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Targeting progesterone receptors in newborn males and females: From the animal model to a new perspective for the treatment of apnea of prematurity?

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ABSTRACT

The steroid hormone progesterone is well-known for its role in neuroprotection, in the pre- and postnatal brain development, and is also recognized as a potent respiratory stimulant that reduces the frequency of sleep apnea in adult female subjects. Over the past few years, we have used newborn rats or mice to provide convincing evidence that the respiratory effect of progesterone involves a balance between excitation mediated by progesterone receptors, and an inhibition due to the fast conversion of progesterone to allopregnanolone, a positive allosteric modulator of $GABA_A$ receptors. This review focuses on the sex- and age- specific roles of nuclear and membrane progesterone receptors (nPR or mPR), and highlight the clinical potential of these receptors for the treatment of apnea of prematurity. We present original data showing that in newborn rats, selective nPR or mPR agonists are more efficient to reduce apnea frequency at postnatal days 12 than at postnatal day 1, and appear more efficient in males than in females. Furthermore, new results obtained by using intra-cisternal injection of specific siRNA targeting mPRα, mPRβ (two mPR with high brain expression) or nPR suggest that mPRβ regulates the stability of the breathing pattern in males, while effects of nPR appear in females. While several important questions remain to be addressed before a safe clinical use could be proposed, these results highlight the potential role of these drugs as complementary, and sex-specific tools for the treatment of apnea in preterm neonates.

1. Introduction

The postnatal development of a mature respiratory control system involves complex interplays between neurotransmitters as well as anatomical maturation in brainstem areas and in the carotid body that control the respiratory motor output (Bairam and Carroll, 2005; Brady et al., 1999; Carroll, 2003; Ohtake et al., 2000). Respiratory stimulant drugs are essential tools of neonatal medicine, and for more than 40 years, xanthine molecules (caffeine, theophylline, aminophylline or theobromine) have been used as a treatment against apnea of prematurity. However, while the long-term safety of this treatment has been well established in young infants (18–21 months) and up to 11 years of age (Murner-Lavanchy et al., 2018; Schmidt et al., 2017, Schmidt et al., 2007), a non-negligible fraction of patients present persistent apneas (Spitzer, 2012), and it has been emphasized that new therapy should be developed (Finer et al., 2006). A further reason to develop new pharmacological treatments for apnea of prematurity is that theophylline and caffeine in preterm infants promote sleep fragmentation and induce a sleep-debt (Hayes et al., 2007). The steroid hormone progesterone is known as a potent respiratory stimulant in adult mammals (Behan and Wenninger, 2008; Boukari et al., 2017; Loiseau et al., 2018) and this effect likely underlies the well-known male prevalence of sleep apnea that has been consistently reported in the literature (Bixler et al., 2001; Block et al., 1979; Heinzer et al., 2015). It is also noteworthy that hormonal replacement therapy based on conjugated estrogens and medroxyprogesterone reduces the frequency of sleep apnea in women after menopause (Bixler et al., 2001; Shahar et al., 2003). Based on these evidence, it has been suggested that progestin-based drugs should be considered as an alternative for the treatment of apnea of prematurity (AOP) (Finer et al., 2006).

The present review will first consider some basic aspects of circulating levels of progesterone and some of its functions around birth; then, it will focus on studies performed by our group showing the effects of acute or chronic administration of progesterone on the

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respiratory control system in newborns rats. We will also discuss the evidence showing that progesterone acts as an endogenous respiratory stimulant during postnatal development, and present new results concerning the identity of the progesterone receptors involved in these effects. Obviously, there are still several important questions that we need to address before a clinical translation of these findings, but so far, the evidence are encouraging. Importantly, it appears that the effects of progesterone are sex-specific and are apparently more important in newborn males than in females.

