different efficacy. PregS-mediated stimulation of TRPM3 channels requires the sulfate group positioned at ring A and the cis (β) configuration of the side group, both of which epipregnanolone sulfate possess. In fact, the structure of epipregnanolone sulfate only differs from that of PregS in that it lacks the double bond in ring B and this structural difference renders epipregnanolone sulfate less effective in stimulating TRPM3 channels. In HEK cells expressing TRPM3 channels, epipregnanolone sulfate was shown to be less potent than PregS at increasing intracellular Ca^{2+} (Majeed et al., 2010). Consistent with these findings, we observed that epipregnanolone sulfate significantly

increased the frequency of AMPA-mEPSCs in neonatal PCs (Fig. 4.1), but this effect was of lower magnitude than that induced by the same concentration of PregS (Fig. 4.4). Importantly, similar to the PregS-induced increase in AMPA-mEPSC frequency, this effect was blocked by the TRP channel blocker, La^{3+} (Zamudio-Bulcock and Valenzuela, 2011).

Progesterone is a major neurosteroid shown to be inactive at TRPM3 channels (Wagner et al., 2008, Majeed et al., 2010). *In-vitro* and *in-vivo* studies have shown that progesterone promotes dendritic growth and spine formation in PCs via modulation of its nuclear receptor, although a role of progesterone membrane receptors in this trophic effect has not been ruled out (reviewed in (Tsutsui et al., 2003). Progesterone is known to modulate the release of norepinephrine, serotonin, dopamine, and glutamate. In the prefrontal cortex, progesterone inhibits the dopamine-evoked release of glutamate while it does not affect spontaneous release (reviewed in (Zheng, 2009). Here, we report that progesterone neither affected the frequency nor the amplitude of AMPA-mEPSCs. These results are consistent with the lack of activity of this neurosteroid on TRPM3. Additionally, these results argue against acute modulation of spontaneous glutamatergic transmission as a mechanism responsible for some of the trophic effects of progesterone on neonatal PCs.

4.5.3 Nifedipine mimics the effect of PregS

Nifedipine is an L-type calcium channel blocker clinically used for the treatment of conditions such as cardiac arrhythmias, angina, hypertension, and preterm labor (Hirasawa and Pittman, 2003, Conde-Agudelo et al., 2011). Recently, nifedipine was

shown to paradoxically activate TRPM3 channels in recombinant experiments and in pancreatic islets cells with potency similar to that of PregS (Wagner et al., 2008). It is noteworthy that activation of voltage-gated Ca^{2+} channels is not involved in the effect of PregS on neonatal PCs, as bath application of Cd^{2+} does not prevent the PregS-induced increase in AMPA-mEPSC frequency (Zamudio-Bulcock and Valenzuela, 2011). In this study, we show that nifedipine mimics the previously reported PregS-induced increase in glutamate release onto neonatal PCs and that, like the effect of PregS, this effect is sensitive to TRP channel blockade by La^{3+}. Ca^{2+} channel blockers, including nifedipine, are used in obstetrics and gynecology for the management of hypertensive disorders of pregnancy and preterm labor. The teratogenicity of these agents has been reported in animals; however, human data are inconclusive (Tranquilli and Giannubilo, 2009). Nevertheless, a recent systematic review linked the use of nifedipine and other Ca^{2+} channel blockers with significant higher risks for a wide range of feto-maternal adverse events, including cardiac, respiratory, and renal alterations (Khan et al., 2011). Given that nifedipine can readily cross the blood brain barrier (Janicki et al., 1988), and that human TRPM3 channels are also strongly modulated by PregS and other TRPM3 channel activators (Majeed et al., 2010), our results suggest that using nifedipine during the third-trimester of pregnancy should be avoided, as it may cause alterations in cerebellar neuronal circuit formation. Importantly, this effect may not be limited to cerebellar neurons, as another study showed that nifedipine increases glutamate release in magnocellular neurons of the supraoptic nucleus via a Ca^{2+} channel-independent mechanism (Hirasawa and Pittman, 2003).

In conclusion, the present study provides evidence indicating that TRPM3 channels are novel modulators of glutamatergic transmission. A mature PC receives glutamatergic input from numerous granule cell projections, the PFs, and from a single axonal projection of an inferior olivary neuron, the CF. The one to one CF-to-PC association is preceded during development by multinumerary CF-to-PC synapses and it is achieved at the end of the second postnatal week in rodents (approximately equivalent to end of the third trimester of human pregnancy) via synaptic competition among CFs and between CF and PFs. In addition, CF synapse formation starts during embryonic development, while PF-to-PC synapse formation starts at the end of the first postnatal week (Bosman and Konnerth, 2009, Cesa and Strata, 2009). During the developmental period at which the TRPM3-mediated enhancement of glutamate release occurs, the multinumerary CF-to-PC synapses are functionally differentiated and the process of early synapse elimination starts (Kano and Hashimoto, 2009). While the mechanisms controlling the late stages of CF synapse elimination are rather well understood (Bosman and Konnerth, 2009, Kano and Hashimoto, 2009), the early stages of CF synapse elimination have not been fully characterized. CF plasticity mechanisms may play a central role in CF functional differentiation and elimination. It has been shown that, in response to the same stimuli, strong CF inputs undergo long-term potentiation while weak ones undergo long-term depression (Bosman et al., 2008, Ohtsuki and Hirano, 2008, Valenzuela et al., 2010). The mechanisms underlying the differential synaptic properties among CFs are not well understood. We previously showed that the PregS-induced enhancement of glutamate release occurs at developing CF-to-PC synapses (Zamudio-Bulcock and Valenzuela, 2011). Therefore, the interesting possibility that

TRPM3 channel activation by PregS-like neurosteroids released from Purkinje neurons is involved in the differential strengthening of CF inputs needs to be further assessed.

Acknowledgements

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FIGURE LEGENDS

Figure 4.1. Pseudocolored confocal images of TRPM3 expression profile in the neonatal cerebellar cortex. (A) TRPM3 (blue), calbindin (red) and VGlut2 (green) expression in a 2 μm-thick sagittal cerebellar confocal section at P8. Channels are shown separately and a merged image is shown in the bottom right division. Similar results were obtained in sections from 4 additional rats. (B) Competition with the immunizing peptide eliminates TRPM3 protein staining. Similar results were obtained in sections from another rat. External granule cell layer (EGC; nascent molecular layer located immediately above PC layer is not shown for clarity), Purkinje cell layer (PC), internal granule cell layer (IGC). Scale bars: 50 μm.

Figure 4.2. Confocal images and colocalization masks for TRPM3, VGlut2 and calbindin in the PC layer. (A) Top, merge of calbindin (red) and TRPM3 (blue). Bottom, colocalization of calbindin and TRPM3 is illustrated by masking of regions with no overlap between these proteins. Confocal images correspond to a 1 μm thick sagittal cerebellar confocal section at P8. (B) Top, merged image of TRPM3 (blue), calbindin (red) and VGlut2 (green). Bottom, colocalization of calbindin, TRPM3, and VGlut2 is illustrated by masking of regions with no overlap between these proteins. Scale bars: 50 μm. See text for quantification of results from 5 separate experiments.

Figure 4.3. Mefenamic acid inhibits the PregS-induced increase of AMPA-mEPSC frequency. (A) Sample traces of AMPA-mEPSC recordings at P7 in a control PC (calibration: 20 pA, 0.5 s) and corresponding average mEPSC traces (calibration: 8 pA, 3 ms). (B) Corresponding cumulative probability plots; PregS significantly decreased the inter-event interval ($p < 0.01$ by K–S test) but not the amplitude. (C) Sample traces of AMPA-mEPSC recordings in the presence of mefenamic acid (Mef) at P6 (calibration: 20 pA, 0.5 s) and corresponding average mEPSC traces (calibration: 8 pA, 3 ms). (D) Corresponding cumulative probability plots. (E) Time courses of AMPA-mEPSC frequency corresponding to the control and mefenamic acid recordings shown in A and C. (F) Summary of the effect of PregS on AMPA-mEPSC frequency. In the presence of mefenamic acid the effect of PregS was significantly reduced (Mann-Whitney test, ** p <0.05; n = 5).

Figure 4.4. Epipregnanolone sulfate increases AMPA-mEPSC frequency. (A) Sample traces of AMPA-mEPSC recordings at P6 in a control PC (calibration: 20 pA, 0.5 s) and corresponding average traces (calibration: 6 pA, 8 ms). (B) Corresponding cumulative probability plots; epipregnanolone sulfate significantly decreased the inter-event interval ($p < 0.01$ by K–S test) but not the amplitude of these events. (C) Sample traces of AMPA-mEPSC recordings in a La^{3+} treated cell at P7 (calibration: 20 pA, 0.5 s) and corresponding average traces (calibration: 8 pA, 12 ms). (D) Corresponding cumulative probability plots; in the presence of La^{3+} neither the inter-event-interval nor the amplitude of these events was affected by epipregnanolone sulfate. (E) Time courses of AMPA-mEPSC frequency corresponding to the control and the La^{3+} recordings shown

in A and C. (F) Summary of the effect of epipregnanolone sulfate (EpipregnanS) on AMPA-mEPSC frequency. In the presence of La^{3+} the effect of epipregnanolone sulfate was significantly reduced (Mann-Whitney test, ** p <0.05; n = 8).

Figure 4.5. Progesterone changes neither the frequency nor the amplitude of AMPA-mEPSCs. (A) Sample traces of AMPA-mEPSC recordings at P9; calibration: 20 pA, 0.2 sec. Average traces calibration: 4 pA, 3 ms. (B) Corresponding cumulative probability plots for amplitude (left panel) and inter-event interval (right panel). (C) Time course of AMPA-mEPSC frequency for the recordings shown in A. (D) Summary of the effect of progesterone on AMPA-mEPSC frequency (n = 6).

Figure 4.6. Nifedipine increases AMPA-mEPSC frequency. (A) Sample traces of AMPA-mEPSC recordings at P6 in a control PC (calibration: 50 pA, 0.5 s) and corresponding average traces (calibration: 4 pA, 10 ms). (B) Corresponding cumulative probability plots; nifedipine significantly decreased the inter-event interval ($p < 0.01$ by K–S test) without affecting the amplitude. (C) Sample traces of AMPA-mEPSC recordings in La^{3+} at P8 (calibration: 50 pA, 0.2 s) and corresponding average traces (calibration: 4 pA, 4 ms). (D) Corresponding cumulative probability plots illustrating the lack of a significant effect of nifedipine in presence of La^{3+}. (E) Time courses of AMPA-mEPSC frequency corresponding to the control and the La^{3+} recordings shown in A and C. (F) Summary of the effect of nifedipine on AMPA-mEPSC frequency. In the presence

of La^{3+}, the effect of nifedipine was significantly reduced (Mann-Whitney test, *** p <0.0001; n = 9).

Figure 4.1. Pseudocolored confocal images of TRPM3 expression profile in the neonatal cerebellar cortex.

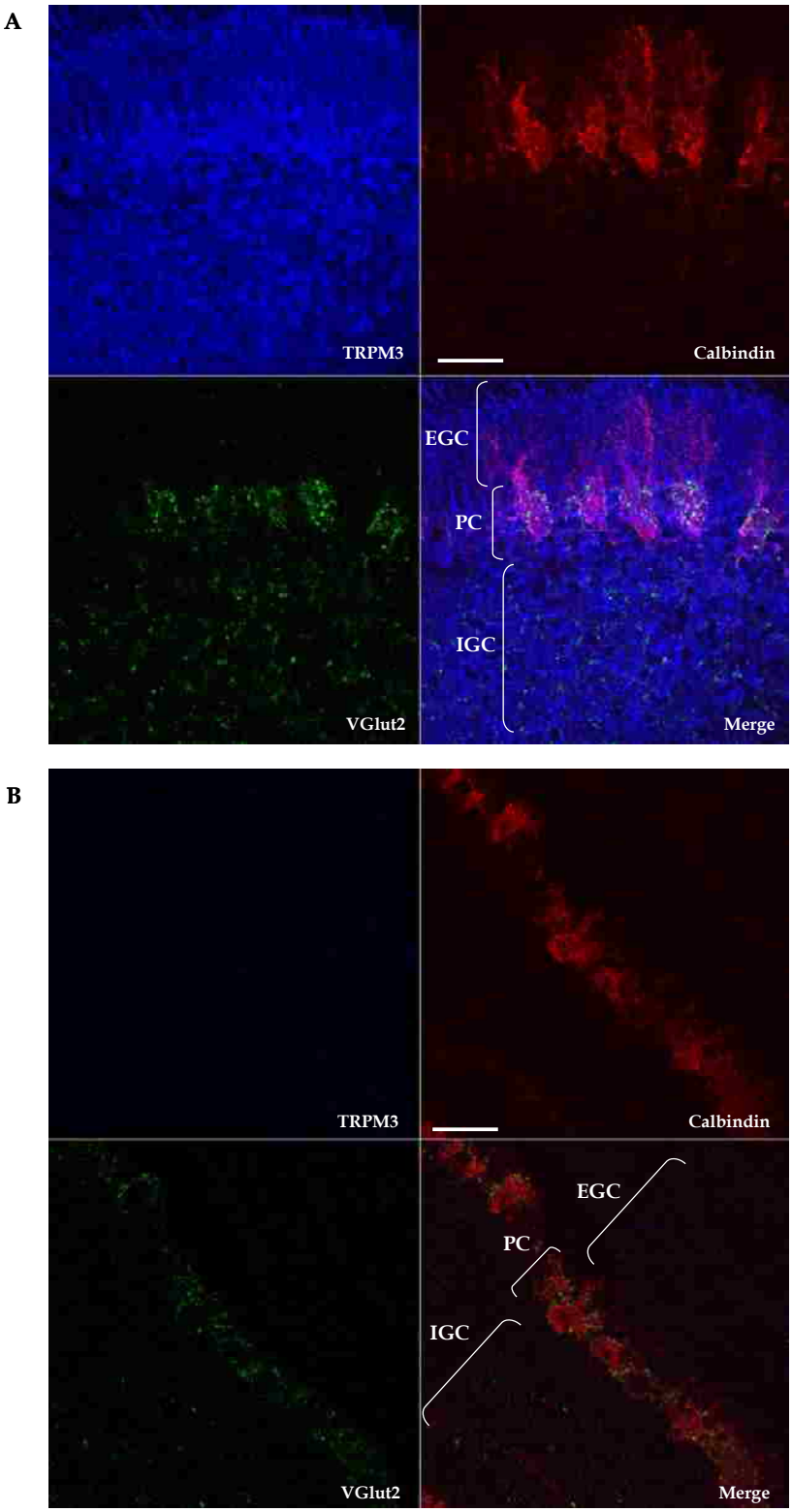


Figure 4.2. Confocal images and colocalization masks for TRPM3, VGlut2 and calbindin in the PC layer.

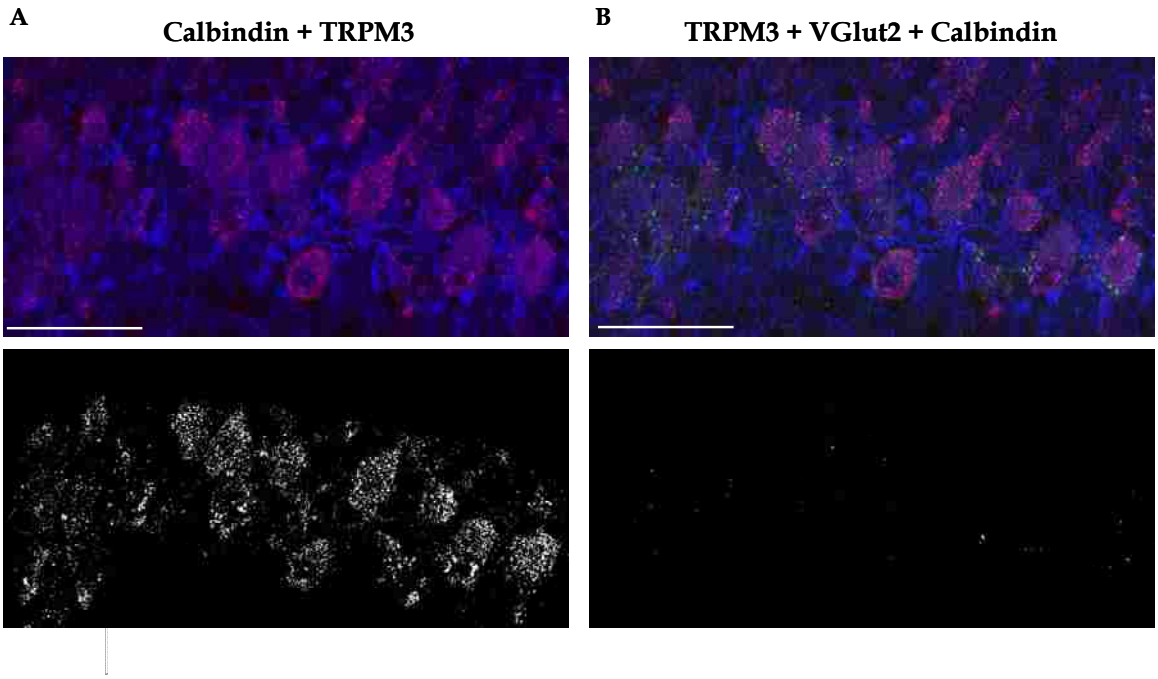


Figure 4.3. Mefenamic acid inhibits the PregS-induced increase of AMPA-mEPSC frequency.

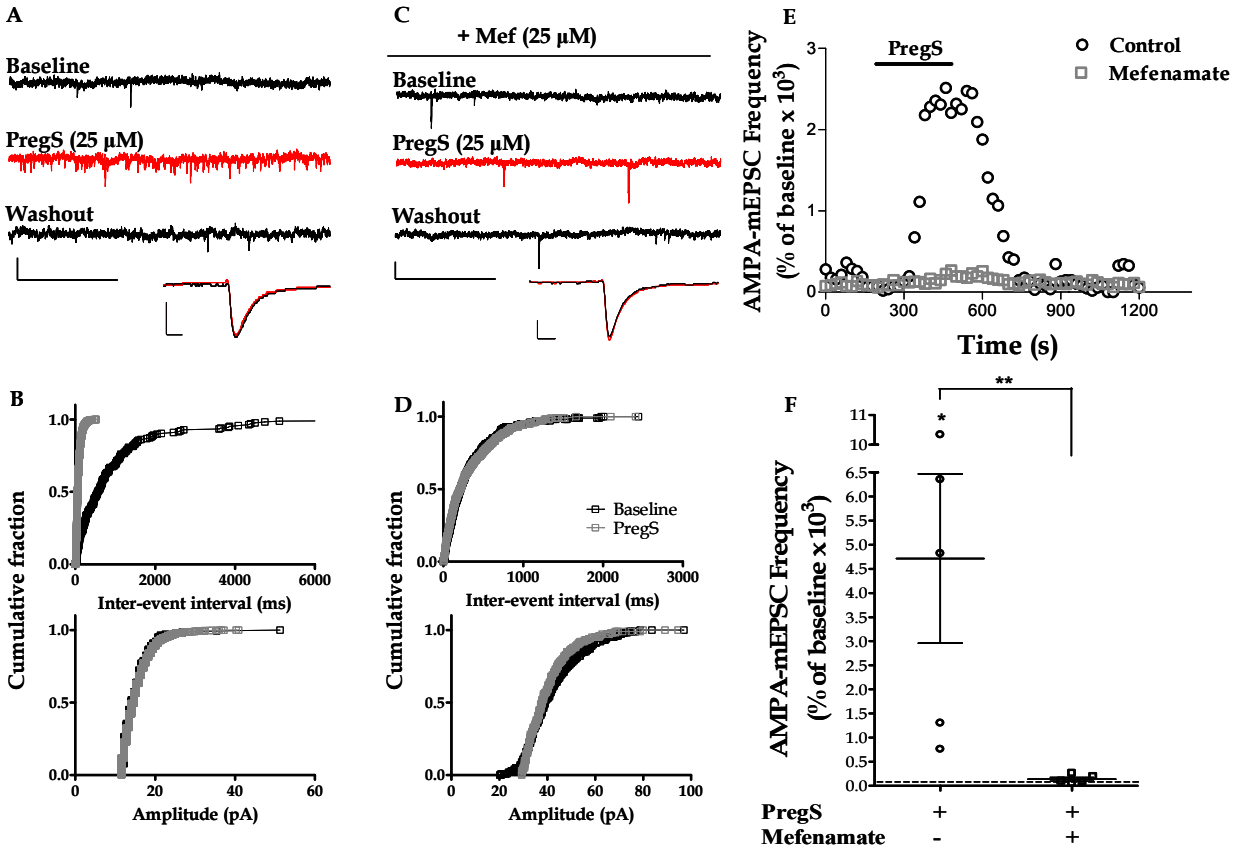


Figure 4.4. Epipregnanolone sulfate increases AMPA-mEPSC frequency.

