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BREATHING CONTROL AND SEROTONIN IN EPILEPSY:
from neonate to adult and implications for sudden death

Belo Horizonte

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Paloma Graziele Bittencourt da Silva

BREATHING CONTROL AND SEROTONIN IN EPILEPSY: FROM NEONATE TO ADULT AND IMPLICATIONS FOR SUDDEN DEATH

Thesis submitted to the Graduate Program in Physiology and Pharmacology at the Federal University of Minas Gerais, in partial fulfillment of the requirements for the degree of Doctor Biological Sciences - Physiology.

Advisor: Dr. Glauber dos Santos Ferreira da Silva
Co-advisor: Dr Matthew R. Hodges

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ATA DA DEFESA DA TESE DE DOUTORADO N° 388 DE PALOMA GRAZIELE BITTENCOURT DA SILVA

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Às 13:30 horas do dia 31 do mês de outubro de 2024, Transmitida através da Plataforma Google Meet, realizou-se a sessão pública para a defesa da Tese de **Paloma Graziele Bittencourt da Silva.** A presidência da sessão coube ao **Prof. Dr. Glauber dos Santos Ferreira da Silva,** orientador. Inicialmente, o presidente fez a apresentação da Comissão Examinadora assim constituída: **Prof. Dr. Glauber dos Santos Ferreira da Silva,** ICB/Universidade Federal de Minas Gerais, **Prof. Dr. Daniel Breseghello Zoccal,** UNESP, **Profa. Dra. Vivian Biancardi,** University of Alberta, Canadá, **Profa. Dra. Ana Carolina Thomaz Takakura,** ICB/USP, **Profa. Dra. Aline Priscila Pansani,** UFG e **Prof. Dr. Matthew R. Hodges, Coorientador -** Departament of Physiology Medical College of Wisconsin, EUA. Em seguida, a candidata fez a apresentação do trabalho que constitui sua **Tese de Doutorado**, intitulada: **"BREATHING CONTROL AND SEROTONIN IN EPILEPSY: FROM NEONATE TO ADULT AND IMPLICATIONS FOR SUDDEN DEATH".** Seguiu-se a arguição pelos examinadores e logo após, a Comissão reuniu-se, sem a presença da candidata e do público e decidiu considerar **APROVADA** a Tese de Doutorado. O resultado final foi comunicado publicamente a candidata pelo presidente da Comissão. Nada mais havendo a tratar, o presidente encerrou a sessão e lavrou a presente ata que, depois de lida será assinada pela Comissão Examinadora.

Belo Horizonte, 31 de outubro de 2024.

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Dedico esta tese à minha mãe, Dona Ida.
(I dedicate this thesis to my mother, Mrs. Ida)

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Só posso levantar as mãos pro céu
Agradecer e ser fiel ao destino que Deus me deu
Se não tenho tudo que preciso
Com o que tenho, vivo
De mansinho, lá vou eu
Se a coisa não sai do jeito que eu quero
Também não me desespero
O negócio é deixar rolar
E aos trancos e barrancos, lá vou eu
E sou feliz e agradeço por tudo que Deus me deu
"Zeca Pagodinho"

Ninguém é dono da verdade,
Mas pode ter sua posse,
Ciente da verdade,
Eu faço o que eu posso
"Forfun"

RESUMO

As crises convulsivas afetam a atividade respiratória, tanto durante quanto após eventos convulsivos, induzindo desde apneias leves e transientes à apneias fatais. Ademais, crises recorrentes induzem alterações na quimiorrecepção central ao CO_2/pH. Estes fatores estão relacionados à morte súbita em epilepsia (SUDEP), entretanto os mecanismos de controle respiratório relacionados ainda não foram elucidados. As crises convulsivas podem acometer crianças e adultos, tendo alta incidência durante o período neonatal. Evidências sugerem a importância da serotonina para atividade respiratória durante crises convulsivas. Dito isso, o objetivo geral desta tese foi estudar o controle respiratório em modelos animais de epilepsia (neonatos e adultos), avaliando mecanismos serotoninérgicos (5-HT). As abordagens experimentais utilizadas foram a pletismografia de corpo inteiro para medidas de ventilação pulmonar, medidas de consumo de O_2 e temperatura corporal. Adicionalmente, foram utilizados métodos neuroquímicos, imunohistoquímicos e farmacológicos para avaliar a participação de mecanismos 5-HT. Nos capítulos 2 e 3, foram utilizados ratos da linhagem Wistar Audiogenic Rats (WAR), um modelo animal de epilepsia suscetível à crise convulsiva audiogênica. No capítulo 2, nossos resultados mostraram que, comparado ao grupo controle Wistar, os ratos neonatos WAR apresentam mais apneias e uma resposta ventilatória à hipóxia diminuída apenas nos primeiros dias de vida (P1-3, P-dias pós natais). Além disso, essas alterações respiratórias não persistem durante o desenvolvimento (P12-14 e P21-23). Ademais, nas três idades estudadas não houveram diferenças nas respostas ventilatórias à hipercapnia. Interessantemente, a atenuação da quimiossensibilidade central foi observada em adultos apenas após crises convulsivas repetidas. Também foi observado que ratos WAR em P12-14 apresentam uma maior susceptibilidade a convulsões induzidas por hipóxia. Dessa maneira, no capítulo 3 exploramos crises convulsivas hipóxicas durante o período neonatal no modelo WAR. Para avaliar a atividade respiratória em neonatos durante crises convulsivas agudas, foi utilizado o modelo crise induzida por exposição à hipóxia em neonatos P10, e ambos Wistar e WAR foram submetidos ao protocolo. Durante crises convulsivas neonatais, ratos WAR mostraram uma maior susceptibilidade às crises mioclônicas e uma ventilação pós-hipóxia reduzida em comparação com os ratos Wistar, ressaltando a vulnerabilidade respiratória dessa linhagem. A atividade respiratória no protocolo de hipóxia foi mediada pelos receptores serotoninérgicos tipo 2, sugerindo um papel excitatório da transmissão 5-HT na resposta fisiológica às crises convulsivas. No capítulo 4, em outro modelo animal de crises audiogênicas (ratos adultos $SD^{Kcnj16-/-}$) foi observado que as crises convulsivas tônico-clônicas generalizadas (GTCS) estavam associadas à apneia ictal, disfunção ventilatória e redução na expressão de 5-HT após crises repetidas. Apesar dessas alterações, não foi observada mortalidade relacionada às crises nesses ratos. A depleção de serotonina com o fármaco PCPA, em modelo similar (ratos adultos $SS^{Kcnj16-/-}$) foi associada a um aumento na mortalidade, o que reforça a importância da serotonina na regulação das respostas durante as crises. Esses achados destacam a complexidade das interações entre crises convulsivas, função respiratória e neurotransmissores como a serotonina, oferecendo novas perspectivas para o estudo de condições graves como SUDEP e SIDS, além de abrir caminho para futuras investigações sobre as bases neurofisiológicas da atividade respiratória nas convulsões e suas complicações.
Palavras chaves: crises convulsivas; atividade respiratória; quimiorreflexo; morte súbita.

ABSTRACT

Seizures affect respiratory activity, both during and after convulsive events, inducing mild and transient apneas to fatal apneas. Furthermore, recurrent seizures induce changes in central chemoreception to CO_2/pH. These factors are related to sudden death in epilepsy (SUDEP), however the respiratory control mechanisms related to this have not yet been elucidated. Seizures can affect children and adults, with the highest incidence during the neonatal period. Evidence suggests the importance of serotonin for respiratory activity during seizures. That said, the general objective of this thesis was to study respiratory control in animal models of epilepsy (neonates and adults), evaluating serotonergic mechanisms (5-HT). The experimental approaches used were whole-body plethysmography for measurements of pulmonary ventilation, O2 consumption and body temperature. Additionally, neurochemical, immunohistochemical and pharmacological methods were used to evaluate the participation of 5-HT mechanisms. In Chapters 2 and 3, we used Wistar Audiogenic Rats (WAR), an animal model of epilepsy susceptible to audiogenic seizures. In Chapter 2, our results showed that, compared to the Wistar control group, neonatal WAR rats present more apneas, and a decreased ventilatory response to hypoxia in the first days of life (postnatal (P) days P1-3). Furthermore, these respiratory alterations do not persist during development (P12-14 and P21-23). Moreover, at the three ages studied, there were no differences in ventilatory responses to hypercapnia. Interestingly, attenuation of central chemosensitivity was observed in adults only after repeated seizures. We also observed that WAR rats at P12-14 present an increased susceptibility to hypoxia-induced seizures. Thus, in Chapter 3 we explore hypoxic seizures during the neonatal period in the WAR model. To evaluate respiratory activity in neonates during acute seizures, the seizure model induced by exposure to hypoxia in P10 neonates was used, and both Wistar and WAR rats were subjected to the protocol. During neonatal seizures, WAR rats showed a greater susceptibility to myoclonic seizures and reduced post-hypoxia ventilation compared to Wistar rats, highlighting the respiratory vulnerability of this strain. Respiratory activity in the hypoxia protocol was mediated by type 2 serotonin receptors, suggesting an excitatory role of 5-HT transmission in the physiological response to seizures. In chapter 4, in another animal model of audiogenic seizures ($SD^{Kcnj16-/-}$ adult rats), it was observed that generalized tonic-clonic seizures (GTCS) were associated with ictal apnea, ventilatory dysfunction, and reduced 5-HT expression after repeated seizures. Despite these changes, no seizure-related mortality was observed in these rats. Serotonin depletion with PCPA in a similar model ($SS^{Kcnj16-/-}$ adult rats) was associated with increased mortality and significant changes in postictal ventilatory response, reinforcing the importance of serotonin in regulating responses during seizures. These findings highlight the complexity of the interactions between seizures, respiratory function and neurotransmitters such as serotonin, offering new insights into the study of severe conditions such as SUDEP and SIDS, and paving the way for future investigations into the neurophysiological basis of respiratory activity in seizures and their complications.

Keywords: Seizures; respiratory activity; chemoreflex; sudden death.

LIST OF ABBREVIATIONS

5-HT – 5-hydroxytryptamine, serotonin

CNS – Central Nervous System

cpm – cycles per minute

fR – respiratory frequency

GFP – green fluorescent protein

GPCR - G protein coupled receptor

GTCS – Generalized Tonic-Clonic Seizure

HCVR - hypercapnic ventilatory response

HIE - hypoxic-ischemic encephalopathy

HPLC - high performance liquid cromatography

HVR - hypoxic ventilatory response

NTS – Nuclei of tract solitary

PB – Parabrachial nuclei

PCPA - Para-chlorophenylalanine, tryptophan hydroxylase inhibitor

RA - room air

RMg - Raphe Magnus

ROb - Raphe Obscurus

Rpa/Rpy - Raphe Pallidus and Raphe Pyramidal

RTN - Retrotrapezoid nuclei

SD - Sprague Dawley rats

SIDS - Sudden Infant Death Syndrome

SS - Salt Sensitive Dahl rats

SUDEP – Sudden Unexpected Death in Epilepsy

SUS - united health system

V_E – pulmonary ventilation

VO_2 - oxygen consumption

VRC – Ventral Respiratory Column

V_T - tidal volume

WAR - Wistar Audiogenic Rats

5-HIIA - 5-Hydroxyindoleacetic

LIST OF FIGURES

Sumário

Chapter 1: LITERATURE REVIEW

1.1. General view

Breathing is an essential rhythm for life. Some pathologies may alter breathing and this disruption can lead to life-threatening consequences. In this study, we evaluated respiratory activity in animal models of epilepsy in order to better comprehend how seizures or predisposition to seizures may promote breathing alterations, focusing on the neural control of breathing, serotonergic neurotransmission and epilepsy. Herein we assessed breathing prior to the occurrence of seizures throughout development (i.e., in neonatal and adult periods) during hypercapnia and hypoxia challenges and during acute seizure events. In addition, the participation of serotonin was evaluated with different methodological tools, focusing on its involvement in the brainstem. This thesis is divided into four chapters. The first chapter provides a general review, while the remaining three chapters present collected data in Brazil at the ICB/UFMG (Chapter 2 and 3) and in the United States at the Medical College of Wisconsin (Chapter 4). Chapter 1 will cover the topics of control of respiratory activity, seizures, the relationship between convulsive seizures and respiratory activity and the role of serotonin in this context. Chapter 2 main objective is to evaluate the predisposition of breathing alteration throughout development in rats susceptible to audiogenic seizure. In chapter 3, breathing was evaluated during acute seizures induced by hypoxia in neonatal rats. And in chapter 4, breathing was evaluated in mutant rats for *Kcnj16* gene, a audiogenic seizure animal model.

1.2. Breathing control

Breathing control system can be simplified into three main components: effectors, central controller and sensors (Greer & Funk, 2013). These three components work together to form eupnea, which is the normal respiratory activity, capable of adapting to external changes (e.g., ambient temperature, gas concentration) and internal changes (e.g., sleep, exercise). Respiratory neural control

acts to generate pulmonary ventilation determined by respiratory rate and tidal volume, the main variables measured in this study. The lung performs gas exchange due to the airflow in the lungs generated by these components. The effectors of respiratory activity, which are the muscles, are controlled by the central nervous system (CNS), which receive and modulate information from the sensors (figure 1.1). This system determines the fundamental rhythm of life, which is respiratory activity, and therefore failures in this control system can be life-threatening.

The diaphragm is the main respiratory muscle. It is a muscle that acts as a pump, in which inspiration is determined by contraction and (passive) expiration is determined by its relaxation (Del Negro et al., 2018). Other muscles, such as those of the upper airways and abdominal muscles, are also important for respiratory activity, and their synchrony is perfectly coordinated by the CNS (Greer & Funk, 2013). Motor nerves such as the phrenic nerve (PN), hypoglossal nerve (HN) and vagus motor nerve (cVN) are components of the CNS and are responsible for activating the diaphragm muscles, the tongue muscles and the larynx muscles, respectively (Bittencourt-Silva et al., 2020; Del Negro et al., 2018). The generation of the respiratory pattern and rhythm is governed by a hierarchy, where pattern-generating neurons modulate the activity of premotor neurons, which in turn are responsible for activating motor neurons that will activate the muscles (Greer & Funk, 2016). The central controller, which controls respiratory activity, is located in regions of the medulla and pons, with regions such as pre-Bötzinger standing out, which contains neurons responsible for generating the rhythm and respiratory pattern and is considered the primary oscillator of respiration (Smith et al., 1991). The rVRG and cVRG groups contain premotor neurons, responsible for inspiratory and expiratory activity, respectively (Del Negro et al., 2018; Dhingra et al., 2020). These regions are located in the ventral region of the medulla and together are called the ventral respiratory column (VRC) (Del Negro et al., 2018; Greer & Funk, 2016). The dorsal region of the medulla contains the NTS (Nuclei of Solitary Tract), which receives information from sensors, including the carotid body, baroreceptors, and pulmonary mechanoreceptors (Guyenet, 2014). In this way, the information from the sensors is integrated into the NTS. The NTS projects to the pons, and this connection between the NTS and the pons is extremely important for determining the end of inspiration, and is therefore important for adjustments to the respiratory pattern (Dutschmann & Dick, 2012). The pons contains two regions, the

Kolliker Fuse (KF) and the Parabrachial Region (PB), which are responsible for connecting more prosencephalic regions to these respiratory regions, coordinating behaviors such as speech and swallowing (Del Negro, Funk, and Feldman 2018; Dutschmann and Dick 2012). Among the sensors that can modulate respiratory activity, the following stand out: 1) lung distension sensors, responsible for the Hering-Breuer reflex, an important sensory afferent signal for determining the end of inspiration (Dutschmann and Dick 2012); 2) baroreceptors, which are arterial blood pressure sensors (Baekey et al., 2010); 3) the carotid body, also called peripheral chemoreceptors, which is responsible for monitoring the partial pressure of O_2 and CO_2 in the arterial bloodblood (Kumar & Prabhakar, 2012); and 4) regions in the CNS that are also sensitive to CO_2/pH and are called central chemoreceptors (Nattie & Li, 2012). These chemoreceptors, both central and peripheral, are crucial for maintaining blood gas homeostasis and are responsible for chemoreflex responses. This means that the reduction in PaO_2 and/or the increase in $PaCO_2$ generates a reflex response of increased ventilation and the failure of this reflex can lead to death.

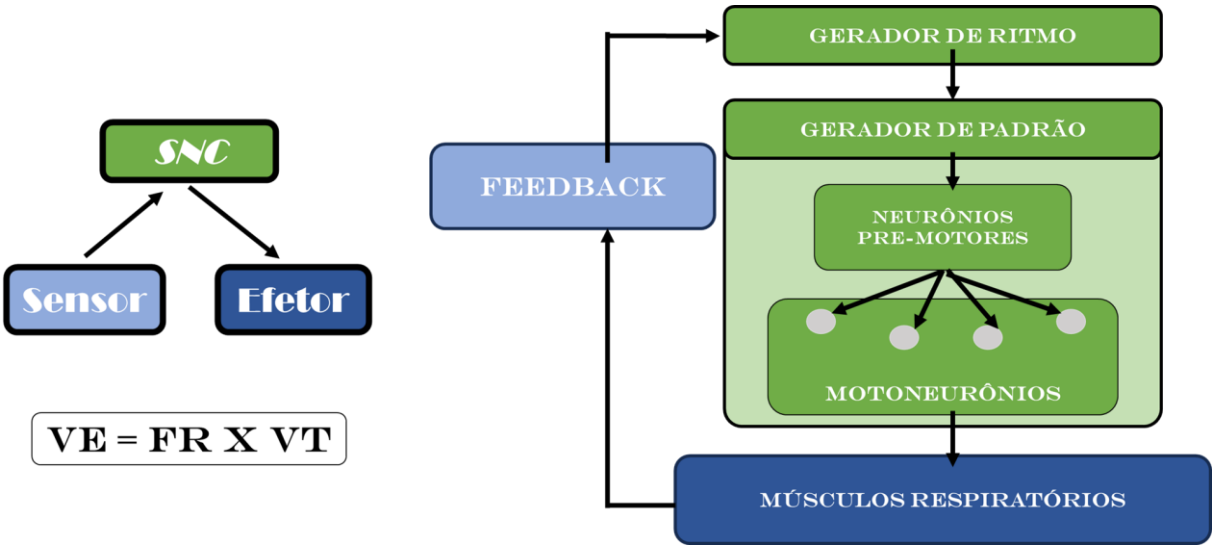

Figure 1.1 Schematic of respiratory control components. In green, components of the central nervous system (CNS), in dark blue, effectors, and in light blue, sensors. This figure was adapted from Greer and Funk 2016.

Peripheral chemoreceptors are located at the bifurcation of the carotid arteries and in the aortic arch (Kumar & Prabhakar, 2012). They are small, highly vascularized organs containing specialized cells (type 1 glomus cells) that detect oxygen through cellular mechanisms that are not yet fully elucidated (Conde et al., 2024; López-Barneo

et al., 2001). What is known about the mechanism in these cells is that a reduction in O_2 promotes the closure of potassium channels and membrane depolarization, leading to neurotransmitter release and signaling the O_2 concentration to the CNS (Conde et al., 2024; P. Kumar & Prabhakar, 2012; López-Barneo et al., 2001). In hypoxic situations, there is an increase in the firing frequency of the neuron that sends this information to the NTS (Del Negro et al., 2018; Kumar & Prabhakar, 2012). The NTS acts as a hub for inputs, coordinating excitatory and inhibitory projections to the VRC and the pons, which alters the respiratory pattern, forming part of a system that adjusts ventilation moment by moment (Costa-Silva et al., 2010). In a hypoxic situation, these sensors are quickly responsible for increasing ventilation in response to the stimulus. In cases of prolonged hypoxia, other parallel mechanisms are activated, such as metabolic reduction and paracrine neurotransmitter release by astrocytes, which modulate respiratory activity (Rajani et al., 2018; Teppema & Dahan, 2010; Zoccal et al., 2024).

As for central chemoreceptors, their location is diffuse. Several regions containing cells capable of causing respiratory changes under acidic conditions can be found in the medulla, pons, and even the hypothalamus (Nattie & Li, 2012). These regions share the presence of cellular mechanisms sensitive to pH, as the CO_2 reacts with water to form a weak acid that dissociates and produces H+, promoting a reduction in pH. The criteria that determine a chemosensitive region are related to the ability of neurons in this region to increase fire and promote an increase in respiratory activity in response to elevated CO_2 (Guyenet et al., 2016; Kumar et al., 2015; Wang et al., 2013), determined, for example, by mechanisms involving proteins sensitive to protons, such as membrane proteins like TASK2 (potassium channel) or GPR4 (G protein) (Kumar et al., 2015; Wang et al., 2013). A notable chemosensitive region is the Retrotrapezoid Nucleus (RTN), identified by some researchers as the primary and sometimes the sole area responsible for central chemoreception (Guyenet et al., 2016). This region is indeed important, and ablation of this area leads to a decrease in the ventilatory response to hypercapnia (Souza et al., 2018; Takakura et al., 2014). However, other regions, such as the Locus Coeruleus (de Carvalho et al., 2014; Gargaglioni et al., 2010) and the Medullary Raphe (Bradley et al., 2002; Cerpa et al., 2017; Da Silva et al., 2011) also meet the criteria that determine chemosensitive capacity.

1.3. Development of Respiratory Control

The respiratory neural network begins to form during the embryonic period, and even during intrauterine life, episodic respiratory movements can already be observed (Greer et al., 2006). However, this network is not fully mature at birth. Various developmental processes occur in the brain as a whole, including in the respiratory neural network, related to synaptic programming and pruning (Hilaire & Duron, 1999; Lindhout et al., 2024). Birth is a crucial event, as before it, in the intrauterine environment, the fetus receives oxygen through the placenta, not performing pulmonary gas exchanges independently, and is exposed to relatively low levels of oxygenation (Hilaire & Duron, 1999; Mouradian, Lakshminrusimha, et al., 2021). At birth, the first breath draws air into the lungs, opening the alveoli for the first time, and oxygen levels are higher than previously accustomed, requiring metabolic adaptation (Greer et al., 2006). Therefore, the first days of life are critical for the organism's development. In rats, between postnatal day 1 (P1) and P3 occurs a crucial initial adaptation phase (Borday et al., 1997; Wong-Riley et al., 2019). Studies characterizing the development of respiratory regions observed that between postnatal days 3 and 4, there is a period of synaptic and cellular metabolism adjustment through cytochrome oxidase, which increases in regions such as the NTS and VRC (Liu & Wong-Riley, 2003). These changes are physiological and part of system maturation. However, alterations at this age can have long-term consequences.

At P10, there is a significant shift in the balance between excitation and inhibition, marking a critical period with several physiological changes (Lindhout et al., 2024). These alterations are associated with modifications in membrane potential and protein expression (Pfeffer et al., 2009). The GABA-A receptor, which is excitatory early in life, becomes inhibitory. The chloride current generated by this receptor is initially an outward current and after P10 becomes an inward current (Ben-Ari, 2014; Cherubini et al., 1991). The GABA -A receptors are crucial since the balance between excitation and inhibition in the respiratory neural network is influenced by GABAergic and glycinergic activity (Del Negro et al., 2018; Wollman et al., 2018). Glutamatergic transmission also undergoes changes during this period (~P10), due to modifications in the expression of AMPA and NMDA receptors (Berger, 2011; Liu & Wong-Riley, 2002).