2. Circulating levels of progesterone during the perinatal period

During pregnancy, the main sources of steroid synthesis for the fetus are the placenta and the adrenal cortex (Albrecht and Pepe, 1990; Seron-Ferre and Jaffe, 1981), and there is a gradual rise of circulating estradiol (E_2) and progesterone levels in the maternal and fetal circulation. During human gestation the plasma concentrations of progesterone are high in the maternal circulation at term (100–200 ng/ml), and are further elevated in the umbilical vein (up to 700–800 ng/ml) and arteries (300–400 ng/ml) (Runnebaum et al., 1975). Therefore, the fetus develops in an environment characterized by elevated concentrations of circulating progesterone. During the first 24–48 h after birth, there is a physiological drop of progesterone to very low levels (Runnebaum et al., 1975; Trotter et al., 1999) and the main source of circulating steroids in newborn switches to the adrenal glands. Preterm birth is therefore characterized by an early deprivation from this rich hormonal milieu, and compared to term infants, preterm neonate may suffer from a lack of the neurotrophic and neuroprotective functions of these hormones for brain development (Berger and Soder, 2015; Hirst et al., 2008). In rats, a similar pattern is observed, and plasma progesterone levels dramatically decrease after birth and remain low during the first days of life (Dohler and Wuttke, 1975) with similar levels in both males and females (Dohler and Wuttke, 1975; Weisz and Ward, 1980). After 20 days of age, the circulating levels of progesterone increase slightly in both sexes (Dohler and Wuttke, 1975) and become significantly higher in females than in males at puberty (around the 5th or 6th postnatal week) (Dohler and Wuttke, 1975). This developmental pattern of plasma progesterone contrasts with the profile of estradiol and testosterone that are also high in-utero but remain elevated throughout the early postnatal development (Dohler and Wuttke, 1975). Furthermore, plasma levels of testosterone are sex-specific, and males have higher levels of circulating testosterone both in-utero and during early postnatal development in rats (Weisz and Ward, 1980).

Interestingly, plasma progesterone levels have been shown to be elevated in sick newborns compared to healthy ones (Anandi and Shaila, 2017; Chennuri et al., 2013). In adult and prepubertal rats it is well acknowledged that activation of the hypophysis-pituitary-adrenal axis under stress exposure leads to increased levels of progesterone and its neuroactive metabolite allopregnanolone in the brain (Purdy et al., 1991; Romeo et al., 2005). In cases of brain injuries these hormones have important neuroprotective roles (Guennoun et al., 2015; Schumacher et al., 2014). All steroid hormones easily cross the blood brain barrier and are present in the cerebrospinal fluid shortly after a systemic administration (Backstrom et al., 1976), therefore changes in plasma progesterone levels affect the central nervous system. Progesterone and other steroids, such as progestin-based contraceptives are also transferred to the newborn through the milk during lactation (Betrabet et al., 1987).

3. Utilization of progesterone in preterm infants?

Postnatal bone deficiency and respiratory insufficiency are important concerns in preterm infants, and Trotter' group tested the hypothesis that sex-hormones positively influence bone growth and favor synthesis of alveolar surfactant (Trotter et al., 1999, 2007). Progesterone and estradiol were administered to preterm infants (< 30 weeks of gestation) for a maximum of 6 weeks after birth to restore in-utero fetal plasma levels. These two small randomized and placebo-controlled trials reported a non-significant trend towards better bone accretion and fewer chronic lung disease in treated babies. Although, the follow-up of these infants at 5 years of age, demonstrated a positive correlation between the duration of the hormonal treatment and neurodevelopmental outcomes (Trotter et al., 2012), such treatment is not yet introduced in clinical practice and further explorations are needed as suggested by the investigators.

To the best of our knowledge, however, the hypothesis that hormonal treatment might reduce apnea in preterm neonates has not been addressed so far. On the other hand, sporadic case-reports in patients suffering from central congenital hypoventilation show that medroxyprogesterone acetate had restored the central chemosensitivity in 2 young infants (1 and 20 months old) (Milerad et al., 1985) and in adult woman desogestrel, a synthetic progestin, had also restored central chemosensitivity (Straus et al., 2010).

4. Newborn rats as an animal model for neonatal respiratory studies

To better understand the role of progesterone as a respiratory stimulant in newborns, and its potential use against apnea of prematurity, we have performed experiments in newborn rats. This is a convenient model since rats have short gestation length, and at birth the behavioral and neurological maturation roughly correspond to a human preterm around 24–30 weeks of gestational age (Clancy et al., 2007; Rice and Barone, 2000). Furthermore, there are important similarities when comparing the developmental pattern of respiratory response to hypoxia between newborn humans and rats (Bissonnette, 2000; Niane and Bairam, 2011), and the maturation of the respiratory control system in rats also include a gradual decrease of the number of apneas and respiratory instabilities during the first 15 days of life (Niane and Bairam, 2012). It is also noteworthy that there are similarities concerning the postnatal changes affecting the circulating levels of progesterone between rat pups and human neonates – as noted above.