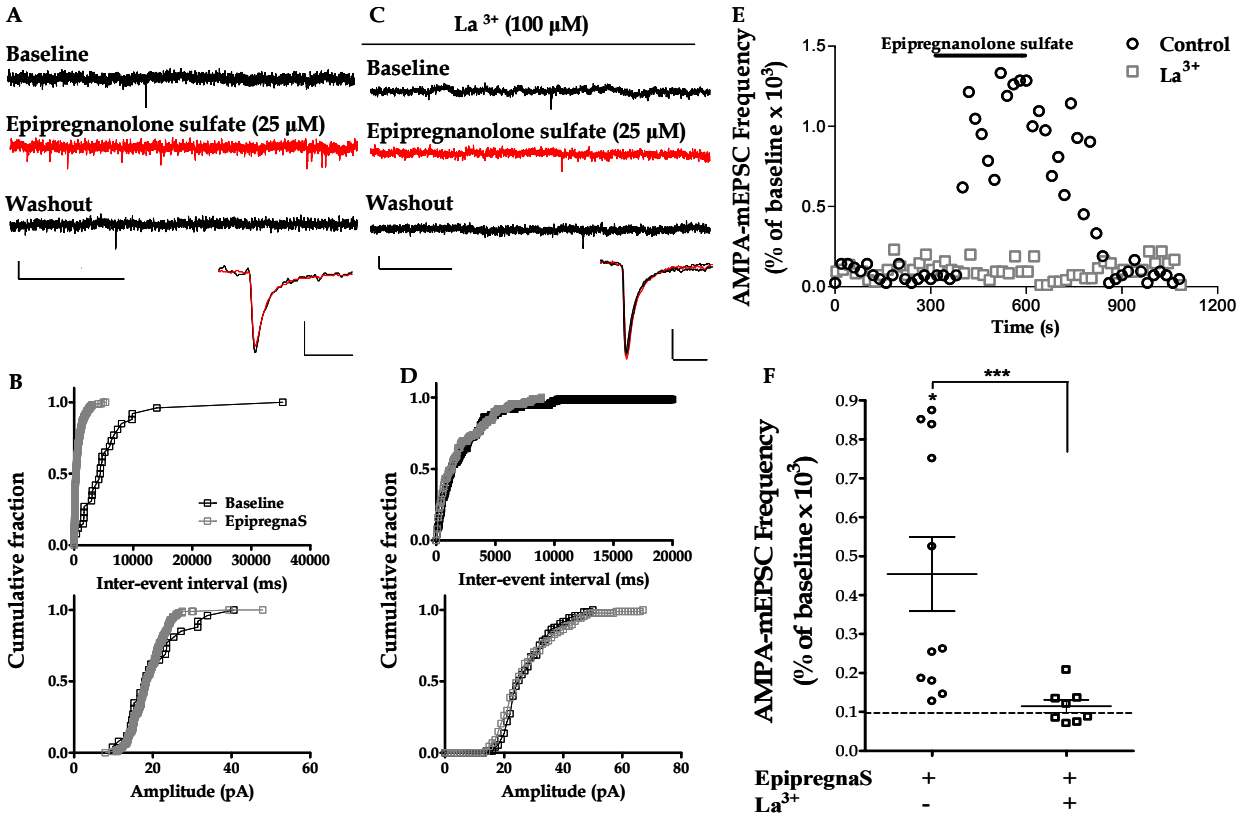


Figure 4.5. Progesterone changes neither the frequency nor the amplitude of AMPA-mEPSCs.

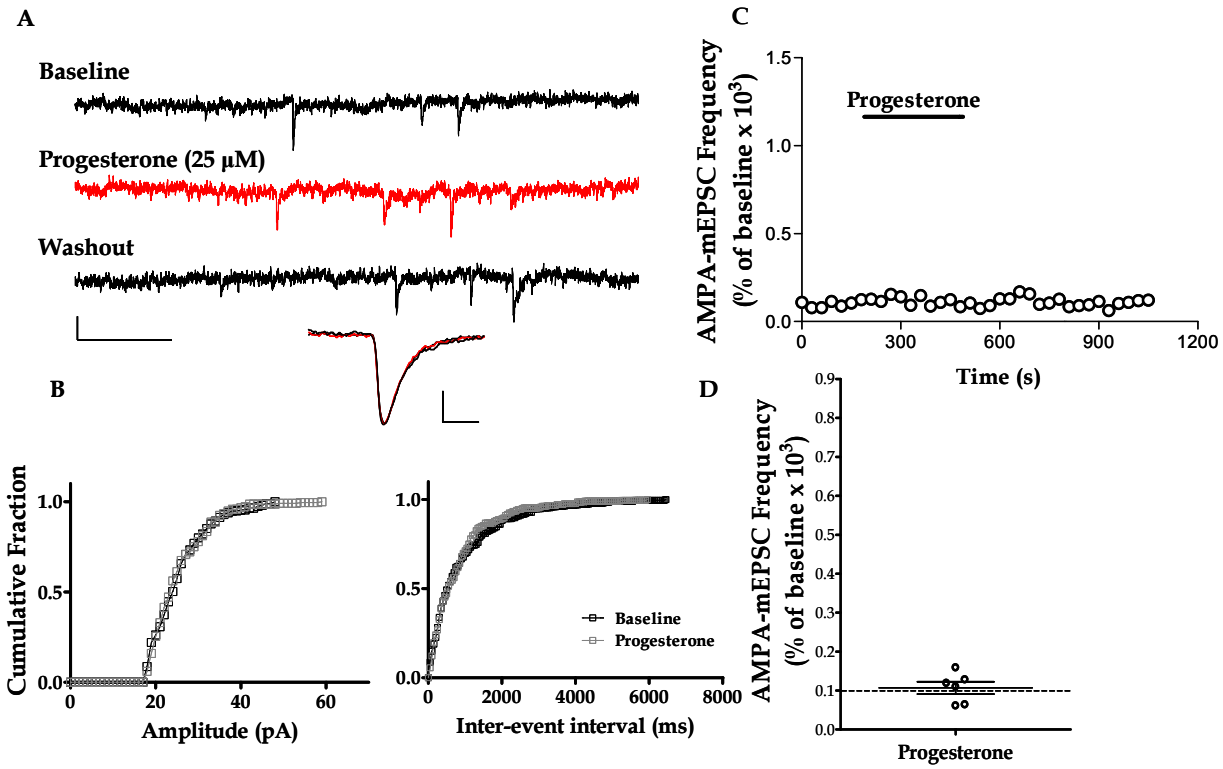
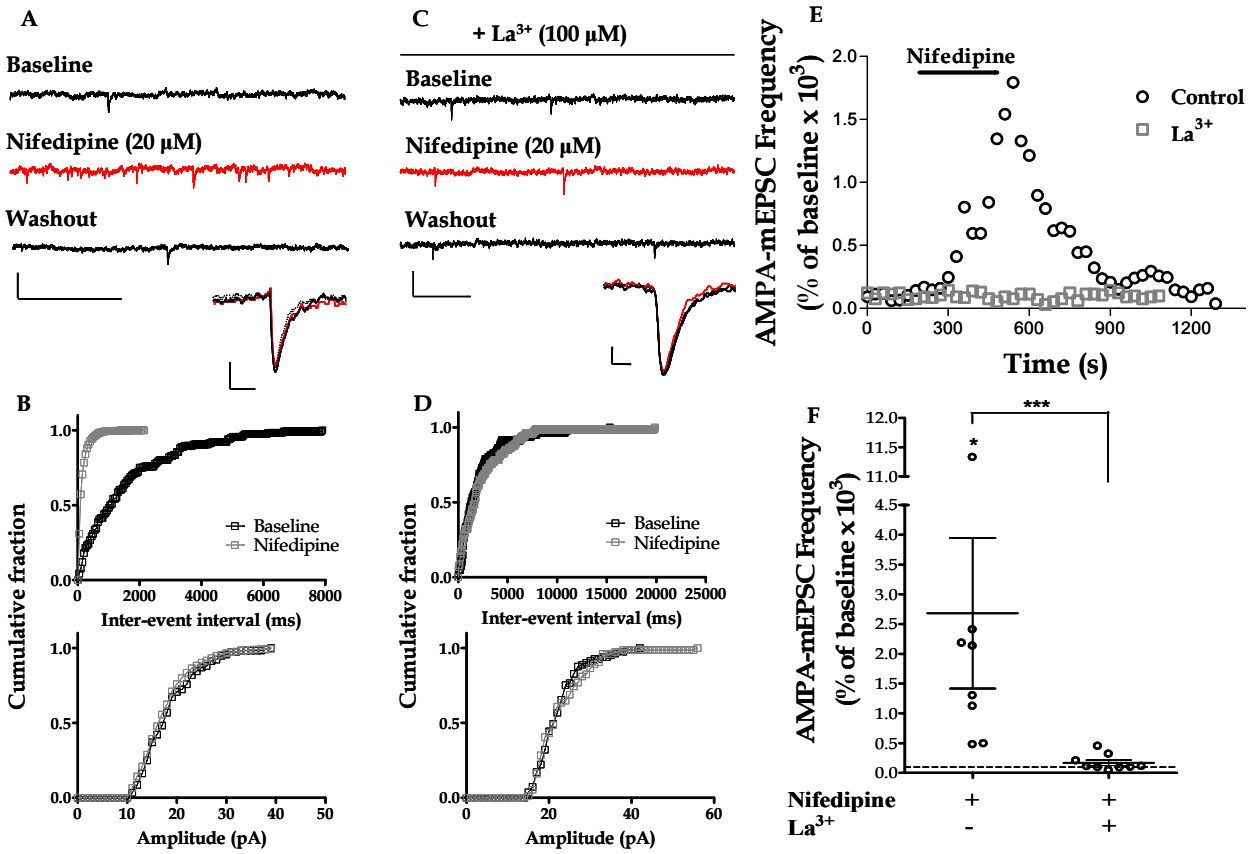


Figure 4.6. Nifedipine increases AMPA-mEPSC frequency.



5. Preventing the degradation of sulfated steroids enhances glutamatergic transmission at developing cerebellar Purkinje cells in hindbrain organotypic cultures

5.1 Abstract

Immature and mature cerebellar Purkinje cells (PCs) have been shown to synthesize the steroid precursor, pregnenolone. Pregnenolone metabolites such as progesterone and estradiol have been shown to modulate the development of PC morphology. However, the role of sulfated steroids on the development of PCs is unknown. Recently, we have reported that exogenous bath application pregnenolone sulfate (PregS) potently enhances glutamate release onto neonatal PCs in acute slice preparations. Here, we show that increasing the levels of endogenous sulfated neurosteroids during development via sulfatase inhibition, enhances glutamatergic transmission at PCs in organotypic hindbrain cultures. In addition, we show that prolonged exposure to DU-14 has deleterious effect on PC viability. Our results indicate that endogenous PregS-like neurosteroids can modulate glutamatergic transmission at developing PCs.

5.2 Introduction

We have demonstrated that exogenous application of the sulfated neurosteroid, pregnenolone sulfate (PregS) potently and reversibly enhances glutamate release at developing Purkinje cells (PCs) (Zamudio-Bulcock and Valenzuela, 2011), via modulation of transient receptor potential M3 (TRPM3 channels) during an active period

of synapse formation. However, whether endogenous PregS-like neurosteroids regulate the development of glutamatergic synapses at PCs remains unknown. We showed that the effect of PregS on glutamatergic transmission was present at neonatal (P4-10), but not at juvenile (P15 to P21) climbing fiber (CF)-to-PC synapses. In juvenile parallel fiber (PF)-to-PC synapses, PregS did not affect glutamate release (Zamudio-Bulcock and Valenzuela, 2011). While CFs contact PCs at about embryonic day (E)19 (reviewed in (Bosman and Konnerth, 2009)), PF-to-PC synapse formation is believed to start at the end of the first postnatal week. Additionally, the migration of granule cells, which give rise to PFs, is not complete until the end of the third postnatal week (Jiang et al., 2008), indicating the ongoing presence of newly formed PF-to-PC synapses during the second and third postnatal weeks. This suggests that if PregS modulated glutamatergic transmission at developing PF-to-PC synapses, this effect could be detected in juvenile PCs. Nonetheless, it remains uncertain if before P15 newly formed PF-to-PC synapses are sensitive to PregS. Thus, it is of interest to study the physiological role of endogenous PregS at both PF and CF-to-PC synapses. While PF inputs arise from the axonal terminals of granule cells located within the cerebellar cortex, CFs arise from the axonal projections of cells in the inferior olive, which is located in the medulla oblongata. Therefore, to study the potential effect of PregS at both types of glutamatergic synapses, we cultured hindbrain explants from rat embryos, which contain the cerebellar anlage as well as the inferior olive. In this organotypic hindbrain culture system, PCs have been shown to be innervated by both PF and CF terminals ((Chedotal et al., 1997, Letellier et al., 2009), also see Appendixes 14 and 15). We used electrophysiological and immunohistochemical techniques to test the effect of increasing the levels of endogenous

sulfated neurosteroids on glutamatergic transmission at PCs in organotypic hindbrain cultures.

5.3 Methods

All experiments were approved by the University of New Mexico Health Sciences center institutional animal care and use Committee and conformed to National Institutes of Health guidelines. Unless specified, all chemicals were from Sigma (St. Louis, MO), Tocris Bioscience (Ellisville, MO), or Invitrogen (Madison, WI).

5.3.1 Organotypic cultures

Sprague-Dawley pregnant rats were anesthetized with Isofluorane and rat embryos were quickly removed at embryonic day (E) 15-16. As previously described (Chedotal et al., 1997, Letellier et al., 2009), embryos were placed in ice cold Gey's balanced salt solution supplemented with glucose (6.5mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (1:1000) and HEPES (25 mM) and brains were dissected out. The region between the tecto-cerebellar and medullo-spinal junctions, which includes the cerebellar anlage and the inferior olive, was isolated. The rostrally fused cerebellar plates were separated and the explants were positioned with their ventral side down in contact with the membrane insert (pore size 0.4 μm) (Millipore, Billerica, MA) (Fig. 5.1). The explants were kept in an atmosphere of humidified 5% CO2 at 37oC for three weeks in a culture media containing 50% Earle's basal medium, 25% Hank's balanced salt solution, 25% heat-inactivated horse serum, 1 mM L-glutamine, 5 mg/ml glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (1:2000). Culture media was replaced every 2 days. From day *in-vitro* (DIV) 14 to 21, (DIV 0 was

the plating day), explants were either exposed to 5 μM DU-14 (generously provided by P.-K. Li, College of Pharmacy, Ohio State University HSC), vehicle, dimethyl sulfoxide (0.025%), or were left untreated.

5.3.2 Electrophysiology

Whole-cell patch-clamp recordings were performed on PCs at DIV20-21 as previously described for acute cerebellar slices (Zamudio-Bulcock and Valenzuela 2011). The explants were placed in the recording chamber with their ventral side up and were allowed to stabilize in the artificial cerebrospinal fluid (ACSF) at 34^{o}C, for at least half an hour before recordings were performed. PCs were recognized based on morphology and location. An internal solution containing (in mM): 150 CsCl, 1.5 $MgCl_2$, 10 HEPES, 0.1 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Calbiochem, La Jolla, CA), 2 Na_2-ATP, 0.4 Na-GTP, 5mM N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314) at pH 7.3 was used. The ACSF was equilibrated with 95% O2/5% CO2 and contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH_2PO4, 1 $MgSO_4$, 26 NaHCO3, 2 $CaCl_2$, 10 glucose, 10 μM 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid (SR 95531). Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 0.5 μM tetrodotoxin (TTX; Calbiochem, La Jolla, CA). If the access resistance changed >30%, the recording was discarded. The membrane potential was held at -70 mV.

Data were acquired with pClamp 10 (Molecular Devices, Sunnyvale, CA) at a filtering frequency of 2 kHz and an acquisition rate of 10 kHz. Miniature events were analyzed using Mini Analysis (Synaptosoft, Decatur, GA). Statistical analyses of pooled

data were performed with GraphPad Prizm 4 (GraphPad Software, San Diego, CA). Data shown as mean ± standard error of the mean.

5.3.3 Immunohistochemistry

Free-floating explants were immersion-fixed in 4% paraformaldehyde overnight. Then, explants were washed 3 times in phosphate buffered saline (PBS) and permeabilized with 0.5% Triton X-100. Simultaneously, nonspecific binding was blocked with 10% normal donkey serum, 10% normal goat serum and 10% bovine serum albumin. Primary antibodies (polyclonal guinea pig anti-VGlut2; 1:500, AB5907 Millipore, Billerica, MA and monoclonal mouse anti-calbindin 1:50, sc-70478, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), were incubated at 4^{o}C for 24h. Secondary antibodies (donkey anti-mouse alexa 555, for calbindin detection, and goat anti-guinea pig alexa 488 for VGlut2 detection) , were incubated overnight at 4^{o}C. Explants were then rinsed, placed on micro glass superfrost slides (VWR international, West Chester, PA) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunostainning was examined with a Zeiss LSM510 confocal microscope (Carl Zeiss, Germany). The intensity of calbindin stainning was quantified using the LSM510 Image Examiner software (Carl Zeiss, Germany).

5.4 Results

5.4.1 Prolonged exposure to DU-14 reduces PC viability

To investigate whether glutamatergic transmission was affected by increasing the levels of endogenous PregS-like neurosteroids, the explants were treated with the

sulfatase inhibitor DU-14 (5 μM). Initially, hindbrain explants were exposed to DU-14 (5 μM) from DIV 12 to 20-28, chronologically equivalent to P7 to 23. When electrophysiological experiments were attempted in these explants at DIV20-28, we noticed that in the DU-14 treated group the number of viable PCs was dramatically decreased and electrophysiological recordings from these cells were not feasible. Therefore, we examined the morphology of PCs using immunohistochemistry. Calbindin immunostainning showed that, in DU-14 treated explants, the morphology of PCs was visibly deteriorated at DIV 24 (Fig. 5.2A). Quantification of the intensity of calbindin immunostainning in randomly selected regions of the cerebellar plates showed a significant reduction in calbindin expression in DU-14 treated explants when compared to dimethyl sulfoxide controls (Fig. 5.2B).

5.4.2 Shorter DU-14 exposure increases the frequency but not the amplitude of activity-dependent and independent glutamatergic transmission at PCs

For these experiments, the cultures were divided into three groups, namely: untreated controls, vehicle (dimethyl sulfoxide, 0.025 %) treated, and DU-14 (5 μM) treated. The treatment time was reduced to DIV 15 to 21. Recordings were performed at DIV 20-21. We found that DU-14 significantly increased the baseline frequency of both activity-dependent sEPSCs and mEPSCs. DU-14 significantly increased sEPSC frequency when compared to untreated controls and vehicle controls, (19.98 ± 6.8 Hz in DU-14 treated explants, n=5, compared to 2.14 ± 0.39 Hz in untreated controls, n=4, and 2.71 ± 0.27 Hz in vehicle controls, n=4) (Fig. 5.3A and B). sEPSC amplitude was not significantly affected by DU-14 treatment (Fig.5.3C). Similarly, baseline mEPSC frequency was increased by DU-14. However, this increase was only significant when

compared to untreated controls (0.39 ± 0.11 Hz in untreated controls, n=6 and 8.68 ± 4.54 Hz in DU-14 treated explants, n=7), but there was no significant difference between untreated controls and vehicle controls (1.98 ± 1.2 Hz, n=5). Moreover, there were no significant changes in the frequency of sEPSC in vehicle treated controls when compared to untreated controls (Fig. 5.4A and B). mEPSC amplitude was not affected by DU-14 treatment (Fig. 5.4C). Time to rise and half-width of sEPSCs and mEPSCs were unaffected by DU-14 treatment (data not shown).

5.4.3 DU-14 exposure does not affect the size of PCs

In whole-cell patch-clamp experiments, rupture of the cell membrane and introduction of a voltage step allows for the measurement of membrane capacitance and resistance. The cell capacitance can be used as an indicator of cell surface area. The membrane resistance represents the number of resting ion channels in the membrane and their conductances. We compared the cell capacitance values across the three experimental groups and found no significant differences between the DU-14 treated PCs and the control groups (Fig. 5.5A). Similarly, there were no significant differences between the three groups in the membrane resistances of PCs (Fig. 5.5B). Given the lack of an effect of DU-14 on cell size, and assuming that the overall shape of the membrane did not change, we can conclude that the average density of resting ion channels in the membrane was not affected by DU-14 treatment. However, this is not consistent with the DU-14 increase in sEPSC and mEPSC frequency, since cells of equal size an increased amount of open channels should be reflected in a decrease in the membrane resistance.