Furthermore, there are notable changes in CO_2 sensitivity during development (Darnall, 2010; Stunden et al., 2001). Chemosensitivity to CO_2/pH is elevated in neonates one day after birth (P1) and decreases over the first week, reaching a minimum around P8 (Stunden et al., 2001). At P14, a significant change is observed in the activation profile of serotonergic neurons related to the chemoreflex (Cerpa et al., 2017; Mouradian, Kilby, et al., 2021). Locus coeruleus contributes to the whole animal hypercapnic ventilatory response adult and neonate Wistar as well (Biancardi et al., 2008; Stunden et al., 2001), and show distinction of chemosensitivity before and after P10 in slices (Gargaglioni et al., 2010).

The development of the hypoxic ventilatory response (HVR) is a complex process shaped by interactions between excitatory and inhibitory influences. Hypoxia (due to asphyxia, ischemia, etc.) can be common in the first days of life due to birth complications (Samaiya et al., 2021). These situations are alarming because of their acute and long-term effects (Russ et al., 2021). Initially, the ventilatory response to hypoxia in early life is low compared to adults (Liu et al., 2006; Moss, 2000; Mouradian, Lakshminrusimha, et al., 2021). In rats, until postnatal day 10 (P10), the carotid body shows low sensitivity to O2; after P10, it begins to undergo modifications and is established as a mature organ that lasts throughout life (Kumar & Prabhakar, 2012).

During hypoxia, the ventilatory response follows a biphasic pattern in neonates and adults. Initially, there is an increase in ventilation (Phase I) driven by excitatory inputs, but this is followed by a ventilatory depression (Phase II), which is attributed to a withdrawal of excitatory inputs and an increase in inhibitory neurotransmitters and neuromodulators like GABA and adenosine (Day & Wilson, 2023; Reeves & Gozal, 2005). These molecules play a central role in mediating hypoxic ventilatory depression. The biphasic HVR undergoes significant changes during postnatal development. In neonates, the initial rise in ventilation during Phase I is brief, and the ventilatory depression in Phase II is more pronounced than in adults, often leading to a reduction in ventilation below baseline levels (Day & Wilson, 2023; Moss, 2000). A key contributor to this hypoxic ventilatory depression is a time-dependent decline in the oxygen sensitivity of the carotid body (Day & Wilson, 2023; Kumar & Prabhakar, 2012), as well as central mechanisms, including a reduction in thermoregulatory set-point and body temperature, leading to decreased oxygen consumption (Mortola & Naso, 1998;

Saiki et al., 1994). Another contributor is the purinergic signaling responsible for modulating the glutamatergic inspiratory synapse in the preBötzinger Complex (Reklow et al., 2019). These central and peripheral factors combine to depress ventilation during prolonged hypoxia. Thermoregulation development occurs around the P10 stage, highlighting the importance of this period in rats (Bravo et al., 2017; Mortola & Naso, 1998). It is worth noting that the P10 to P14 phase in rats corresponds to the first months of life in humans, extending up to approximately one year (Ben-Ari, 2014; Sun et al., 2016). Weaning in rats occurs around P21 and marks a transition to the adult period, where respiratory activity and body temperature control are already mature. Puberty in rats occurs between P30-45 and constitutes another developmental window, heavily modulated by hormonal influences, important for brain and organism development (Klein & Romeo, 2013).

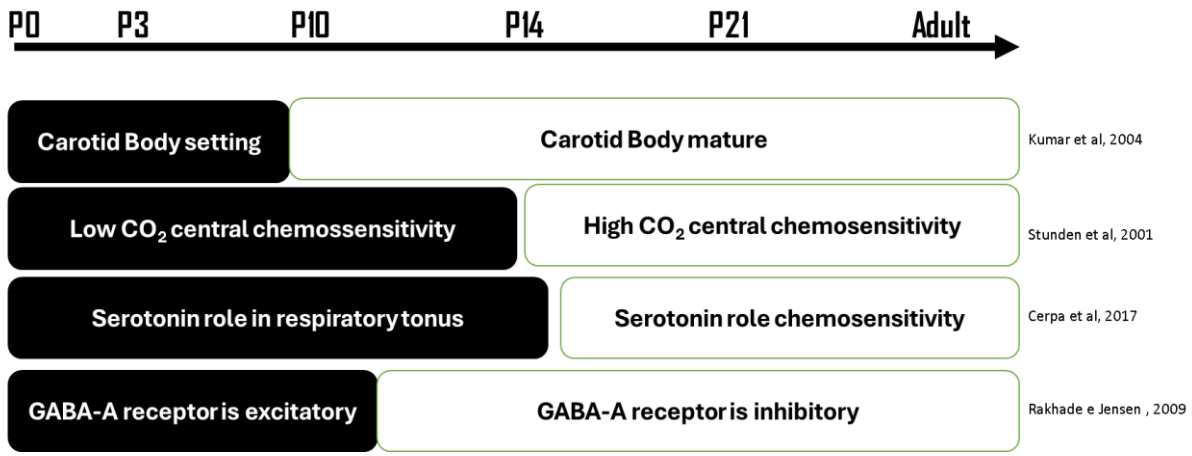

Figure 1.2. Schematic illustrating the ontogeny of components of the respiratory control system. The development of the carotid body, central CO2 chemosensitivity, the role of serotonin in respiratory tone and chemosensitivity, and the transition from excitatory to inhibitory function of GABA-A receptors are shown at different ages.

Serotonin (5-HT) plays a pivotal role in respiratory control during development. It is a monoamine synthesized from tryptophan via the action of the enzymes tryptophan hydroxylase 1 and 2 (TPH1 and TPH2), and kynurenic acid (Bravo et al., 2017; Deneris & Gaspar, 2018; Okaty et al., 2019). The serotonergic system has broad effects on respiratory activity, particularly in maintaining eupnea (normal breathing) in the early stages of life (Cerpa et al., 2017; Hodges et al., 2009). In knockout mice lacking the serotonin precursor gene (*Lmx1b*), the absence of serotonin leads to

frequent and severe apneas during the neonatal period, highlighting its critical role in respiratory stability (Hodges et al., 2009). However, between postnatal days (P12 and P21), the role of serotonin shifts, no longer providing tonic respiratory drive but instead contributing to respiratory chemoreception (Cerpa et al., 2017).

The development of the serotonergic system undergoes significant changes throughout early life (Cummings & Hodges, 2019; Liu & Wong-Riley, 2010a, 2010b; Wong-Riley et al., 2019). Notably, the expression of TPH and the serotonin transporter (SERT) decreases significantly around P12 in key brainstem regions involved in respiratory control, such as ventral respiratory column (VRC) (Liu & Wong-Riley, 2010b). These changes may correspond to shifts in serotonin's role in modulating breathing over time. For instance, the 5-HT1A receptor, which plays a role in inhibitory neurotransmission, also shows a significant drop in expression at P12, marking a developmental shift in serotonergic influence on respiratory control (Liu & Wong-Riley, 2010a).

The predominant effect of 5-HT on respiratory control is excitatory, with its influence varying by age and experimental conditions. Serotonin receptors, specifically 5-HT2, 4–7, are G protein-coupled receptors, while 5-HT3 receptors function as ligand-gated ion channels (Pytliak et al., 2011). Serotonergic projections from the medullary raphe extend to several respiratory-related nuclei (Figure 1.3), including the nucleus tractus solitarius (NTS), nucleus ambiguus, retrotrapezoid nucleus (RTN), pre-Bötzinger complex (pre-BötC), parabrachial nucleus (PB), and motor nuclei that control the phrenic and hypoglossal nerves (Cummings & Leiter, 2020). These projections also contain neuropeptides such as substance P (SP) and thyrotropin-releasing hormone (TRH), emphasizing serotonin's broad influence on respiratory regulation (Richerson, 2004; Szereda-Przestaszewska & Kaczyńska, 2020).

Serotonin is particularly important in responding to hypoxia, especially in terminating hypoxic apnea and triggering gasping, a survival mechanism during severe oxygen deprivation (Cummings & Leiter, 2019; John et al., 1985; Nuding et al., 2024a). Studies show that in neonatal animals, 5-HT, particularly through 5-HT2A and 5-HT2C receptors, facilitates gasping, which is crucial for autoresuscitation (Cummings, 2021; Peña & Ramirez, 2002). Deficiencies in the serotonergic system during early postnatal life, such as in Pet1$^{-/-}$ mice, lead to delayed gasping and a decrease in survival rates

due to the inability to effectively respond to hypoxia (Cummings, Hewitt, et al., 2011; Erickson & Sposato, 2009). Serotonin originating from the caudal raphe nucleus is critical in reversing hypoxic apnea and bradycardia, helping to restore normal respiratory rhythms during and after hypoxic events (Cummings, Commons, et al., 2011; Nuding et al., 2024b; Yang & Cummings, 2013). The maturation of serotonergic neurotransmission is crucial for effective respiratory control during early life, with serotonin playing key roles in both eupneic breathing and hypoxic recovery.

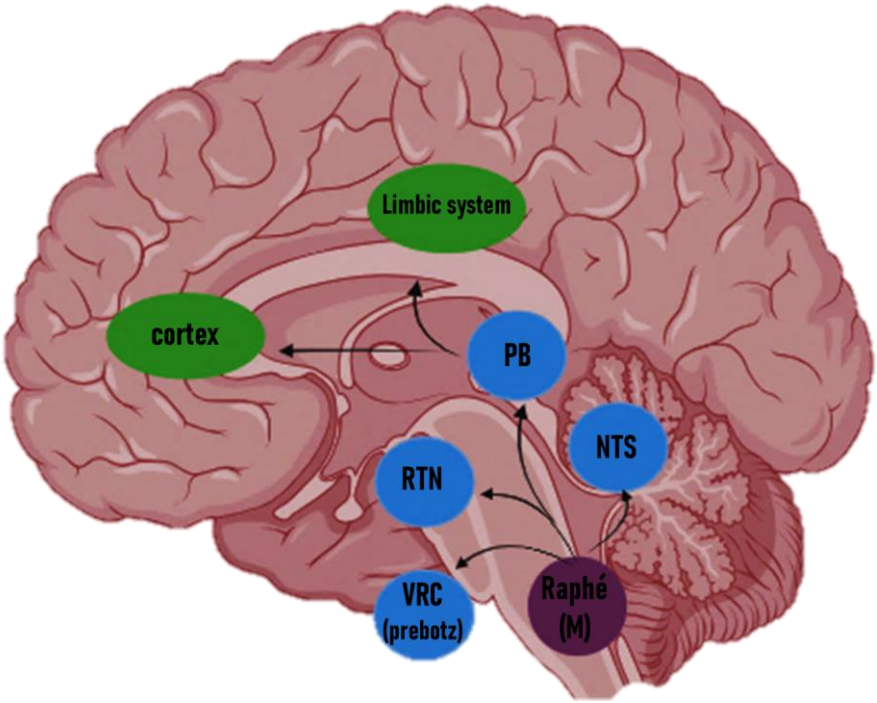

Figure 1.3. Medullary raphe projections to brainstem breathing regions. Serotonin originating from the medullary raphe interacts with different nuclei within the brainstem to organize the sequential processes that are essential to eupnea and hypoxic and hypercapnic breathing responses. Adapted Cummings & Leiter, 2020**.**

1.4. Seizures and epilepsy

Seizures are a brain disorder predominantly characterized by recurrent and unpredictable disruptions of normal brain function, typically initiated by an imbalance between excitation and inhibition in neural circuits (Devinsky et al., 2018; Dichter, 1994). According to the International League Against Epilepsy (ILAE), the definition of seizures is a transient occurrence of signs and/or symptoms due to excessive and/or synchronous neuronal activity in the brain (Fisher et al., 2014). Moreover, according to

the ILAE, to classify seizures as epilepsy, they must: 1) be spontaneous and occur at least twice in less than 24 hours, 2) and/or present a probability of a second occurrence of at least 60% in the next 10 years, and 3) and/or have a diagnosis of an epileptic syndrome (Fisher et al., 2014). The etiology of epileptic seizures can be related to structural, genetic, infectious, metabolic, immunological, and unknown factors (Devinsky et al., 2018).

The socioeconomic importance of epilepsy lies in its chronic nature and its impact on individuals' quality of life. Epilepsy affects approximately 5.8 per 1,000 people in developed countries (Bell et al., 2014; Devinsky et al., 2018) and around 17.8 per 1,000 people in Latin America (Burneo et al., 2005). In Brazil, about 2.7 million people have epilepsy (Noronha et al., 2007). Certain populations, such as an indigenous group in Mato Grosso, have higher incidence and prevalence of seizures, likely due to familial risk factors, as other factors were not significant (Borges et al., 2002). It is worth noting that the Unified Health System (SUS) is responsible for providing medical care to a large portion of this population. Through SUS, it is possible to have electroencephalograms, MRI scans, medical consultations, and receive medications free of charge. The most commonly used drugs in SUS are first-line, first-generation medications (e.g., sodium valproate), which provide effective control in up to 60% of cases. For the remaining cases, second-line (e.g., levetiracetam) and third-line drugs (e.g., phenytoin) are used (Noronha et al., 2007). However, approximately 30% of patients are refractory to treatment, both in Brazil and worldwide (Ferreira & Silva, 2009; Kwan et al., 2010). This is particularly concerning, as repeated seizures have a significant impact on the patient's biopsychosocial life and are associated with higher mortality (Hesdorffer et al., 2011).

Types of epilepsy are classified based on the origin of the seizures, which can be focal, generalized, or unknown (Fisher et al., 2005; Scheffer et al., 2017). Focal seizures begin in a specific part of the brain and can spread, while generalized seizures affect both hemispheres from the outset (Scheffer et al., 2017). Neuronal hyperexcitability is a key feature and plays a crucial role in the development of seizures (Rakhade & Jensen, 2009). In cases of genetic mutations, changes in ion channel functions and neurotransmitters can lead to neuronal hyperexcitability, as in mutations in the *Scn1A* gene (Dravet syndrome), which cause alterations in sodium channels, generally increasing neuronal excitability. In this case, an increase in temperature is

enough to trigger generalized seizures (Teran et al., 2023). Mutations in potassium channels, such as the *Kcnq2* (Grinton et al., 2015) or *Kcnj16* genes (Manis et al., 2023), also alter membrane biophysics and increase excitability. In *Kcnj16* knockout rats, hyperexcitability is related to audiogenic stimuli, indicating that it has a well-defined origin in auditory-related neuronal activation, spreading through the brain via synaptic mechanisms and/or extracellular potassium increase, also causing generalized seizures (Manis et al., 2021; Ross & Coleman, 2000).

Regarding the age group with the highest prevalence of seizures, childhood stands out, with rates ranging from 1 to 3.5 per 1,000 live births (Aicardi & Chevrie, 1970; Saliba et al., 1999). This period represents a sensitive phase of development and is vulnerable to excitability changes (Sun et al., 2016). One of the most common seizures is febrile seizures, affecting about 2-5% of all children (Stafstrom, 2002). These seizures usually occur due to febrile hyperthermia and are associated with infections (Barrett et al., 2024; Stafstrom, 2002). Two-thirds of children who experience a febrile seizure never have another episode (Holm et al., 2012).

Seizures during the neonatal period, regardless of etiology, develop into epilepsy in only about 17.9% of cases (Glass et al., 2016; Pisani et al., 2015). The number and duration of seizures can promote long-term neurological changes, such as cognitive delay or epileptogenesis processes (Castelhano et al., 2015; Shellhaas et al., 2021). The most common etiologies of repeated seizures are hypoxic-ischemic encephalopathy (HIE, 38%), ischemic arterial or venous stroke (18%), and intracranial hemorrhage (12%) (Glass et al., 2016). The incidence of seizures in these cases is high, with 59% of individuals experiencing seven or more electrographic seizures and 16% presenting with status epilepticus (Glass et al., 2016). High magnitudes of seizures, as seen in these cases, are associated with worse outcomes (Rakhade & Jensen, 2009), and the absence of medical treatment can be fatal. It is postulated that sudden neonatal deaths may be related to unreported/untreated seizures (Alharbi et al., 2023; Hesdorffer et al., 2011). This finding reinforces the importance of detecting and characterizing neonatal epileptic seizures. However, few studies in animal models evaluate seizures during the neonatal period (Haut et al., 2004).

In relation to experimental models of seizure induction, there are methodologies that use electrical, chemical, or sensory stimuli (or combinations), as well as those

produced with a known genetic background, usually through inbreeding or genetic engineering, such as transgenics and knockouts (Kasahara et al., 2018; Lopes-aguiar et al., 2014; Ross & Coleman, 2000). In rodents susceptible to audiogenic seizures, intense sound stimuli can cause changes in brain function, induce epileptogenesis, and mimic temporal lobe epilepsies (Dutra Moraes et al., 2000; Garcia-cairasco et al., 2017). This model is interesting for studying epilepsy characteristics under controlled conditions, and significant progress has been made in understanding the mechanisms of epileptogenesis. Regarding respiratory changes in audiogenic models, the DBA, WAR, and KCNJ16 knockout models stand out (Faingold et al., 2016; Garcia-cairasco et al., 2017; Granjeiro et al., 2016; Manis et al., 2021).

1.5. Relationship between Seizures and Respiration: Implications for SUDEP

Seizures are often accompanied by autonomic changes that can manifest in cardiovascular, gastrointestinal, urogenital, and respiratory systems (Blum, 2009; Freeman, 2006; Wannamaker, 1985). The changes caused by seizures in respiratory activity can be acute or prolonged. Acutely, seizures can even stop respiratory activity, depending on the type of seizure (Bateman et al., 2008). In absence seizures, there are no significant changes in the respiratory pattern (Umezu et al., 2024), but in generalized tonic-clonic seizures (GTCS), ictal apneas and severe bradycardia are common (Manis et al., 2023; Seyal & Bateman, 2009; Teran et al., 2023; Umezu et al., 2024; Wenker et al., 2022). These apneas are associated with temporal lobe epilepsy, and recent evidence points to specific regions of the amygdala (e.g., basolateral amygdala), whose activation in patients inhibits respiratory activity and induces GTCS (Dlouhy et al., 2015; Harmata et al., 2023; Rhone et al., 2020). Along with ictal apneas, there is a reduction in blood oxygen concentration and an increase in CO_2 levels, with prolonged apneas causing more severe alterations in blood gasses (Bateman et al., 2008; Lacuey et al., 2018; Seyal & Bateman, 2009).This prompts the question of how seizures may disrupt respiratory patterns and potentially influence chemoreflex mechanisms—an evolutionarily conserved system typically regarded as independently to sustain eupnea.

In the long term, repeated seizures are associated with changes in chemoreflex and cardiovascular functions (Akyüz et al., 2021; Hampson et al., 2022; Sainju et al., 2019). Although respiratory dysfunctions in individuals with different types of epilepsy are not uncommon, they are under-investigated and, consequently, underdiagnosed (Devinsky et al., 2016). Recently, evidence has emerged showing the relationship between respiratory changes and sudden death in individuals with epilepsy (Sudden Unexpected Death in Epilepsy, SUDEP). The occurrence of sudden unexpected death in individuals with epilepsy has been reported in studies dating back nearly a century (Blum, 2009). Sudden unexpected and unexplained death is substantially more common in individuals with epilepsy than in the general population, even leading to the creation of the term SUDEP. Its incidence ranges from 1/1000/year in well-controlled epilepsy patients to 1/100/year in patients with severe, treatment-refractory epilepsy (Abdel-Mannan et al., 2019; Hesdorffer et al., 2011).

SUDEP represents a significant concern, especially considering the lack of knowledge about SUDEP among healthcare professionals, which is alarming (Kroner et al., 2014; Mosini et al., 2022). In Brazil, A study revealed that 20% of healthcare professionals in the Epilepsy League do not know what SUDEP is, and 18.2% admitted to being unaware of potential risk factors for this condition (Mosini et al., 2022). Furthermore, 84.1% of respondents do not discuss, or discuss with only a few of their patients, the risk factors for SUDEP (Mosini et al., 2022). These numbers are concerning, as 31.4% of epilepsy patients and their caregivers have never heard of SUDEP, as evidenced by (Kroner et al., 2014). This lack of awareness may contribute to underreporting and inadequate prevention of preventable deaths among young people with epilepsy in Brazil.

Although knowledge of the pathophysiological mechanisms involved in SUDEP is undoubtedly important for its prevention, little is known about this phenomenon. The few clinical and experimental studies on SUDEP published so far do not provide conclusive results, adding very little to the understanding of its pathophysiology (Harden et al., 2017). While the pathophysiology of SUDEP remains largely unknown, various clinical and animal studies point to the involvement of the respiratory system as a key component in SUDEP. It is now known that seizures occur immediately before the cascade of events leading to cardiorespiratory arrest in GTCS. This data suggests that the main mechanism leading to SUDEP begins with a severe, centrally mediated

alteration of both respiratory and cardiac functions following GTCS (Ryvlin et al., 2013). Depending on its intensity, postictal neurovegetative collapse, characterized by ictal apnea and bradycardia, may lead to immediate death or delayed terminal cardiorespiratory arrest after several minutes of altered cardiorespiratory function, most likely exacerbated by deep hypoxia (Ryvlin et al., 2013). One hypothesis is that intrinsic mechanisms that lead to seizure termination (or are associated with it) cause this collapse and the generalized postictal EEG suppression.

It is important to highlight that 20% of cases occur before the age of 20 (Ferreira & Silva, 2009) (olhar outra referencia , pq essa é em portugues). In reality, it is postulated that SUDEP cases in children are underreported and determined as Sudden Infant Death Syndrome (SIDS). Among SIDS cases, 31.7% had a history of febrile seizures, of which 74.4% presented simple febrile seizures. In these SIDS cases, the prone position at the time of death, death during sleep, and unwitnessed deaths predominated (Hesdorffer et al., 2011). Preventing the risk of asphyxia in the sleep environment should be combined with efforts to understand intrinsic biological pathways, some potentially associated with other categories of infant and perinatal mortality (Goldstein et al., 2016a). The serotonergic hypothesis for SIDS proposed by (Kinney & Haynes, 2019) suggests that a significant subset of infants with SIDS exhibit abnormalities in the serotonergic system, resulting from a "central lesion" in the medullary reticular formation, where serotonin neurons are located (Duncan, 2018; Haynes et al., 2023). This lesion may lead to the failure of brainstem protective responses to homeostatic challenges during sleep at a critical developmental period, resulting in sleep-related sudden death. The model suggests that SIDS occurs when three factors combine: (i) a vulnerable infant with an underlying pathophysiological abnormality that places them at risk for sudden death; (ii) a critical developmental window, which is the first few months of life; and (iii) an external stressor that triggers sudden death during sleep, such as prone sleeping position, excessive blankets, soft bedding, and bed-sharing (Duncan, 2018). These external factors can cause homeostatic/metabolic imbalances, such as hypoxia, asphyxia, hypercapnia, and hyperthermia, challenging the brainstem's homeostatic responses. Interestingly, these factors are shared by SUDEP (Buchanan, 2019a). The serotonin system, which is involved in arousal, exhibits defects in the caudal medullary reticular formation, contributing to this failure. The progression of asphyxia leads to loss of consciousness

and hypoxic coma, followed by extreme bradycardia and hypoxic gasping, a process that would normally attempt to restore breathing. However, in cases of sudden death, this autoresuscitation response fails, leading to death.