5. Considering sex in neonatal respiratory studies

It is largely acknowledged that there are appreciable sex-specific differences in morbidity and mortality following hypoxemic/ischemic insults in human neonates (Mage and Donner, 2004), and male sex remains a well-known risk factor for sudden infant death syndrome (Mehboob et al., 2017). Furthermore, the long-term consequences of these adverse neonatal events are sex-specific: a recent meta-analysis reported that boys have more severe long-term IQ impairments than girls, and in animal models of neonatal hypoxic-ischemia there are sex-specific cognitive deficits (Smith et al., 2014). The most common and convenient animal model of apnea of prematurity is exposure to intermittent hypoxia during early postnatal development, and in rats this induces sex-specific long-lasting consequences, with adult males but not females showing working memory deficits following exposure to intermittent hypoxia during postnatal days 10–30 (Kheirandish et al., 2005). Finally, studies on apnea and respiratory instabilities in rat pups show a clear sex-specific effect, with males presenting more apneas than females (Fournier et al., 2013; Kouchi et al., 2017). Earlier studies in a clinical cohort of 1023 term infants (between 2 and 28 weeks of postnatal age) showed that obstructive apneas are found in 44.5% of the boys studied and in 36.0% of girls, with marked sex-specific differences between 8 and 11 postnatal weeks (Kato et al., 2000). Moreover, our group recently reported data collected from a large cohort of 24,387 preterm neonates between 24–34 weeks of gestational age (Canadian Neonatal Network), showing that there were more preterm girls with a diagnosis of apnea of prematurity, but girls had fewer days of caffeine therapy than boys, thus suggesting faster maturation of the respiratory control system (Bairam et al., 2018; McDonald et al., 2018).

It is also noteworthy that recent investigations on the neuro-protective effect of steroids in newborn animal models clearly showed sex-specific preventive effects for progesterone with protective effects in males but not in females after a hypoxic-ischemic insult (Dong et al., 2018). Accordingly, progesterone has sex-specific effects in the brain, and this should be considered when assessing the effects of progesterone on respiration and apnea frequency.

6. Respiratory effects of chronic and acute progesterone administration in newborn rats

Our first series of experiments were performed in newborn male rats that have been exposed to progesterone through the milk of lactating mothers between postnatal days 2–10 (Lefter et al., 2007). The results showed that this treatment increased the ventilatory response to hypoxia and reduced the frequency of apneas under normoxia or during hypoxia. This respiratory phenotype likely involved a reduction of the inhibitory effects of dopamine at the level of the peripheral chemoreceptors, because an i.p. injection of domperidone (a D2 dopamine receptor antagonist that does not cross the blood-brain-barrier) increased minute ventilation in control rats, but not in rats treated with progesterone. More recently, we showed that chronic progesterone administration (by daily gavage between postnatal days 3–12 – 4 mg/kg/day) reduces the frequency of spontaneous and post-sigh apneas in males, while in females there is only a modest reduction of the frequency of spontaneous apneas (Joseph et al., 2018).

To assess if these effects of progesterone on the hypoxic ventilatory response could be observed on a shorter time frame (rather than following long-term exposure), we used newborn male rats that received a single i.p. injection of progesterone at 0, 2, 4 and 8 mg/kg (Hichri et al., 2012). In 12-days old male rats, progesterone injection had no effect on minute ventilation under normoxia and on hypoxic ventilatory response. However, in 4-days old male rats, progesterone enhanced the initial and the late phase of the hypoxic response with the dose of 4 mg/kg, while there was no effect with the other doses. Based on these experiments, we used the dose of 4 mg/kg in all subsequent studies. Interestingly, the frequency of apnea was decreased by an acute injection of progesterone in 1-day old rats, but in 12-days old rats it increased the frequency of apneas (Bairam et al., 2013). This dose also enhanced the hypoxic ventilatory response in the youngest rats (1, 4, and 7 days of age), but not in 12 days old rats (Bairam et al., 2013).