5.5 Discussion

We have previously shown that in acute cerebellar slices exogenous application of PregS potently enhanced the frequency of mEPSCs at developing PCs (Zamudio-Bulcock and Valenzuela, 2011). In humans, PregS has been shown to be present in the cerebellum (reviewed in (Schumacher et al., 2008)). In the rodent, however, although pregnenolone has been shown to be synthesized in PCs, the synthesis of its sulfate ester has not been confirmed (Tsutsui et al., 2003). This study provides evidence suggesting that sulfated neurosteroids could be synthesized in the cerebellar cortex and that, pharmacologically-induced increases of the endogenous levels of this neurosteroid leads to the strengthening of glutamatergic transmission in PCs in-vitro. In neonatal brain slices, radioimmunoassay studies have been shown that DU-14 (5 μM) significantly increases the levels of PregS-like neurosteroids (Caldeira et al., 2004). DU-14 inhibits the enzymatic activity of 3-β hydroxysteroid sulfatases; therefore, in addition to PregS, DU-14 could also increase the production of other sulfated 3-β hydroxysteroids, which could contribute to the increase in glutamatergic transmission. For instance, dehydroepiandrosterone sulfate has also been shown to activate TRPM3 channels, although with lower efficacy than PregS (Wagner et al., 2008, Majeed et al., 2010). Therefore, the findings reported here could be, in part, mediated by an increase in endogenous dehydroepiandrosterone sulfate levels.

In the neonatal hippocampus it was shown that postsynaptic depolarization induced the release of a PregS-like neurosteroid which, acting as a retrograde messenger, potentiated glutamatergic transmission. It is possible that a similar mechanism takes place in the developing PC. Thus, DU-14 treatment may set the PC glutamatergic circuit into an ongoing excitatory loop in which increased levels of endogenous PregS-like

neurosteroids are retrogradely released upon PC excitation by CF and /or PF activity stimulating a further enhancement of glutamate release onto PC, resulting in increased PC excitability thus leading to an ongoing increase in the retrograde release of PregS.

The decrease in PC viability caused by prolonged exposure to DU-14 could be a consequence of excitotoxicity via the PregS-induced increase in glutamatergic transmission. Developing, but not mature, PCs dendrites express Ca^{2+}-permeable GluR2-lacking AMPA receptors (Douyard et al., 2007). NMDA receptors are expressed in both, developing and mature PCs, with a transient absence during the third postnatal week (reviewed in (van Welie et al., 2011)). Therefore, in developing PCs, excessive Ca^{2+} entrance through AMPA and NMDA receptors, could lead to excitotoxicity and eventual cell death.

Our findings that chronic exposure to DU-14 significantly increases the average baseline frequency of mEPSCs and sEPSCs can be interpreted in two ways. In control PCs, at DIV15 (chronologically equivalent to P10) PregS increases the frequency of mEPSCs in PCs (526.5 ± 171.7 % of baseline, n=2, data not shown). The K-S test showed a significant decrease in the inter-event interval. Additionally, in acute slices the effect of PregS was specific to CF-to-PC synapses and disappeared after P15 (Zamudio-Bulcock and Valenzuela, 2011). Therefore, although it is uncertain if, after DU-14 washout, the steroid sulfatase enzymatic activity is recovered and PregS levels are decreased, we expect that at DIV20-21 glutamatergic synapses at PCs are no longer sensitive to PregS. Making this assumption, we can state that treatment with DU-14 induces a persistent enhancement of glutamatergic transmission. Such persistent enhancement of glutamatergic transmission could be explained by unsilencing of

synapses or stabilization of multinumerary CF inputs. The lack of an effect in the amplitude of both sEPSCs and mEPSCs argues that long term exposure to elevated PregS levels does not change the density or the conductance of postsynaptic AMPA receptors. Alternatively, it is possible that chronic exposure to elevated levels of PregS sensitizes these synapses and if PregS levels are still high in the explant at the time of the recordings, our results could reflect an ongoing effect of elevated levels of PregS. This would imply that these synapses do not develop tolerance to PregS.

The ultimate physiological outcome of this pharmacologically-induced increase in sulfated neurosteroid levels on the development of glutamatergic synapses is yet to be tested. Preliminary experiments showed that while in control explants, PF and CF-like currents could be evoked, in DU-14 treated PCs, only PF-like responses could be elicited (see Appendix 15). These results could indicate that chronic exposure to elevated levels of sulfated neurosteroids results in the total elimination of CF-to-PC synapses or in a change in the properties of these synapses. These findings could be relevant in cases of abnormal elevations of sulfated neurosteroids. In a model of fetal alcohol spectrum disorders, studies from our laboratory, suggest that prenatal alcohol exposure increases the levels of PregS, but not of dehydroepiandosterone sulfate, in the fetal brain ((Caldeira et al., 2004) reviewed in (Valenzuela et al., 2008)). The results presented here suggest that the level of endogenous PregS-like neurosteroids can be manipulated in culture and suggest a potential physiological role for these neurosteroids in the development of glutamatergic synapses onto PCs. Future experiments will make use of the hindbrain culture system and will aim to decrease endogenous levels of PregS using short hairpin RNA dependend downregulation of the specific PregS sulfotransferase Sult2b1a and will

study the development of CF and PF-to-PC synapses (for details see future directions section).

5.6 Figure legends

Figure 5.1. Schematic representation of dissection and plating of hindbrain cultures from rat embryos at E15-16. The location of the cerebellar plates (Cb) and the inferior olive (IO) are indicated in the lateral and the frontal views. Dash lines indicate cuts. Adapted from Chedotal et al (1997) and Letellier et al (2009).

Figure 5.2. Prolonged inhibition (DIV12-28) of steroid sulfatases decreases PC viability. (A) Confocal images of DIV24 hindbrain explants at a magnification of 63X, the vehicle control is shown on the left and the DU-14 treated is on the right. Scale bars 20 μm. (B) Calbindin fluorescence intensity is significantly lower in DU-14 treated explants (* $p<0.05$ by Mann-Whitney test n=3).

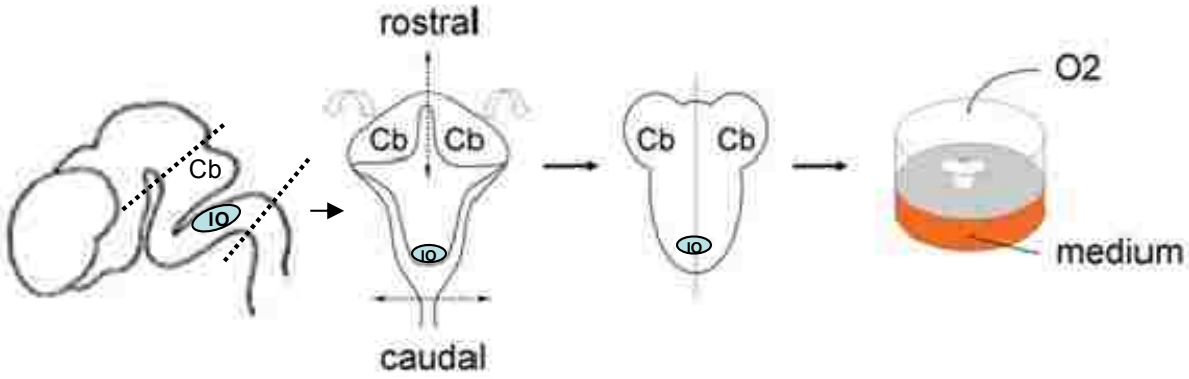
Figure 5.3. Shorter inhibition of steroid sulfatases increases the frequency but not the amplitude of sEPSCs in PCs. (A) Sample traces of sEPSCs recordings at DIV20-21 PCs. Scale bar: 50 pA, 0.5 s. (B) DU-14 significantly increases sEPSC frequency when compared to both, untreated controls and vehicle controls (*$p<0.05$ by one-way ANOVA followed by Dunn's multiple comparison test n=4-5). (C) DU-14 treatment does not significantly affect the amplitude of sEPSC.

Figure 5.4. DU-14 treatment increases mEPSC frequency. (A) Sample traces of mEPCs recordings at DIV20-21 PCs. Scale bar: 20 pA, 0.2 s. (B) DU-14 significantly

increases mEPSC frequency when compared to untreated controls (*p<0.05 by one-way ANOVA followed by Dunn's Multiple Comparison Test, n=5-7). There was no significant difference between untreated controls and vehicle controls. (C) DU-14 treatment does not significantly affect the amplitude of mEPSC.

Figure 5.5. DU-14 treatment does not affect PC membrane capacitance or resistance.

Figure 5.1. Schematic representation of dissection and plating of hindbrain cultures from rat embryos at E15-16.



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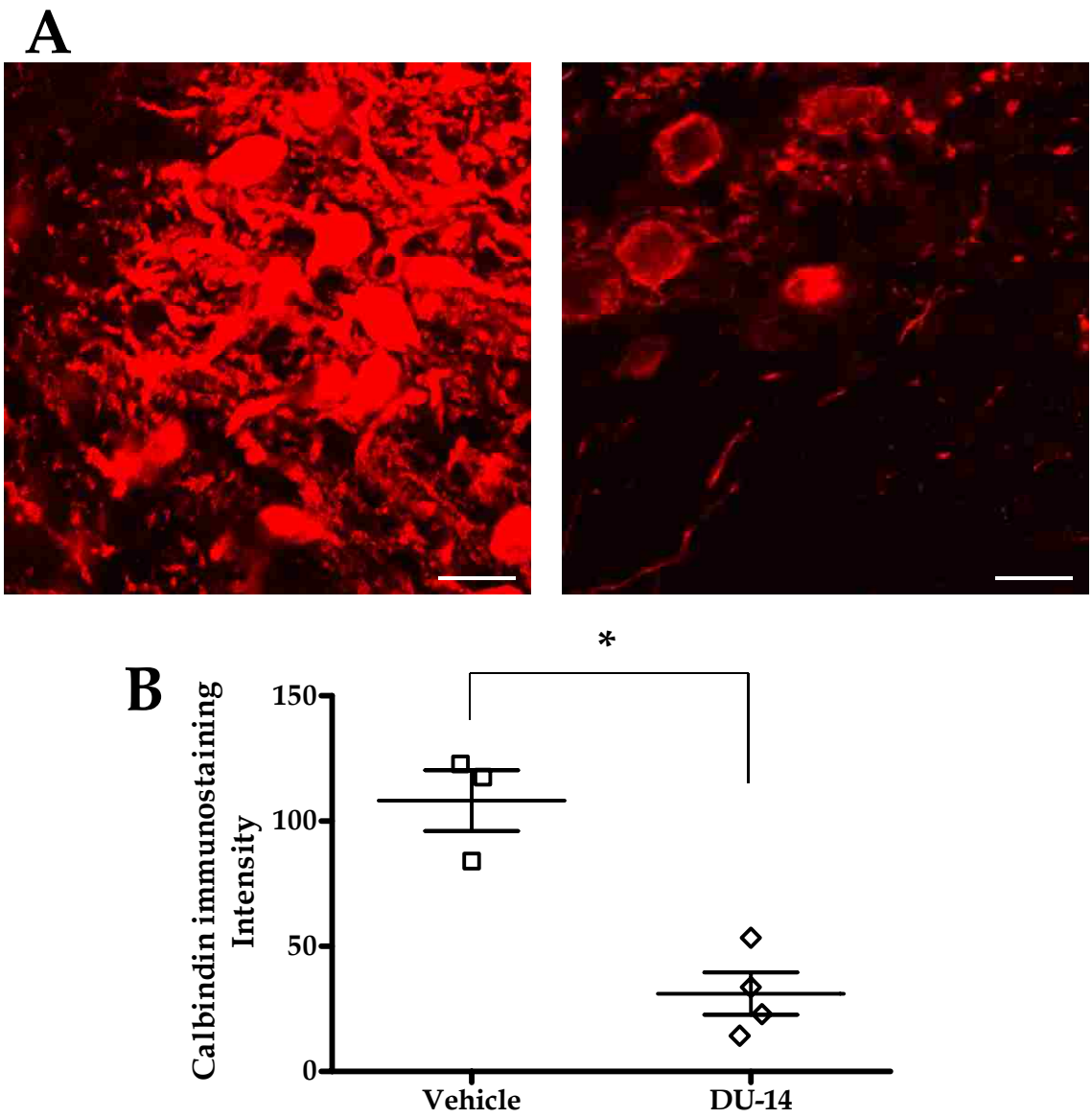

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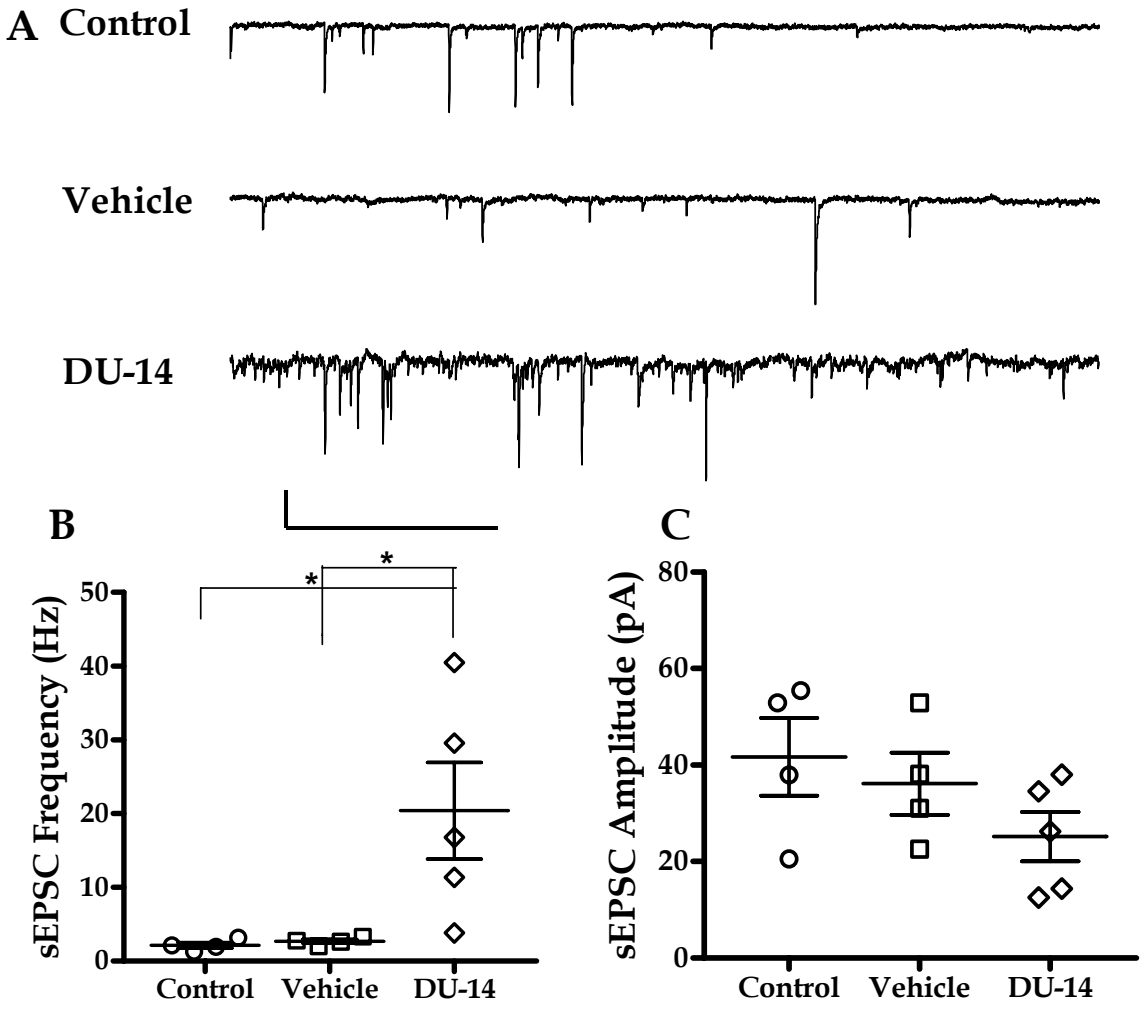
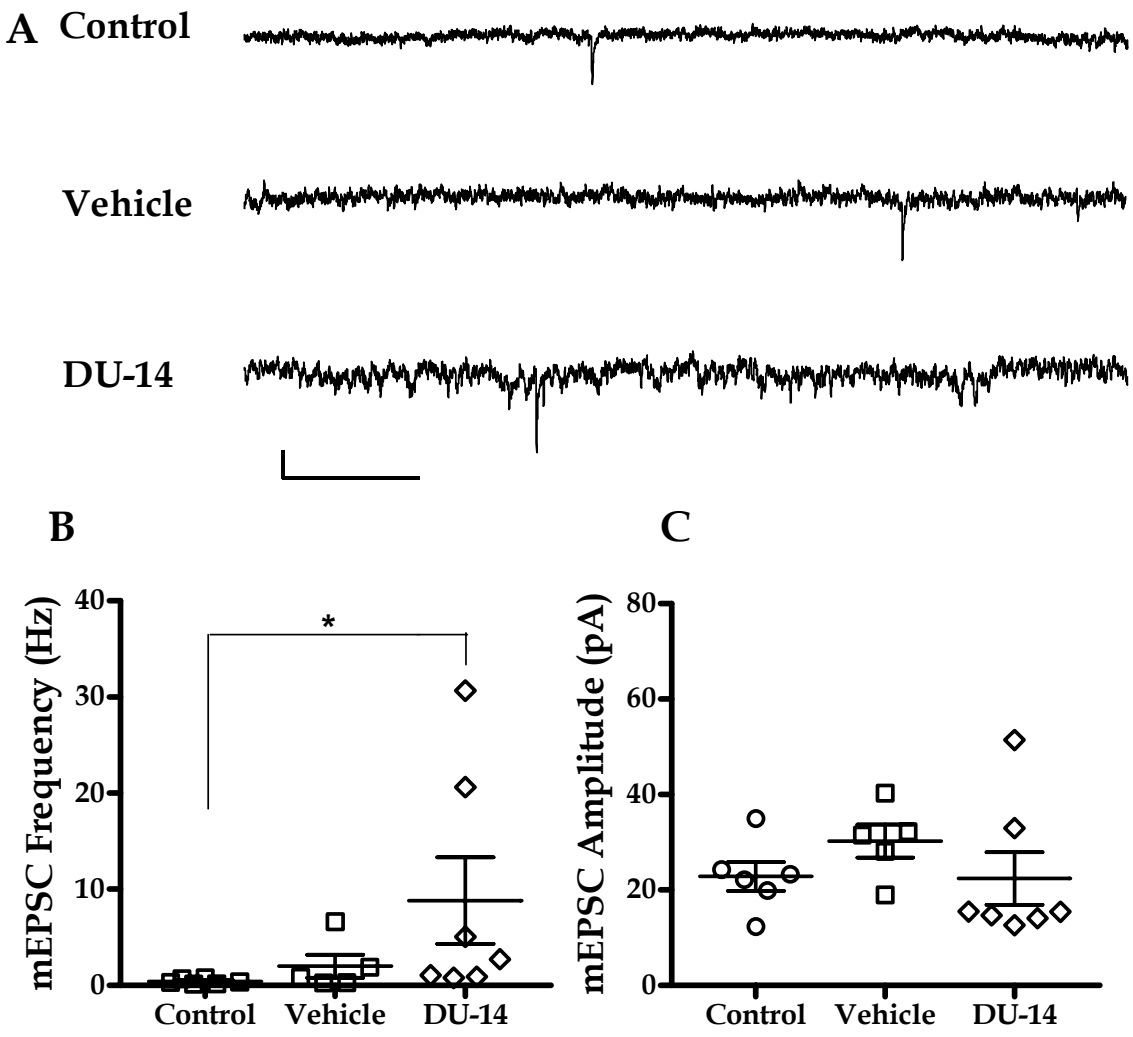


Figure 5.4. DU-14 treatment increases mEPSC frequency.