Chapter 2: GENETIC SUSCEPTIBILITY TO EPILEPSY AND RESPIRATORY CHANGES DURING NEONATAL DEVELOPMENT

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2.1 INTRODUCTION

Neonatal seizures are a common manifestation of neurological disorders in newborns (Glass et al., 2016; Shields, 2006). In fact, the highest prevalence of seizures occurs during infancy (Devinsky et al., 2018). Etiologies of neonatal seizures, such as genetic epilepsies, brain malformations, and hypoxic-ischemic encephalopathy, are particularly notable due to the severity of seizures, as a higher frequency or more severe seizures are associated with an increased risk of mortality (Devinsky et al., 2016; Glass et al., 2016; Hesdorffer et al., 2011). Many studies show that seizures are associated with changes in respiratory patterns in adult models, especially tonic-clonic seizures (Manis et al., 2023; Umezu et al., 2024; Wenker et al., 2022). Among the respiratory changes, ictal and post-ictal apnea are particularly notable, requiring careful clinical observation due to hypoxemia (Bateman et al., 2008; Lacuey et al., 2018; Seyal & Bateman, 2009). However, respiratory changes related to seizures throughout neonatal development remain unknown.

Sudden Unexpected Death in Epilepsy (SUDEP) is a significant health concern, recognized as the most common cause of death in patients with epilepsy (Hesdorffer et al., 2011). Recent evidence indicates that SUDEP is more frequent in pediatric cases than previously thought (Hefti et al., 2016; Sveinsson et al., 2017). In fact, SUDEP in children is often mislabeled as sudden infant death due to the similarities, as both diagnoses are exclusionary, without clear evidence of the underlying cause of death (Buchanan, 2019b; Richerson & Buchanan, 2011). For SUDEP, evidence shows that seizures result in terminal apnea preceding cardiac asystole, with oxygen desaturation resulting from apnea and the absence of resuscitation (Ryvlin et al., 2013). In the case of SIDS, there are indications of oxygen desaturation, likely exacerbated by the lack of arousal when lying prone in the crib (Duncan, 2018; Li et al., 2018). The failure of

autoresuscitation occurs in both situations, illustrating the critical importance of ventilatory responses to hypoxia as a life-preserving mechanism.

Another similarity between SUDEP and SIDS is the reduced levels of serotonin in the brain. Evidence shows this characteristic in the brainstem of both newborns and adults with sudden and unexpected deaths (Duncan, 2018; Goldstein et al., 2016a; Haynes et al., 2023; Patodia et al., 2018). Its importance stems from the role of serotonergic neurons in regulating many physiological functions, including mood, respiration, body temperature, and sleep (Bravo et al., 2017; Hodges et al., 2009). In clinical practice, serotonin reuptake inhibitors are widely used as antidepressant medications, and the literature provides evidence of the anticonvulsant effects of 5-HT-enhancing drugs, which can raise the seizure threshold and reduce seizure-related mortality (Buchanan et al., 2014). In respiratory control, the role of serotonin is particularly pronounced during the neonatal period. In the early postnatal stages, these neurons contribute to the continuous drive of the respiratory network (Cerpa et al., 2017). During low oxygen conditions, serotonin is released to regulate respiratory and cardiovascular responses (Herman et al., 1999; Yang & Cummings, 2013), making it relevant in the context of SIDS (Haynes et al., 2023). In adults, serotonergic modulation of respiration plays a more specific role in increasing ventilation during hypercapnia (Da Silva et al., 2011).

Notably, many animal models of epilepsy show decreased serotonergic presence in the dorsal and medullary raphe (Joyal et al., 2023; Lin et al., 2013; Manis et al., 2023; Totola et al., 2017). In the case of the Wistar Audiogenic Rat (WAR) strain, which exhibits audiogenic seizures characterized by tonic-clonic activity in response to acoustic stimuli, there is a significant reduction in the number of 5-HT neurons identified, specifically in the raphe obscurus and raphe pallidus regions (Totola et al., 2017). Additionally, adult WAR rats exhibit a notable alteration in their ability to adapt ventilation to changes in blood gases (elevated $PaCO_2$ or reduced PaO_2) (Granjeiro et al., 2016), making them a suitable model for further investigation into the respiratory risks associated with epilepsy. However, it is unknown whether these characteristics are intrinsic to their genetic background and whether they may be related to respiratory changes during development.

In the present study, we explored the hypothesis that the WAR strain exhibits respiratory alterations during the neonatal period, suggesting that the genetic background that makes the WAR strain susceptible to seizures also determines respiratory changes. To this end, we evaluated respiratory and metabolic parameters, along with monoamine levels in the ventral respiratory column, in Wistar and WAR animals at three different age stages, under ambient air, hypoxia, and hypercapnia conditions. Additionally, we examined innate reflexes as a means of assessing neurodevelopment. Our results show that during the early stages of development, WAR rats exhibit alterations in innate reflexes, apnea duration, hypoxic ventilatory responses, and monoamine release, thus suggesting disrupted maturation or altered neural activity. Another interesting finding was the seizure susceptibility in WAR rats aged P12-14 during hypoxic stimuli. This may represent a promising new tool for investigating hypoxia-induced seizures as an experimental dynamic to gain deeper insights into SUDEP and SIDS.

2.2 OBJECTIVE

2.2.1 General Objective

Characterize the ventilatory pattern of WAR rats during development.

2.2.2 Specific Objectives

1. Evaluate the respiratory pattern of neonates at P1-3, P12-14, and P21-23 from the WAR and Wistar strains under baseline conditions.
2. Evaluate the respiratory responses of neonates at P1-3, P12-14, and P21-23 from the WAR and Wistar strains when subjected to acute exposure to hypoxia (10% O2) and hypercapnia (4% and 7% CO_2).
3. Assess the content of monoamines (serotonin, norepinephrine, and dopamine) in respiratory regions of the brainstem using high-performance liquid chromatography (HPLC).
4. Compare the CO_2 ventilatory response of adult Wistar and WAR rats before and after audiogenic seizure events.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Male and female rats at different developmental stages from Wistar and Wistar Audiogenic Rats (WAR) were used in this study. The Wistar animals (control group) were obtained from the Bioterism Center at the Federal University of Minas Gerais (CEBIO/UFMG), and the WAR animals were obtained from the colony at the Department of Physiology and Biophysics at UFMG. The WAR colony was maintained by breeding male and female WARs with a high incidence of severe audiogenic seizures. The audiogenic seizure was conducted as previously described (Dutra Moraes et al., 2000; Garcia-cairasco et al., 2017). Briefly, acoustic stimulation was performed as a screening test to verify the susceptibility to audiogenic seizures. This stimulation consisted of exposing the animals, over three consecutive days, to a 120 dB sound stimulus and evaluating the presence and severity of audiogenic seizures (Garcia-Cairasco et al., 1996). The acoustic stimulation was performed when the animals were 60 days old. The behavioral seizure progression involved: (1) running, (2) running-jumping-falling, (3) generalized limb seizure (clonus), and (4) limb tonic seizure. The animals were classified using a seizure severity score (SI; severity index) ranging from 0 to 1 (where SI=0 represents no seizure and SI=1 represents maximum severity). All WARs used in this study had an SI > 0.85. The breeding pairs were maintained through inbreeding, ensuring the continuity of the lineage with the characteristic of seizure susceptibility (Garcia-cairasco et al., 2017). In the study, characteristics were evaluated at three developmental ages: postnatal days 1-3, 12-14, and 21-23. Male and female data were aggregated once we determined that there was no significant difference between sexes within the group. The neonates were kept with their mothers until the day of the experiment or weaning at 21 days in both the WAR and Wistar strains. The litters were not acoustically stimulated, meaning all animals were naive in this study. However, all WAR neonates used in this study were born from parents with high seizure severity scores (>0.85). Both animal groups were kept in the animal facility of the Department of Physiology and Biophysics at UFMG with food and water ad libitum and a 12h/12h light-dark cycle (7 am–7 pm). All procedures were previously approved by the Animal Experimentation Ethics

Committee of UFMG (CEUA 78/2021). Each experimental group at the different ages consisted of neonates from at least two different litters.

2.3.2 Righting and Chewing Reflexes

Innate reflexes, such as the chewing reflex and the righting reflex, were evaluated in newborns (6h to 18h after birth, P0). The chewing reflex involves triggering chewing by placing a piece of polyethylene tubing (P50) in the animal's mouth and counting how many times the animal opens its mouth in 20 seconds (Biancardi et al., 2022; Lund & Kolta, 2006). The righting reflex involves placing the animal in a supine position on a surface and recording the time (in seconds) it takes for the animal to right itself with all four limbs on the surface in a prone position (Biancardi et al., 2022; Motta-Teixeira et al., 2018).

2.3.3 Pulmonary Ventilation Recording

Ventilatory measurements were performed on awake rats between 9 am and 5 pm. The setup was adjusted according to the animal's age and size. The pressure oscillation signals corresponding to breathing were acquired through a differential pressure transducer (Model DP45-14-2114, Validyne, Northridge, CA, USA), amplified (Model CD15, Validyne, Northridge, CA, USA), converted from analog to digital signal (PowerLab System, ADInstruments®, Sydney, Australia), and recorded in the LabChart signal acquisition program (version 7.3, ADInstruments®, Sydney, Australia). Respiratory frequency (fR) and tidal volume (VT) were measured from the pressure signal. Minute ventilation (VE) was calculated as the product of fR a V_T. The animals were weighed before the experiment, and the variables were normalized by body mass (kg).

In P1-3, ventilatory measurements were evaluated by pressure plethysmography as previously described by Biancardi et al. 2022. We used a custom-made double-cylinder head-out chamber (35 mL/35 mL), in which a plastic film separates the head chamber from the body chamber. The ambient temperature was

maintained at 33°C. The front chamber had openings for air intake and output, and the airflow was kept at 160 mL/min. The rear chamber had an opening used to monitor pressure oscillations related to breathing and another for calibration. In this system, the pressure signal (mV) is directly proportional to the volume.

In P12-14 and P21-23, ventilatory measurements were evaluated by whole-body plethysmography with continuous airflow. The animals were placed in acrylic chambers (Bonther, Ribeirão Preto) of 200 mL or 1200 mL for P12-14 and P21-23, respectively. The airflow was kept constant and balanced (400 mL/min or 600 mL/min for P12-14 or P21-23, respectively) using a positive pressure pump and a negative pressure suction pump. Ambient temperature was maintained as described by Biancardi et al. 2022, at 29°C for P12-14 and 27°C for P21-23. Problems inherent to system leakage were mitigated by the high resistance at the air intake and output, minimized to approximate a closed system behavior. Thus, calibration volume was injected at increasing rates to compensate for leaks, allowing the conversion of the pressure signal generated by breathing into tidal volume (V_T, mL) to be calculated according to Bartlett & Tenney, 1970.

For adult animal ventilation recording, whole-body plethysmography was performed in a chamber for adult rats (5 L). In this experiment, the airflow was stopped for about 2 minutes to record ventilatory variables. VT was calculated according to the formula of Bartlett & Tenney, 1970:

$$VT = \frac{VK \times \left(\frac{PT}{PK}\right) \times TC \times (PB - PC)}{TC \times (PB - PC) - TA \times (PB - PR)}$$

Where: V_T is the tidal volume; VK is the volume of air injected into the animal's chamber for calibration; PT is the pressure associated with each tidal volume; PK is the pressure associated with the calibration volume; TC is the body temperature (in Kelvin); TA is the air temperature inside the animal's chamber; PR is the water vapor pressure in TC; PB is the barometric pressure, and PC is the water vapor pressure in the animal's chamber.

2.3.4 Body Temperature and Oxygen Consumption

Body temperature was not measured in P1-3. In P12-14 and P21-23, temperature was assessed before and after the experimental protocols using a thermocouple rectal probe (0.7 mm outer diameter) and thermometer reader (Omega, model HH12B). Oxygen consumption (VO2) was evaluated by indirect calorimetry using open respirometry. In this method, air samples from the chamber's intake and output were collected by a gas analyzer (Gas Analyzer ML206, ADInstruments). VO_2 was calculated using the following formula:

$$VO2 = \frac{[Ve \times (FiO2 - FeO2)]}{1 - FiO2} \times STPD$$

Where: Ve is the intake flow, FiO2 is the fraction of O2 in the intake, and FeO2 is the fraction of O2 in the output. VO2 was corrected by body mass in Kg, and values were presented normalized to STPD (standard temperature, pressure, and dry). Ventilation and O2 consumption were used to calculate the ventilatory equivalent of O2 (VE/VO2).

2.3.4 Experimental Protocols

Each age group consisted of different animals, so that each animal was subjected to only one stimulus. The animals were weighed and placed in the plethysmography chamber, which was heated and maintained at the age-appropriate room temperature by means of a heat source (lamp), and was constantly monitored. Each animal remained in the chamber for habituation for 20 minutes. Then, the basal recording in room air conditions was recorded for 15 minutes. Then, the animals were divided into groups exposed for 15 minutes to hypoxic (10% O_2) or hypercapnic (4% CO_2 and 7% CO_2) gas mixtures, where the gas mixtures were performed using a gas mixer (GSM-3 Gas Mixer, CWE). Immediately after the experimental protocol, the animals were decapitated and euthanized, and the brains were collected for quantification of monoamines. The raw values of the initial period are shown in the table, and the values of the ventilatory responses are presented as a percentage (%) of the baseline.

The ventilatory response to CO_2 in adults was assessed at two times: before and after screening (acoustic stimulation to induce audiogenic seizures). For this

hypercapnia ventilatory response experiment, the animals were placed in a habituation chamber for 30 minutes, followed by a baseline recording period in room air for two minutes with the chamber closed. Then, the gas mixture was changed to a hypercapnic mixture (7% CO_2), in which the animals remained for 20 minutes before a new recording with the chamber closed. After two weeks, the Wistar and WAR animals were subjected to the acoustic stimulation test. Exposure to acoustic stimulation did not alter the behavior of the Wistar animals, while the WAR animals demonstrated audiogenic seizures. Only the WAR animals that presented a behavioral score greater than or equal to 0.85 in the induced susceptibility assessment were included in the analysis. After two weeks, the animals were again placed in the plethysmography chamber to assess ventilation in room air and in hypercapnia.

2.3.5 Monoamines quantification

With the brain collected, microdissections of the respiratory ventral column were performed on dry ice and homogenized, respectively, in 100 µL of 0.15 M perchloric acid (PCA) 15 containing 0.1 mM EDTA and 57 nM 3,4-dihydroxybenzylamine (DHBA; Sigma-Aldrich, Milwaukee, WI), used as an internal standard. All samples were dosed in the same analysis and the same dilution solution was used for all of them. Samples in solution were sonicated until complete homogenization and centrifuged at 12.000 rpm for 20 min, 4 ºC. The pellet was directed to protein dosage by Bradford and the supernatant was removed to evaluate the concentrations of 5-HT, 5-HIAA, NA and DA as previously described (Rocha et al., 2022; Silva et al., 2020). 5-HT, 5-HIAA, NA and DA were identified according to their elution time and quantified using calibration curves by the internal standard method (DHBA).

2.3.6 Data analysis and statistics

Innate reflex analysis was compared using the Student's t-test. Apnea analysis was performed during a 15-min period of room air (normoxia), defined as the absence of ventilation for more than two respiratory cycles (Patterson et al. 2016). The number and duration of apneas were quantified and compared using Student’s t-test. Analyses

of ventilatory variables (fR, VT and VE) were performed during ventilation without animal movement artifacts. Ventilation during hypoxia was assessed every 3 minutes to distinctly analyze the components of the biphasic hypoxic ventilatory responses. The ventilatory response to hypercapnia was analyzed 5 minutes prior the end of the hypercapnic stimulus. Two-way ANOVA for repeated measures was used to compare “group” and “time”, and Tukey's multiple comparison post-test was performed to evaluate statistical differences, where *$p<0.05$.

2.4 RESULTS

2.4.1. Body weight

Figure 2.1A shows representative images of Wistar and WAR animals at different ages. WAR lineage animals exhibit significantly lower body weight compared to the Wistar group throughout development, from P1-3 to P21-23 (Figure 2.1, Wistar n=48 vs. WAR n=49, $p < 0.0001$). Reduced body weight in the WAR strain has been previously reported in adults (Granjeiro et al., 2016), but this is the first time it has been reported during development.

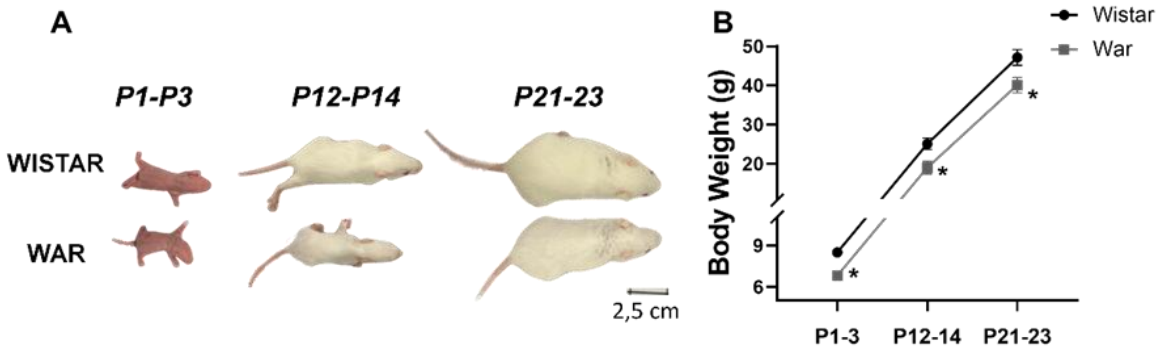


Figure 2.1. WAR animals are smaller compared to Wistar throughout development. A) Photographs of rats at P1, P13, and P21 of Wistar (top, n=48) and WAR (bottom, n=49) at the same scale. B) Body weight (g) across ages. Data are presented as mean ± standard error. Two-way ANOVA, Tukey post-test, *$p < 0.05$ WAR vs. Wistar.

2.4.2 Innate Reflexes

Wistar animals exhibited an average of 18.23 ± 0.68 chews in 20 s, approximately 1 chew per second, while WAR animals showed a reduction in this chewing reflex (8.33 ± 0.74 chews/20 s). Regarding the righting reflex, the time taken by WAR was longer to return to the dorsal position compared to Wistar rats (Wistar vs. WAR: 1.98 ± 0.26 vs. 3.56 ± 0.49 s, $p = 0.0042$). The absence and/or reduction of response to stimuli in WAR animals indicate that innate reflexes are impaired in this group (Figure 2.2).

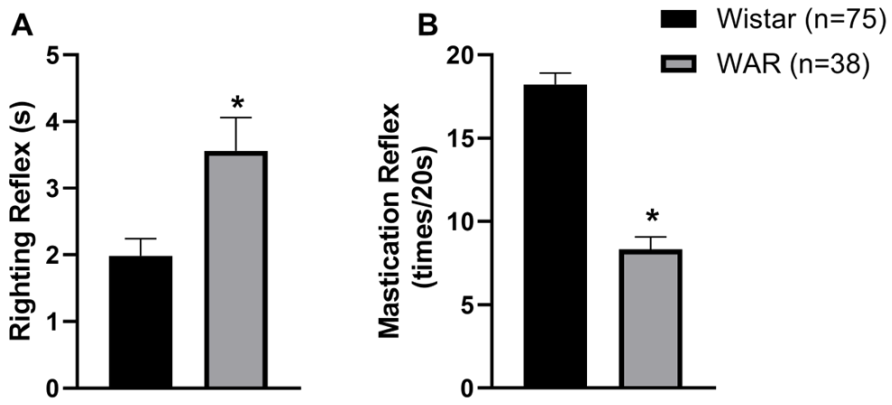


Figure 2.2. The WAR lineage exhibits attenuation of innate reflexes after birth. A) Righting reflex and B) chewing reflex. Student's t-test, *p < 0.005 WAR vs. Wistar.

2.4.3 Ventilation in Room Air

In ambient air, the absolute values of ventilatory variables fR, V_T, VE, and VO_2 can be seen in the table 1, in which there is no statistical difference between Wistar and WAR variables under baseline conditions.

Table 1. – Absolute ventilation values in ambient air. Student's t-test, p > 0.05.

	P1-P3		*P12-14*		*P21-23*	
Raw Values	**Wistar (n=16)**	**War (n=17)**	**Wistar (n=16)**	**War (n=17)**	**Wistar (n=15)**	**War (n=15)**
fR (cpm)	134.3 ± 4.0	138.1 ± 6.9	173.0 ± 5.2	188.5 ± 9.4	149.2 ± 4.6	155.0 ± 11.5
VT (mL/kg)	10.53 ± 0.56	10.46 ± 0.43	11.60 ± 0.66	10.31 ± 0.78	12.00 ± 0.50	11.64 ± 1.08
VE (ml/min/kg)	1377.52 ± 81.30	1409.38 ± 80.90	2004.76 ± 113.85	1955.66 ±188.06	1795.74 ± 81.48	1757.94 ± 123.14
VO_2 (ml/min/kg)	47.85 ± 2.80	48.32 ± 2.51	60.21 ± 1.33	57.16 ± 4.01	42.51 ± 4.98	45.82 ± 6.26

2.4.4 Apneas

Apneas were observed at all ages, as shown in the representative traces in Figure 2.3 (Figures 2.3A, E, and I). At postnatal days 1 to 3 (Figures 2.3A, B, C, and D), WAR animals exhibited a greater number of apneas compared to the Wistar group (4.55 ± 0.70 vs. 9.60 ± 3.02, Wistar n=16 vs. WAR n=17, p < 0.05), which had a longer

duration (Wistar vs. WAR: 1.62 ± 0.10 vs. 5.73 ± 0.62 s, Figure 3C, $p < 0.05$), with some lasting over 6 seconds (Figure 2.3A). At postnatal days 12 to 14, the number of apneas and duration was statistically similar between Wistar (n=16) and WAR (n=17) (Figures 2.3E, F, and G), and at postnatal days 21 to 23, no difference in the number of apneas was observed (Figure 2.3J); however, WAR rats still had longer apneas than Wistar (P21-23: Wistar n=15 vs. WAR n=15: 1.74 ± 0.19 vs. 3.07 ± 0.54, $p < 0.05$, Figures 3K). In Figure 2.3D, H, L shows the distribution of apnea events durations categorized into three intervals: 1 - short-duration apneas (up to 1.5 s, interval 1.5); 2 - medium-duration apneas (between 1.6 and 4.4 s, interval 3.0); and 3 - long-duration apneas (over 4.5 s, interval 4.5). Longer apneas are more prevalent in the WAR group at all ages (Figures D, H and L), especially during the earliest age assessed (P1-3), and tend to decrease during development.