7. Inhibitory effects of progesterone on the respiratory control system are mediated by its neuroactive metabolite allopregnanolone

The contrasting effect of acute and chronic progesterone injections on apnea frequency and hypoxic ventilatory response in 10–12 days-old rats could be explained by the fast conversion of progesterone to allopregnanolone. Allopregnanolone is a metabolite of progesterone synthesized by successive reductions at the 5- and 3- positions of the steroid A-ring by the 5α-reductase and the 3α-hydroxysteroid oxidoreductase enzymes that are both present in the brain (Reddy, 2010; Reddy and Estes, 2016; Schumacher et al., 2014). It is noteworthy that progesterone administration (4 mg/kg, ip) increases the circulating level of allopregnanolone in 12-days-old male rats (Uppari et al., 2016), thus suggesting that the inhibitory effects of acute progesterone administration are at least partially attributable to allopregnanolone. The presence of a reduced OH group at the C3 position in the steroid A-ring confers binding affinity to specific sites of the $GABA_A$ receptor within the transmembrane domains of the α and β subunits. Binding of allopregnanolone on the α subunit site occurs at the observed physiological concentrations of endogenous allopregnanolone in the brain ($0.1 - 0.3$ μM) and enhances GABA-evoked responses by increasing the open probability of the chloride channel (Reddy and Estes, 2016). This effect occurs at synaptic $GABA_A$ receptors (phasic inhibition) and at extra-synaptic sites, therefore enhancing tonic inhibition of neuronal networks (Reddy, 2010). The inhibitory effects of allopregnanolone on neuronal activity are considered as a potential avenue for the treatment of many neurological diseases such as epilepsy or mood disorders including anxiety, depression, or alcohol withdrawal syndromes (Reddy, 2010). At supra-physiological doses (> 1μM) allopregnanolone binds to another site on the $GABA_A$ receptor and acts as a direct agonist (Reddy and Estes, 2016).

Interestingly, the levels of allopregnanolone in the brain during the last days of gestation are elevated (around 5 ng/g in the whole brain, and up to 15–20 ng/g in the forebrain), reaching values that are 5–10 times higher than the levels of brain progesterone (Grobin and Morrow, 2001; Kellogg and Frye, 1999). This likely indicates an intense conversion of progesterone to allopregnanolone during late fetal life. During the early postnatal days, allopregnanolone gradually decline to low levels in the brain, but increases thereafter between the 10th and 14th postnatal days, following the increased synthesis of progesterone and sustained expression of enzymes for neurosteroid synthesis during the same developmental window (Carre et al., 2001). Through these effects on the GABA receptor, allopregnanolone decreases the respiratory activity of the isolated brainstem/spinal cord preparation in-vitro and minute ventilation in newborn rats in-vivo (Ren and Greer, 2006). These effects are however very strong before birth: at gestational age 20 allopregnanolone (300 nM in vitro, 4 mg/kg in-vivo) depresses the respiratory activity by more than 80%, and this inhibitory effect becomes gradually less important. At postnatal day 4, allopregnanolone decreases the respiratory activity by 20–30% both in vitro and in-vivo (Ren and Greer, 2006).

In-vivo, it is possible to block the conversion of progesterone to allopregnanolone with finasteride. In 10–12 days-old male and female rats, a single injection of finasteride reduces the frequency of apneas and an injection of progesterone with finasteride could not further increase the frequency of apnea (Joseph et al., 2018). These experiments indicate that, similarly to what has been described in other brain regions, there is a rapid and dynamic equilibrium between progesterone and allopregnanolone that plays an important role for the stability of the respiratory control system (see summary in Fig. 1). This implies that to take full advantages of the respiratory stimulant effects of progesterone it is necessary to avoid the conversion to allopregnanolone. Our most recent data indicate that this could be achieved by identifying and targeting specific progesterone receptors involved in respiratory control (Joseph et al., 2018) (and see below).