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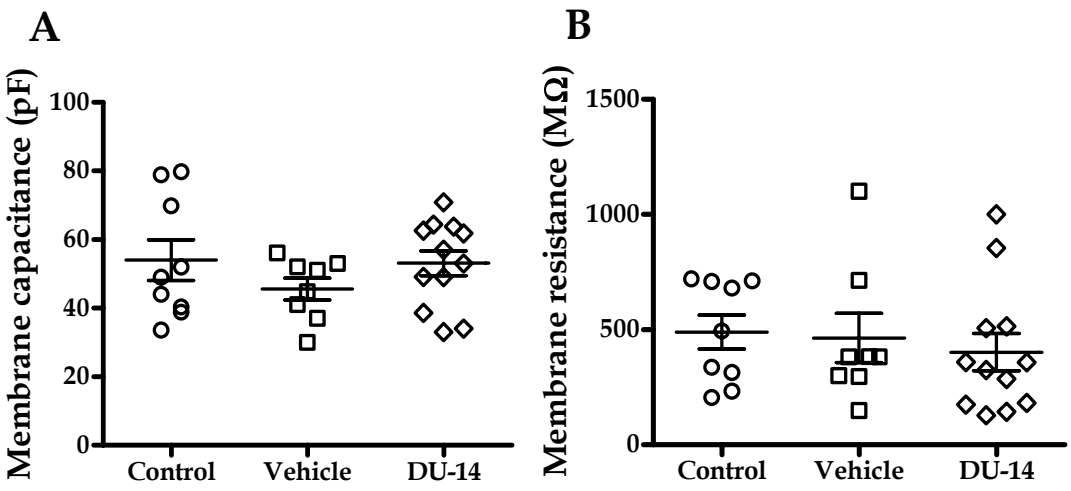

6. Conclusions

6.1 Summary of main findings

The main findings of the studies presented in this dissertation can be summarized as follows. First, it was demonstrated that the neuroactive steroid pregnenolone sulfate (PregS) reversibly modulates GABA and glutamate release at the developing cerebellar Purkinje cells (PCs). The effect on glutamate release, however, was significantly greater than that on GABA release. Therefore, the studies were focused on further characterizing the effect of PregS on glutamate release. This effect was found at climbing fiber (CF)-to-PC synapses from neonatal, but not juvenile rats. On the other hand, developing parallel fiber (PF)-to-PC synapses were not affected in juvenile PCs. Second, the effect of PregS on glutamate release at immature PCs was Ca^{2+}-dependent and was mediated by activation of steroid-sensitive transient receptor potential melastatin 3 (TRPM3) channels. Importantly, this finding identified TRPM3 channels as novel modulators of glutamatergic transmission in the developing brain. Third, pharmacologically-induced increases in endogenous PregS-like neurosteroids induce a long-lasting increase in glutamatergic transmission at PCs in organotypic hindbrain cultures. This effect may damage PCs via an excitotoxic mechanism and also trigger elimination of CF-PC synapses.

6.2 Overall conclusions and functional implications

The development of PCs and the formation of GABAergic and glutamatergic synapses onto these cells are known to be influenced by several factors including neurotrophins, neurosteroids, and activation of neurotransmitter receptors and calcium

channels. Nevertheless, the role of the pregnenolone sulfate (PregS)- induced potentiation of glutamatergic transmission via modulation of the transient receptor potential M3 channel proposed in this dissertation is unprecedented.

During early postmitotic development, remodeling of PC dendrites is controled by multiple intrinsic factors, including retinoid-related orphan receptor α, a transcription factor whose expression in PCs modulates early stages of dendritic tree outgrowth and remodeling (reviewed in (Sotelo and Dusart, 2009)). However, the majority of PC development events, including migration, survival, dendritic development, and synapse formation and stabilization depend on interactions with other neuronal and glial cell types. The migration of PCs, via interactions with radial glia, is modulated by the extracellular matrix glycoprotein, reelin, released by cells within the external granule cell layer (reviewed in (Sotelo and Dusart, 2009)). We found that TRPM3 channels are highly expressed in the external granule cell layer (EGL), where immature granule cells and radial glia fibers are located. Therefore, it would be interesting to investigate whether these channels also play a role in the migration of PCs.

Dendritic development and synaptogenesis in PCs are the result of a spatially and temporally coordinated concert of cell-to-cell interactions in which neurotrophins, neurosteroids, and activity-dependent mechanisms have been shown to play a role. PCs receive excitatory inputs from axonal projections of the inferior olive, the CFs, and from the granule cell projections, the PFs. The first ones to develop are the CF-to-PC synapses, which contact PCs by E17 (reviewed in (Liu et al., 2008b)). During early development, the survival of PCs has been suggested to depend on CF synaptic input. In organotypic slice cerebellar cultures lacking the inferior olive, which give rise to the axonal CF

projections, the number of PCs is dramatically reduced, an effect that can be rescued by co-culturing these slices with inferior olive-containing slices. The trophic effect of the CF input can be mimicked by pharmacologically-induced depolarization of cultured PCs (Ghoumari et al., 2006). Interestingly, the PregS-induced increase in glutamate release at CF-to-PC synapses could contribute to the role of CF in PC survival.

Elongation and migration of CF projections from the inferior olive starts at around embryonic day E13 and has been proposed to be mediated by brain-derived neurotrophic factor (BDNF) and neurotrophins 3/4 (NT3/4), and their receptors (tropomyosin-related receptor kinases B and C; TrkB and TrkC, respectively). The levels of these neurotrophins and their receptors are developmentally regulated, being highest during embryonic and early postnatal development. NT3 and NT4 are synthesized in the developing cerebellum, but not in the inferior olive. However, NT3 and NT4 peptides have been shown to be accumulated in this brain region, suggesting retrograde transport from the cerebellum. On the other hand, BDNF is synthesized within both the cerebellum and the inferior olive, and has been proposed to stimulate axonal elongation by anterograde transport. In BDNF- or NT3- deficient mice, ataxia and behavioral deficits, consistent with CF-to-PC synapse dysfunction, have been reported (reviewed in (Sherrard and Bower, 2002)). Interestingly, the expression of neurotrophins is reduced before the onset of CF synapse elimination and the activation of TrkB decreases in the inferior olive at the onset of CF regression (Sherrard et al., 2009).

During early CF-to-PC development (first postnatal week), multiple CF inputs are functionally differentiated via synaptic competition among themselves, resulting in simultaneous strengthening of one CF input and weakening of the rest. Subsequently, the

strongest CF translocates from the soma to the proximal dendrites of the emerging dendritic tree, and the weak CF inputs are eliminated. The mechanism responsible for such functional differentiation and subsequent elimination is not well understood. It has been proposed that Ca^{2+} entrance through α1 -containing P/Q type Ca^{2+} channels ($C_{av}2.1$), facilitates the strengthening of the winning CF. In mice lacking α1 -containing P/Q type Ca^{2+} channels ($C_{av}2.1$), multi-innervation by CF persists. Moreover, PFs normally synapse onto distal regions of the dendritic tree and also innervate the proximal dendrites, suggesting that CF functional differentiation and translocation to proximal dendrites failed to occur (Miyazaki et al., 2004). Additionally, while strong CFs undergo long-term potentiation, weak CFs display long-term depression in response to the same stimulation paradigm (Bosman et al., 2008, Ohtsuki and Hirano, 2008). It has been proposed that the spatial arrangement of the CFs relative to the sites of Ca^{2+} entry determines whether they are strengthen or weakened. During the period of functional differentiation and CF translocation to the dendrites, L-type Ca^{2+} channels disappear from the soma and P-type Ca^{2+} channels start to appear in the emerging dendritic tree (reviewed in (Bosman and Konnerth, 2009)). PregS has been proposed to be retrogradely released at CA1 hippocampal pyramidal cells upon application of a depolarization protocol that mimicked early network-driven oscillatory bursts known to increase intracellular Ca^{2+} (Garaschuk et al., 1997, Mameli et al., 2005). In the hippocampus, increases in intracellular Ca^{2+} were shown to increase PregS levels (Kimoto et al., 2001). Additionally, although it is unknown whether the release of PregS from these cells involves vesicular release or transporter-mediated extrusion, it is known that dendritic release of retrograde messengers is generally Ca^{2+}-dependent and can be spatially

restricted (reviewed in (Regehr et al., 2009)). Therefore, it is possible that in PCs the compartmentalized Ca^{2+} levels could favor the production and retrograde release of PregS at the sites with highest intracellular Ca^{2+}. Thus, PregS could contribute to the strengthening of those CF inputs located at such privileged sites. Our studies on the PregS-induced increase of glutamate release at these synapses focused on the largest evoked CF-induced current, which includes the responses of both strong and weak CF inputs. Therefore, studies evaluating the effect of PregS in strong and weak CFs separately would be needed to test this hypothesis.

Activation of TrkB receptors was also shown to be required for CF synapse elimination, as mice lacking this BDNF receptor display persistent CF multi-innervation. Additionally, in these mice the number of GABAergic synapses is reduced and the postsynaptic currents are prolonged, suggesting that the $GABA_A$ receptors do not undergo the developmental switch from α3 to α1 subunits (Takayama and Inoue, 2004b, Bosman et al., 2006). Given that both CFs and a subset of molecular layer interneurons synapse onto the proximal part of the PC dendritic tree, it has been proposed that GABAergic interneurons could modulate CF-to-PC synapse formation via synaptic competition for postsynaptic sites (Bosman and Konnerth, 2009). Thus, in TrkB mutants, the empty postsynaptic sites could be occupied by CFs. Therefore, it is uncertain whether TrkB modulates CF-to-PC synapse development directly or indirectly by modulating the formation of GABAergic inputs onto the PC.

If, endogenous PregS release is favored at sites with high intracellular Ca^{2+} GABAergic interneuron-to-PC synapses would also be potentiated by PregS. Consistent with this, we found that GABAergic transmission at neonatal PCs was potentiated by

PregS, but to a lesser extent than glutamatergic transmission (Zamudio-Bulcock and Valenzuela, 2011). Although, the mechanism mediating the effect of PregS at GABAergic synapses remains unknown, given the high expression of TRPM3 in the EGL, it is possible that this effect of PregS is also mediated by TRPM3. Thus, the difference in the potency of the effect of PregS at glutamatergic versus GABAergic synapses could be explained by a different level of expression of TRPM3 channels at these synapses.

The development of PF-to-PC synapses starts at the end of the first postnatal week when NT3, BDNF, TRKB, and TRKC, are expressed in both developing PC and granule cells. Therefore, these neurotrophic factors are thought to be involved in granule cell development and PF-to-PC maturation (reviewed in (Sherrard and Bower, 2002)). In developing PCs, the production of BDNF is modulated, at least in part, by the steroid, estrogen, which is also produced in the PC, and has been linked to PC dendritic growth, spinogenesis and synaptogenesis. Estrogen deficient mice display altered dendritic morphology and synaptogenesis and these effects are correlated with decreased BDNF production. Conversely, estrogen administration to these mice increased BDNF levels in the cerebellum and improved dendritic growth, spinogenesis, and synaptogenesis (reviewed in (Tsutsui, 2008)). A proposed mechanism for BDNF- and estrogen- mediated synaptogenesis is the production of synaptic vesicle proteins, whose expression is regulated by both estrogen and BDNF (reviewed in (Tsutsui, 2008)). Activation of glutamate receptors has been shown to upregulate BDNF expression in granule cells (Lindholm et al., 1993). Therefore, it is also possible that, if PregS increases glutamate release at neonatal PF-to-PC synapses, the resulting enhancement of postsynaptic AMPA

receptor activation in PCs could in turn modulate BDNF expression, contributing to the formation of these synapses.

Synaptic competition between PF and CF inputs has been suggested by a number of studies where granule cell survival is compromised or functional PFs are absent. These include studies where mitotic granule cells are ablated by X-irradiation and natural occurring mutants, the Weaver, Reeler, and the Staggerer mice (reviewed in (Bosman and Konnerth, 2009)). The glutamate receptor type δ2 (GluR δ2) plays a key role in PF to PC competition. In the absence of this receptor, which is exclusively expressed in PCs, the number of spines contacted by PFs is reduced by 50% and the vacant sites are occupied by CFs, resulting in persistent multiple CF innervation (reviewed in (Bosman and Konnerth, 2009)). PF-CF synaptic competition could be used to elucidate whether PregS primarily modulates the development of CFs. If this is the case, decreases of endogenous PregS levels, as proposed in the future directions section, could result in persistent multi-innervation of weak CFs and failure of CF-PC synapses to translocate to the dendrites; PFs would occupy the empty sites, producing stronger postsynaptic currents. On the other hand, if PregS affects PF synapse formation as well, decreased PregS levels would result in the weakening of both PF and CF inputs.

In summary, the development of synapses onto PCs has been shown to be modulated by a plethora of factors. However, the precise mechanisms leading to the appropriate development of glutamatergic and GABAergic transmission are not understood. The demonstration that PregS and TRPM3 are potent modulators of glutamatergic transmission contributes another piece to the puzzle of PC synapse development (Zamudio-Bulcock and Valenzuela 2011, chapter 4). Moreover, the

widespread expression profile of steroid-sensitive TRPM3 channels in the cerebellar cortex (Chapter 4 Fig 1) suggests that both PregS and TRPM3 channels could also be key modulators of granule and PC cell migration, Bergmann glia maturation and PF elongation.

6.3 Critique and Future Directions.

The effect of PregS on glutamate release at CF-to-PC synapses was found to be age-dependent, being present in PCs from neonatal (P4-10), but not in juvenile (P15-21) rats. Moreover, it was not present at PF-to-PC synapses from juvenile rats. However, additional recordings showed that in juvenile rats (P18-P27) PregS significantly increases the frequency of mEPSCs (see Appendix 7.8). This increase was considerably smaller than that observed in neonatal PCs. Maturation of both CF- and PF-to-PC synapses is completed at the end of the third postnatal week (reviewed in (Valenzuela et al., 2010)). Therefore, it is possible that after P15, the effect of PregS decreases to levels undetectable in evoked CF currents, given that the massive release of glutamate nearly saturates the postsynaptic AMPA receptors (Konnerth et al., 1990). Future experiments under conditions of low glutamate release probability (induced by low extracellular Ca^{2+} levels) may unmask an effect of PregS on CF responses in older animals.

It remains uncertain whether before P15 PregS modulates glutamate release at PF-to-PC synapses. Although, in agreement with the literature (Scelfo and Strata, 2005), PF responses were not found in P6-8 PCs, after P8 small PF responses could be detected. Therefore, it should be revisited whether PregS modulates glutamatergic transmission at these synapses from P8 to P15.

Since there are no available specific TRPM3 channel antagonists, the identification of TRPM3 channels as modulators of glutamatergic transmission required the use of a number of non-specific pharmacological agents that might affect glutamatergic transmission by blocking various targets. Mefenamic acid blocks cyclooxygenases in addition to blocking TRP channels, hence its clinical use as a non-steroidal inflammatory drug. Interestingly, cyclooxygenase 2 has been shown to regulate depolarization-induced suppression of inhibition and excitation in hippocampal cells via modulation of endocannabinoid signalling (Straiker et al., 2011). La^{3+} is a known non-specific cation channel blocker. In the hippocampus, however, it has been shown to potentiate $GABA_A$ receptor-mediated currents and to increase the affinity of this receptor for GABA (Boldyreva, 2005). This finding makes the use of La^{3+} unsuitable to test the involvement of TRP channels in the PregS-induce increase of GABA release at developing PC. Nifedine is a Ca^{2+} channel blocker that acts predominantly on L-type Ca^{2+} channels. These channels are abundantly expressed in the soma of developing PCs (reviewed in (Bosman and Konnerth, 2009)) and mediate Ca^{2+} oscillations. Thus, nifedipine could dramatically affect postsynaptic responses.

The potential implication of PregS and TRPM3 channels in developmental events such as granule cell and PC migration, Bergmann glia maturation and PF elongation needs to be experimentally tested. The presence of many other PregS targets within the developing cerebellar cortex such as, NMDA, σ1, glycine, AMPA, and $GABA_A$ receptors, and voltage-gated Ca^{2+} channels, indicate that PregS is an important modulator of its development. For instance, activation of NMDA receptors has been shown to be essential for the migration of granule cells (Mancini and Atchison, 2007). Also, electron

microscopy studies have shown that migrating granule cells are in close association with PC dendrites and Bergmann glia (Yamada et al., 2000); thus, PregS released from PC dendrites could regulate the migration of granule cells. Alternatively, PregS-mediated activation of TRPM3 channels on the Bergmann glia could modulate granule cell migration.

Although numerous studies have provided evidence for the biological relevance of PregS, the endogenous synthesis of this neurosteroid is controversial. The reason for this controversy is that direct analytical methods have failed to detect PregS in rat and mouse brain. Nonetheless, the presence of 3β-hydroxysteroid sulfates in human plasma and brain, including the cerebellum, has been confirmed. It is noteworthy, however, that hydroxysteroid sulfotransferases have not been detected in human (reviewed in (Schumacher et al., 2008)). A number of studies have reported elevated levels of this steroid in rat and mouse brain and plasma based on indirect methods of analysis, such as radioimmunoassays and tandem gas chromatography and mass spectrometry. These methods require prior solvolyzis/hydrolyzis of PregS, and subsequently, the released free pregnenolone is measured (reviewed in (Schumacher et al., 2008)). A recent study showed that cholesterol appears to be the main precursor of pregnenolone detected by tandem gas chromatography and mass spectrometry analysis of the rat brain. This study suggested that during sample treatment, pregnenolone forms from cholesterol autoxidation (Liere et al., 2009).

It has been suggested that PregS has not been detected with direct analysis methods, despite the efforts for improving sample purification and fractionation, because elevated levels of PregS may only be present within limited regions or compartments

such as the synaptic cleft. Thus, high local levels of PregS could be diluted in large tissue samples (Schumacher et al., 2008).

Although PregS *per se* has not been detected in the rodent brain, there is compelling evidence of its existence. First, the enzymes involved in its synthesis are present in the brain. Pregnenolone is sulfonated by cytosolic sulfotransferases (Sult) that catalyze the transfer of a sulfonate group from the universal sulfonate donor, 3'-phosphoadenosine-5'-phosphosulfate, to the hydroxyl group on ring A. Sult2b1 efficiently sulfonates pregnenenolone and, of their two isoforms, Sult2b1a seems to be specific to pregnenolone, whereas Sult2b1b sulfonates dehydroepiandrosterone, cholesterol and a number of oxysterols (reviewed in (Schumacher et al., 2008)). The mRNA for both Sult2b1 isoforms has been detected in whole mouse embryos as early as E8.5 (Shimizu et al., 2003). The sulfatase that catalyzes the hydrolysis of steroid sulfates has been shown to be present in mouse brain throughout postnatal development. In the cerebellum, this sulfatase was present from E18.5 to P9 (Compagnone et al., 1997). Second, the production of PregS in the brain has been inferred from a number of studies using pharmacological approaches to increase the levels of endogenous PregS using sulfatase inhibitors. For instance, administration of the sulfatase inhibitor, DU-14, enhanced learning and memory in rats and mimicked the PregS-induced enhancement of paired-pulse facilitation within the hippocampus (reviewed in (Schumacher et al., 2008)). In the immature hippocampus, alcohol was shown to increase the efficacy of glutamatergic synapses. This effect was mimicked by application of DU-14 and prevented by the antibody scavenger that binds pregnenolone-related steroids, suggesting that this alcohol effect is mediated by the production of PregS-like neurosteroids (Mameli

and Valenzuela, 2006). In addition, the enhancement of glutamatergic transmission in the neonatal hippocampus, induced by exogenous application of PregS, was mimicked by postsynaptic depolarization and this was blocked in the presence of the antibody that binds to pregnenolone-related steroids. This finding suggests the retrograde release of a PregS-like neurosteroid at developing hippocampal synapses (Mameli et al., 2005). Lastly, the DU-14-mediated enhancement of glutamatergic transmission described in chapter 5, suggests that the degradation of sulfated steroids can be prevented in PCs in organotypic cultures and this mimicked the enhancing effect of PregS on glutamatergic transmission at PCs.

Studies using acute slice preparations provided essential information about the main characteristics of the effects of PregS in neurotransmission at developing PCs. However, these preparations only provide information on the effect of PregS in the partially intact cerebellar cortex circuitry. During the preparation of parasagittal slices the external inputs to the cerebellar cortex, the deep cerebellar nuclei and inferior olive, are cut. Thus, it is not possible to evaluate potential effects of PregS on these inputs and how those get integrated in the context of the whole circuit. Moreover, the short-term viability of the acute slices does not allow the study of long term effects of PregS on PC neurotransmission.

The functional consequences of the PregS-induced increase in glutamate release reported in this dissertation will be tested using the hindbrain organotypic culture system implemented in Aim 3. In these cultures, the endogenous levels of PregS will be reduced in PCs by downregulation of the steroid cytosolic sulfotransferase SULT2b1 gene (reviewed in (Schumacher et al., 2008)). To this end, we will use the RNA interference

and biolistic transfection methods. Specifically, we will use a short hairpin RNA plasmid for rat Sult2b1 under the control of the U1 promoter and co-expressing the green fluorescence protein (GFP) gene, which will allow for the identification of transfected cells. After amplification in bacteria, the plasmid for Sult2b1, or its negative control, will be biolistically transfected into the explants. During this procedure, the explants will be bombarded with high velocity gold particles coated with the plasmid (bullets). Cells where the bullet comes to rest in the nucleus will be transfected. This method allows for the transfection of a sparse number of cells (Woods and Zito, 2008), such that we will be able to examine individual PCs in isolation. Non-transfected PCs and PCs transfected with the negative control plasmid will be used as controls. The timeline of redundant CF synapse elimination, the achievement of monoinnervation, the developmental increase in the amplitude of CF and PF responses and cell size will be compared between controls and PCs transfected with the short hairpin RNA plasmid for Sult2b1.