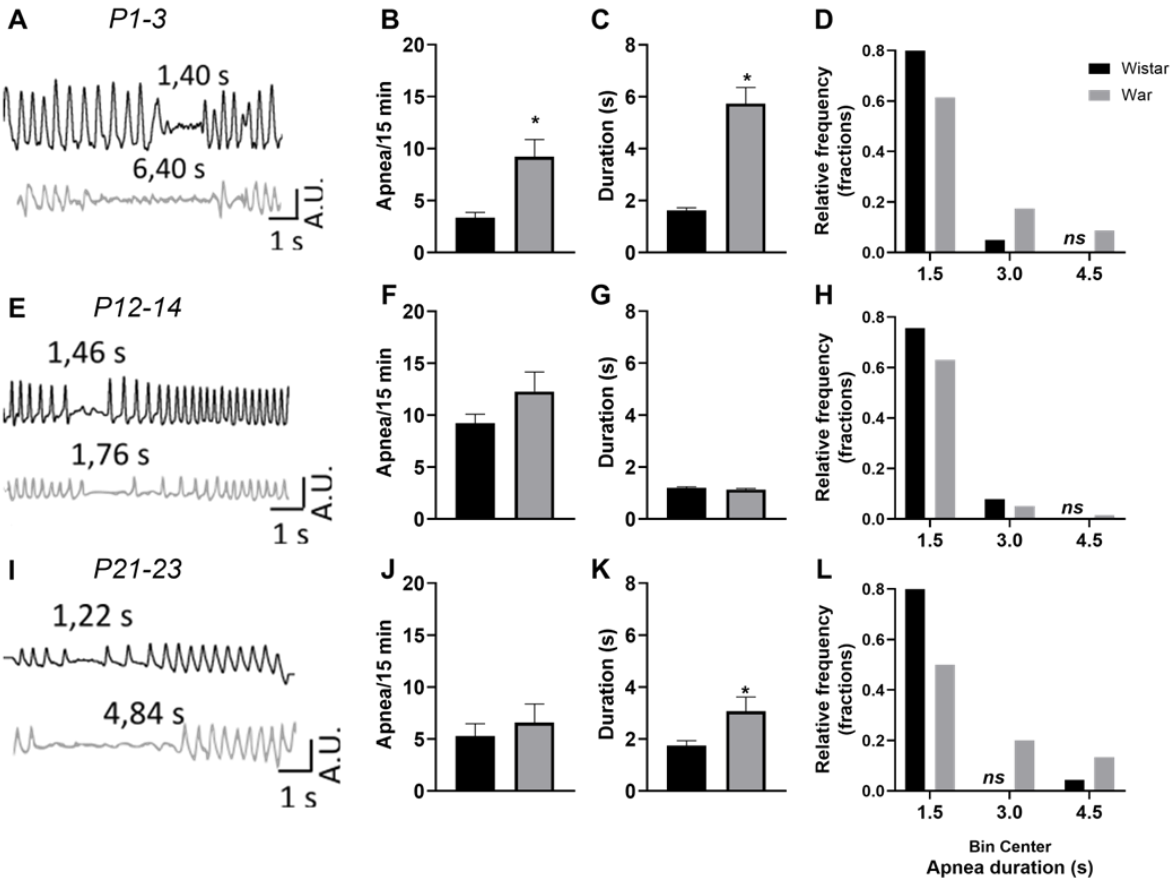


Figure 2.3. Presence of apneas across ages. Characterization of apnea patterns at P1-3 (A, B, C, and D), P12-14 (E, F, G, and H), and P21-23 (I, J, K, and L). A, E, and I are representative figures of apneas and duration. B, F, and J are group averages of the number of apneas, and C, G, and K show the duration at P1-3, P12-14, and P21-22, respectively. Ns: not shown. Student's t-test, *$p < 0.05$ Wistar vs. WAR.

2.4.5 Respiratory Variability

Regarding respiratory variability, Figures 2.4A, B, and C correspond to representative graphs (scatter plots) of the variability in respiratory cycle duration, correlating one cycle (Breath-to-breath, BB, x-axis) with the next (BB+1, y-axis). The values of group means are given by the standard deviation (SD) of the point dispersion, with SD1 being the dispersion along the horizontal diagonal and SD2 along the vertical diagonal. In this aspect, we observed that there was no difference between Wistar and WAR animals in the variability of respiratory cycle duration ($p > 0.05$, Figure 2.4D, E, and F). These data suggest that there are no significant respiratory irregularities between WAR and Wistar groups.

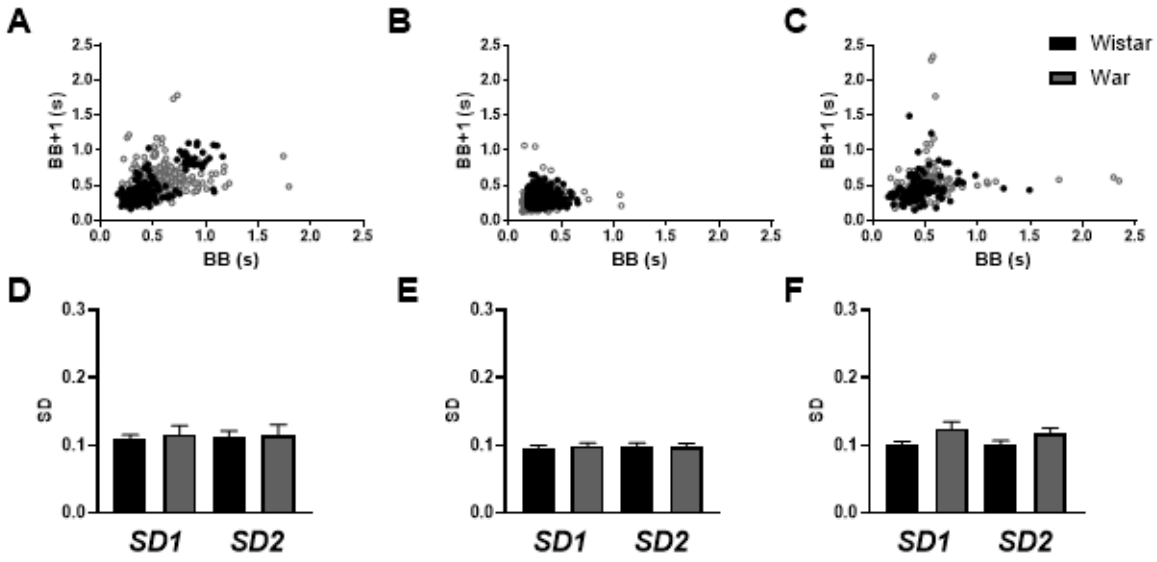


Figure 2.4. Variability in ambient air of WAR is similar to that of Wistar. Figures A, B, and C are representative graphs of each animal's dispersion at ages P1-3 (Wistar n=16 and WAR n=17), P12-14 (Wistar n=16 and WAR n=17), and P21-23 (Wistar n=15 and WAR n=15), respectively. Figures D, E, and F are the group averages of the standard deviation of respiratory cycle duration (SD). Here, SD1 is the distribution of dispersion along the horizontal diagonal, and SD2 is along the vertical diagonal. Data are presented as the mean ± standard error of the mean. Student's t-test, $p > 0.05$.

2.4.6 Ventilatory Response to Hypoxia

The reduction of oxygen generates a rapid response of increased ventilation at all ages (Figure 2.5). Here we highlight that in P1-3, Wistar (n=8) animals present an increase in fR, V_T and consequent increase in VE from the beginning of exposure to hypoxia over the 15 minutes (Figure 2.5B, C and D). WAR (n=9) animals at the same

age do not present an increase in frequency in response to hypoxia (Figure 2.5B, C and D). The V_T of WAR animals increased in a similar way to the V_T of Wistar animals in P1-3. This gives WAR an attenuated ventilatory response to hypoxia when compared to the control at the end of the 15 minutes at this age (Figure 2.5D). In addition, the reduction in VO_2 at the end of hypoxia was attenuated and the ventilatory equivalent (VE/VO_2) was also lower compared to animals in the Wistar group (Figure 2.6A and D). In contrast, WAR (n=7) and Wistar (n=8) animals at P12-14 did not show any difference in the ventilatory response to hypoxia (Figure 2.5E, F and G). The increase in VE in response to hypoxia occurred due to a combination of increased fR and V_T in both groups. VO_2 was similar between the two groups (Figure 2.6). Interestingly, body temperature was lower in WAR than in controls at the end of hypoxia (Figure 2.6G). Behavioral changes characteristic of convulsive seizures were observed only in WAR animals at this age (P12-14) during exposure to hypoxia (10% O_2). Three of the seven WAR rats studied presented head shaking behavior, characterized by head myoclonus (Figure 2.7). These events were not observed at any other age. Furthermore, this behavior was not observed in any Wistar animal. This is interesting because it shows that WAR animals are more likely to have myoclonic seizures with stimuli that do not affect Wistar at this specific age.

At P21-23, the ventilatory response to hypoxia was similar between Wistar (n=7) and WAR (n=7), due to a combined increase in fR and VT (Figure 5H, I and J). The VO_2 of Wistar animals was lower at the end of hypoxia when compared to WAR, while the body temperature of WAR is lower at the end of hypoxia. However, VE/VO_2 did not show significant differences between the groups (Figure 2.6 C, F and H).

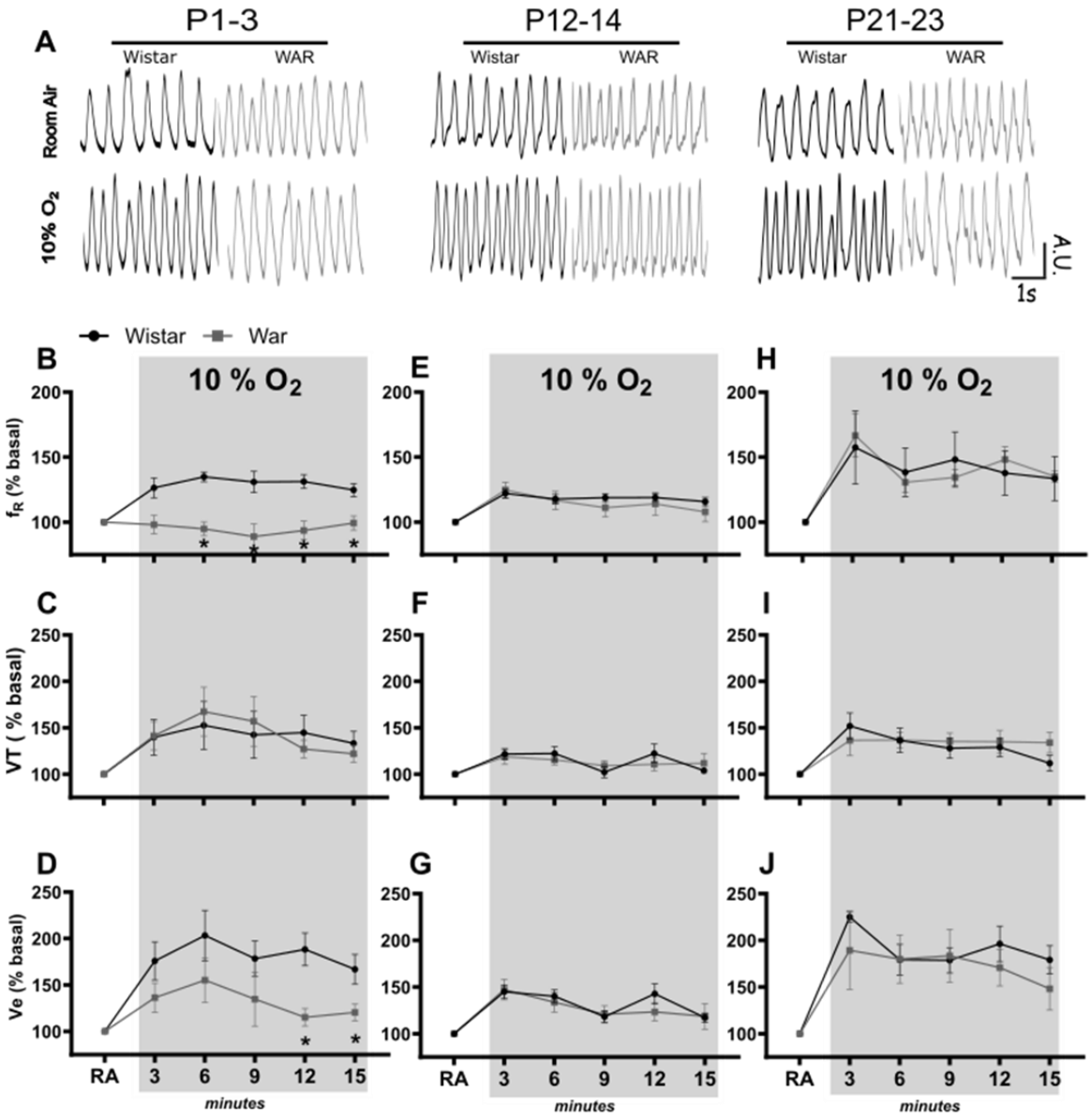


Figure 2.5. Ventilatory response to hypoxia in WAR rats is attenuated at P1-3, but not at other ages. A) Representative recordings in room air and hypoxia in Wistar (black) and WAR (gray) lines. Values presented as % relative to baseline in room air (RA). Gray bar corresponds to hypoxic condition over 15 minutes. The different panels demonstrate the different ages and ventilatory variables. P1-3 (left column; B, C and D), P12-14 (middle column; E, F and G), P21-23 (right column, H, I and J). fR (top row; B, E and H), VT (middle row, C, F and H) and VE (bottom row; D, G and J). Data represented as mean + standard error of the mean. Two-way ANOVA repeated measures, post Tukey test. *p<0.05 Wistar vs WAR.

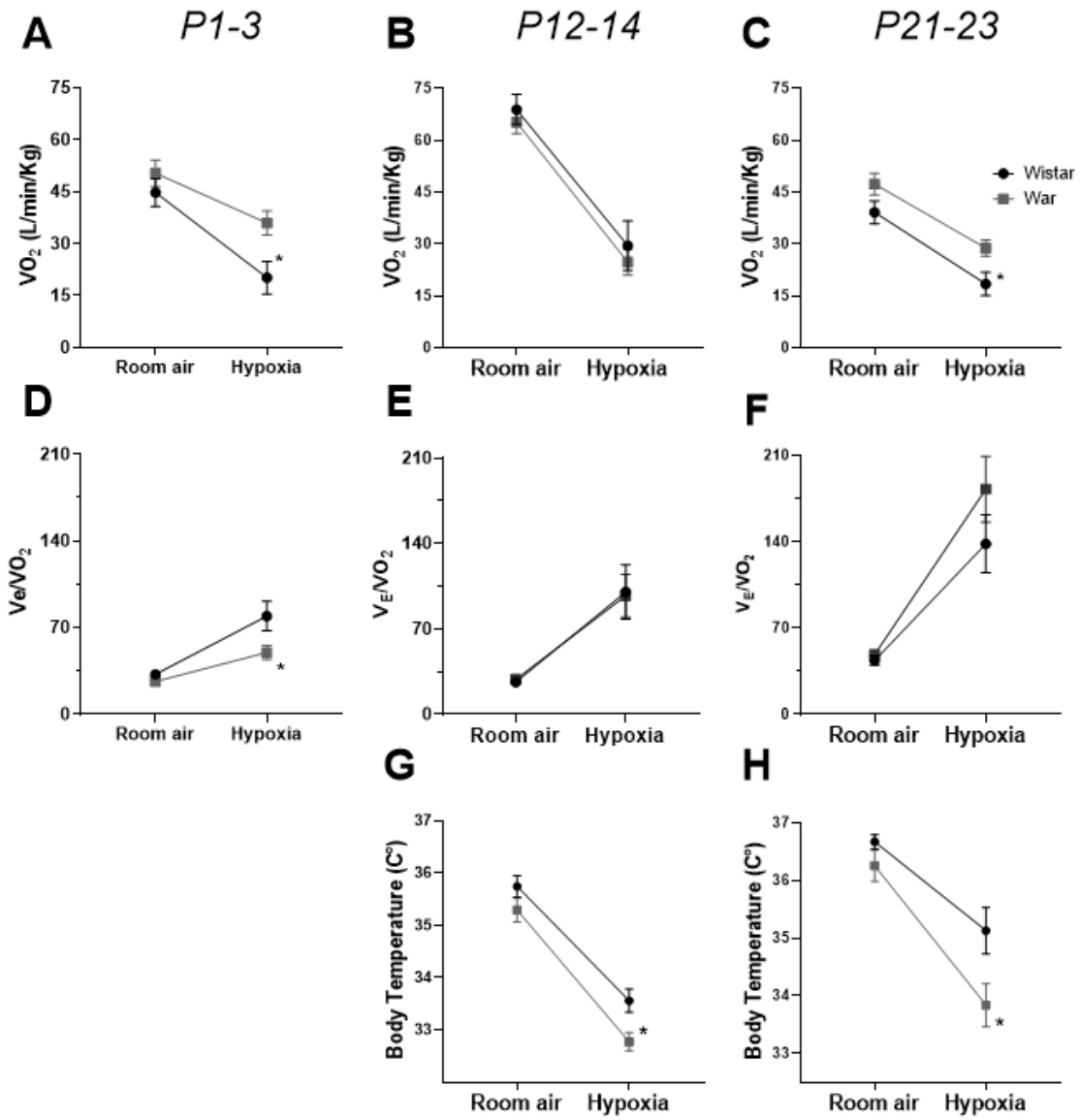


Figure 2.6. VO_2, VE/VO_2, and body temperature during hypoxia. A, B, and C) Oxygen consumption (VO_2); D, E, F) Respiratory equivalent (Ve/VO2) of ages P1-3 (left column), P12-14 (middle column), and P21-23 (right column) in room air and hypoxia. G and H) Body temperature of ages P12-14 and P21-23. Temperature at P1-P3 was not measured. Data represented as mean + standard error of the mean. Two-way repeated measures ANOVA. *p<0.05 Wistar vs WAR.

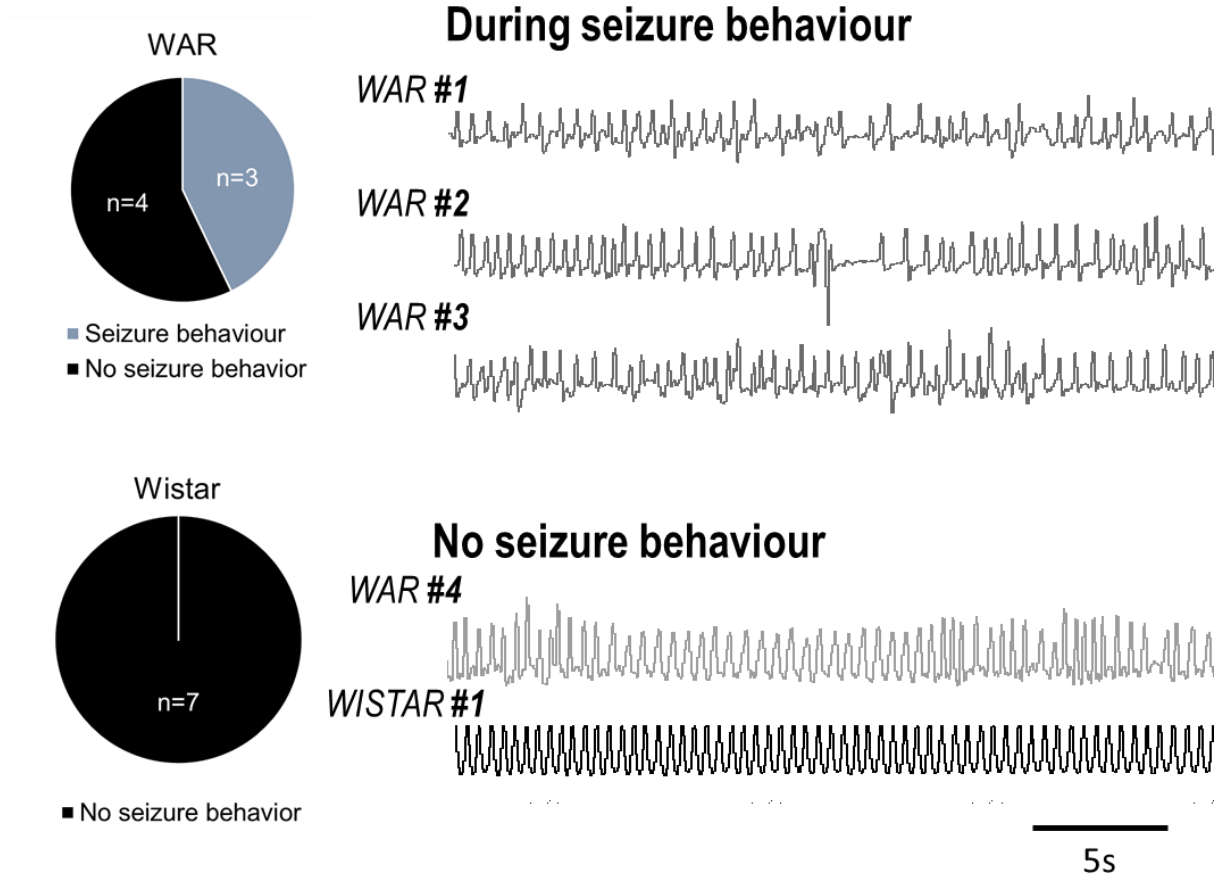


Figure 2.7. WAR animals presented seizures during the hypoxia protocol at P12-14. Representative recordings of ventilation during seizure behavior (WAR #1, 2, and 3). Representative recordings of ventilation of animals that did not present seizures (WAR#4 and Wistar#1).

2.4.7 Quantification of Monoamines (Normoxia and Hypoxia)

The quantification of monoamines was performed to evaluate possible neurochemical changes in the medulla under experimental conditions where altered respiratory phenotypes were identified. The quantification of serotonin (5-HT), its metabolite (5-HIAA), and norepinephrine (NA) in the ventral surface of the medulla under room air conditions throughout development was similar between Wistar and WAR animals (Figure 2.8A, B, and C). However, exposure to hypoxia led to an increase in 5-HT, 5-HIAA, and NA in P1-3 (Figure 2.8E, F, and G) in Wistar animals, but not in WARs. In other words, under hypoxic conditions, WAR neonates showed reduced contents of these monoamines in the ventrolateral medulla compared to Wistar neonates. This effect was not observed at other ages (P12-14 and P21-23). Dopamine (DA) showed no significant changes in Wistar and WAR animals during

ambient air or after hypoxia throughout development (Figure 2.8D and H). Interestingly, in P1-3, the concentration of 5-HT, 5-HIAA, and NA during hypoxia was positively correlated with the ventilatory response to hypoxia ($p<0.05$, Pearson correlation: $R^2 > 0.60$, Figure 2.9), in which the higher the concentration of these monoamines, the greater the ventilatory response to hypoxia.