8. Respiratory stimulant effects of nuclear and membrane progesterone receptor agonists in newborn male and female rats

As discussed above, the stimulatory effects of progesterone in the respiratory control system are apparently masked by the influence of allopregnanolone on $GABA_A$ receptors. On the other hand, it is well established that biological functions of progesterone in the central and peripheral nervous system are mediated by specific nuclear and membrane progesterone receptors (nPR and mPR). There are at least two isoforms of nPR, encoded by a single gene through alternative splicing. nPRs belong to the family of steroid receptors that upon binding with their ligands are translocated to the nuclei and act as transcription factors to activate or repress genes that possess a specific binding sequence in their promoter region. nPR have been identified in the carotid body of fetuses, newborn and adult rats (Joseph et al., 2006), are localized in the hypothalamus of adult and newborn mice and rats, and in the nucleus tractus solitarius in adult rats (Brinton et al., 2008; Haywood et al., 1999; Helena et al., 2009; Romeo et al., 2005). On the other hand, there are five different mPRs (mPRα, mPRβ, mPRγ, mPRδ and mPRε) encoded by different genes that belong to the family of Progestin and AdipoQ receptor (PAQR) (Guennoun et al., 2015; Schumacher et al., 2014). mPRs are localized in the central and peripheral nervous system (Castelnovo et al., 2017; Guennoun et al., 2015;

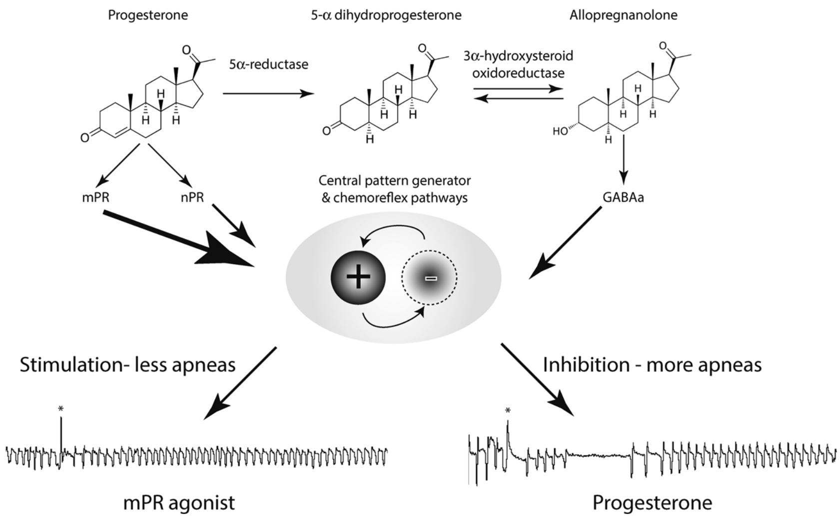

Fig. 1. Simplified illustration showing the balance between allopregnanolone and progesterone receptors for the control of respiratory activity and apnea frequency in newborn rats. The "central pattern generator" in the brainstem highlights the reciprocal interactions between excitation (+, left side, pool of glutamatergic neurons) and inhibition (-, right side, including GABAergic neurons) that generate and shape the respiratory rhythm and integrate the drive from the chemoreflex pathways – simplified from (Guyenet, 2014).
The respiratory effects of progesterone on this network likely result from interactions with specific nuclear and membrane receptors (nPR and mPR) acting as respiratory stimulant, therefore reducing the frequency of apneas. On the other hand, the conversion of progesterone to allopregnanolone facilitates inhibitory mechanisms through the GABAergic system, increasing the frequency of apneas. Respiratory traces at the bottom have been obtained in 10–12 days old rats, showing the contrast between an acute injection of a mPR agonist that reduces the frequency of apneas, and progesterone that increases the frequency of apneas following conversion to allopregnanolone (Joseph et al., 2018). Note that the traces present a typical sigh (*), followed by an apnea after progesterone administration.

Schumacher et al., 2014), and might therefore contribute to mediate the effects of progesterone on the nervous system.

To assess the relative roles of the nPR and mPR on the stability of the breathing pattern in 10–12 days old male and female rats, we used the specific agonists for nPR (R5020, 4 mg/kg ip) or mPR (Org-od-02-0, 4 mg/kg ip) (Joseph et al., 2018). Interestingly our results showed that the mPR agonist was more efficient to reduce the frequency of spontaneous apneas in male and female rats than the nPR agonist. Here we present the total frequency of apnea (spontaneous + post-sigh) recorded in one-day old (P1) male and female rats that received ip injections of R5020 and Org-od-02-0 (both at 4 mg/kg) or vehicle, and for comparisons the values in 10–12 days old rats (Fig. 2 – values for 10–12 days old rats present the sum of the frequency of spontaneous and post-sigh apneas from (Joseph et al., 2018)). All values at P1 have been obtained following a similar protocol than at P12, and all experiments have been approved by our local committee on animal care and utilization in research (permit#: 2012 – 269). At P1, the nPR agonist (R5020) and the mPR agonist (Org-od-02-0) reduced the frequency of apneas in males, but not in females. These data also show that the mPR agonist is more efficient to reduce the frequency of apnea at P12 than at P1 in males and females, while the nPR agonist apparently loses its effect between P1 and P12 in males.