The retrograde release of PregS also needs to be assessed. Studies performed in the hippocampus showed that a rabbit anti-PregS IgG blocks the depolarization-induced increase of mEPSC frequency. A more specific approach for future studies in the immature PCs could be used. Once it has been established that postsynaptic depolarization mimics the effect of PregS on glutamate release, the involvement of PregS can be tested in the hindbrain culture system. Thus, in PCs, where Sult2b1a expression has been impaired, depolarization-induced enhancement of glutamate release should also be impaired.

Although knocking down Sult2b1 in specific PCs are necessary to validate the synthesis of PregS-like neurosteroids in PCs and can provide important information about

the role of endogenous PregS on the development of synapses onto PCs; given that TRPM3 was found to be expressed in all layers of the immature cerebellar cortex, the results could be difficult to interpret. For instance, if the release of PregS from PCs plays a role in granule cell migration, this would result in reduced or abnormal PF-to-PC synapse formation, which is known to result in the persistence of multiple CF inputs (reviewed in (Bosman and Konnerth, 2009)). Therefore, in order to study the effect of PregS on CF-to-PC development in isolation, the expression of TRPM3 on inferior olivary neurons could be manipulated. Knock out of TRPM3 channels in inferior olivary neurons with a RNA interference plasmid coupled with expression of the light-activated ion channel rhodopsin gene could be used. This would allow us to selectively stimulate CFs that lack TRPM3.

Given that the development of the cerebellar cortex in hindbrain cultures resembles *in vivo* development (Chedotal et al., 1997, Letellier et al., 2009), manipulations of PregS and TRPM3 expression in hindbrain explants are suitable for elucidating the physiological roles of endogenous PregS and TRPM3 channels in the development of the cerebellar cortex. Nonetheless, *in vivo* studies would be needed for their validation and to study the behavioral outputs of these manipulations. Downregulation of PregS synthesis in developing PCs could be achieved by specifically downregulating Sult2b1 using a promoter specific to the PC, such as the gene encoding the Purkinje specific protein Pcp2 (Iscru et al., 2009). Using this approach, functional and morphological studies can be performed. The behavioral test commonly used to evaluate cerebellar circuitry network function is the conditioning of the eye blink reflex paradigm, which consists in the temporal pairing of a conditioned stimulus, a tone, with an

unconditioned stimulus, an air puff. The integration of conditioned and unconditioned stimuli has been shown to be dependent on activation of the mossy fiber to granule cell pathway and the CF-to-PC pathway, respectively (reviewed in (Bracha et al., 2009)). Therefore, it would be interesting to study if developmental PregS downregulation would affect the outcome of this behavioral test. It would also be interesting to test whether PregS downregulation throughout the cerebellum would affect PC development differently than localized downregulation in PCs.

A recent study postulated TRPM3 channels as heat nociceptors (Vriens et al., 2011). In this study, TRPM3 knockout mice were used. Surprisingly, it was reported that these animals displayed no obvious deficits in fertility, gross anatomy, body weight, core body temperature, locomotion, exploratory behaviors, and general somatosensation, excluding the nocifensive responses to TRPM3 activation (Vriens et al., 2011). In contrast, mutant mice where PC and granule cell survival, migration, dendritic development, and synapse formation is impaired, show obvious deficits in cerebellar-dependent functions (reviwed in (Sotelo and Dusart, 2009)). Therefore, it would be of interest to investigate the morphological state of the cerebellar cortex in these animals as well as further analyzing cerebellar function.

Lastly, the findings presented in this dissertation significantly contribute to our understanding of cerebellar cortex development. It constitutes the first demonstration of neurosteroid-mediated modulation of neurotransmission in this brain area. The presence of the effect of PregS in such an active period of synapse formation, dendritic remodeling, and cell migration, and the potency of such effect, postulates this neurosteroid as a key modulator of PC development. The PregS involvement in PC

synapse development could help elucidate the mechanisms underlying cerebellar malfunctions leading to ataxia and movement disorders in Niemann-Pick type C disease, a developmental neurodegenerative disease caused by defective intracellular cholesterol trafficking, which disrupts neurosteroidogenesis, and that is characterized by PC loss, among other histopathological lesions (Griffin et al., 2004). Since PCs are highly sensitive to developmental ethanol exposure (Servais et al., 2007), chronic prenatal ethanol exposure increases the levels of PregS-like neurosteroids in the developing brain, and ethanol increases the efficacy of immature hippocampal synapses in a PregS-like neurosteroid-dependent manner (Caldeira et al., 2004, Mameli et al., 2005, Mameli and Valenzuela, 2006, Valenzuela et al., 2008), the findings presented here could help elucidate the pathophysiology of the cerebellar alterations that characterize fetal alcohol spectrum disorders.

The involvement of TRPM3 in the PregS-induced increase in glutamatergic transmission is the first indication of the role of TRPM3 channels in brain neurotransmission. Moreover, the abundant expression of TRPM3 in the developing cerebellar cortex opens up a new area of research to elucidate the roles of these channels in cerebellar and brain development, broadening our understanding of the processes involved in neuronal circuit refinement in the central nervous system.

7. Appendix

7.1 Activity-independent NMDA receptor-mediated increase in $[Ca^{2+}]_i$ in presynaptic terminals of the neonatal CA1 hippocampal area

Purpose of the study

Studies from our laboratory suggested that PregS increases glutamate release in the hippocampal CA1 region by increasing presynaptic $[Ca^{2+}]$ via activation of NR2D-containing NMDA receptors (Mameli et al., 2005). We performed Ca^{2+} imaging measurements in presynaptic Schaffer collateral terminals to study the NMDA receptor activation-mediated increases in presynaptic $[Ca^{2+}]$ in the absence of activity.

Method

In hippocampal slices from P4-5 Sprague-Dawley rats, the Ca^{2+} indicator FURA2-AM (10 μM) was injected into Schaffer collaterals with a tip-broken glass pipette. Then, the indicator was allowed to reach the presynaptic terminals for 1 hour and fluorometric measurements were performed in a distal region from were the dye had been injected (Figure 7.1A). NMDA was used to activate the receptors. All experiments were performed in the presence of TTX (0.5 μM)

Results

We found that bath application of NMDA increased the presynaptic intracellular Ca^{2+}, as expected. Application of the NMDA receptor blocker DL-APV in the presence

of NMDA antagonized this increase. Interestingly, ifenprodil, a blocker for NR2B-containing NMDA receptors significantly decreased the effect of NMDA ($p< 0.0001$). As a control for the terminals' viability, KCl was applied at the end of every experiment. However, in 9 out of 10 experiments, NMDA elicited a significant increase in intracellular Ca ($p<0.05$ by Wilcoxon Signed Rank Test vs 100).

Conclusion

These results indicate that activation of NMDA receptors in neonatal Schaffer collateral terminals indeed increases presynaptic $[Ca^{2+}]_i$. However, NR2B-containing receptors play an important role in this increase. Nonetheless, the significant increase seen in the presence of ifenprodil could account for activation of PregS-sensitive NR2D-containing NMDA receptors. Future experiments would also need to evaluate the involvement of voltage-gated Ca^{2+} that could activate downstream of NMDA receptors.

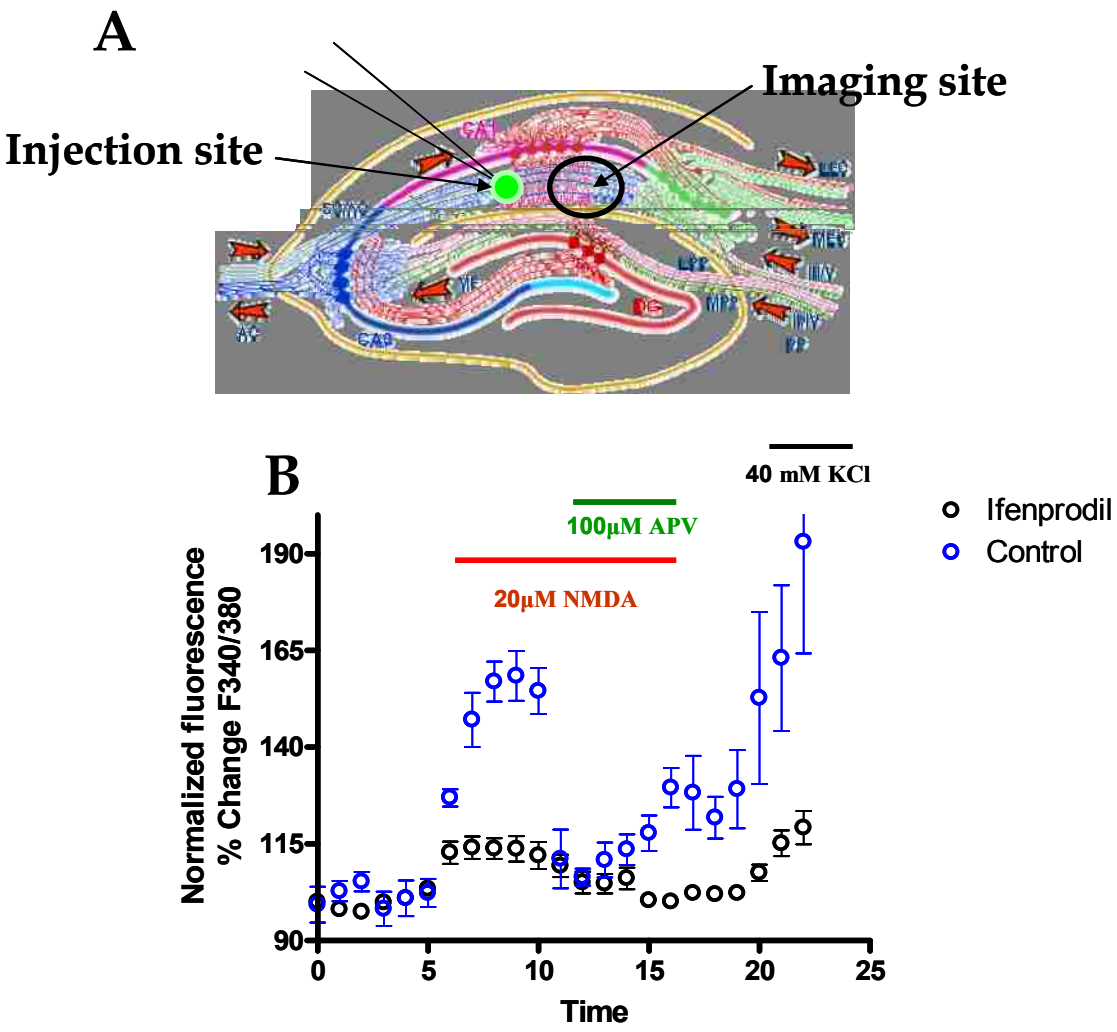

Figure 7.1. (A) Schematic representation of experimental paradigm. (B) NMDA-induced increase in $[Ca^{2+}]_i$ in the presence (n=10) and the absence (n=7) of ifenprodil. Time is given in minutes.

7.2 G-1-mediated activation of the estrogen-sensitive GPR30 receptor does not modulate giant depolarizing potential frequency and $[Ca^{2+}]_i$ in hippocampal CA3 pyramidal cells in P7 rats.

Purpose of the study

Activation of the estrogen-sensitive seven transmembrane G protein-coupled receptor GPR30 increases $[Ca^{2+}]_i$ mobilization (Revankar et al., 2005). Neonatal hippocampal CA3 pyramidal cells display giant depolarizing potentials (GDPs), which are the result of neuronal network activity and are thought to be involved in the refinement of developing hippocampal synapses (Ben-Ari, 2001). GDPs trigger transient increases in $[Ca^{2+}]_i$ (reviewed in (Galindo et al., 2005)). GPR30 expression has been detected in the neonatal hippocampus (Brailoiu et al., 2007). The specific ligand for GPR30, G-1, has been shown to induce similar increases in $[Ca^{2+}]_i$ to those induced by estrogen. Therefore, we investigated if activation of estrogen-sensitive GPR30 receptors, can modulate GDPs in neonatal CA3 pyramidal cells and increase $[Ca^{2+}]_i$ in the CA3 region.

Methods

GDPs were electrophysiologically recorded as previously described (Galindo et al., 2005). For calcium imaging experiments, slices were pre-incubated with FURA2-AM (10 μM) for 10 minutes and subsequently used for fluorometric calcium imaging. The Ca^{2+} signal was obtained from IR-DIC-identified CA3 pyramidal and interneuron cell bodies by first subtracting the background fluorescence from the 350 and 380 nm images, and subsequently performing a ratiometric analysis of these exposures. G-1 (1 μM) or the

vehicle DMSO was bath applied and GPDs frequency and Ca^{2+} signals were compared to baseline.

Results

G-1 application did not affect the frequency of GDPs. Also, G-1 did not significantly increase $[Ca^{2+}]_i$ in the neonatal hippocampal CA1 region (Fig 8.2).

Conclusion

These results indicate that GPR30 activation does not modulate neonatal neuronal network activity in the CA3 hippocampal region. Furthermore, these results suggest that the non-genomic, rapid, estrogen-induced increases in intracellular calcium via GPR30 receptors do not play a role in the modulation of GDPs.

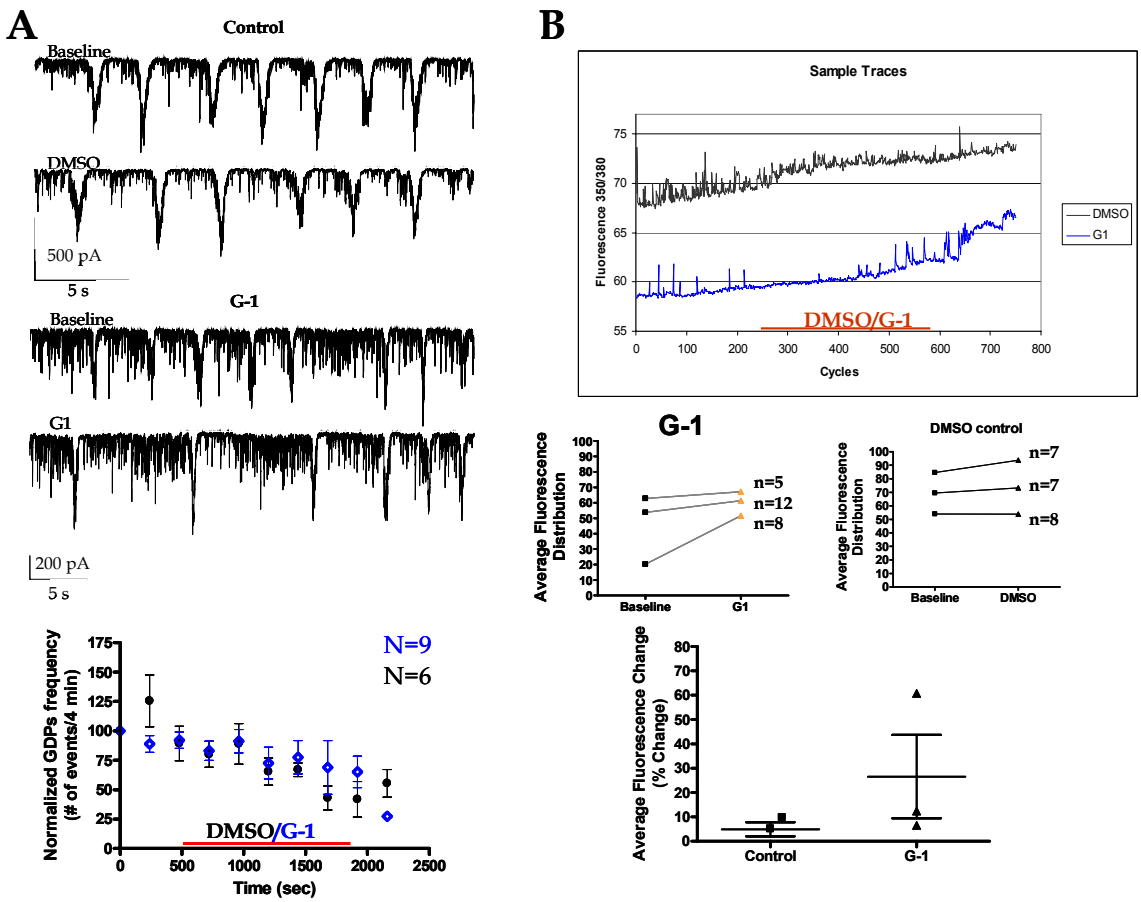

Figure 7.2. **(A)** Recordings of GDPs in the presence of DMSO or G-1. Bottom panel shows time course graph of GDPs normalized frequency **(B)** Ratiometric calcium measures in the CA3 region. Unpaired t-test showed no significant difference between DMSO and G-1 –induced Ca^{2+} fluorescent changes. Each group represents 1 recording and each recording includes 5 to 12 cells.

7.3 PregS reversibly increases the frequency and decreases the amplitude of mPSCs in PCs in an age dependent manner

Purpose of the experiment

To study the effects of PregS on combined glutamatergic, and GABAergic-mPSCs.

Method

Electrophysiological recordings were made in the whole-cell patch-clamp configuration from cerebellar PCs at P4 to P12. The cells were divided into three groups according to their developmental stage P4-5, P7-8 and P9-12. mPSCs were recorded at a holding potential of -70 mV using an internal solution containing a high Cl^- concentration. Under these conditions, both cationic currents mediated by AMPA receptors as well as Cl^- currents mediated by $GABA_A$ receptors were inward.

Results

A five-minute bath application of PregS (25 μM) reversibly increased the frequency of mPSCs at P4-5 and P7-8 but not at P9-12 (Fig. 7.3). This effect was most robust at P4-5, declined, but was still significant, at P7-8, and was not statistically significant at P9-12. PregS started to increase mPSCs frequency within 1.5 minutes after bath application began. The frequency went back to baseline values after approximately 8 minutes of washout (Fig 7.3). In addition to the increase in frequency, PregS also induced a decrease in the amplitude and half-width of mPSCs at P4-5 and P7-8 but not at P9-12.

Conclusions

These experiments reflected the PregS-mediated increases in both glutamate and GABAergic transmission. The PregS effect on the amplitude of these events reflects the inhibition of $GABA_A$ receptors by PregS, as it was later confirmed that this effect on the amplitude is exclusive to $GABA_A$-mPSCs (Zamudio-Bulcock and Valenzuela., 2011). However, the decrease in half-width could not be seen in isolated $GABA_A$ currents and therefore it remains unexplained. The age dependency of the PregS-induced increase in mPSCs could be due to an age induced reduction in the PregS potency for AMPA-mPSC modulation. Alternatively, a decrease in the ability to detect AMPA-mPSCs under these recording conditions might explain such age dependency, as, paradoxically, isolated AMPA-mPSCs did not show an age-dependent increase in frequency or amplitude (appendix 6), suggesting that there could be an age-dependent decrease in our ability to detect these events under these recording conditions. This could be due to increased complexity of dendritic arborizations with the consequent movement of synapses to more distal locations, away for the recording electrode.