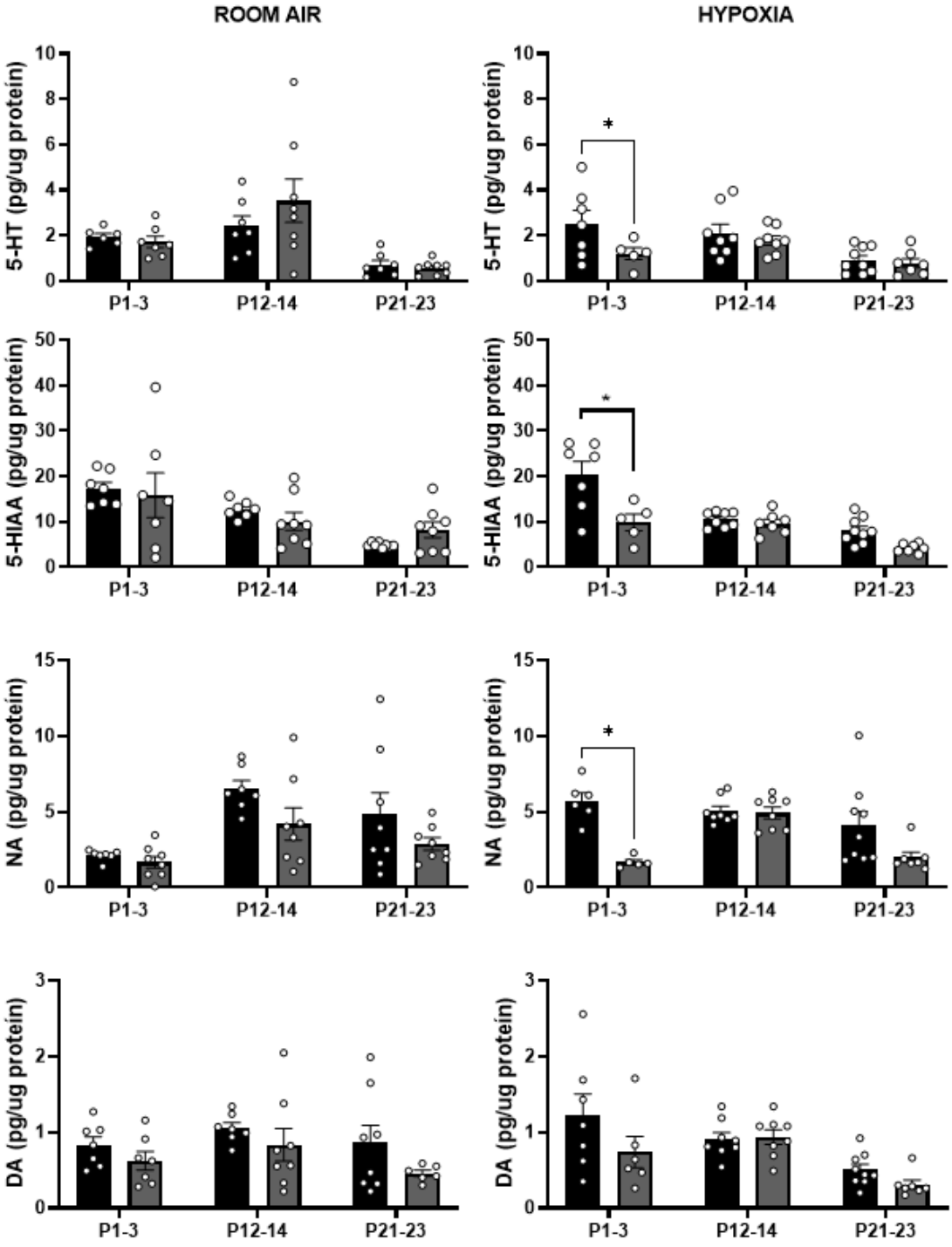


Figure 2.8. WAR shows attenuation of neurotransmitter release after hypoxia. Quantification of monoamines in the ventral respiratory column under ambient air and hypoxia at three ages. Wistar in black and WAR in gray. A and E) Serotonin (5-HT); B and F) 5-hydroxyindoleacetic acid (5-HIAA; metabolite of 5-HT); C and G) Norepinephrine (NA) and D and H) Dopamine (DA). Data are represented as mean ± standard error of the mean. Student's t-test, *p<0.05 Wistar vs WAR under the same condition and age.

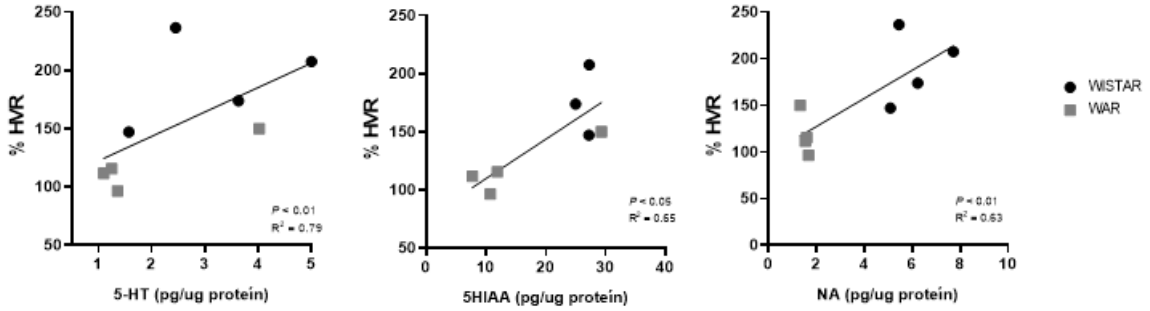


Figure 2.9. Correlation between serotonin content (A), 5-HIAA (B), and norepinephrine (C) in the ventral medulla and the ventilatory response to hypoxia (HVR) at the age of P1-3. Pearson correlation, $p<0.05$, $R^2>0.60$.

2.4.8 Ventilatory Responses to Hypercapnia

The ventilatory response to hypercapnia was evaluated by exposing animals to 4% and 7% CO_2. At all ages, the hypercapnia stimulus resulted in increased fR (Figure 2.10A, B, and C), increased VT (Figure 2.10D, E, and F), and consequently increased VE (Figure 2.10G, H, and I) in a similar manner in both groups (Wistar: P1-3 n=8; P12-14 n=8; P21-23 n=8; WAR: P1-3 n=9; P12-14 n=10; P21-23 n=8, p> 0,05). The increase in these ventilatory variables was progressive and correlated with the increase in CO_2 concentration at 4% and 7%. In other words, the ventilatory response to CO_2 in WAR animals was similar to that of Wistar animals at all three ages studied (Figure 2.10).

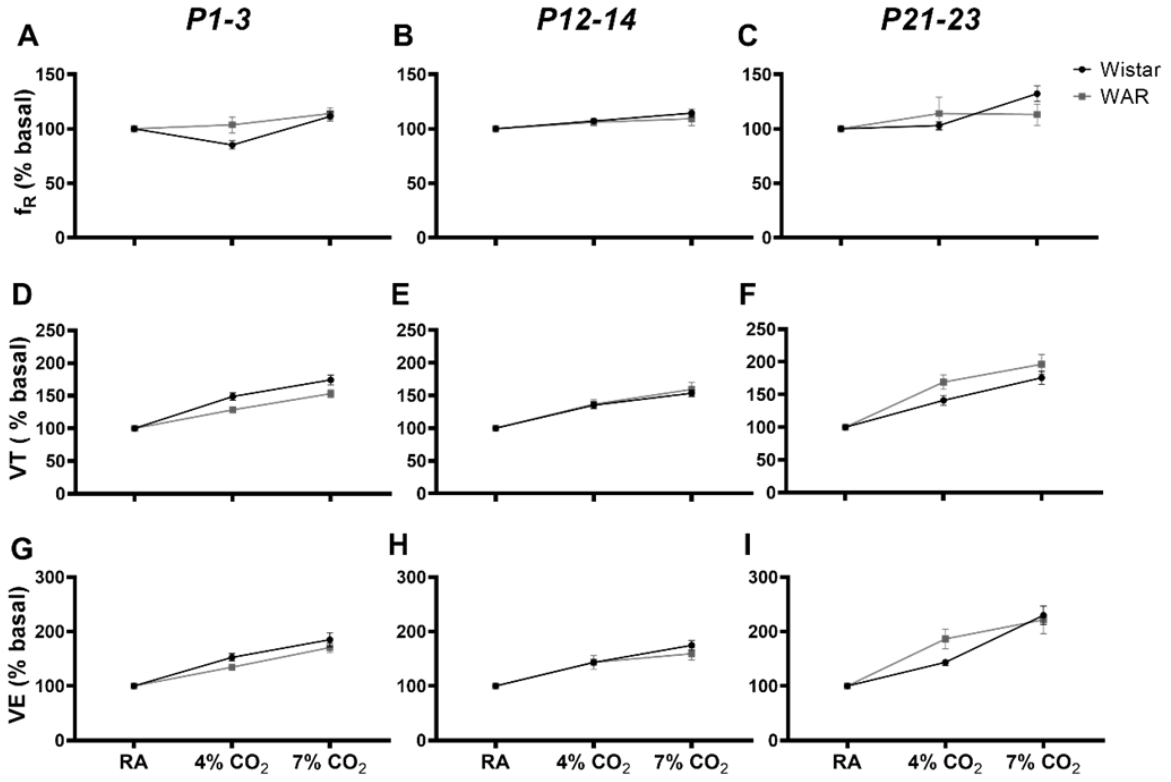


Figure 2.10. Ventilatory response to hypercapnia in Wistar and WAR neonates. Values are presented as a percentage relative to baseline in ambient air (RA). The different panels demonstrate different ages and ventilatory variables. P1-3 (left column; A, D, and G), P12-14 (middle column; B, E, and H), P21-23 (right column; C, F, and I). fR (top row; A, B, and C), VT (middle row; D, E, and F), and VE (bottom row; G, H, and I). There was no difference between Wistar and WAR groups in ventilatory responses to hypercapnia. Two-way ANOVA, $p>0.05$.

The oxygen consumption (VO_2) of WAR animals in response to hypercapnia exhibited the same response profile compared to Wistar as the ages progressed, except for a specific difference at P1-3 (Figure 2.11A). At this age, the VO_2 of WAR increased in response to 4% CO_2 compared to baseline (62.75 ± 3.91), while in Wistar it remained stable (40.36 ± 7.37). The ventilatory response to hypercapnia was also expressed as VE/VO_2 (ventilatory equivalent of O_2), and similarly, no significant differences were observed between WAR and Wistar groups, except for one point at 4% CO_2, correlated with the increase in VO_2 at the same point.

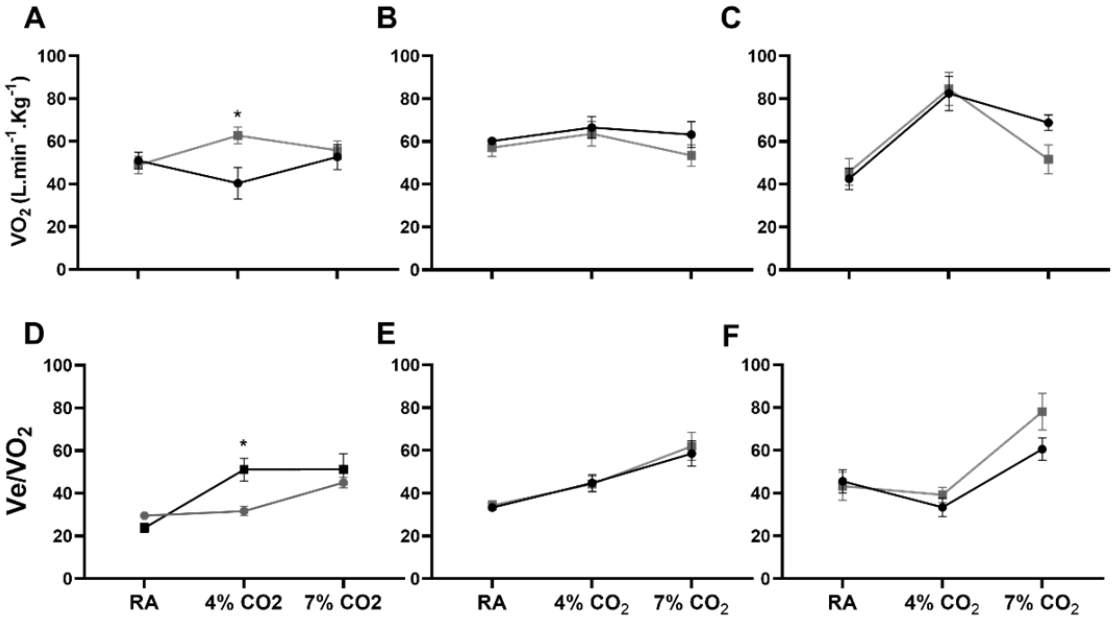


Figure 2.11. Oxygen consumption and ventilatory equivalent in response to hypercapnia. Values are presented as raw values. Panels A and D correspond to P1-3, B and E: P12-14, and C and F: P21-23. * $p<0.05$ Wistar vs WAR.

Figure 2.12 shows the chemosensitivity to CO_2 assessed from the slope of the VE curve ($\Delta VE/\Delta CO_2$; mL/min/kg/%CO_2). From this information, it was possible to compare the sensitivity of the response to CO_2 among ages, similar to the study by Stunden et al. 2001. It can be observed that throughout development there is an increase in sensitivity to hypercapnia, with P1-3 and P12-14 showing lower sensitivity than P21-23. However, the comparison of curves demonstrates that there was no difference between WAR and Wistar groups. This result contradicts the expectation that there would be changes in chemoreception to CO_2/pH, given that WAR animals show reduced chemoreflex when adults.

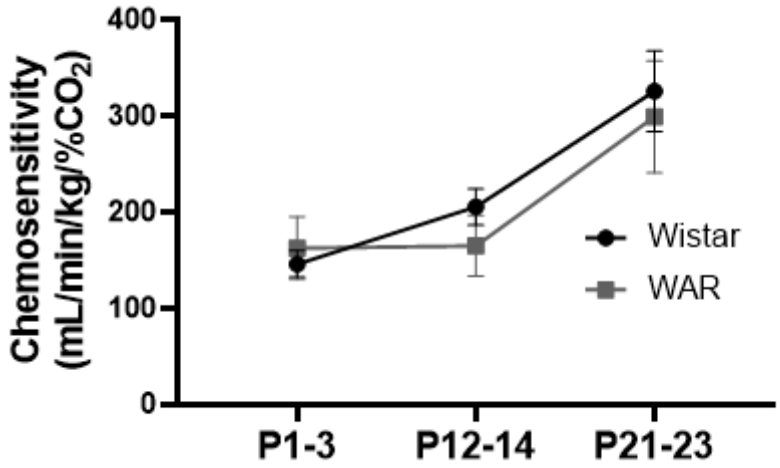


Figure 2.12. The sensitivity of the ventilatory response to CO_2 in WAR rats is not different from Wistar during development. Sensitivity was calculated through the ratio $\Delta VE/\Delta CO_2$; mL/min/kg/%CO_2. No differences were observed between Wistar and WAR groups. Two-way ANOVA, $p>0.05$.

2.4.9 Ventilatory Response to Hypercapnia in Adults

Based on literature evidence reporting an attenuated ventilatory response to hypercapnia in adult WARs (Totola et al. 2017; Granjeiro et al. 2016), additional experiments were conducted to test the hypothesis that seizure events are necessary to induce this attenuation in chemoreception to CO_2/pH. Thus, the ventilatory response to hypercapnia in Wistar and WAR rats was evaluated before and after audiogenic seizure events (Naive vs Post Seizure test, Figure 2.13A). Wistar adult rats maintained the same pattern of increased fR, V_T, and VE in response to the 7% CO_2 stimulus (Figure 2.13C, D, and E) before and after the acoustic stimulus event. Even though Wistar animals underwent the same sound stimulation, they did not exhibit seizure events. However, our results revealed that WAR animals, after experiencing audiogenic seizures, exhibited an attenuation in the ventilatory response to hypercapnia when compared to before the seizures stimulus (figure 2.13B), with fR not increasing in response to hypercapnia and a reduction in the increase of V_T (Figure 2.13C e D).This result suggests the importance of seizure events in generating consequences for respiratory control. Thus, the attenuated chemoreflex in adult WAR animals is a consequence of the seizure event and not due to genetic alterations.

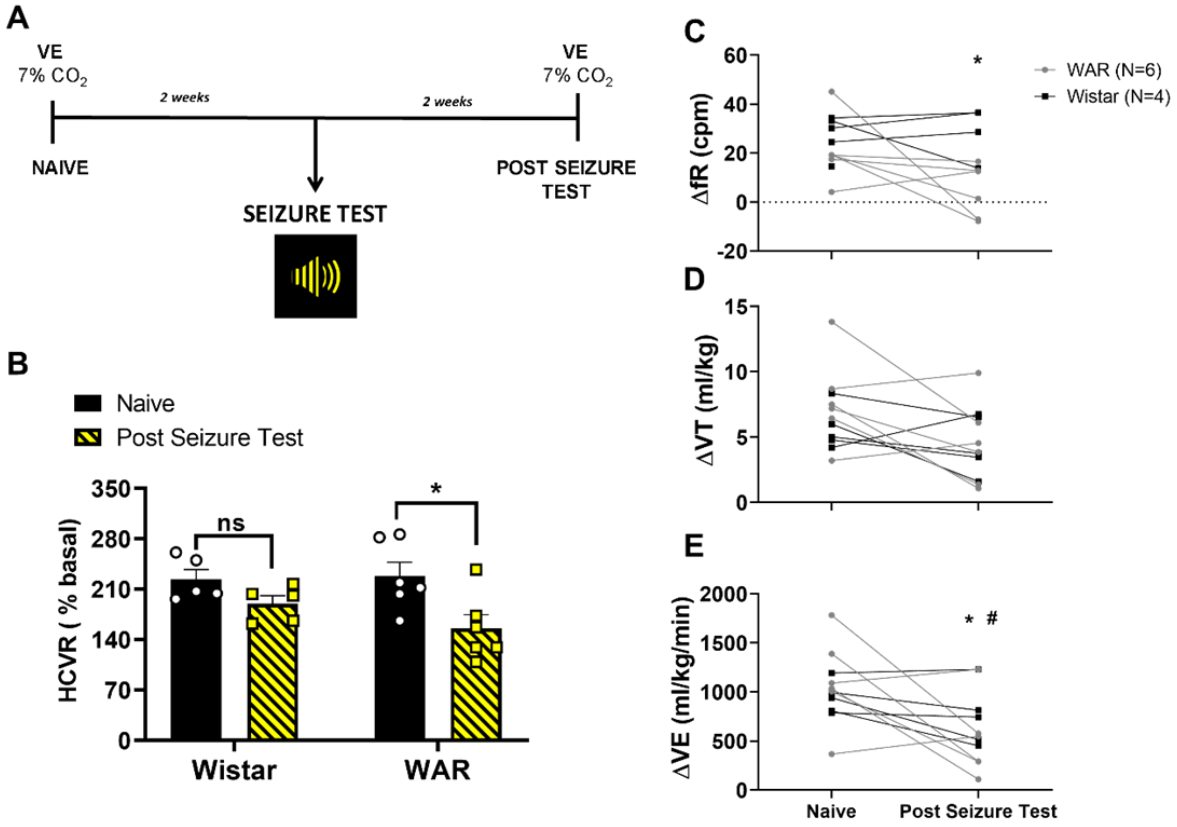


Figure 2.13. Attenuation of the response to hypercapnia in adults is due to the audiogenic seizure stimulus in WAR animals. A) Experimental protocol in adult Wistar and WAR animals. WAR animals experienced convulsive seizures while Wistar did not experience seizures. B) Ventilatory response to hypercapnia (HCVR) before (Naive) and after (Post Seizure Test) the seizure test. C, D, and E present the comparison of the chemoreflex response to CO_2 of the same animal before and after the test. Two-way ANOVA, repeated measures, Bonferroni post-test, *p<0.05 Naive vs Post Seizure, # p<0.05 Wistar vs WAR.

2.5 DISCUSSION

2.5.1 Initial Stage of Development

The first days of life are an interesting period for the WAR lineage, as it shows many alterations when compared to Wistar control animals of the same age. Innate reflexes attenuation may be indicative of brain maldevelopment. The attenuated righting reflex is related to impairment in the development of the central nervous system (CNS) (Motta-Teixeira et al., 2018). This reflex is a vestibular proprioceptive response involving neural circuits in the brainstem and motor organization (Lubics et al., 2005). The attenuation of this reflex is not inherently detrimental but may be associated with reduced myelination (Kim et al., 2022) or decreased proprioceptive synapses (Fletcher et al., 2017). In this regard, the WAR lineage can be characterized by the identification of a gene variant *Vlgr1* with malfunctioning (Damasceno et al., 2018a), a gene that encodes a receptor of the G protein-coupled adhesion receptor family (aGPCR), and among other functions, regulates myelination and increases the stability of this protein in myelin-forming cells (Shin et al., 2013). Changes in myelin production alter the brain's ability to conduct electrical impulses properly, and in early in life this can affect innate reflexes.

The jaw opening reflex, also known as the mastication reflex, is related to the innate breastfeeding reflex. And since swallowing must be closely coordinated with breathing, a lack of coordination can lead to aspiration. Thus, the importance of this reflex in the first days of life, whereas victims of sudden infant death syndrome (SIDS) have demonstrated an unusual amount of apnea/obstruction during feeding or apneic pauses during sleep (Curran et al., 2005). The lack of control of the laryngeal muscles is a significant cause of regurgitation, as failures in the protective airway responses contribute to suffocation, especially when sleeping prone (Duncan, 2018; Thach, 2000). This reflex indicates the motor organization of the upper airway (including the tongue and pharynx) during the first day of life (Lund & Kolta, 2006). Coordination of this reflex occurs in the brainstem and in regions that actively participate in regulating the laryngeal reflex and breathing (Del Negro et al., 2018). The attenuation of this reflex in WAR neonates may indicate alterations in these respiratory brain sites that regulate rhythm and pattern, especially to coordinate the muscles of the upper airway. Our data represent a correlation, for the first time, between an animal model of epilepsy with

orofacial impairment in the first days of life.

Regarding respiratory activity, an important feature demonstrated by the WAR lineage in the early stages of development is the presence of prolonged apneas. The origin of the apneas is unclear, possibly being obstructive or central. Reduced airway tone significantly contributes to obstructive apneas (Paton & Dutschmann, 2002), and due to the attenuation of the jaw opening reflex, we could speculate it might be a case of obstructive apneas related to alteration in upper airway control. However, central apneas are common in the first days of life (Abu-shaweesh et al., 2020; Martin & Fanaroff, 1998). Our results indicate that there was no alteration in neurotransmitter levels in the ambient air situation in the brainstem of WAR, suggesting that the increase in the number of apneas in this group is not related to the release of these neurotransmitters to the respiratory control centers, particularly serotonin. However, we do not rule out that alterations related to these monoamines (receptors, ion channels, second messenger signaling, etc.) could be responsible for the apneas. The presence of abnormal apneas may represent another manifestation of impairment in the development of the WAR lineage. In a knockout mouse of gene *Mecp2*, which also presents alteration in myelination, exhibit prolonged apneas are also observed during the first days of life (Patterson et al., 2016; Ramirez et al., 2013).

2.5.2 Ventilatory Response to Hypoxia

In the present study, the WAR lineage showed an absence of the tachypneic component with attenuation of the ventilatory response to hypoxia in the first days of life (P1-3). Along with the results of the metabolic response during hypoxia, it is possible to affirm that WAR hypoventilate during hypoxia. The first days of life (P1-3) are an interesting period for the maturation of the respiratory system and for the hypoxic ventilatory response (HVR). During this phase, the carotid body has lower sensitivity to oxygen (Kumar & Prabhakar, 2012), and the development of the respiratory neural network occurs alongside the adjustment of these sensors (Day & Wilson, 2023). In the brain, neurotransmitters like serotonin and norepinephrine also play an important role during hypoxia (Cummings & Hodges, 2019; Dzal et al., 2020). Norepinephrine is released from regions of the pons during hypoxia to modulate breathing (Taxini et al., 2021). Interestingly, during the first days of life, norepinephrine

may be released into the bloodstream during hypoxic conditions by the adrenal glands, which may cross the brain blood barrier and also modulate respiratory regions (Prabhakar et al., 2012). Regarding serotonin, its role has proven vital for survival in hypoxic/anoxic conditions, adjusting breathing and cardiovascular responses at early ages (Cummings & Leiter, 2019; Erickson & Millhorn, 1994; Yang & Cummings, 2013). Thus, the decrease in HVR only in P1-3 in WAR may be related to changes in many of these aspects of respiratory development. One of our hypotheses was that the content of serotonin and norepinephrine in the respiratory centers could be correlated with the respiratory response (figure 2.9). Our results indicate that the lower the levels of neurotransmitters (5-HT and NA), the lower the HVR response in P1-3 ($p<0.05$). Overall, this corroborates the evidence in humans, as premature infants exhibit reduced HVR that may persist until the second year of life (Berkowitz, 2012), and are at higher risk of SIDS than term infants (Malloy, 2013). The inability to self-resuscitate in cases of hypoxemia are related to cases of SIDS and SUDEP (Bateman et al., 2008; Goldstein et al., 2016), highlighting the importance of studying the chemoreflex in these animals susceptible to audiogenic seizures during development.