9. Respiratory stimulant effects of endogenous progesterone in newborn male and female rats

All the experiments described above have been performed using exogenous administration of progesterone, or specific nuclear or membrane PR agonists. It is largely acknowledged that steroid hormones are also synthesized at high levels in the central and peripheral nervous system, playing key developmental roles in fetuses and newborn (Beyer, 1999; Hirst et al., 2008), including for neurogenesis and neuroprotection (Berger and Soder, 2015; Liu et al., 2010). Assessing the role of endogenous progesterone production in our models is however a delicate task. Due to its position as an intermediate for synthesis of all steroids, it is nearly impossible to selectively inhibits progesterone synthesis. Likewise, there is only a limited choice of progesterone receptor antagonist. Mifepristone is one of the most often used, and while it does have an effect on the postnatal maturation of the peripheral chemoreceptors in male rats (Joseph et al., 2012), it is also an antagonist of the glucocorticoid receptor, and exerts rapid effects independent of nPR by blocking voltage-dependent Ca^{2+} channels (Perusquia et al., 2009) and could therefore not reveal a selective role of activation of nPR by endogenous progesterone synthesis.

To circumvent this issue, we used the available model of PRKO mice that have a constitutive deletion of nPR (PRKO mice) and we measured basal breathing and the ventilatory response to hypoxia in 1-, 4-, and 10-days old male and female mice. In these animals, there was no alteration of ventilation or apnea frequency under baseline conditions (for males and females) compared to their wild-type littermates, but PRKO mice had a reduced ventilatory response to hypoxia with marked sex-specific effects at 1 and 10 days of age, while there was no effect in 4 days old mice (Potvin et al., 2014). At P1, the nPR deletion had stronger effects in males than in females on the hypoxic ventilatory response. At P10 the nPR deletion induced a lower tidal volume response to hypoxia in males, but a lower respiratory frequency response to hypoxia in females. These results are consistent with the sex-specific nPR expression in the central nervous system during postnatal development (Quadros and Wagner, 2008), with the elevated level of circulating and brain progesterone at birth (Dohler and Wuttke, 1975;

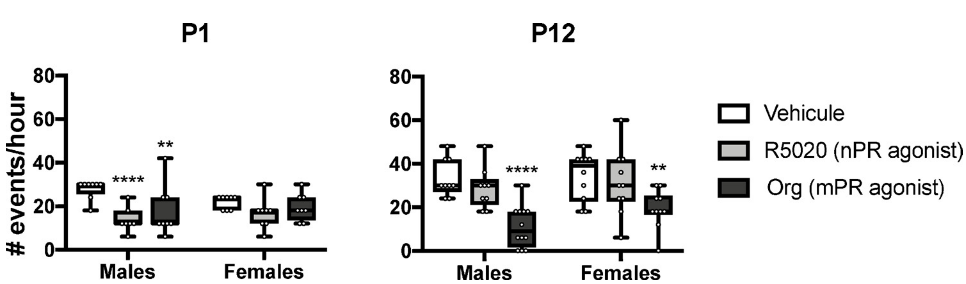

Fig. 2. Frequency of apneas (events/hour) recorded at postnatal days 1 (left panel) and 10–12 (right panel) in male and female rat pups following ip injection of vehicle (Veh), the nPR (R5020 - 4 mg/kg) or mPR (Org-od-02-0 - 4 mg/kg) agonist 1 h before the recording. The plots show the median, 25th-75th percentiles (box), min and max values (whiskers), and individual data points. ****: $p < 0.0001$, **: $p < 0.01$ vs Veh. For each age, we performed a two-way ANOVA (with sex and drug as grouping variables) followed by a Fisher's LSD post-hoc test.
Number of animals into each group at P1 males: Veh (n = 8), R5020 (n = 7), Org (n = 8) - females (n = 8 each). Number of animals into each group at P12 males Veh (n = 9), R5020 (n = 9), Org (n = 12) - females (n = 10 each).