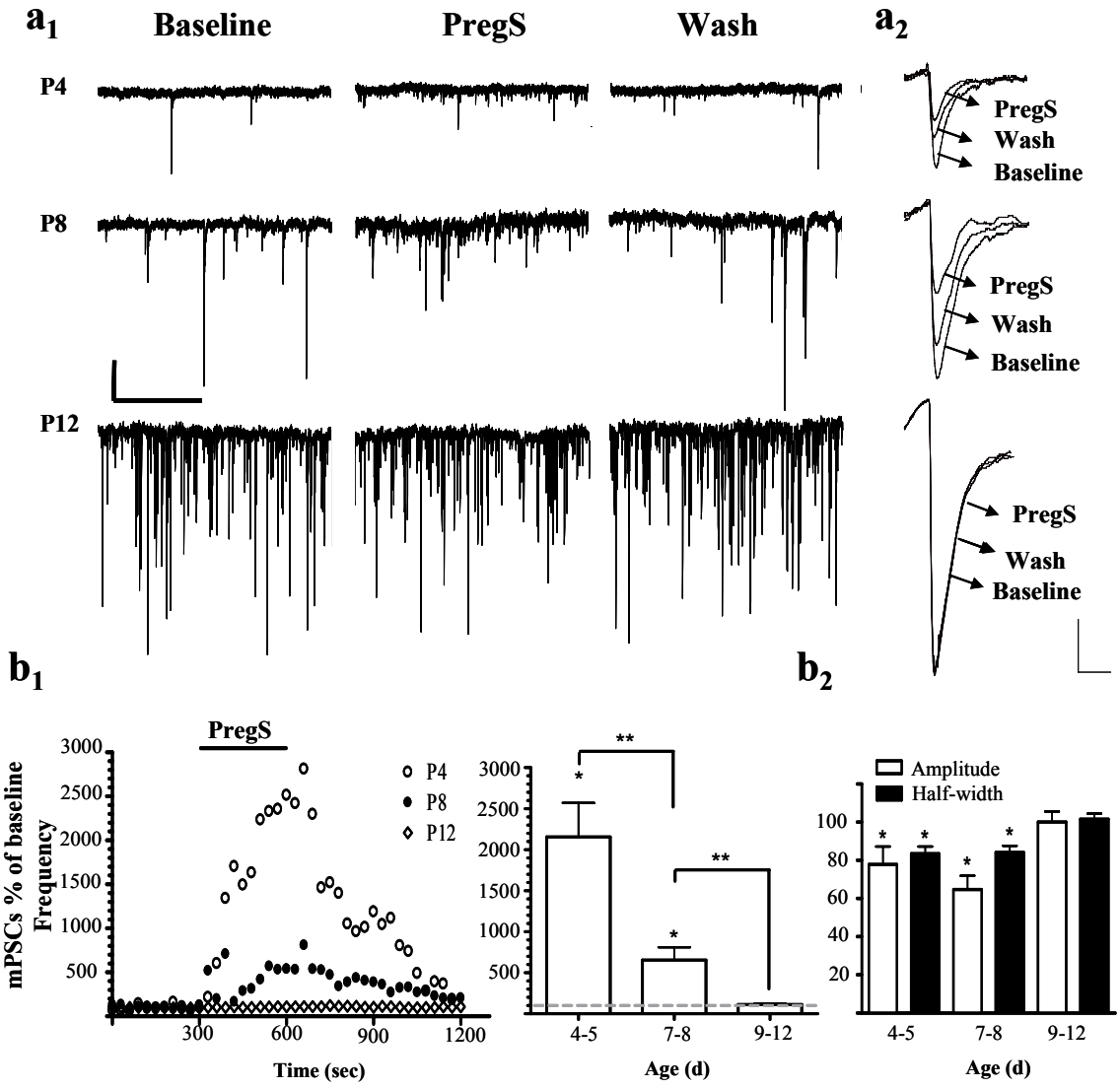

Figure 7.3. Pregs reversibly increases the frequency and decreases the amplitude and half-width of mPSCs in PCs in an age-dependent manner. 25 μM Pregs increases the frequency (a1) and decreases the amplitude (a2) of mPSCs in PCs at P4 and P8 but not at P12. Calibration: a1: 20 pA, 1 sec; a2:.10.2 pA, 13msec. b1, Left panel, time course of the effect of 5-min application of 25 μM Pregs on mPSC frequency in individual PCs at P4, P8 and P12. Right panel, PregS increases mPSC frequency at P4-5 (n=6) and P7-8 (n=7) but not at P9-12 (n=8). b2, PregS significantly decreases mPSC amplitude and half-width of mIPSCs at P4-5 and P7-8 but not at P12. (* $p<0.05$, ** $p<0.01$).

7.4 NMDA receptor activation increases mPSC frequency in an age dependent manner in developing PCs

Purpose of the experiment

Activation of presynaptic NMDARs enhances GABA release onto developing cerebellar PCs (Glitsch and Marty, 1999, Duguid and Smart, 2004, Glitsch, 2008). In the immature hippocampus, PregS enhances glutamatergic transmission via modulation of presynaptic NMDA receptors (Mameli et al., 2005). Therefore, we tested if NMDA bath application could mimic the age dependency of the effect of PregS on the frequency of mPSCs in P4-12 PCs.

Method

mPSCs were recorded using a CsCl internal solution in the presence of TTX (0.5 μM) at a holding potential of -70 mV; 100 μM DL-APV was used to block NMDA receptors. Recordings were grouped according to age in three groups P4-5, P7-8 and P9-12.

Results

NMDA bath application induced an increase in mPSCs. Similarly to the Pregs effect this effect was also age-dependent. NMDA did not have an effect on the amplitude of these events.

Conclusion

This results are in agreement with reports showing that NMDA (15 μM) bath application significantly enhances the frequency of mIPSCs by about 140% in rats aged P11-14 (Glitsch and Marty, 1999). We show that in recordings of combined $GABA_A$- and AMPA-mediated mPSCs from younger PCs (P4-5), the same concentration of NMDA can induce increases higher than 1,000 %. This suggests that in neonate PCs, NMDA receptors act as potent modulators of synaptic neurotransmission, although based on this result we cannot discern whether glutamatergic and/or GABAergic synapses are affected by NMDAR activation. It has also been shown that at this age CF stimulation increases $[Ca^{2+}]_i$, thereby increasing GABA release onto PC via modulation of NMDARs. Therefore, if a similar glutamate-to-GABA synapse modulation can occur in activity-independent neurotransmission, it is possible that in the absence of glutamate receptor blockers glutamatergic synapses contribute to the NMDA-induced increase in GABA release onto PC.

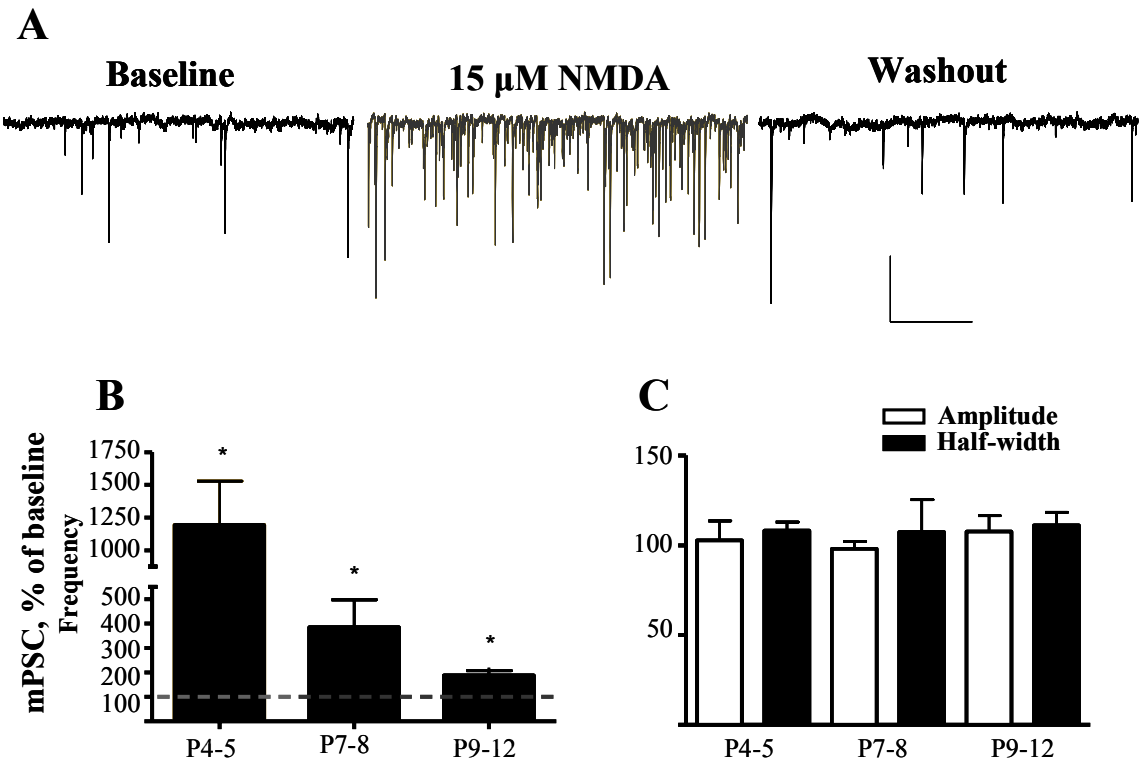

Figure 7.4. A. Sample traces illustrating the effect NMDA application on mPSCs . **B.** NMDA-induced increase in mPSCs frequency at P4-5 (1,193 ± 335 % of baseline, n=4), P7-8 (384.7 ±112.3 % of baseline, n=4) and P9-12 (187.6 ± 20.08 % of baseline, n=5). Note age dependency of the increase. **C.** NMDA did not affect mPSC amplitude or half-width .

7.5 $GABA_A$ receptor- and AMPA receptor-mediated events differentially contribute to mPSCs in PCs in an age-dependent manner

Purpose of the experiment

Under the recording conditions described in appendix 7.3, a mixture of action potential-independent $GABA_A$ receptor as well as AMPA receptor-mediated events were studied. In order to determine whether the effect of PregS was specific to GABAergic or to glutamatergic synapses, we first determined the percent contribution of $GABA_A$ receptor- and AMPA receptor-mediated events at the different ages studied.

Method

We recorded a 5 minute mPSC baseline (as previously described in appendix 7.3) followed by a 5 min exposure to 10 μM gabazine to block $GABA_A$ receptor-mediated events ($GABA_A$-mPSCs) in P4-5, P7-8 and P9-12 PCs. Subsequently, AMPA receptor-mediated events were blocked by adding 50 μM GYKI-53655 to the solution. At all ages, when GYKI-53655 was added to the solution in presence of gabazine all events disappeared, suggesting that at these ages all miniature events recorded resulted exclusively from activation of $GABA_A$ and/or AMPA receptors on PCs. Therefore, application of gabazine resulted in isolation of AMPA receptor-mediated events (AMPA-mEPSCs).

Results

At P4-5, $GABA_A$-mPSCs and AMPA-mEPSCs contribute equally to the frequency of mPSCs. However, as the cells grow, AMPA-mEPSC contribution decreases. Also, the percent amplitude of AMPA-mEPSCs with respect to the total amplitude of mPSCs decreases with age. This result could be explained by the developmental increase in $GABA_A$-mPSC amplitude. In our recording conditions, the detection of Cl^- ions currents through $GABA_A$ receptors is favored. Additionally, it is possible that the developmental increase in the amplitude of AMPA-mEPSC is not well detected under our experimental conditions (see appendix 6).

Conclusion

When simultaneously recording $GABA_A$- and AMPA- mediated mPSCs in developing PCs using a high Cl- internal solution, and holding the membrane potential at -70 mV, the majority of the events are mediated by $GABA_A$ channels at P7-12. However, at younger ages (P4-5) half of the mPSCs are mediated by AMPA channels. This could be due to the small size of PCs at this age and to the somatic location of glutamatergic synapses at this age, but as the cell grows and some synapses are translocated to the newly formed dendritic tree, filtering of AMPA mediated events might occur.

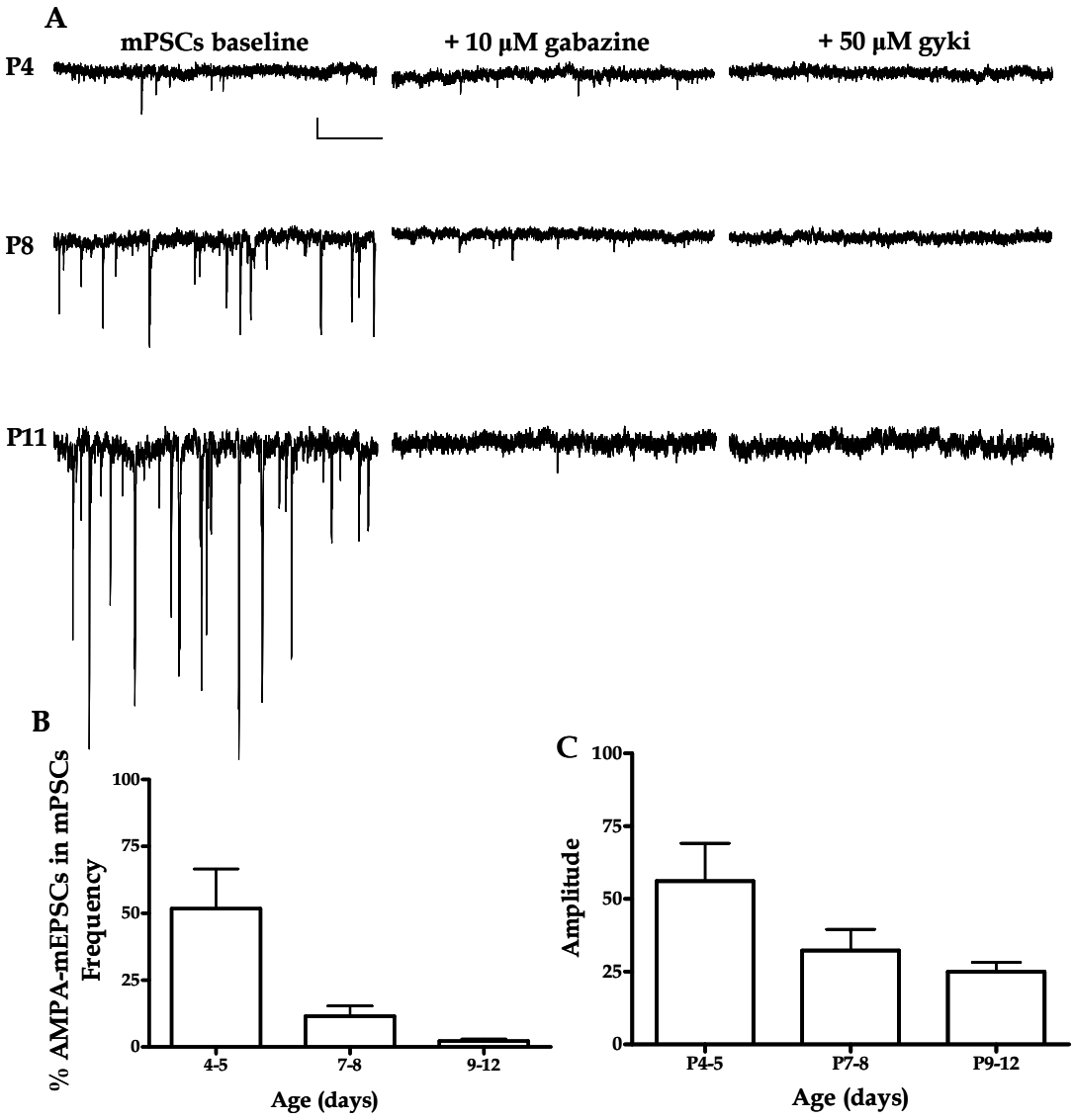

Figure 7.5. $GABA_A$-mPSCs and AMPA-mEPSCs differentially contribute to mPSCs in an age-dependent manner. **A.** sample traces of recordings from PCs at P4, 8 and 11 upon consecutive 5 min application of gabazine and GYKI-53655 (gyki), calibration: 20 pA, 500 ms. **B.** The percent contribution of AMPA-mEPSCs to mPSC frequency varies with age at P4-5 it is 51.73 ±14.91% of mPSC baseline (n=6), at P7-8 decreases to 11.52 ± 3.87% (n=4), and P9-12 it decreases even further to 2.28 ±0.75% (n=7). **C.** The percent contribution of AMPA-mEPSCs to mPSC average amplitude also decreases with age. At P4-5 56.11 ± 13.02 %, at P7-8 32.26 ± 7.27 % and at P9-12 24.94 ± 3.25 %.

7.6 The action potential-independent glutamatergic and GABAergic synaptic activity recorded in developing PCs gradually increases with respect to age.

Purpose of the experiment

We noticed that the amplitude, the frequency and the half-width of mPSCs increased with age, reflecting the synapse formation and stabilization that occurs during development. Therefore we generated a table of the average frequency and amplitude of mPSCs and isolated $GABA_A$- and AMPA- mediated mPSCs between P4 and 12.

Method

mPSCs recordings from PCs were divided into three age groups; namely, P4-5, P7-8 and P9-12. The average frequency, amplitude and half-width were calculated for each group and the table was generated. Isolated $GABA_A$-mPSCs and AMPA-mPSCs were recorded at P4-5 and P7-8.

Results

The amplitude and frequency of mPSCs increased with age. Additionally, recording of isolated AMPA-mPSCs or $GABA_A$-mPSCs revealed that while the frequency and the amplitude of $GABA_A$-mPSCs significantly increased from P4-5 to P9-12 (p <0.05 by unpaired t test); the frequency and the amplitude of AMPA-mPSCs did not significantly change with age.

Conclusions

The increases and amplitude and frequency of $GABA_A$-mPSCs reflect the synaptic development of MLI-to-PC synapses during the neonatal period. The increase in the frequency of these events is in agreement with the previously reported increase in the frequency of $GABA_A$-mPSCs at PCs at P2-12 (Kawa, 2003). However, this author found that the amplitude of these events increased up until P10, but decreased by P12 (Kawa, 2003). The lack of a developmental increase in both the frequency and the amplitude of AMPA-mPSCs is, however, unexpected. Kawa (2003), also found a developmental increase in the frequency of AMPA-mPSCs in P2 to 12 PCs. On the other hand, he found that the amplitude of these events increased from P4 to P10, but decreased back to P4 levels by P12 (Kawa, 2003). We also found a trend toward a decrease in the amplitude of these events at P9-12. The decrease in the amplitude of both $GABA_A$-mPSCs and AMPA-mPSCs could be explained by enhanced filtering in the growing dendrites of PCs. Nonetheless, we did not see such developmental decrease in the amplitude of $GABA_A$-mPSCs, and this could be explained by the increase in the driving force of Cl^- ions caused by our CsCl based internal solution. The recording conditions between our experiments and those of Kawa (2003) were different and could explain the discrepancies in the developmental changes in AMPA-mPSCs amplitude and frequency. This author used a Cs-methenasulfonate based internal solution and simultaneously recorded $GABA_A$-mPSCs and GABA-mPSCs at a holding potential of -40 mV, at which $GABA_A$-mPSCs and AMPA-mPSCs appear as outward and inward currents, respectively. At -40 mV the driving force for cations currents mediating mEPSCs is lower than under the holding potential we used, -70 mV. Consistent with this, while Kawa (2003) detected amplitudes

of AMPA-mPSCs to be around 9 pA at P4, we detected these currents to be around 25 pA at the same age. Also, under our recording conditions we found slightly higher frequencies of these events at P4-5 than those reported in this study. This would be consisted with the increased capability to detect AMPA-mPSCs at this age. Nevertheless at P7-8 and P9-12, this apparent advantage to detect AMPA-mPSC is not consistent with our inability to detect the developmental increases in the frequency and the amplitude of AMPA-mPSC. Nonetheless, the developmental decrease in the amplitude of these events, could at least in part, explain the lack of an increase in their frequency. That is, as the events became smaller, we were unable to detect them.

Frequency

	mPSCs			$GABA_A$-mPSCs			AMPA-mPSCs		
	mean	**Std. error**	**n**	**mean**	**Std. error**	**n**	**mean**	**Std. error**	**n**
P4-5	0.4316	0.1653	6	0.7954	0.2364	5	0.7916	0.4903	5
P7-8	2.343	0.7102	7	2.947	1.726	5	0.6032	0.3052	5
P9-12	12.94	1.984	8	8.982	1.917	5	0.8133	0.1227	4

Amplitude

	mPSCs			$GABA_A$-mPSCs			AMPA-mPSCs		
	mean	**Std. error**	**n**	**mean**	**Std. error**	**n**	**mean**	**Std. error**	**n**
P4-5	26.92	1.851	6	70.16	14	5	25.75	2.392	5
P7-8	56.95	13.61	7	90.97	11.89	5	23.96	3.152	5
P9-12	108.2	13.75	8	136.5	21.62	5	19.98	2.367	4

Fig 7.6. Postnatal development of miniature synaptic currents frequency and amplitude in PCs.

7.7 The magnitude of the PregS-induced increase in mEPSC frequency in P9-12 PCs is not different at 10, 20 and 50 μM.

Purpose of the experiment

To test the concentration dependency of the PregS-induced increase in the frequency of mEPSCs in immature PCs.

Method

Recordings of mEPSC were performed using a Cs-Gluconate based internal solution and in the presence of the $GABA_A$ receptor blocker, bicuculline (20 μM) and TTX (0.5 μM) at a holding potential of -65 mV. PregS was bath applied at 3 different concentrations 10, 20, and 50 μM . Frequency and amplitude changes with respect to baseline were analyzed.

Results

PregS significantly and reversibly increased the frequency of mEPSCs at concentrations of 10, 20, and 50 μM. However, this effect was not significantly different between the concentrations tested (p >0.05 by Dunn's multiple comparison test). PregS did not change the amplitude of these events at any of the concentrations tested (Fig 8.7).

Conclusions

In the hippocampus, 17 μM PREGS is the lowest concentration that induces a significant increase in glutamate release (Mameli et al., 2005). Here in, we found that 10 μM PREGS induces a significant increase in the frequency of mEPSCs, indicating that PregS potency is higher in the cerebellum than in the hippocampus. In separate experiments in P4-10 PCs, we found that an even lower concentration of PregS (5 μM) significantly enhanced mEPSC frequency (401.1±114.7% of baseline), but this effect was smaller than the effect of 25 μM PregS (Zamudio-Bulcock and Valenzuela., 2011). Together these results indicate that at a concentration higher than 5 μM, but lower than 10 μM, the effect of PregS on glutamatergic transmission in the developing cerebellar cortex reaches a plateau.