With development progress, the respiratory network, synapses, and system become more mature (Putnam et al., 2005). The maturation of respiratory neurons and motoneurons dramatically increases excitability to neuromodulators, such as serotonin, norepinephrine, and substance P, through receptor expression during development (Day & Wilson, 2023; Dzal et al., 2020; Revill et al., 2019), which may explain the contrasting HVR results from P1-3 and other ages. Although at P12-14 and P21-23, the WAR lineage presents HVR equivalent to Wistar, it is interesting to note that a reduction in metabolism (VO_2 and body temperature) may play an important role in hypoxia (Figure 2.6). The hypoxia-induced anapyrexia is more pronounced in the WAR group than in Wistar, both at P12-14 and P21-23. The reduction in heat production related to homeostatic temperature control decreases oxygen consumption, which is crucial in a hypoxic scenario (Gautier, 1996; Mortola & Naso, 1998). For the age range of P12-14, the reduction in body temperature was sufficient to decrease VO2 similarly between Wistar and WAR, but not at P21-23. This final stage of development (P21-23) represents an age when systems are more mature and similar to adulthood, and the attenuated reduction in VO_2 demonstrated by WAR suggests an altered metabolic response to hypoxia. In this regard, studies show that adult WARs exhibit high mitochondrial density and activity, associated with oxidative

damage and positive regulation of glucose metabolism pathways (Dechandt et al., 2019), and in transcriptional analysis most differentially regulated genes indicate metabolic alterations in the model(Damasceno et al., 2018b). Altogether, our results corroborate with the hypothesis of metabolic alterations in WAR strain, that is more evident with development.

*2.5.*3 Threshold for Hypoxia-Induced Seizures

Although WAR P12-14 does not exhibit significant ventilatory changes during hypoxia, it is intriguing to note that hypoxia alone induced seizures in some WAR animals at this age. The observed behavior was a characteristic rapid head movement, similar to the “shaking” of a wet dog, a type of myoclonic activity observed especially in neonatal seizure models (Dickerson et al., 2012). During the protocol, this was not expected. Hypoxia-induced seizures in rats generally occur at ages around P10 (~P8-P12, Jensen et al., 1991), under more severe hypoxic conditions (< 10% O_2) along with higher ambient temperatures (Jensen et al., 1991; Sanchez et al., 2005). The seizures were not further evaluated and quantified in this protocol. However, it is relevant to report the events that occurred, which suggest that the WAR strain has a lower threshold for hypoxia-induced seizures, and this is not related to changes in the chemoreflex. Moreover, the effects of acute convulsive seizure in neonates caused by hypoxia were duly evaluated in experiments that are described in chapter 3.

Hypoxia can induce seizures by increasing brain excitability due to the release of glutamate under low oxygen conditions at the cellular level (Ben-Ari, 2006; Sanchez et al., 2005). This circumstance occurs in this specific developmental window (P8-15) because the excitability of the CNS of the whole brain undergoes changes during this period (Jensen et al., 1991; Rakhade & Jensen, 2009). Between P10-12, an important change for brain maturation occurs, which is the development of the inhibitory property of GABAergic channels that is important for seizures susceptibility (Ben-Ari, 2006, 2014). Previous studies have shown that the WAR strain in addition to to audiogenic seizures. is more susceptible to seizures induced by pilocarpine and PTZ (Garcia-cairasco et al. 2017; Scarlatelli-Lima et al. 2003). The presented data show that low levels of cerebral oxygenation during this special period in WAR animals are sufficient to trigger seizures, which reveals a new possibility of using the WAR model for studies

of seizures during the neonatal period. Nonetheless, this evidence sheds a light regarding how hypoxic seizures during neonatal period may be seen, since the correspondence of the brain development period between P11-14 in rats is around 2-4 human postnatal months, which coincides with the peak incidence of SIDS (Duncan, 2018; Rakhade & Jensen, 2009).

*2.5.*4 Ventilatory response to hypercapnia

During development, ventilatory response to CO2 of WAR was similar to Wistar, including in P21-23, an age by which the CO2 chemoreflex is fully developed (Dzal et al., 2020; Putnam et al., 2005; Stunden et al., 2001). This was a conflicting result compared to the attenuation of chemoreception to CO2 in adult WAR animals seen by two other studies (Granjeiro et al., 2016; Totola et al., 2017). Notably, in the study by Totola et al, where the WAR group was compared after repeated seizures (kindling) to the “naive” WAR group. These results were important for formulating our initial hypothesis that WAR animals would present alterations in the chemoreflex during development due to intrinsic characteristics of the lineage. Remarkably, these two studies contain a similar methodology to identify the susceptibility to seizures. Since this lineage does not present a clear genotypic characteristic, as in knockout animal models of specific genes, the susceptibility to seizures is a phenotype that needs to be evaluated. Therefore, the WAR animals in both studies were subjected to screening, which consisted of at least 3 days of acoustic stimuli to evaluate the susceptibility to audiogenic seizures according to the severity of the seizure presented. In the study by Totola and collaborators, the animals called “naive” were also subjected to this screening procedure.

In the present study, during development, WAR and Wistar animals were not subjected to the screening process. And in adults, our results showed that HCVR in naïve WAR rats is similar to that of Wistar rats. While after the seizure event (induced by the acoustic test), HCVR is attenuated as in the studies of Totola et al. and Granjeiro et al. This reinforces the relevance of audiogenic seizures causing long term alterations in chemoreflex, and is consistent with evidence of attenuated chemoreflex after repeated seizures in humans and other animal models (Sainju et al., 2019; Teran et

al., 2023; Totola et al., 2019). Any prior screening procedure to assess the audiogenic susceptibility of WAR, or other animal model of seizure, could potentially contaminate results. According to Dutra Moraes et al, 2000, repetitive sound stimulation of WARs leads to a gradual electrographic recruitment of limbic structures, leading to facilitation of the epileptogenic circuitry of the forebrain, similar to a kindling phenomenon (Dutra Moraes et al., 2000). In light of this, our study indicates that repeated seizure events during screening may cause changes in brainstem regions, especially regions related to the chemoreflex, also altering serotonin levels in the brainstem (showed by Totola et. al). Although, further experiments to quantify serotonin in naïve animals and animals subjected to seizures after sound stimulation would need to be performed to confirm this hypothesis.

2.6 CONCLUSION

The WAR strain presents important respiratory alterations at P1-3, such as the presence of apneas and reduced ventilatory response to hypoxia, but not to hypercapnia. During development, at the ages studied (P12-14 and P21-23), the WAR strain does not present chemoreflex alterations, suggesting that genetic susceptibility to epilepsy alone is not sufficient to trigger severe respiratory alterations. Experiments performed before and after a seizure event suggest that the occurrence of a seizure event is necessary for alterations in chemosensitivity to CO2. Another interesting finding was the susceptibility to seizures during hypoxia in WAR animals at P12-14. This audiogenic seizure model, without additional action, shows a predisposition for respiratory impairment in the neonatal period and susceptibility to hypoxic seizures. This represents a promising new tool to investigate the relationship between SIDS and SUDEP.

Chapter 3: VENTILATION AND BEHAVIOR DURING HYPOXIA-INDUCED SEIZURES IN WAR AND WISTAR NEONATES

3.1. INTRODUCTION

The neonatal period represents a phase of life with high vulnerability to the occurrence of seizures (Glass et al., 2016). An interesting fact is that the incidence of seizures is higher during this period than at any other time, ranging from 1 to 3.5 per 1000 live births (Aicardi & Chevrie, 1970; Saliba et al., 1999). This is because the neonatal period represents a critical phase of brain development (Ben-Ari, 2006). It is a key developmental window, during which internal factors, such as genetic mutations and malformations, and/or external factors, such as environmental conditions and infections, can disrupt the normal condition of the brain and induce seizures (Glass et al., 2016; Pisani et al., 2015). Seizures during the neonatal period, regardless of etiology, develop into epilepsy in about 17.9% of cases (Pisani et al., 2015). However, more severe clinical conditions can occur in cases of repeated seizures (Glass et al., 2016).

In infants who present with neonatal seizures that progress to epilepsy, 32% of these individuals are resistant to treatment (Shellhaas et al., 2021). The number and duration of seizures can lead to long-term neurological changes, such as cognitive delay or epileptogenesis processes (Castelhano et al., 2015; Shellhaas et al., 2021). Furthermore, sudden neonatal deaths may be related to unreported/untreated seizures (Alharbi et al., 2023; Hesdorffer et al., 2011). This finding underscores the importance of early detection and characterization of neonatal epileptic seizures.

The presence of seizures is correlated with the presence of hypoxia (Bateman et al., 2008). However, during the neonatal period this might be challenging to access because hypoxia may not be a consequence of seizures, but perhaps the cause. The study by Wertheim and colleagues conducted measurements of physiological parameters associated with EEG and showed that out of eleven neonates, nine

experienced an acute drop in oxygen saturation (SpO_2) of at least 5% during at least one seizure (Wertheim et al., 2024). Yet, associated apneas were observed in only three neonates (Wertheim et al., 2024). This is an important difference related to neonatal seizures when compared to seizures in adults. The most prevalent etiologies of seizures in neonates and infants are those related to cerebral hypoxemia (Blum, 2009; Glass et al., 2011; Russ et al., 2021). Basic demographic data show that the most common etiologies of repeated seizures in neonates are hypoxic-ischemic encephalopathy (HIE, 38%), arterial or venous ischemic stroke (18%), and intracranial hemorrhage (12%) (Glass et al., 2016). The incidence of seizures in these cases is high, with 59% of subjects experiencing seven or more electrographic seizures and 16% presenting with *status epilepticus* (Glass et al., 2016). This etiology is particularly notable due to the magnitude of seizures generated (Rakhade & Jensen, 2009), and the absence of medical treatment can be fatal (Pisani et al., 2009).

The absence of oxygen for brain tissue is extremely damaging. The brain is an organ that requires a lot of energy, and about 20% of total O_2 consumption is cerebral (Masamoto & Tanishita, 2009). Thus, the lack of oxygen disrupts the ATP production chain, and anaerobic mechanisms are insufficient to provide energy for the brain (Nieber et al., 1999). In this situation, neurotransmitters, including glutamate, are released, causing widespread excitation (Jensen & Wang, 1996; Nieber et al., 1999). During development, the brain is more susceptible, which leads to seizures (Ben-Ari, 2006; Sun et al., 2016). Moreover, the situation is so alarming for the CNS that it triggers important inflammatory processes and can lead to cell death (Samaiya et al., 2021; Shaw et al., 2023). Several studies show that neonatal seizures related to hypoxemia contribute to long-term neurological changes (Alharbi et al., 2023), even after exposure to anti-seizure medication. These individuals show low cognitive and language scores 18 months after the seizure episode (Alharbi et al., 2023). The sequelae can include neuromotor and neurocognitive deficits (Shaw et al., 2023; Shellhaas et al., 2021). Furthermore, seizures that occur in this context are often refractory to conventional therapy with antiepileptic drugs (Shellhaas et al., 2021).

Evidence from animal models is important for understanding and developing therapeutic strategies in pediatric clinics, as they help elucidate the factors contributing to the high susceptibility of the immature brain to seizures and the age-specific mechanisms of epileptogenesis. Among the numerous models, the global hypoxia

model described by Jensen and collaborators stands out (Pitkänen et al., 2005). In this protocol, rats within a narrow age range during the neonatal period, between P8-P11, are subjected to decremental hypoxia exposure and exhibit epileptiform, behavioral, and electroencephalographic activity (Sun et al., 2016). Although this model is widely studied (Sun et al., 2016), little is known about the physiological mechanisms related to breathing in this model. Respiratory dysfunctions in individuals with different types of epilepsy are not uncommon (Bateman et al., 2008; Dlouhy et al., 2015; Ryvlin et al., 2013), but they are under-investigated and, consequently, underdiagnosed (Freeman, 2006), especially in the neonatal period. In the previous chapter, we showed that WAR animals at P12-14 presented seizures when exposed to 10% O_2, a milder hypoxia situation than the one used in Jensen's protocol. Therefore, our hypothesis is that WARs are more susceptible to hypoxia-induced seizures in the decremental hypoxia protocol at P10 compared to Wistar rats. Additionally, our aim includes evaluating breathing patterns during the hypoxia protocol, since hypoxia-induced seizures are directly related to respiration due to the direct effects of oxygen deprivation and the chemosensitive response to this challenge. Thus, studying respiratory control in hypoxia-related neonatal seizures allows for the development of more precise and effective intervention strategies. In this regard, our work is pioneering in studying respiratory mechanisms in this model of neonatal seizure induction.

This includes identifying therapeutic targets and formulating approaches to minimize the adverse effects of seizures on respiratory function, and vice versa. In this context, serotonin neurotransmission has significance in the hypoxic ventilatory response and seizure behavior (Buchanan et al., 2014; Herman et al., 1999; Shin et al., 2009). Additionally, 5-HT_2 receptors stand out for their role in seizures and sudden infant death (Brennan et al., 1997; Duncan, 2018; Haynes et al., 2023; Patterson et al., 2016). Hence, in this work, serotonin expression in the brainstem was evaluated through 5-HT immunostaining and the involvement of 5-HT_2 receptors was assessed through non-specific blockade using ketanserin (5-HT_2 antagonist) in WAR and Wistar rats subjected to the decremental hypoxia protocol..

3.2 OBJECTIVES

3.2.1 General objective

To characterize respiratory activity and seizure behavior induced by decremental hypoxia in neonate rats and explore the possible serotonergic mechanisms involved in this phenomenon.

3.2.2 Specific objectives

1. To characterize the behavioral and respiratory response of Wistar and WAR rats at P10 during hypoxia-induced seizures.
2. To evaluate serotonin expression in the brainstem of Wistar and WAR rats at P10 during hypoxia-induced seizures through immunohistochemistry.
3. To assess the involvement of 5-HT_2 receptors in the respiratory response of Wistar and WAR rats at P10 to the hypoxia-induced seizure protocol.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Ten-day-old (P10) WAR and Wistar rats of both sexes were used. The Wistar rats were obtained from the Bioterium Center at ICB/UFMG (CEBIO), while the WAR (Wistar Audiogenic Rats) were obtained from the colony of the Department of Physiology and Biophysics at UFMG. As in the previous chapter, the WAR neonates used were naive (weren't submitted to screening) and were derived from crossings of breeding pairs highly susceptible to audiogenic seizures. The animals were kept in the animal facility of the Department of Physiology and Biophysics at UFMG, with access to food and water *ad libitum* and a 12-hour light-dark cycle (7:00 AM to 7:00 PM). These neonates were kept with their mothers until the time of the experiment. Each experimental group consisted of neonates from at least three different litters. The experimental procedures were approved by the Animal Experimentation Ethics Committee (CEUA 21/2020) at UFMG.

3.3.2 Hypoxia-induced seizures in neonates

The neonates (P10) from both groups were individually placed in a plethysmography chamber and allowed to acclimate in room air for 20 minutes. The animals were then subjected to the progressive hypoxia protocol, as described by Jensen and colleagues (Jensen & Wang, 1996; Sun et al., 2016). It consists of a total of 15 minutes of hypoxia exposure, during which the inspired oxygen fraction (FiO_2) is rapidly reduced from 21% O_2 to 7% O_2, maintained for 8 minutes, followed by a reduction to 5% O_2 for 6 minutes, and finally to 4% O_2 for 1 minute. After the hypoxia exposure, the animals remained in the chamber for an additional 15 minutes in room air (21% O_2) for recovery breathing assessment. The ambient temperature was maintained at 32.0–32.4°C. The image below illustrates the protocol used and a recording of a representative animal during a seizure event.

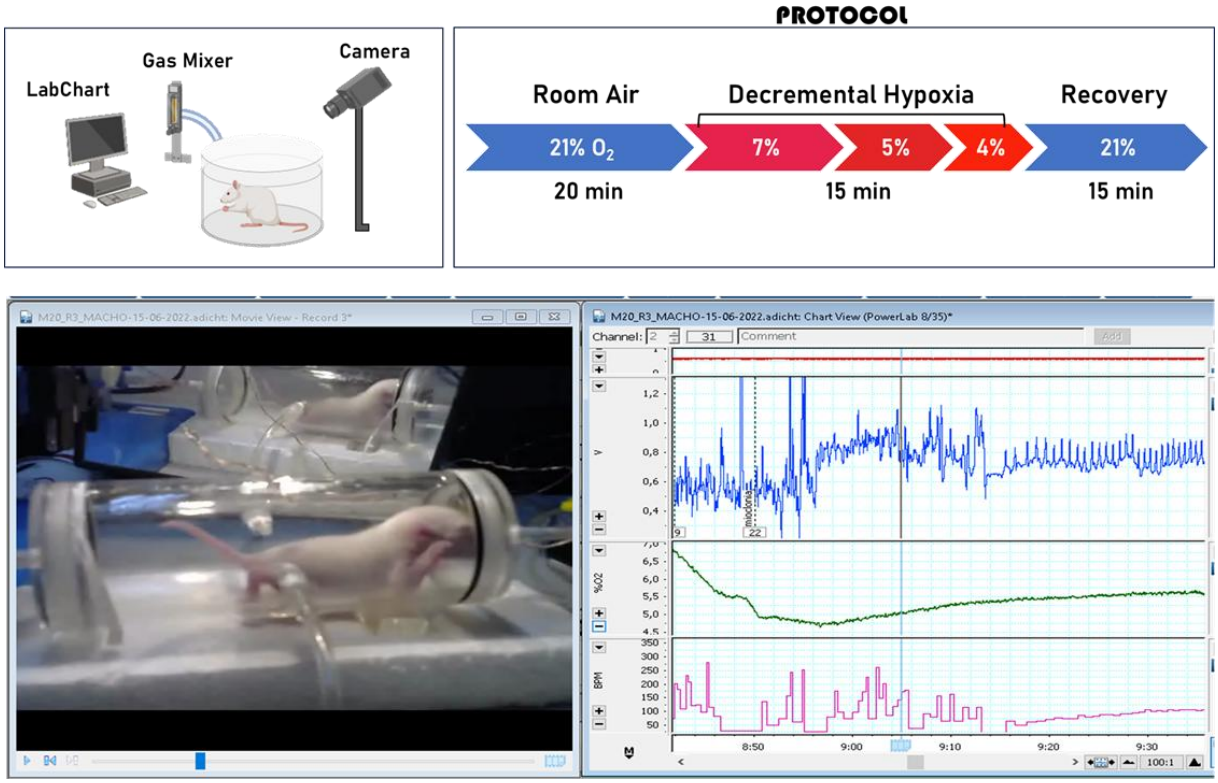


Figure 3.1. Experimental setup and design. A) Setup.B) Experimental protocol.C) Representative image of a P10 Wistar during myoclonus and its corresponding ventilation recording. On the right, the first channel (red) shows the flow inside the chamber, the second channel (blue) records ventilation, the third channel (green) represents the oxygen concentration in the chamber, and the fourth channel (pink) shows respiratory rate. The light blue vertical bar correlates the moment in the video with the recording. Note that animal movement interferes with the ventilation recording during the ictal period, but not during the postictal period.

3.3.3 Behavioral analysis

For behavioral analysis, the animals were recorded on video throughout the entire protocol, including the initial acclimatization period, during, and after exposure to the hypoxia protocol. The video analysis for behavior quantification was performed blindly to avoid experimental bias. Three main behaviors were identified and related to seizures in neonatal rats were: jerk (rapid involuntary contractions), galloping (alternating paw movements), and myoclonus (intense movements of the head or limbs (forelimbs and/or hindlimbs). The latency (time to the first seizure event) and the total number of occurrences for each type of behavior during the hypoxia protocol were quantified.

3.3.4 Pulmonary ventilation measurements

The method used to measure pulmonary ventilation in this study was the same as in the previous chapter for animals aged P12-14: whole-body plethysmography with continuous flow. The variables of fR, VT, and VE were measured as previously described. Data were presented as a percentage relative to baseline values (% of baseline). In this experiment, oxygen consumption (VO_2) was not measured due to methodological issues related to the rapid change in flow during alterations in chamber gas levels.

3.3.5 Immunohistochemistry

At the end of the experiment, the animals were removed from the plethysmography chamber, kept in a heated chamber for 1 hour and 30 minutes, and then perfused. Initially, phosphate-buffered saline (PBS 0.01 M) was used for transcardiac perfusion, followed by 4% paraformaldehyde (PFA 4% in PBS). After perfusion, the brains were removed, placed in a fixative solution (PFA 4% in PBS) for 2 to 4 hours, and then transferred to a cryoprotectant solution (30% sucrose in PBS). The brains were then frozen, sectioned (40 µm), and stored in a freezer until immunohistochemistry was performed.

Immunohistochemistry was conducted using the free-floating method, as described in previous studies from our lab (Fernandes et al., 2021; Fonseca et al., 2020). Brainstem slices containing the medullary raphe (Khazipov et al., 2015) were washed with 0.1 M glycine, incubated with 0.4% Triton X-100 and 1% H_2O_2, and 3% bovine serum albumin. The slices were then incubated with the primary anti-serotonin antibody (1:20,000, Sigma, catalog no. S5545, produced in rabbit) for 48 hours at 4°C. Afterward, they were washed with PBS and incubated with a biotinylated anti-rabbit secondary antibody (1:2000, Vector Laboratories, catalog no. BA2000) for 2 hours at room temperature. For detection, the sections were incubated with an avidin-biotin-peroxidase complex (1:500 in PBS; Elite ABC kit, Vector Laboratories) for 1 hour at room temperature and then stained with a solution containing 3,3′-diaminobenzidine-HCl (DAB, 0.2 mg/mL; Sigma-Aldrich), nickel sulfate (Ni, 25 mg/mL), and 0.03% H_2O_2. A negative control was also performed by omitting the primary antibody to ensure no

nonspecific staining. Images were captured at 4x and 20x magnification using an optical microscope and the Axiovision 4.8 software. The analysis of the slices was conducted blindly, and 5-HT immunoreactive neurons were manually counted using ImageJ software. The number of 5-HT neurons was counted in sections corresponding to the interval from -10.20 to -7.40 mm from the bregma, and from -4.40 to -1.60 mm from the lambda (Khazipov et al., 2015), from the beginning of the medullary pyramid to the presence of the elongated facial nucleus, which corresponds to the rostro-caudal extent of the medullary raphe (Morinaga et al., 2019). The raphe was divided into: 1) Parapyramidal Raphe (lateral to the pyramids) and Pallidus (between the pyramids) - Rpa/py; 2) Obscurus Raphe (medial region above the pyramids) - Rob; and 3) Magnus Raphe (most rostral region of the ventral column) - RMg. Each animal had at least three slices from the same region at different coronal coordinates, and the mean of these slices was used to calculate the group mean.