Rhoda et al., 1984) that might have short-lasting stimulatory effects on the hypoxic ventilatory response, and, with the fact that around postnatal day 10 neurosteroid synthesis in the brain is functional and produces progesterone at physiologically relevant concentrations (Carre et al., 2001). However, the PRKO mice model has some limitation as discussed in our papers (Marcouiller et al., 2014; Potvin et al., 2014).

In adult mice, we have used RNA silencing to assess the role of mPRα and mPRβ, two members of the mPR family that are expressed in the central nervous system and in the spinal cord (Guennoun et al., 2015). Our results have shown a remarkable elevation of apnea frequency recorded during sleep, and a reduction of the hypoxic ventilatory response in male and female mice after 2 weeks of continuous infusion of the siRNA against mPRβ in the IVth ventricle (Boukari et al., 2016). Contrastingly, the siRNA against mPRα did not altered the frequency of apnea in males or females, and only slightly reduced the hypoxic ventilatory response. An intriguing effect of the treatment with the siRNA against mPRβ was the important elevation of apnea frequency in male mice, showing that this receptor might contribute to a key, yet unsuspected role of endogenous progesterone synthesis for stability of the respiratory control system in adult males. In newborns, we also choose to use siRNA to assess the respective roles of nPR, mPRα, and mPRβ on the respiratory control system in male and female rats. In these experiments, we used newborn rats rather than mice because of the larger size of the pups, facilitating surgery and intra-cerebral injections, and because the mothers accept much more easily the pups following the surgery. In these experiments, male and female rat pups at 10 days of life have been used, anaesthetized with isoflurane (3%), and have received an injection of 50 μg of specific siRNA (Ambion in vivo siRNA, ThermoFisher Scientific) against mPRα (GCCCUUCUG UCACAAGUtt), mPRβ (GAAGAUCUGUCAAGUGGUAtt), nPR (GAGAU GAGGUCAAGCUAUAtt), or an artificial cerebrospinal fluid (aCSF in mM: 129 NaCl, 3.35 KCl, 0.58 NaH_2PO_4, 1.15 $MgCl_2$, 1.26 $CaCl_2$, 21 $NaHCO_3$, 30 glucose) in the cisterna magna using a protocol described previously (Ballot et al., 2015). We applied standard pre-and post-operative cares, including analgesia, and all protocols have been approved by our local committee on animal care and utilization in research (permit #: 2012 – 023). Since previous experiments have shown that similar siRNA treatment reduces the expression of target genes for at least 48 h in adult mice (Makimura et al., 2002), we performed recordings 24–48 h following the injection into the cisterna magna. Each pup was placed in a whole-body plethysmograph chamber for continuous respiratory and metabolic recordings for 1 h. Respiratory (tidal volume, respiratory frequency, and minute ventilation) and metabolic (O_2 consumption and CO_2 production rates) parameters have been evaluated as previously described (Joseph et al., 2018), and we chose portions of stable breathing pattern of about 20 min typically towards the end of the recording to assess the frequency of sighs, post-sigh apneas, and spontaneous apneas using standard criteria as previously described (Joseph et al., 2018). Respiratory and metabolic parameters obtained during the recordings were similar across all groups, as well as the frequency of sighs (not shown). Apnea frequency (spontaneous + post-sigh) are shown in Fig. 3: the treatment with the siRNA against mPRβ significantly increased the frequency of apneas in males but not in females, the siRNA against nPR significantly increased the frequency of apneas in females but had no significant effect in males. Finally, the siRNA against mPRα had no effect in males or in females. These results are only partially coherent with our previous set of data (cf Fig. 2) showing the mPR agonist efficiently reduces apnea frequency in male and female rat pups, while the nPR agonist had no effect in females (Joseph et al., 2018). Such discrepancy might be due to the different sites of drug injection (systemic *vs* central nervous system), the time frame post-injection, or the mode of action of each treatment (pharmacological agonist *vs* RNA interference). Nonetheless, these results also confirm earlier findings obtained in adult mice showing that mPRβ plays a prominent role to regulate the stability of the breathing pattern, while mPRα has more limited effects (Boukari et al., 2016).