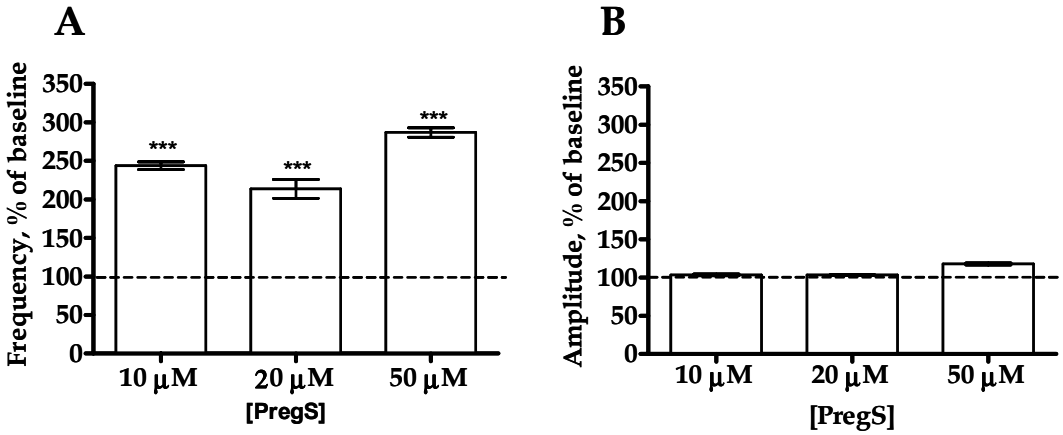

Figure 7.7. A. PregS at concentrations of 10 μM (n=3), 20 μM (n=6) and 50 μM (n=4) increases mEPSC frequency (***p<0.05 by one sample t-test vs. 100). **B.** PregS does not significantly change the amplitude of mEPSCs (p>0.05 by Wilcoxon Signed Rank Test).

7.8 PregS also increases mEPSC frequency in juvenile P18-27 cerebellar PCs.

Purpose of the experiment

To test whether PregS modulates glutamatergic transmission in juvenile PCs.

Method

Recordings of mEPSC were performed using a Cs-Gluconate based internal solution and in the presence of the $GABA_A$ receptor blocker, bicuculline (20 μM) and TTX (0.5 μM) at a holding potential of -65 mV. PregS (20 μM) was bath applied for 5 minutes. Frequency changes with respect to baseline were analyzed.

Results

PregS significantly and reversibly increased the frequency of mEPSCs in juvenile PCs (223.1 ± 24.18% of baseline).

Conclusions

These data suggest that PregS modulates glutamatergic transmission onto PCs beyond the neonatal period. However, this effect is considerably smaller than the effect seen in the neonatal period, suggesting that PregS role on glutamate release is strongest during synapse formation and stabilization. Although this is an important piece of information and it would be interesting to elucidate the physiological role of PregS in the juvenile PCs, comparisons between the results obtained in the neonate vs the juvenile

groups should be made with caution. These data were collected under different experimental conditions i.e. different internal solution, slightly lower PregS concentration, and using a different $GABA_A$ receptor blocker. Therefore, these results need to be validated using the same experimental conditions employed in experiments with neonatal PCs.

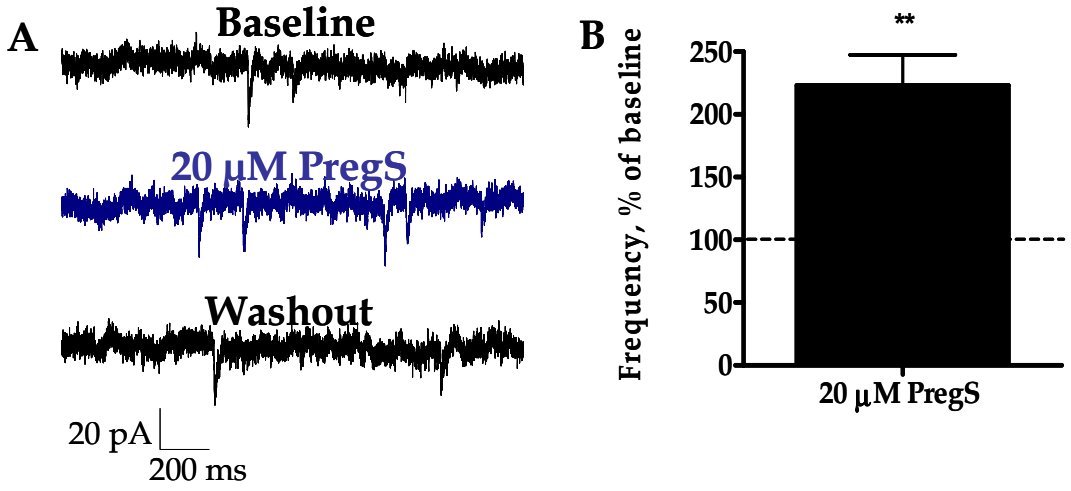

Figure 7.8. A. Sample traces of mEPSC recordings from a P24 cerebellar Purkinje neuron; 20 µM PregS transiently increased the number of events. **B.** PregS significantly increases the frequency of mEPSCs in PCs at P18-27 (**$p < 0.05$ by t-test vs. baseline, n=4).

7.9 The Sigma 1 Receptor agonist PRE-084 does affect glutamate release onto Purkinje cells at P9 to P12.

Purpose of the experiment

In cultured hippocampal neurons, PregS increases glutamate release transiently via modulation of σ1 receptors (Meyer et al., 2002). Therefore, we investigated if activation of σ1 receptors could mimic the PregS-induced increase of mEPSC frequency in P9-12 cerebellar PCs.

Methods

Recordings of mEPSC were performed using a Cs-Gluconate based internal solution and in the presence of the $GABA_A$ receptor blocker, bicuculline (20 μM), and TTX (0.5 μM) at a holding potential of -65 mV. The σ1 receptor agonist 2-(4-Morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride (PRE-084 hydrochloride), at a concentration of 1 μM, was bath applied. Frequency and amplitude changes with respect to baseline were analyzed.

Results

PRE-084 significantly changed neither the frequency nor the amplitude of mEPSCs in P9-12 PCs.

Conclusions

These data suggest that σ1 receptors do not play a role in glutamatergic transmission onto developing PCs and further support the data showing that these receptors are not involved in the PregS effect on glutamate release onto neonatal PCs (Figure 2. (Zamudio-Bulcock and Valenzuela, 2011))

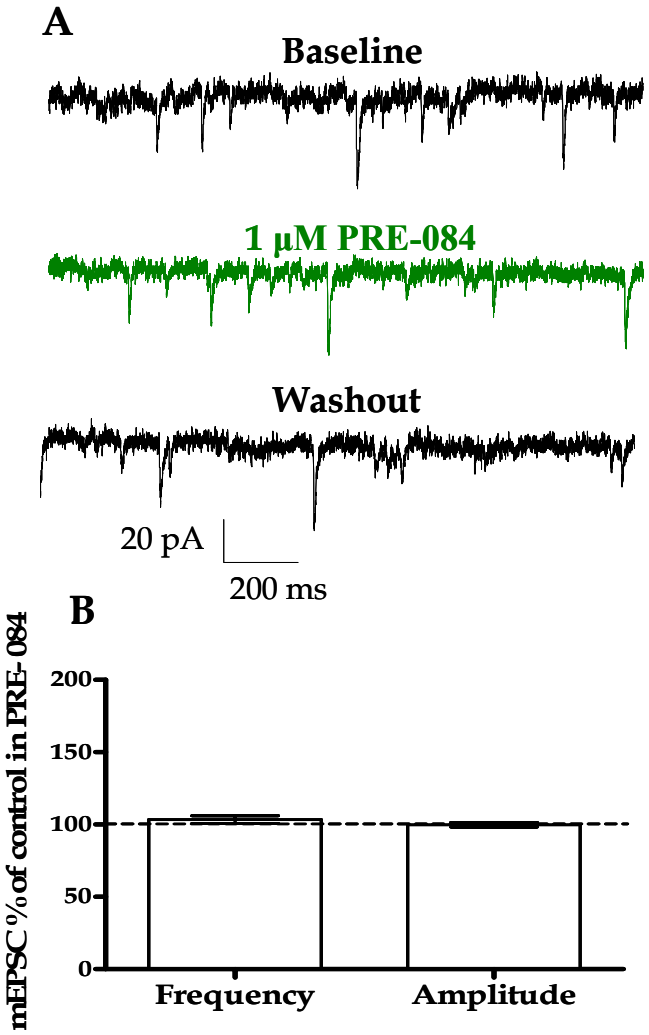

Figure 7.9. σ1 receptor agonism did not mimic the PREGS induced increase in mEPSCs frequency **A**. Sample traces of mEPSCs in a P9 PC. **B**. The amplitude and the frequency of mEPSCs remained unchanged upon PRE-084 application (p>0.05 by Wilcoxon Signed Rank Test vs 100).

7.10 The PregS-induced increase in GABA release onto neonatal PCs could depend on NMDA and/or glycine receptor activation

Purpose of the experiment

Activation of NMDA receptors has been shown to increase GABA release onto developing PCs (Glitsch and Marty, 1999, Duguid and Smart, 2004, Glitsch, 2008). Activation of glycine receptors has also been shown to facilitate both glutamate and GABAergic transmission onto developing PCs (Kawa, 2003). Therefore, we investigated the involvement of these receptors in the PregS-induced increase of $GABA_A$-mPSC frequency in neonatal P4-5 PCs.

Methods

We first investigated the effect of NMDA and glycine receptor blockade on the effect of PregS on combined AMPA and $GABA_A$ –mPSCs (mPSCs) recorded in the presence of TTX (0.5 μM). Then, isolated $GABA_A$-mPSC were recorded from P4-5 PCs in the presence of GYKI 53655 (50 μM) and TTX (0.5 μM). NMDA and glycine receptors were blocked with DL-2-Amino-5-phosphonopentanoic acid (DL-APV) (100 μM) and strychnine (1 μM), respectively. All experiments were performed using the same CsCl based internal solution as in Zamudio-Bulcock and Valenzuela (2011).

Results

In the presence of DL-APV and strychnine the PregS-induced effects on the frequency and amplitude of mPSCs, in P7-8 PCs, was significantly reduced (Figure

8.10A). Additionally, we found that at P4-5, in the presence of the NMDA and glycine receptor blockers, PregS did not significantly increase the frequency of $GABA_A$-mPSC (Figure 8.10B). The K-S test showed that in only 1 out of 5 cells PregS induced a significant decrease in the inter-event interval. Similarly, the average amplitude of these events was not significantly affected by PregS in the presence of NMDA and glycine receptor blockers. K-S test showed that in 2 out of 5 cells PregS induced a significant decrease in the amplitude of $GABA_A$-mPSC.

Conclusions

These results suggest that activation of NMDA and or glycine receptors could be involved in the effects of PregS on mPSC frequency and amplitude. Experiments on isolated AMPA-mPSC showed that NMDA and glycine receptors are not involved in the effect of PregS on glutamatergic transmission at neonatal PCs (Zamudio-Bulcock and Valenzuela, 2011). Therefore, we can deduce that the role of NMDA and glycine receptors on the effect of PregS is specific to $GABA_A$-mPSCs. PregS significantly increases the frequency and decreases the amplitude of $GABA_A$-mPSC at P4 to P8 (Zamudio-Bulcock and Valenzuela, 2011). However, when only P4-5 PCs are considered, the K-S test shows that PregS induced a significant decrease in the inter-event interval in 3 out of 5 cells. Similarly, the K-S test also showed that in only 2 out 5 cells PregS induced a significant decrease in the amplitude of these events. These data suggest that the effect of PregS on GABA release onto developing PCs is highly variable. Therefore, from the experiments presented here, it is not clear whether or not NMDA and/or glycine receptors play a role in this effect. An increase in the number of

experimental values and the inclusion of recordings at P7-8 would be necessary in order to reach conclusive results.

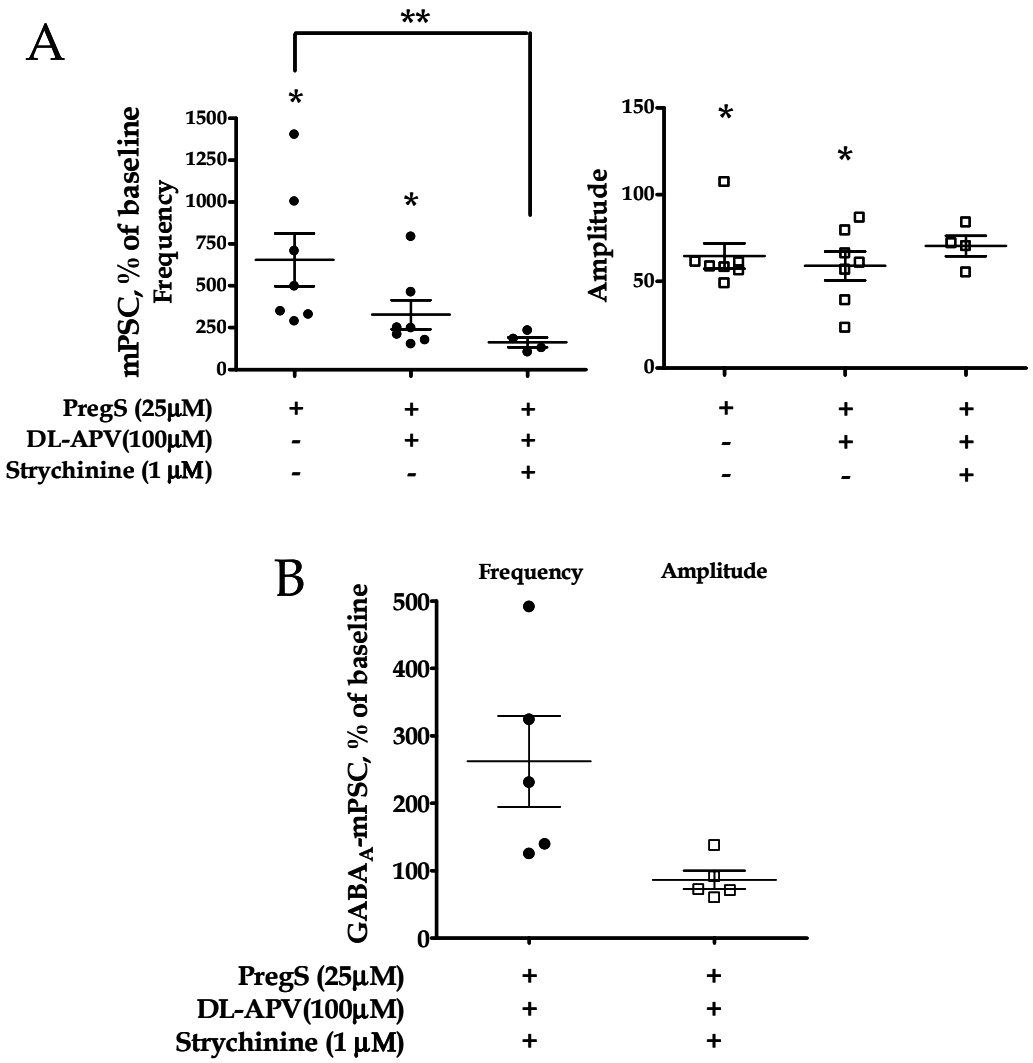

Figure 7.10. A. At P7-8, the presence of both, DL-APV and strychnine significantly reduced the PregS-induced increase in mPSC frequency (left panel, $^{**}p < 0.01$ by Dunn's Multiple Comparison Test, $^{*}p < 0.05$ by Wilcoxon Signed Rank Test vs 100). **B.** In the presence of DL-APV and strychnine, PregS does not significantly affect the frequency or the amplitude of $GABA_A$-mPSCs ($p= 0.0743$ by one sample t-test vs 100).

7.11 The P450scc inhibitor, aminogluthetamide aminoglutethimide, significantly decreases depolarization-induced potentiation of inhibition

Purpose of the experiment

PC depolarization or CF stimulation increase $[Ca^{2+}]_i$ in PC dendrites and induces a short term depression in GABA release followed by a longer and transient enhancement in GABA release onto PCs. This transient enhancement in GABA release is called depolarization-induced potentiation of inhibition (DPI) and has been ascribed to activation of NMDA receptors by retrogradely released glutamate. We tested whether neurosteroids could play a role in this phenomenon.

Methods

In these experiments, we recorded mIPSCs from P9-12 PCs using a CsCl based internal solution in the presence of TTX (0.5 μM) and NBQX (10 μM). To reduce endogenous neurosteroid production, the experimental group was pre-incubated, for 20 minutes at 34^o, with aminoglutethimide (AMG), a specific P450scc inhibitor. After a 5 minute baseline recording, the PC was given five depolarizing pulses from -70 mV to 0 mV; pulse duration was 1 s and the inter-event interval was 10 ms.

Results

We found that blockade of neurosteroid synthesis significantly decreases DPI.

Conclusion

These results suggest that in PCs, which are highly neurosteroidogenic, induction of DPI may be modulated by neurosteroids.

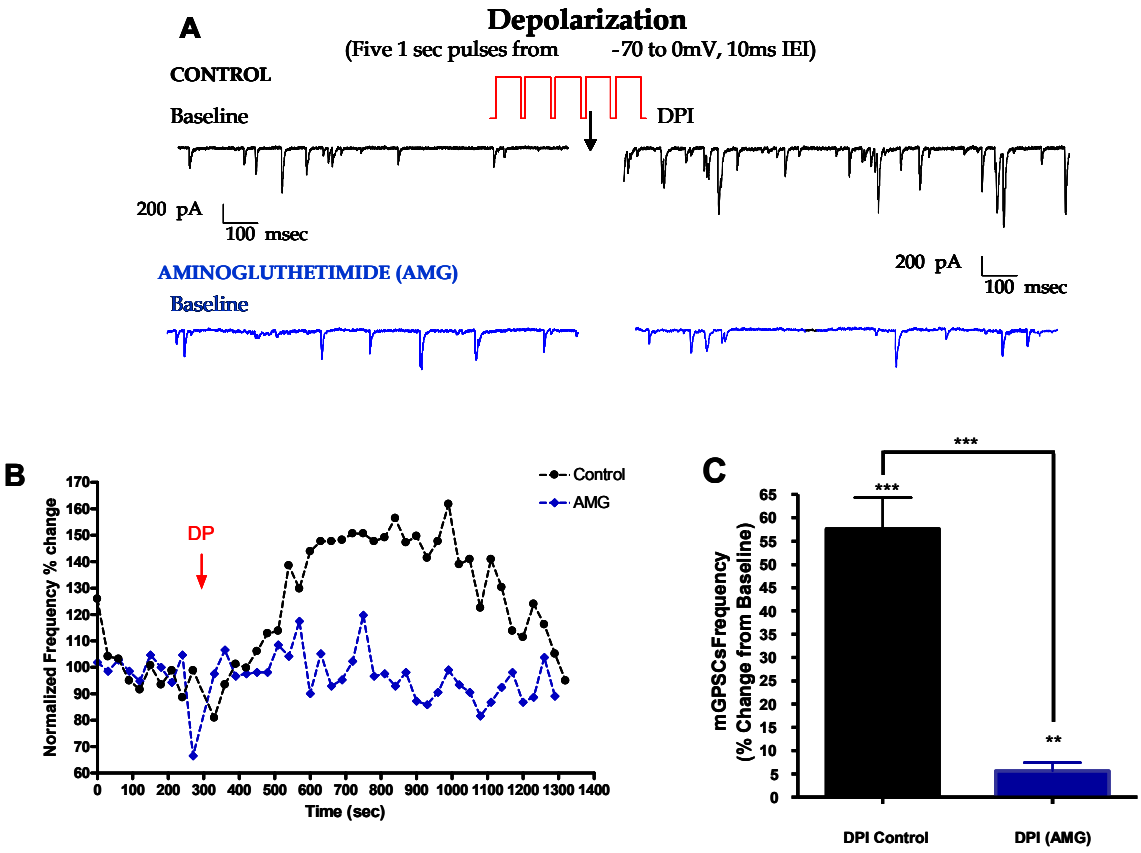

Figure 7.11. A. Sample traces of mIPSC recordings obtained before and after PC depolarization in a control at P9 and an AMG treated PC at P12. B.. mIPSC frequency time course in the absence and presence of AMG. C. Summary of AMG effects on DPI (*** $p < 0.0001$; ** $p < 0.01$, n=4-5).