3.3.6 5-HT_2 receptor blockade

To evaluate participation of 5-HT_2 receptor in neonatal hypoxic seizures, paired groups were used, receiving either a vehicle (0.9% saline) or a non-specific 5-HT_2 receptor antagonist (Ketanserin, Sigma, 5 mg/kg, i.p.) of WAR and Wistar rats. The experimental protocol consisted of acclimating the animals in the chamber, followed by the recording of baseline variables. Intraperitoneal injections (maximum volume: 100 µL) of either the vehicle or the drug were administered. Breathing was then recorded in room air conditions for 20 minutes, followed by the decremental hypoxia protocol. Afterward, the animals were analyzed in room air to evaluate recovery. Two animals that received ketanserin, one from each group, died during the hypoxia protocol.

3.3.7 Statistical Analysis

For the statistical analysis of the data, GraphPad Prism 8 software was used. To compare behavioral results between the Wistar and WAR groups, a Student's t-test was applied. For ventilation results, a two-way ANOVA with repeated measures was

conducted to assess the effect of time and the interaction between the Wistar and WAR groups. A two-way ANOVA was also used for neuron counts. Additionally, to examine the interaction between the Wistar and WAR groups and the drug treatment, a two-way ANOVA was performed. Results were considered statistically significant when $p < 0.05$.

3.4 RESULTS

3.4.1 Behavioral Response during Decremental Hypoxia

The three main behaviors observed throughout the hypoxia protocol were: jerk, gallop, and myoclonus. The jerk behavior was the most common in both groups, with all animals exhibiting at least one jerk event and no statistical differences (Figure 3.2) between Wistar (n=9) and WAR (n=12). The gallop behavior was the second most observed behavior; not all Wistar animals displayed this behavior (n=6 did, n=3 didn't), but all WAR animals (n=12) did show this type of seizure. However, there were no differences in latency or the number of events for this behavior between the groups (Figure 3.2C and D). Regarding myoclonus events (Figure 3.2E and F), WAR animals (n=5) exhibited shorter latency and a greater number of events compared to Wistar (n=4). These data suggest a greater susceptibility of WAR animals to seizures compared to Wistar, as hypothesized.

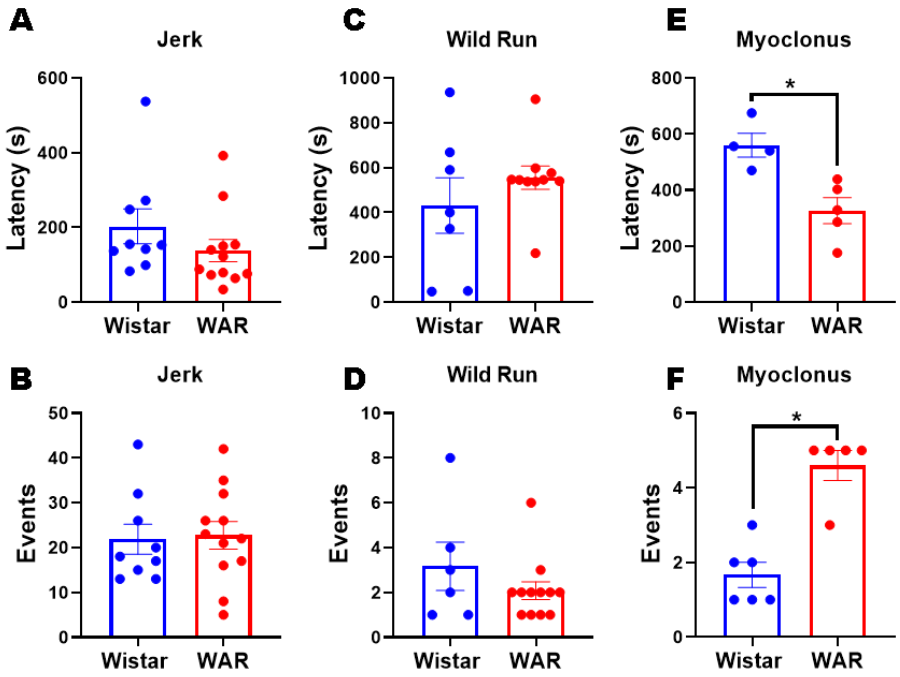


Figure 3.2. Convulsive behavior induced by decremental hypoxia. Latency and number of events of jerk (A and B), gallop (C and D), and myoclonus (E and F) during the hypoxia protocol in P10 Wistar (blue) and WAR (red) rats. Data presented as mean + SEM, Student's t-test, *p<0.05.

3.4.2 Ventilatory Response during Decremental Hypoxia

The ventilatory response during decremental hypoxia differed from the response observed in the previous chapter. This is due to the intensity of the decremental hypoxia and the presence of seizures during the ventilation recording. In the representative compressed recordings from Wistar and WAR during the seizure, movement artifacts can be observed (Figure 3.3B and C). In this specific protocol, these artifacts are largely related to the behaviors associated with seizure activity. Regarding respiratory activity, it was observed that in both groups (Wistar and WAR), fR, V_T, and VE increase at the beginning of the protocol, between minutes 1 and 8 when the oxygen concentration is at 7% O_2 (Figure 3.3E). This trend continued until the oxygen concentration was reduced to 5% O_2, where fR decreased and V_T increased. At 4% O_2, the reduction in fR was so drastic that the animal exhibited gasping (Figure 3.3F), and V_T reached its maximum value. During the protocol, there were no statistical differences in the ventilatory responses between the Wistar and WAR groups (Figure 3.4). Although the drop in fR for WAR is not as pronounced as that of Wistar, it is not statistically different ($p=0.1009$).

During recovery, the control group (Wistar) showed an increase in VE of 34% compared to baseline, primarily due to an increase in V_T. In contrast, WAR animals showed significantly lower V_T during this period compared to Wistar, resulting in reduced VE during recovery (Figure 3.4).

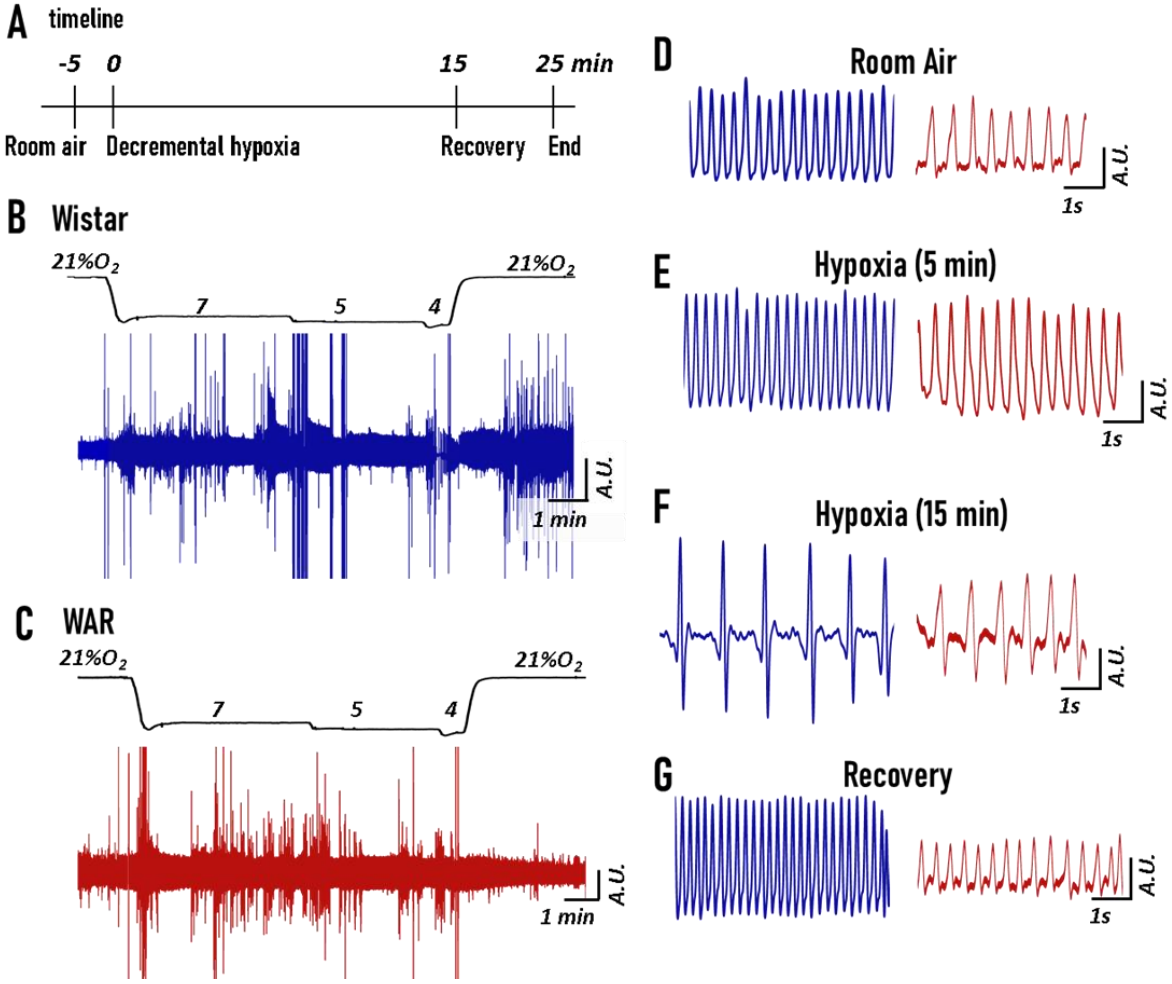


Figure 3.3. Representative recordings from the hypoxia-induced seizure protocol. A) Experimental protocol aligned with representative recordings of breathing. B and C) Representative compressed recording from the Wistar and WAR groups, respectively. The black line represents the concentration of oxygen in the chamber, while the blue and red lines represent recordings of respiratory activity. It can be observed that the compressed breathing recording is marked by movement artifacts related to seizures. In panels D, E, F, and G, we have representative recordings from Wistar (blue) and WAR (red) animals during ambient air (D), the onset of hypoxia (E), the end of the hypoxia protocol (F), and (G) during recovery in ambient air.

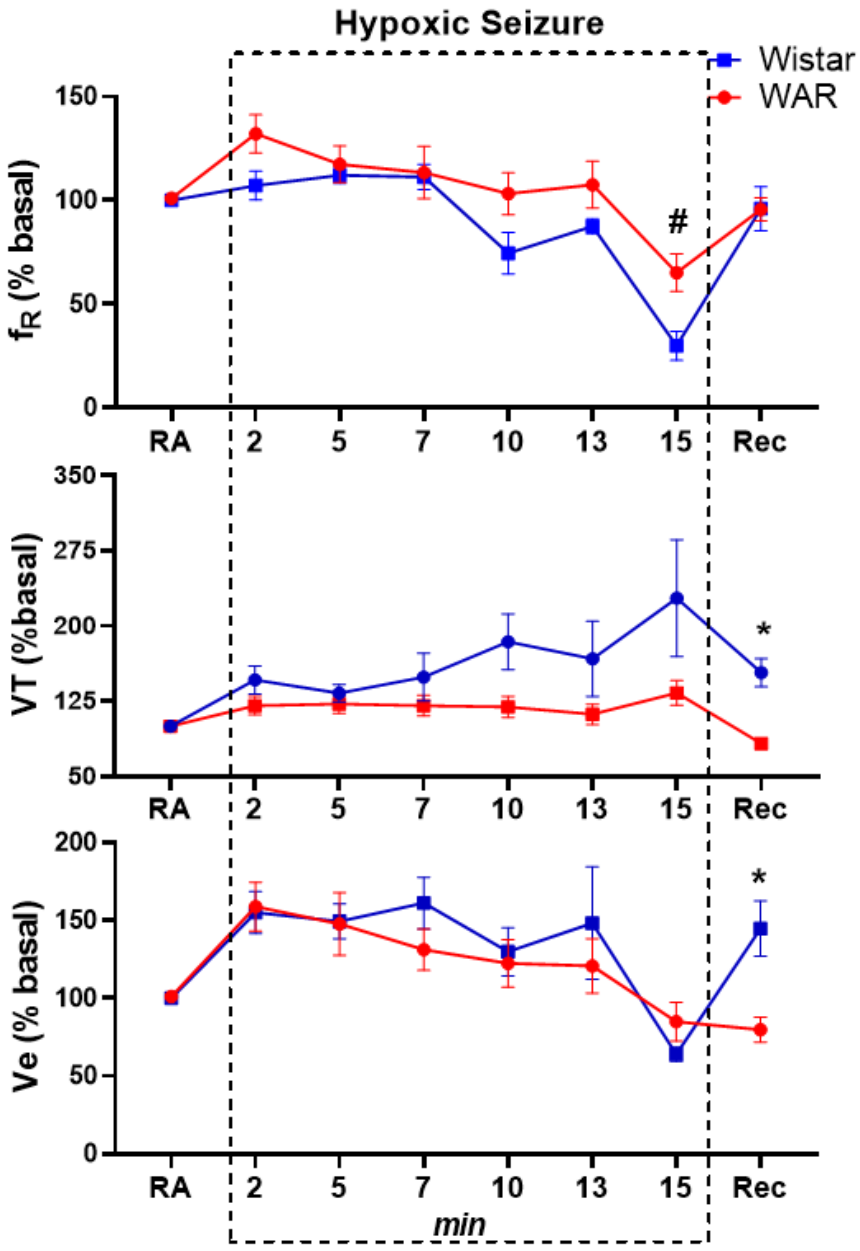


Figure 3.4. Ventilatory response to decremental hypoxia. Values of respiratory frequency (fR), tidal volume (VT), and pulmonary ventilation (Ve) normalized to baseline values. Data presented as mean + SEM, two-way ANOVA, * $p<0.05$ Wistar vs WAR, # $p<0.05$ minute 2 vs minute 15.

The body temperature was measured before and after the hypoxia protocol. After hypoxia, the body temperature decreased by approximately 1°C in both groups (Figure 3.5). However, there were no differences between the Wistar and WAR groups. For the Wistar group in ambient air, the average body temperature was 34.6 ± 0.6 °C

(n=8), and during hypoxia, it dropped to 33.9 ± 0.6 °C. For the WAR group, the average value was 34.07 ± 0.37 °C (n=8), and after hypoxia, it decreased to 33.3 ± 0.3 °C.

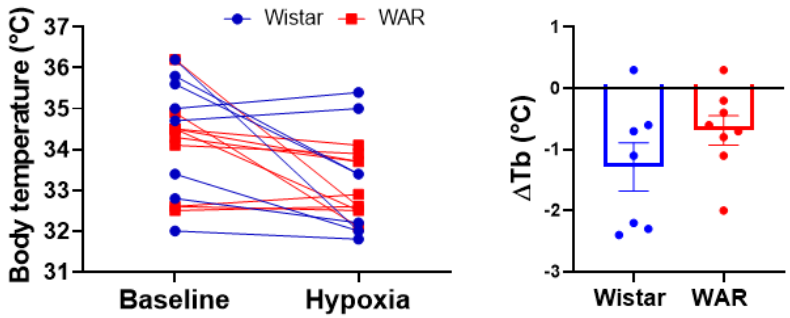


Figure 3.5. Body temperature before and after the hypoxia protocol. Raw values of body temperature before (baseline) and after the hypoxia protocol (hypoxia) for Wistar (blue) and WAR (red) animals. Temperatures of Wistar and WAR were statistically similar, p>0.05.

3.4.3 Serotonin labeling in the brainstem after decremental hypoxia

The serotonin labeling through immunohistochemistry allowed for the visualization of serotonergic neurons in the medullary raphe (Figure 3.6). For this experiment, some animals that did not undergo the hypoxia protocol were used to assess the quantity of serotonergic neurons under control conditions (room air). In this control situation, our results demonstrated that the WAR strain exhibits a reduction in the number of 5-HT neurons in the region of the Obscurus raphe (ROb) when compared to the Wistar (Figure 3.7), but not in other regions (Rpa/Rpy and RMg).

After the hypoxia-induced seizure, serotonin labeling in the ROb region was significantly reduced in both groups (Figure 3.7). This reduction is so pronounced that certain slices exhibit no 5-HT labeling in this region, as shown in the representative figure below (Figure 3.6). However, there was no significant difference in the Rpa/Rpy and RMg regions when compared to ambient air (Figure 3.7).

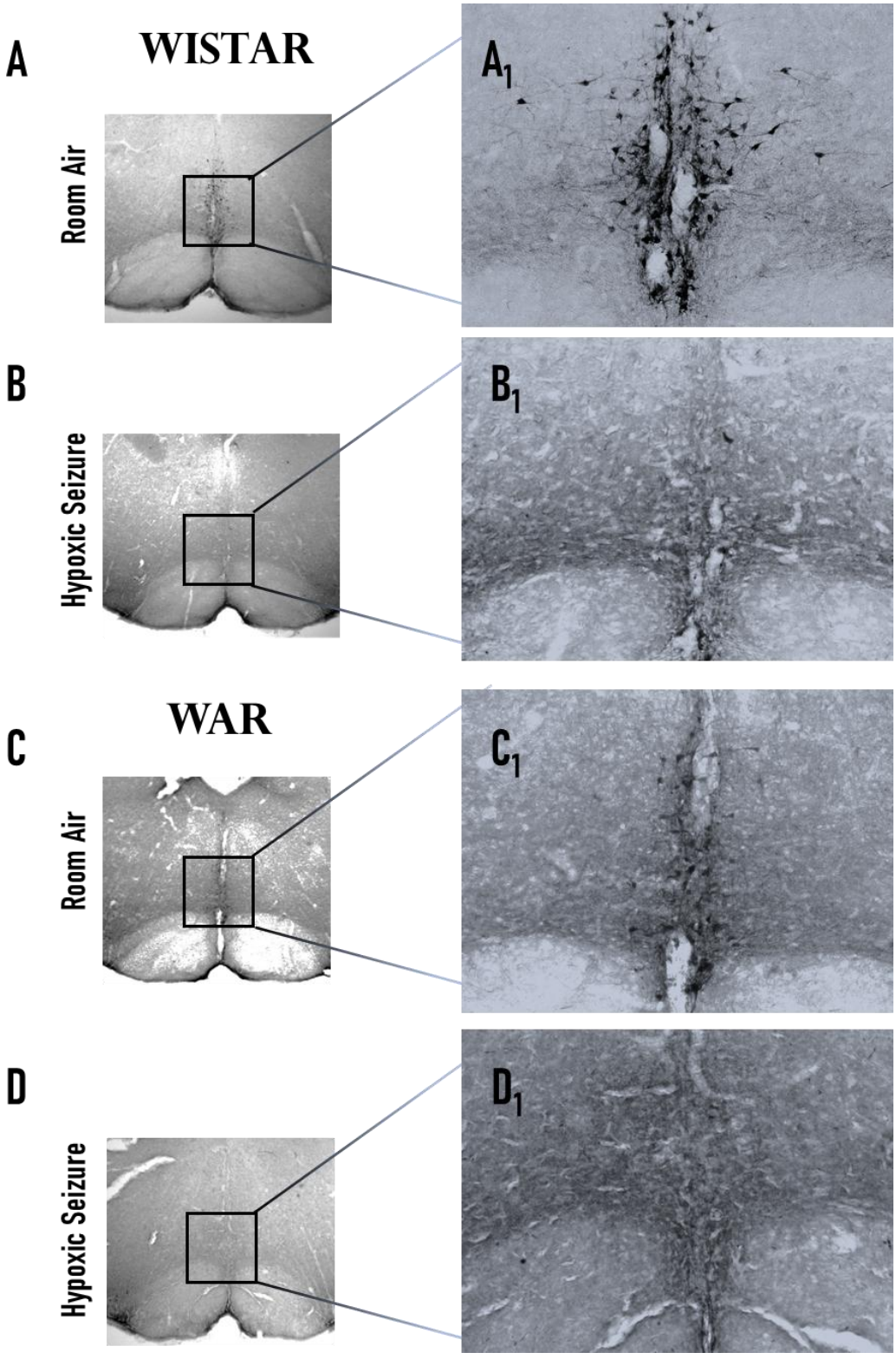


Figure 3.6.1. Neonatal hypoxic seizures deplete serotonin. A) and C) are representative images of the brainstem from Wistar and WAR (control) in ambient air, while B) and D) are from animals subjected to the hypoxia protocol. Some tissues may appear slightly cracked, but at 10x zoom, it is possible to assess the number of neurons. In the obscurus raphe region, detailed in images A1, B1, C1, and D1, we can observe an almost complete depletion of neurons marked for serotonin.

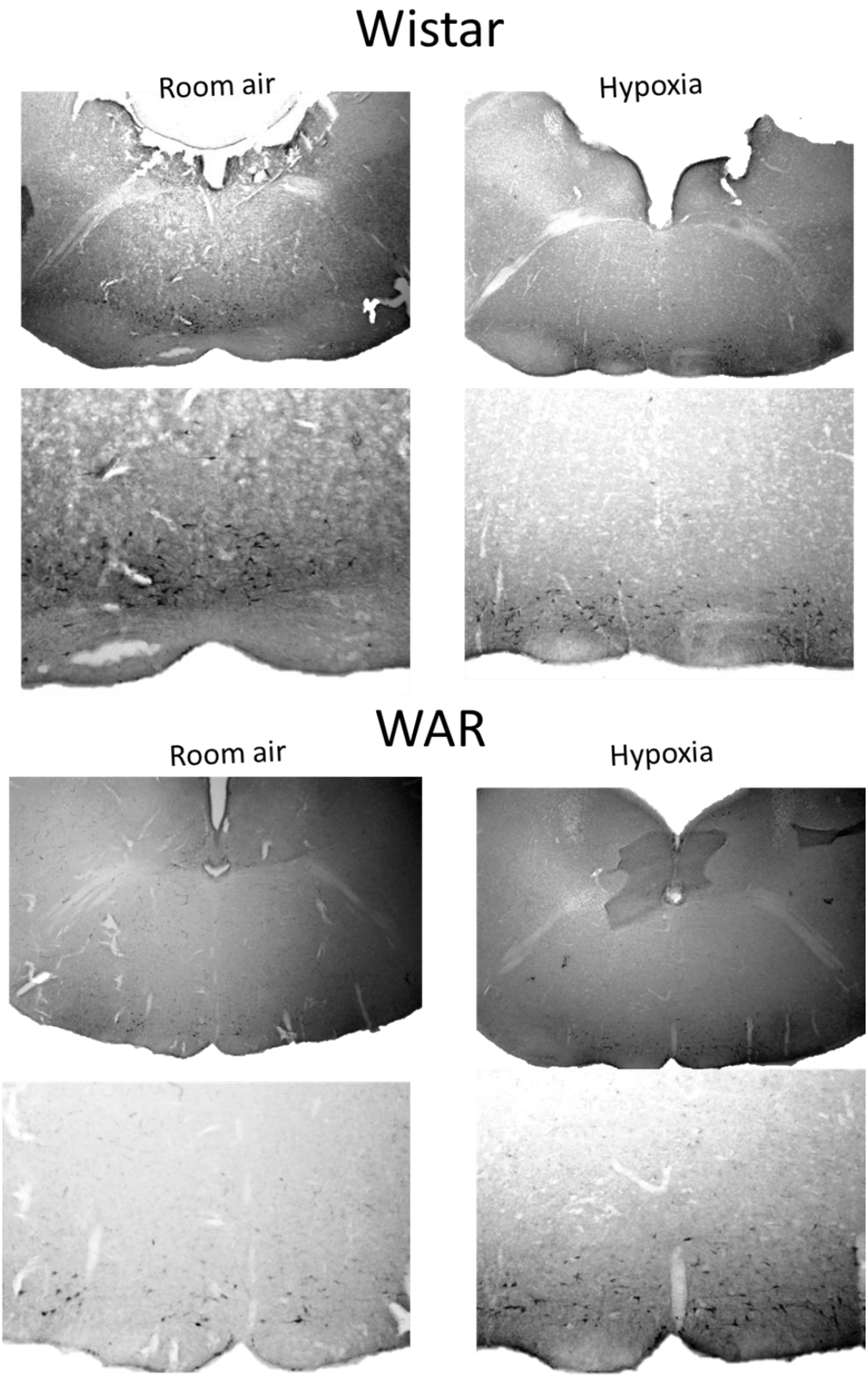


Figure 3.6.2. Images of Raphe Magnus of Wistar and WAR rats in room air or hypoxia.