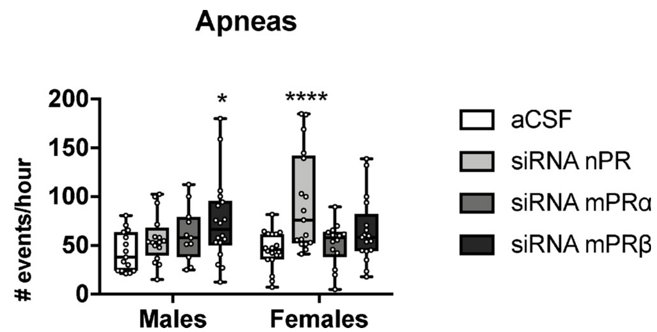

Fig. 3. Frequency of apneas (events/hour) recorded at postnatal day 11–12 in male and female rat pups, 24–48 h following an injection of vehicle, the siRNA against nPR, mPRα, or mPRβ into the cisterna magna. The plots show the median, 25th-75th percentiles (box), min and max values (whiskers), and individual data points. ****: $p < 0.0001$, **: $p < 0.01$ vs Veh. We performed a two-way ANOVA (with sex and siRNA as grouping variables) followed by a Fisher's LSD post-hoc test.
Number of animals in males: aCSF (n = 15), siRNA nPR (n = 16), siRNA mPRα (n = 11), siRNA mPRβ (n = 18) – in females: aCSF (n = 18), siRNA nPR (n = 17), siRNA mPRα (n = 14), siRNA mPRβ (n = 17).

10. Chronic caffeine treatment favors the inhibitory effects of allopregnanolone on respiratory control

Caffeine is widely used as a respiratory stimulant drug to reduce the frequency of apnea in preterm neonates (Abu-Shaweesh and Martin, 2017). The short- and long-term safety has been clearly established (Schmidt et al., 2017, Schmidt et al., 2007), but it is not effective in all treated neonates. Indeed, a recent study documented the persistence of very long apneas (> 60 s) in some neonates treated with caffeine (Mohr et al., 2015). We have reported that chronic caffeine treatment between postnatal days 3–12 in rats (used as a model of long-term caffeine therapy for apnea in preterm neonates) enhances the inhibitory respiratory effects of an acute injection of progesterone, allopregnanolone, or the $GABA_A$ antagonist bicuculline assessed in 12-day-old rats (Uppari et al., 2016), and these effects appear less pronounced in females (Uppari et al., 2017). Being an antagonist of adenosine receptors, caffeine decreases the influence of one of the main inhibitory system in the central nervous system. Because brain inhibition plays a critical role to avoid uncontrolled firing of neuronal networks and the loss of specific neuronal activity (Buzsaki et al., 2007), it is not surprising that a complementary inhibitory system is triggered by prolonged blockade of adenosine receptors by caffeine. Under these circumstances, progesterone loses its appeal as a respiratory stimulant drug for the treatment of apnea in preterm neonates that have previously been treated with caffeine. Our data indicate that mPR agonists could be an efficient option, but several aspects of the respiratory effects of mPRs should be further understood, including the influence of age and sex and the expression and function of these receptors in the respiratory control system.

11. Summary/Perspectives

The data that we have accumulated over the past decade clearly indicate that progesterone exerts important functions in the regulation of the breathing pattern during postnatal development in rodent models. These functions are however complicated by the fact that several families of progesterone receptors could mediate such effects, and the target (or targets) most likely to have an effect on the stability of the breathing pattern for a clinical translation remains to be firmly identified. As also demonstrated by our data, the interactions between progesterone and the GABAergic system could not be ignored, and taking advantage of the respiratory stimulant role of progesterone requires to bypass this inhibitory system. Our results indicate that mPRβ, and to a lower extent nPR could be relevant targets, and further research should help understand by which mechanisms, and at which levels of the respiratory control system these receptors exert their effects. On the other hand, it appears equally important to assess whether neonatal exposure to progesterone or progesterone receptor agonists have long-term effects on the central nervous system to rule-out potential undesired side effects. Hypothetically, another benefit of progesterone or progesterone receptor agonists could be to provide neuroprotection against the detrimental consequences of intermittent hypoxia associated to the repeated apneic episodes. The fact that progesterone has sex-specific neuroprotective properties (Dong et al., 2018) is encouraging and could lead to further research development to assess which progesterone receptor(s) are involved in animal model of apnea of prematurity (such as newborn rats exposed to intermittent hypoxia) and if these effects are clinically relevant. Obviously, the next decade(s) of research offer exciting perspectives to further develop our knowledge in this area.

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