7.12 Brief characterization of organotypic slice cerebellar culture development and sensitivity to PregS.

Purpose of the experiment

The initial objective of these experiments was to implement a culture system that would allow us to study the role of PregS on synaptic development onto PCs by modulating PregS levels in the slices. The set of data shown here corresponds to a brief characterization of synaptic activity development and PregS sensitivity of PCs in this culture system.

Methods

Parasagittal cerebellar slices (400 μm thick) from 1 day old rats were plated and kept at 37°C in 95 % O_2 – 5% CO_2 for up to three weeks. The culture media contained 50% Eagle's basal medium, 25% heat inactivated horse serum, 25% Earle's balanced salt solution and 1% penicillin/streptomycin. Electrophysiological experiments were performed at days *in vitro* (DIV) 8, 10-12 and 19. Spontaneous firing frequency was recorded in the cell-attached mode. Whole-cell voltage-clamp was used to record spontaneous GABA and glutamate-mediated currents (sIPSCs and sEPSCs, respectively) using a CsCl based internal solution in the absence of synaptic blockers. mIPSCs were recorded in the presence of TTX (0.5 μM) and NBQX (10 μM). Complex-like spike activity was recorded in the whole-cell current-clamp mode. PregS was used at a concentration of 25 μM.

Results

In agreement with previous reports (Seil et al., 1978, Drake-Baumann and Seil, 1999) our results show that in cerebellar slice cultures PC electrical activity and synapse formation develop in a way that resembles *in vivo* development. First, PC spontaneous firing frequency increases with age (Figure 8.12A). This finding is in agreement with a previous study demonstrating that, similar to *in vivo* development, spontaneous action potential in the cortex of organotypic cerebellar cultures increases with age (Seil et al., 1978). Second, the frequency of sPSCs (sEPSCs+sIPSCs) increases as the culture matures (Figure 8.12B). The frequency of spontaneous excitatory postsynaptic currents (sEPSCs) as well as inhibitory postsynaptic currents (sIPSCs) has been shown to increase during PC development in acute slice preparations (Kawa, 2002). Additionally, it has been shown that complex spikes are the predominant spike form in PCs in cerebellar cultures (Lonchamp et al., 2006). This spontaneous complex spike-like activity was also seen in cerebellar cultures (Figure 8.12C). Interestingly, this activity does not seem to be dependent on AMPA receptor activation, as NBQX did not block it (not shown). Importantly, we found that developing PCs in organotypic cultures are sensitive to PregS (Figure 8.12D). At DIV 8-9, bath application of 25 μM PregS significantly and reversibly increases mIPSC frequency.

Conclusions

Our data are in agreement with previous reports characterizing the development of PC synapses in organotypic slice cultures. In this system, PCs receive GABAergic as well as glutamatergic synapses. We show that, similar to in acute slice preparations,

PregS enhances GABAergic transmission in developing PCs. In this system glutamatergic inputs to PCs arrive from granule cells projections that develop in a PF-like fashion (Dupont et al., 2006). Granule cell proliferation and migration from the outer granule cell layer to the inner granule cell layer occurs in cerebellar slice cultures (Mancini and Atchison, 2007). However, in this system the inferior olivary inputs to the cerebellar cortex, the CFs, are severed. Therefore, CF-to-PC synapse development cannot be studied in cerebellar slice cultures.

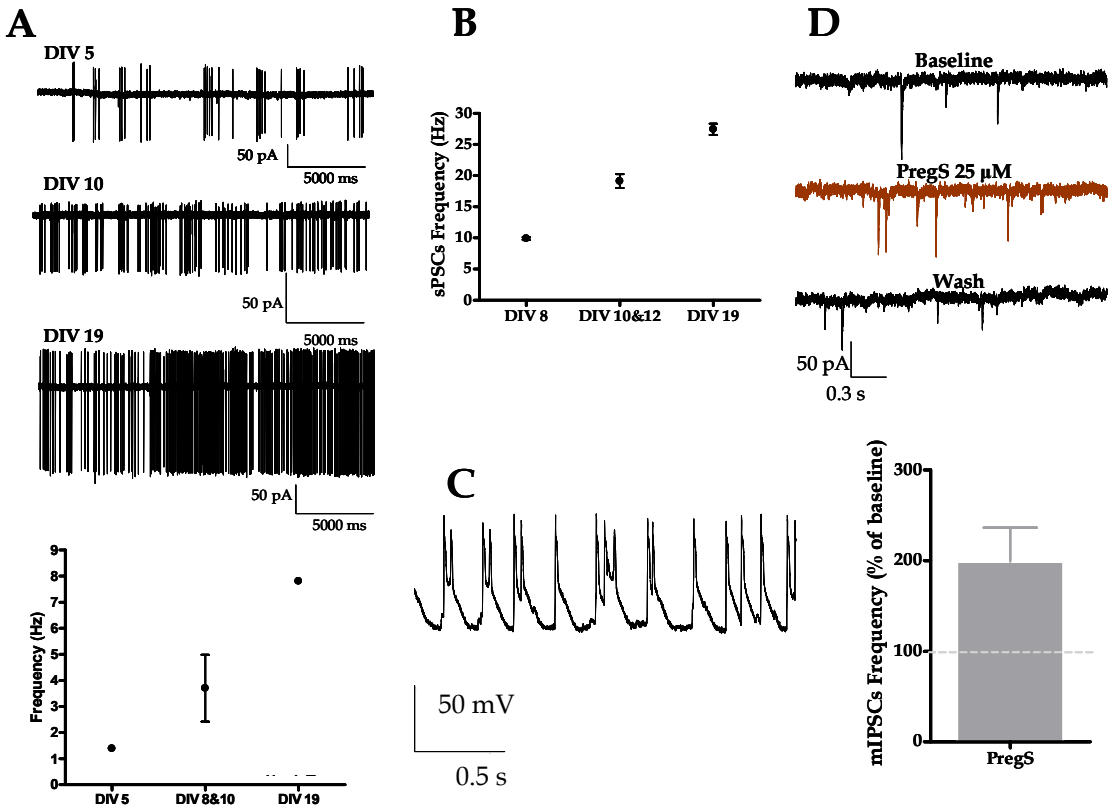

Figure 7.12 . A. In cultured cerebellar slices, the frequency of spontaneous firing in PCs increases with age. Bottom panel shows spontaneous firing frequency values at different ages (n=1-2). **B.** The frequency of GABA + glutamate spontaneous events increases with age (n=2-3). **C.** Complex spike-like activity recorded form a PC at DIV 12. **D.** PregS increases the frequency of mIPSCs in DIV 8 and 10 PCs. The top panel shows mIPSCs recordings in the presence of PregS from a DIV 8 PC. The bottom panel shows bar graph summary of PregS effect in DIV8-10 PCs. K-S test showed a significant decrease in inter-event interval (n=3).

7.13 The TRPC channel blocker SKF96365 does not block the PregS-induced increase in AMPA-mEPSCs

Purpose of the experiment

In acutely isolated dentate gyrus hilar neurons, PregS enhances spontaneous glutamate release by inducing presynaptic Ca^{2+}-induced Ca^{2+} release. Activation of TRPC channels has been proposed to mediate this effect of PregS (Lee et al., 2010). Additionally, the immature cerebellum expresses TRPC1-7 channel proteins as early as P1. Interestingly, TRPC3 is predominantly expressed in the PC layer (Huang et al., 2007a). SKF96365 is an antagonist for TRPC channels as well as store-operated calcium channels and voltage-gated calcium channels and it was shown to block the effects of PregS in the dentate gyrus (Lee et al., 2010). Importantly, this agent is inactive at TRPM3 channels (Grimm et al., 2003). Therefore, we investigated if TRPC channels could mediate the PregS-induced increase of glutamate release onto neonatal PCs.

Method

AMPA-mEPSCs were recorded as previously described (Zamudio-Bulcock and Valenzuela, 2011). PregS (25 μM) was bath applied in the presence of SKF96365 (100 μM) (Tocris Bioscience, Ellisville, MO).

Result

Blockade of TRPC channels does not prevent the PregS effect on glutamatergic synaptic transmission at P4-5 PCs.

Conclusion

This result indicates that TRPC channels do not play a role in the effect of PregS. Nonetheless, given their abundant expression in the PC layer in the neonate (TRPC3), their potential role in the modulation of glutamatergic transmission should be further studied.

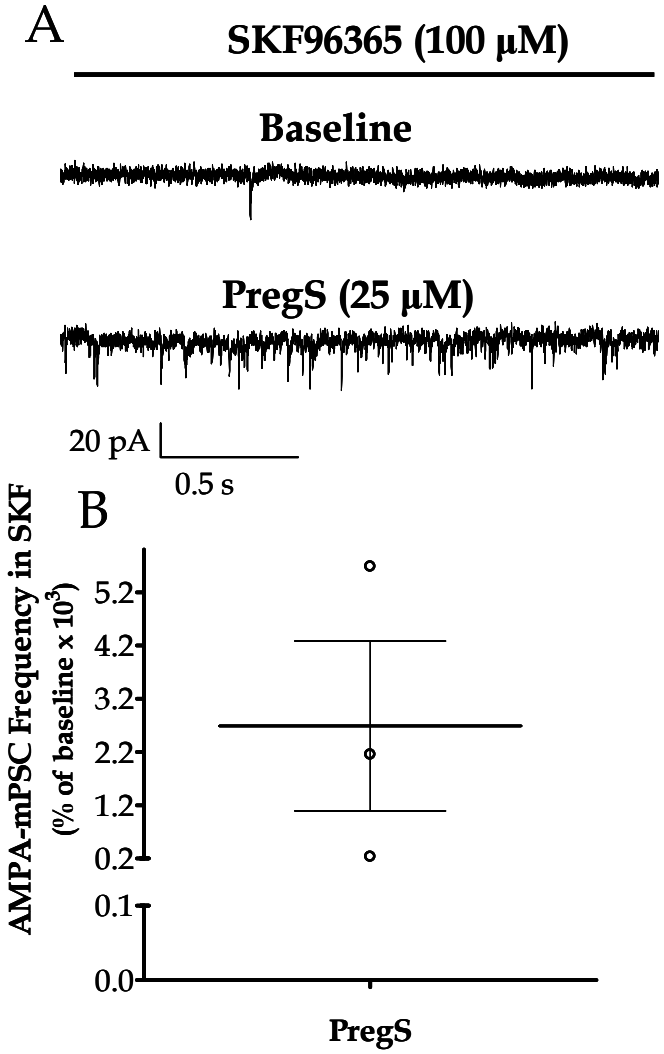

Figure 7.13: **A.** Sample traces of AMPA-mPSCs in the presence of SKF96365 in a P5 PC. **B.** In the presence of SKF96365, PregS increased the frequency of these events (2,691 ± 1,565 % of baseline; raw change from baseline 14.07 ± 4.12 Hz; n=3). The K-S test showed a significant decrease in the inter-event interval in 3 recordings.

7.14 Climbing fiber innervation of Purkinje cells in rat hindbrain cultures

Purpose of the experiment

In mouse hindbrain cultures the development of CF-to-PC synapses has been shown to resemble the *in vivo* maturation of these synapses. First, multiple CF synapse onto PCs and subsequently redundant CF inputs are eliminated (Letellier et al., 2009). In this set of experiments we assessed CF innervation onto PCs in rat hindbrain cultures.

Methods

We cultured hindbrain explants from rat embryos at DIV 15, as described in Chapter 6, and evaluated CF innervation at DIV 18-23, chronologically equivalent to P13-18. In acute slices it has been shown that after P13 the majority of PCs are monoinnervated (Hashimoto et al., 2009). In mouse hindbrain cultures, plated at embryonic day 14, the majority of PCs are monoinnervated at DIV 21, chronologically equivalent to P15. CF-evoked excitatory postsynaptic currents (CF-eEPSCs) were recorded as previously described in Zamudio-Bulcock and Valenzuela (2011). The stimulating electrode was placed in the region near the PC, approximately 200 μm apart. The membrane potential was held at -70mV. Paired-pulses were delivered with an inter-event interval of 50ms. The stimulation intensities varied from 10 to 100μA. Immunohistochemistry experiments were performed as described in Chapter 6; additionally, guinea pig anti-VGlut2 was used to assess the presence of CF terminals in the explants.

Results

We found that in control explants, CF e-EPSCs could elicit paired-pulse depression, indicating the presence of CF inputs at PCs. This result is expected, given the age of the explants (DIV 18 -23). In 3 cells, CF-evoked currents showed a single all or none current step, indicating that these cells were monoinnervated (Fig. 8.14 B, C, D). Additionally, we found VGlut2 expression surrounding the PCs at DIV20-28 (Fig. 8.14 A).

Conclusion

In agreement with previous reports (Chedotal et al., 1997, Letellier et al., 2009), we found that CFs contact PCs in hindbrain cultures. Importantly, at mature stages they show monoinnervation. These results set the groundwork for future studies to investigate the role of sulfated steroids in the achievement of CF-to-PC monoinnervation.

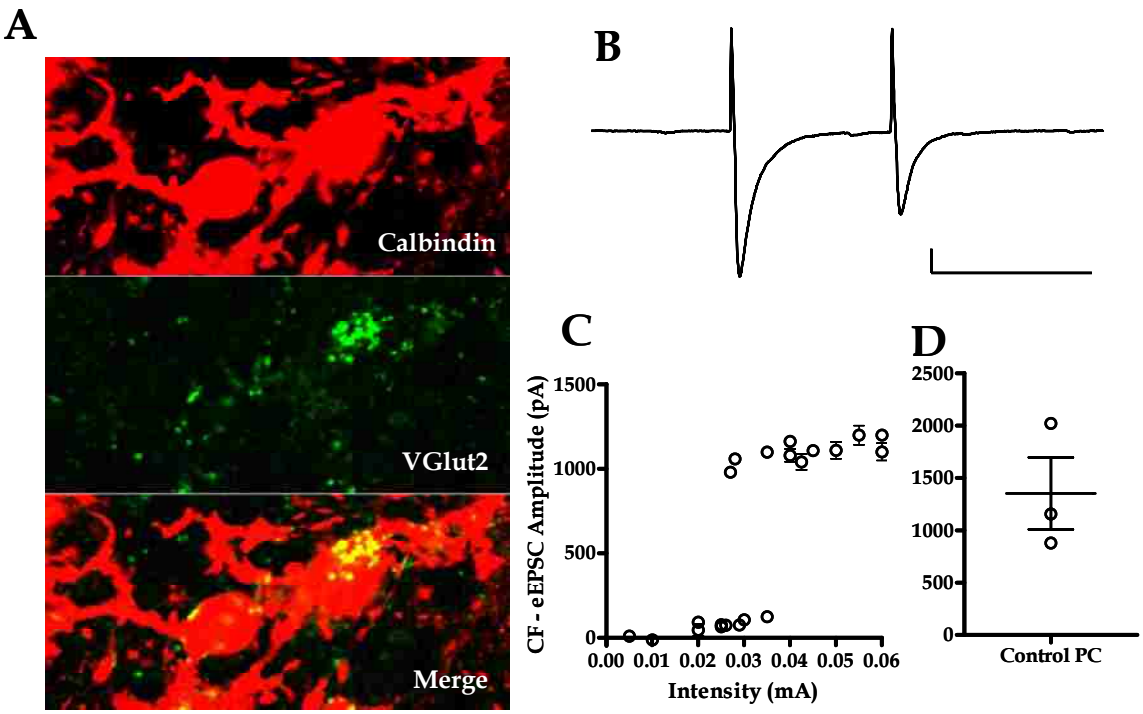

Figure 7.14: **A.** Confocal image showing calbindin positive PC and VGlut2 positive glutamatergic terminals in a rat hindbrain explant at DIV 28. **B.** Sample trace of CF-eEPSC recording, showing paired-pulse depression. Scale bar: 200 pA, 50 ms. **C.** Corresponding plot, showing CF characteristic all or non responses. **D.** Average plot of CF-eEPSC1 amplitudes in 3 PC at DIV18-23.

7.15 Evoked excitatory postsynaptic currents in Purkinje cells from rat hindbrain cultures exposed to the sulfatase inhibitor DU-14

Purpose of the study

To assess whether CF innervation onto PCs is affected by increased levels of endogenous PregS-like neurosteroids via chronic treatment of rat hindbrain cultures with the sulfatase inhibitor DU-14.

Methods

Hindbrain explants were treated from DIV12-24 with either DU-14 (5 μM) or with the vehicle, DMSO. At DIV22-23, whole-cell patch-clamp recordings from PCs and stimulation of eEPSCs were performed as previously described in Zamudio-Bulcock and Valenzuela (2011) and Appendix 14. The paired-pulse ratio (PPR) was calculated as eEPSC2/eEPSC1.

Results

We found that while in the DMSO controls both paired-pulse depression and paired-pulse facilitation could be elicited in two out of 3 cells; the other cell exhibited paired-pulse facilitation only (Fig. 8.15 A), consistent with the presence of CF and PF inputs at this developmental stage. In the DU-14 exposed explants, the paired-pulse depression population was absent in recordings from 3 different cells (Fig. 8.15 B).

Conclusions

These preliminary data suggests that chronic increases in PregS-like neurosteroid levels may induce the loss of CF inputs. Chronic activation of TRPM3 channels in CF terminals and the resulting increases in intracellular Ca^{2+} could result in Ca^{2+} overload and eventual elimination of the terminals. Alternatively, increased PregS levels could induce a change in the probability of glutamate release at CF synapses. Unfortunately, it was difficult to assess the presence of all-or-none responses in both experimental groups. This could be due to contamination of the responses with PF-induced currents, alternating with CF-induced currents. These confounds need to be addressed in future experiments prior to examining the role of PregS-like neurosteroids in the development of glutamatergic synapses at PCs.

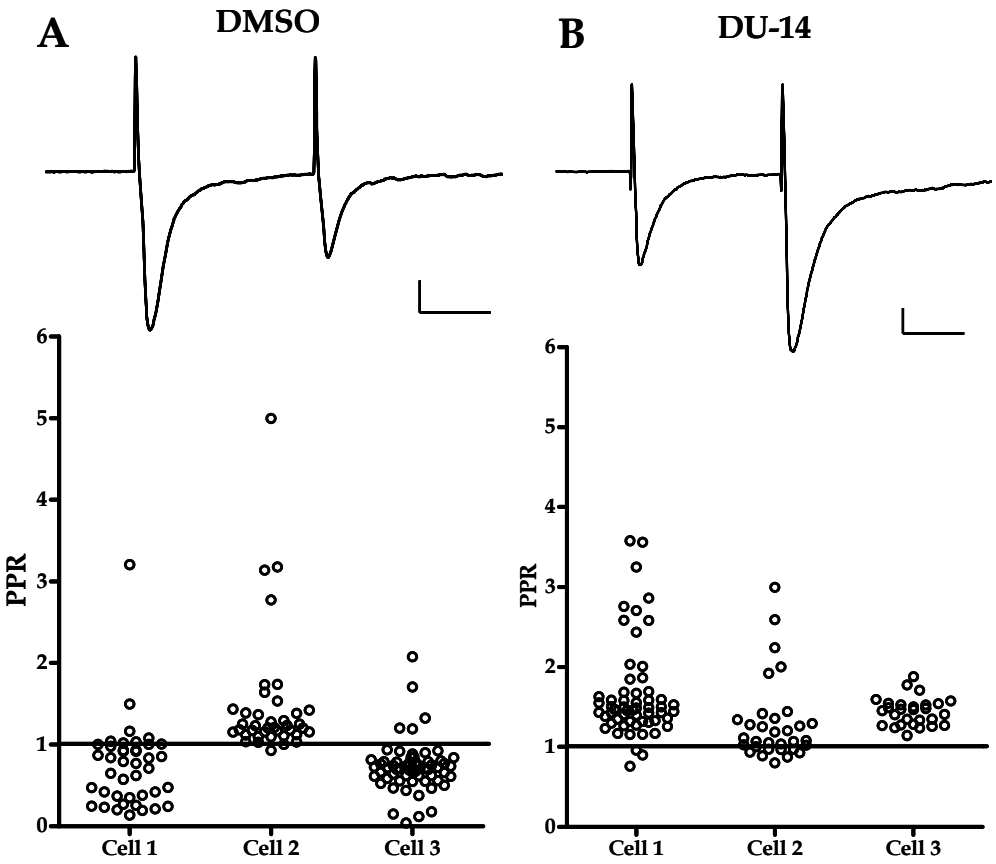

Figure 7.15: **A.** Sample trace of a eEPSC recording in a dimethyl sulfoxide control PC at DIV22 (top). Bottom panel shows PPR graph for 3 DMSO control PCs at DIV 22, note that in 2 out of 3 cells paired-pulse depression is predominant. **B.** Sample trace of an eEPSC recording in a DU-14 treated PC at DIV 23 (top). Bottom panel shows PPR graph for 3 DU-14 treated PC at DIV23. Note that paired-pulse facilitation is predominant in this group. Scale bars: 200 pA, 20 ms.

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