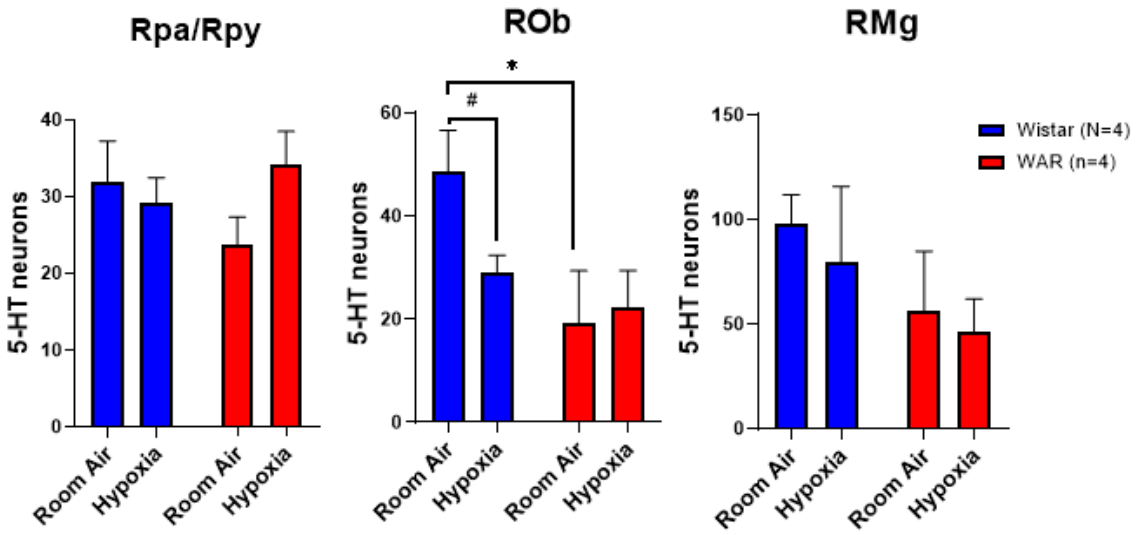
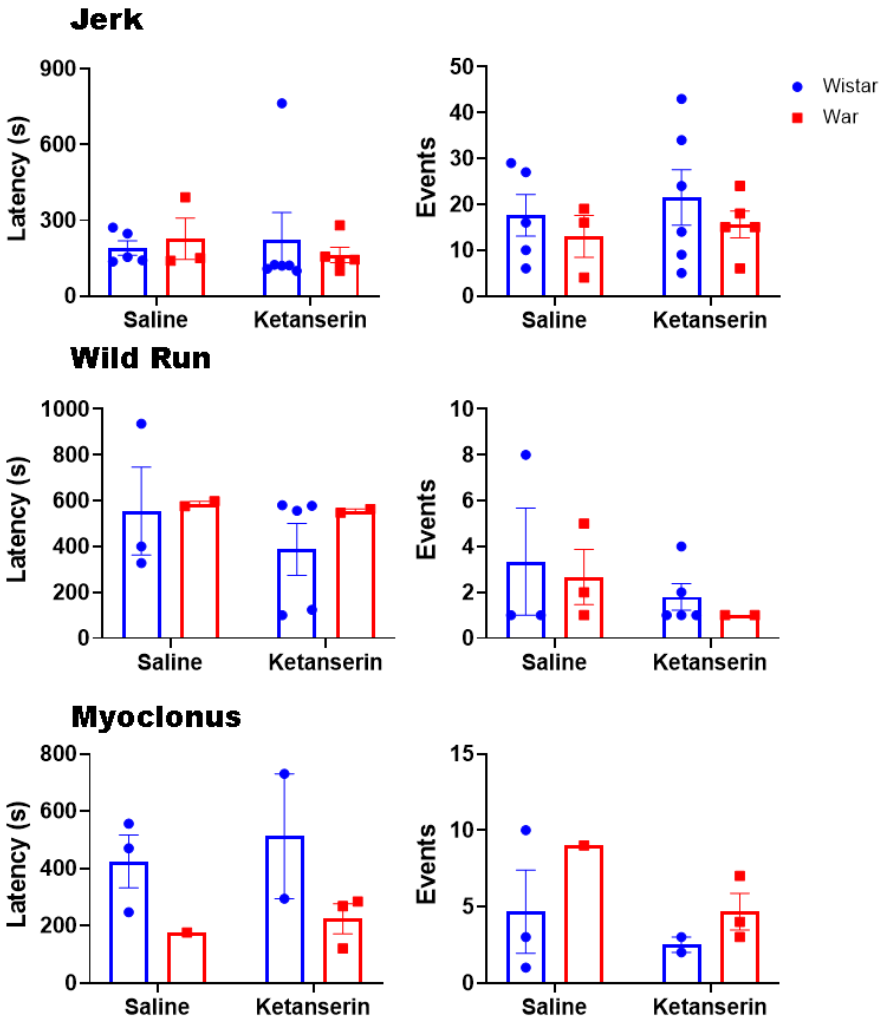


Figure 3.7. Quantification of serotonergic neurons in the brainstem. The regions of the pallidus raphe and parapyramidal raphe (A, Rpa/Rpy), the obscurus raphe (B, ROb), and the magnus raphe (RMg). Two-way ANOVA, *p<0.05 Wistar vs. WAR and #p<0.05 Room air vs. Hypoxia.

3.4.4 Blockade of 5-HT$_2$ Receptors

Antagonism of the 5-HT$_2$ receptor with ketanserin didn't alter behavioral parameters (Figure 3.8). However, two animals that received ketanserin, one from each group, died during the hypoxia protocol. This finding highlights the importance of 5-HT$_2$ receptors in the response to decremental hypoxia. The main finding of this protocol was related to breathing parameters (figure 3.9), in which the presence of gasping occurred earlier in animals that received ketanserin compared to those that received saline, in Wistar and WAR groups. Especifically, when reaching 5% O_2, fR decreases and V_T increases in animals that received ketanserin. This may indicate a greater sensitivity to hypoxia with the blockade of these receptors, triggering gasping mechanisms earlier.



3.8. Behavior activity induced by decremental hypoxia after blockade of 5-HT$_2$ receptors. Latency and number of jerk, galloping, and myoclonic events during the hypoxia protocol in P10 Wistar (blue) and WAR (red) rats. Data are presented as mean ± SEM, Student's t-test.

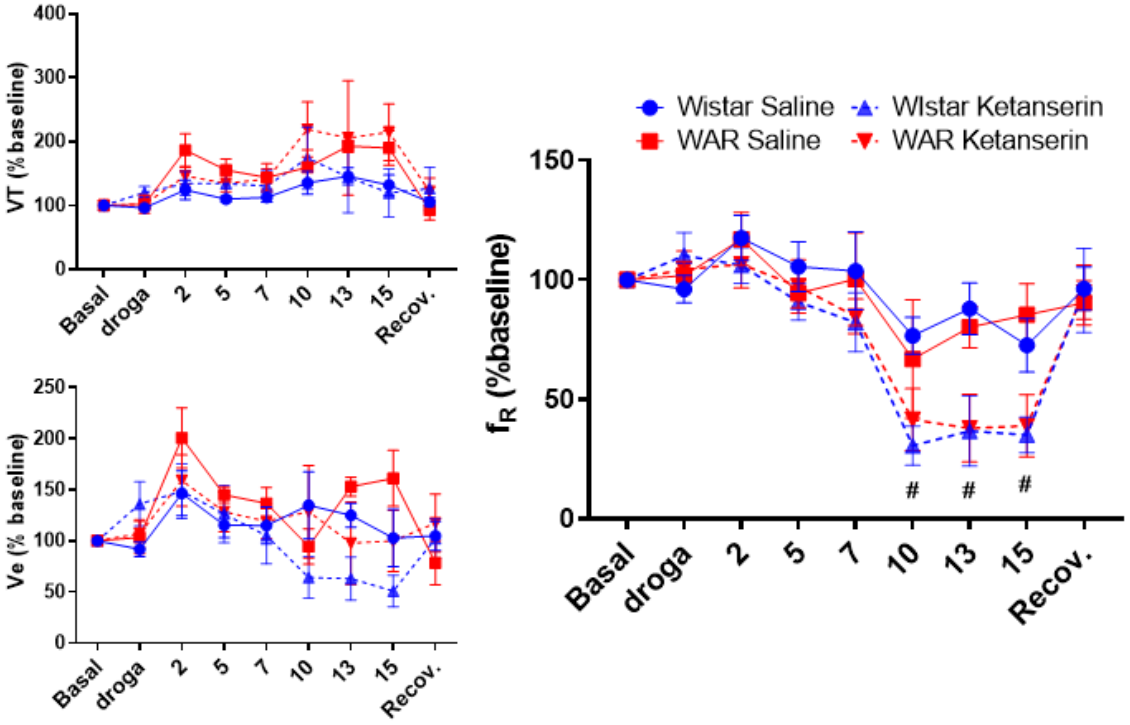


Figure 3.9. Respiratory activity induced by decremental hypoxia after blockade of 5-HT$_2$ receptors. Respiratory activity: Values of respiratory frequency (fR), tidal volume (V_T), and pulmonary ventilation (V_E) normalized to baseline values (in %). Solid lines: saline (0.9%); dashed lines: ketanserin (5 mg/kg). Data are presented as mean ± SEM, two-way ANOVA, *p<0.05 Wistar vs. WAR, #p<0.05 minute 2 vs. minute 15.

3.5 DISCUSSION

3.5.1 Behavior

The behavioral assessment revealed three main behaviors during decremental hypoxia: jerk, galloping, and myoclonus. This deeper analysis of behavior tried to maximize our data analysis, in order to compare between Wistar and WAR groups. The jerk behavior was the most common in both groups, followed by galloping and then myoclonus. These behaviors are commonly featured during neonatal seizures (Aoun et al., 2021; Pisani et al., 2021). They reflect seizure-related brain activity (Dutra Moraes et al., 2000; Racine, 1972). Jerks are associated with more localized electrical activity, while myoclonus involves a broader spread (Aoun et al., 2021; Baram, 1999). As the seizure's electrical activity expands, the behavior progresses from jerks to galloping movements, eventually leading to myoclonus (Anjum et al., 2018; Aoun et al., 2021; Born et al., 2021). When comparing Wistar and WAR behavior during hypoxia, the latency and number of events are similar for jerks and gallops, but not for the myoclonus behavior.

In the neonatal seizures, myoclonus is characterized by head movements, also known as wet dog shaking head movement. This behavior is considered by researchers as a good reference for tonic-clonic seizures (Pisani et al., 2009; Shellhaas et al., 2021; Talos et al., 2013). This protocol of a neonatal model (Jensen et al., 1991; Sanchez et al., 2005) was chosen for being highly common (Sun et al., 2016). In it, WAR strain displayed a shorter latency and a greater number of myoclonus events than Wistar. In the previous chapter, we described that WAR at P12-14 had GTCS in milder hypoxic condition (10%O2). Altogether, neonatal WAR demonstrates a higher susceptibility for GTCS under hypoxic situations. This reinforce previous studies that have shown adult WAR are more susceptible to seizures induced by electroshock, pentylenetetrazol, and pilocarpine (Scarlatelli-Lima et al., 2003), as well as audiogenic seizures (Garcia-cairasco et al., 2017).

Accessing behavior as a variable has difficulties, such as the fact that not all animals exhibit seizures in the same manner or intensity. So, a larger sample size (N) is required to achieve sufficient power to detect a statistically significant difference.

Since there is no definitive genetic marker for susceptibility to seizures in WAR strain (Dutra Moraes et al., 2000), the susceptibility to audiogenic seizure is accessed through screening, because not all rats in the strain have seizures in the acoustic test (Doretto et al., 2003; Garcia-Cairasco & Sabbatini, 1989). Our behavioral results reinforce that the WAR strain is more susceptible to seizures and provide a new perspective regarding the strain: could susceptibility to seizures during the neonatal period be a potential screening methodology? Exploring and better characterizing the behavioral aspects of neonatal seizures in WAR remains an open avenue for future experiments.

3.5.2 Breathing

Alterations during breathing activity in neonatal seizures is well documented in humans (Blum, 2009; Shellhaas et al., 2021), but is less known in animal models (Sun et al., 2016) (REF). Our results show that in the protocol described by Jensen and cols, control rats (Wistar) presented a pattern of VE that initially increased, due to V_T and fR. Then it underwent through adjustments as the level of hypoxia progressed. Breathing frequency decreased, decreasing VE, until finally, the rat exhibited a gasping behavior. Since neonate WAR presented higher myoclonic behavior, breathing could be affected by GTCS. However, when compared to Wistar, WAR presented a similar breathing response, except for the recovery period.

The initial respiratory response to hypoxic stimulus may be mediated by the carotid body (Bavis et al., 2023; Day & Wilson, 2023). This means that NTS could play a role, integrating this information and sending projections to breathing regions (Bavis et al., 2023; Day & Wilson, 2023). At this initial moment, oxygen uptake from the external environment is enhanced, thus the increase in VE at this point. With time and under the decremental hypoxia, VE was adjusted by decrease in fR, especially at 4% O_2, in which there is gasping. This response is not dependent on peripheral chemoreception mechanisms (St John & Knuth, 1981), but rather on central mechanisms (John et al., 1985), such as the activity of the primary oscillator, the pre-Bötzinger complex (Borday et al., 1997; Day & Wilson, 2023; John et al., 1985; St John & Knuth, 1981). The motor component is characterized by intense inspiratory efforts, capable of generating significant subatmospheric intrapleural pressures that result in

elevated tidal volumes and improve coronary perfusion and carotid blood flow to the brain (Nuding et al., 2024a; Ramirez & Bush, 2022; St John & Knuth, 1981). Failures of these mechanisms of breathing during acute situations are related to death, as seen in cases of SIDS(Duncan, 2018; Glass et al., 2011; Kinney & Haynes, 2019). In humans, cyanosis and dyspnea are commonly observed in association with neonatal seizures (Blum, 2009). In which, hypoxic-ischemic encephalopathy (HIE) is a very common cause of seizures in neonates(Alharbi et al., 2023; Shellhaas et al., 2021). However, little is known of the breathing consequences of it.

After acute hypoxia, an increase in ventilation above baseline values is common due to respiratory facilitation mechanisms (Shaw et al., 2023; Teppema & Dahan, 2010). This post-hypoxia response is likely a form to restore homeostasis after hypoxic stress. Interestingly, WAR animals did not exhibit this phenomenon, suggesting that the mechanisms related to post-hypoxia facilitation may be altered in this lineage. In this post-hypoxia condition, the release of serotonin leads to increased respiratory motor activity, and may enhance ventilation in Wistar but not in WAR (Devinney et al., 2016).

3.5.3 Serotonin

We demonstrate that decremental hypoxia causes lower labeling of serotonergic neurons in the ROb region. This reduction was so significant that certain slices show no labeling in this region, as illustrated by the representative figure in the results. In other regions, such as Rpa/Rpy and RMg, neuron counts were similar after hypoxia. We postulate that serotonin is being released, depleting 5-HT of the neuron body in specific regions as ROb, but not in others. In the pilocarpine seizure model, serotonin is released and can be found in the hippocampus after 30 minutes of the seizure event (Lin et al., 2013). It is possible that Rpa/Rpy and RMg released serotonin contents but did not show lower neuron labeling. In this regard, the release of serotonin during decremental hypoxia may result from either hypoxia or seizure activity. More studies are required to evaluate the specific contributions of the raphe regions, as well as serotonergic projections to other regions, such as the NTS and the VRC.

Regarding the comparison between lineages, our results show that WAR animals exhibited a reduction in serotonin labeling in the ambient air compared to Wistar in the ROb area. In the previous chapter, we showed that WAR neonates had lower serotonin content in the VRC after hypoxia in P1-3. However, our results from Chapter 2 also show that at P12-P14, a closer age to P10, there were no significant differences in serotonin levels in WAR strain. A possible explanation could be the severity of hypoxia, as it was used a milder reduction in oxygen (10% O_2) compared to this decremental hypoxia protocol. Moreover, an important factor of decremental hypoxia is the gasping characteristic, which is related to serotonin release and the involvement of 5-HT_2 receptors (Cummings, 2021).

In the brainstem, the actions of 5-HT on 5-HT2A/2C receptors are extensive (Cummings & Hodges, 2019; Cummings & Leiter, 2019). These receptors are located in crucial regions for ventilatory control, such as the NTS and the VRC, and their expression changes with age (Liu & Wong-Riley, 2010b). During rest, they maintain inspiratory motor output in regions such as the hypoglossal nerve and the phrenic nerve (Duncan et al., 2010; Fuller et al., 2005; Haynes et al., 2023). 5-HT can cause the activity of some neurons in the pre-Bötzinger complex (pre-BötC) to shift to a pattern of increased firing, also related to 5-HT_2 receptors (Al-Zubaidy et al., 1996). In vitro studies with transverse slices of a neonatal mouse spinal cord suggest that gasping depends on the activation of 5-HT2A receptors (Cummings, 2021; Cummings & Leiter, 2019). The importance of this serotonergic receptor related to respiratory activity, especially during gasping, is associated with sustaining life, modulating respiratory and cardiovascular effects (Cummings, 2021; Yang & Cummings, 2013). Therefore, the blockage of these receptors during hypoxic protocol would portray the participation of these 5-HT_2 receptors on the breathing response. In fact, our results contribute to the understanding of the role of these receptors, as the (non-specific) blockade of 5-HT_2 receptors with ketanserin resulted in a premature display of gasping when compared to saline treated animals in both strain. Furthermore, 2 animals that received ketanserin died. This finding corroborates with the literature understanding on the importance of these serotonergic receptors to survival.

Serotonin is a neurotransmitter that has been implicated in the causes of sudden infant death syndrome (SIDS) (Kinney & Haynes, 2019). Many studies suggest deficiencies of serotonin in the brainstem as a contributing factor to sudden infant death

(Erickson & Sposato, 2009). SIDS is associated with evidence of hypoxia and failure of self-resuscitation (Duncan, 2018; Goldstein & Kinney, 2017; Kinney & Haynes, 2019). Studies from Professor Haynes' research group suggest that SIDS may be related to alterations in the 5-HT_2 receptor in the brainstem (Cummings et al., 2024; Haynes et al., 2023). This evidence is crucial for understanding the mechanisms involved in SIDS as we work towards comprehending what SIDS is and how to prevent it. In order to understand the connection between SIDS and seizure activity, it is essential to further investigate the role of serotonin in neonatal seizure model.

3.6 CONCLUSION

We characterized respiratory activity during hypoxia-induced neonatal seizures in neonatal rats, exploring the possible serotonergic mechanisms involved. Our results revealed that the respiratory activity of the rats exhibited a self-resuscitation pattern at the end of the decremental hypoxia protocol, and 5-HT_2 receptors were found to be fundamental in modulating the self-resuscitation response to the hypoxia protocol. We also investigated behavioral responses to compare Wistar and WAR lineages during hypoxia-induced seizures. It was observed that WAR rats exhibited greater susceptibility to myoclonic seizures and reduced post-hypoxia ventilation compared to Wistar rats, highlighting the greater vulnerability of WAR rats.

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Chapter 4: POST-ICTAL CARDIORESPIRATORY SUPPRESSION: SEROTONERGIC MECHANISMS IN THE BRAINSTEM AND IMPLICATIONS FOR SUDDEN UNEXPECTED DEATH IN EPILEPSY (SUDEP)

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4.1. INTRODUCTION

Epilepsy is characterized by recurrent seizures and directly affects more than 50 million people worldwide (Devinsky et al., 2018). It is estimated that about one-third of patients are refractory to pharmacological treatments (Fisher et al., 2014). These refractory epilepsy patients have a high incidence of sudden unexpected death in epilepsy (SUDEP) events (DeGiorgio et al., 2019; Harden et al., 2017). Strong evidence points to the involvement of cardiorespiratory neural control in SUDEP (Devinsky et al., 2016). Cardiorespiratory data from the MORTEMUS study showed a consistent pattern of transient or terminal respiratory dysfunction following generalized tonic-clonic seizures (GTCS) (Ryvlin et al., 2013). Although this human data is highly informative regarding the sequence of events leading to SUDEP, the causes and mechanisms of respiratory changes remain unclear.

The evidence linking seizures to respiratory alterations is extensive (Lacuey et al., 2018; Umezu et al., 2024; Wenker et al., 2022). Acutely, generalized tonic-clonic seizures (GTCSs) are associated with the presence of apnea and bradycardia. According to Bateman, Li, and Seyal (2008), ictal central apneas are associated with hypoxemia in 70% of cases, being considered severe in 14% of them (Bateman et al., 2008). Prolonged apneas (≥60 s), which promote severe hypoxemia, are a potential biomarker for SUDEP (Lacuey et al., 2018). Repeated seizures are linked to long-term changes in the CO_2 chemoreflex (Sainju et al., 2019). In this context, evidence shows that the absence of the chemoreflex may be a factor in potential cases of SUDEP (Dragon et al., 2019; Sainju et al., 2019). However, the pathophysiological consequences of repeated seizures resulting in SUDEP remain poorly understood.

What changes do seizures induce to promote respiratory dysfunction? Brainstem regions related to cardiorespiratory control may be key factors in SUDEP. In this regard, serotonergic (5-HT) dysfunction in the brainstem has been implicated in this process (Buchanan, 2019b; Richerson & Buchanan, 2011).

Animal models of epilepsy have been shedding light on the role of brainstem regions in SUDEP. Excessive excitability is a significant feature of seizures (Rakhade & Jensen, 2009; Tang & Thompson, 2010).and mutation in *Kcnj16* gene is linked to epilepsy (Staruschenko et al., 2022; Zhang et al., 2021). The protein encoded by *Kcnj16* gene is a plasma membrane protein (Kir5.1) that forms a heterodimer, creating an inwardly rectifying potassium channel (Brasko et al., 2017; Zhang et al., 2021). Kir channels preferentially allow potassium to flow into rather than out of a cell, facilitating hyperpolarization-based K+ transport (Zhang et al., 2021). In the brain, Kir channels are responsible for membrane hyperpolarization following an action potential (Staruschenko et al., 2022). In the kidneys, Kir5.1 participates in potassium reabsorption in the nephron, essential for maintaining electrolyte balance and the absence of this protein results in systemic hypokalemia (Manis et al., 2020). Furthermore, this protein is associated with pH regulation by chemosensitive cells (D'Adamo et al., 2011; Patterson et al., 2021). In the brainstem, Kir5.1 immunoreactivity is co-localized with 5-HT neurons of the raphe nuclei and higher expression of this protein during development is associated with increased pH sensitivity in 5-HT neurons (Puissant et al., 2017).

In this context, Professor Matthew Hodges' research group has shown that in Dahl salt-sensitive rats (SS) with a global knockout for the *Kcnj16* gene (SS $^{Kcnj16-/-}$) present generalized tonic-clonic seizures induced by acoustic stimuli. Acutely, GTCS causes ictal apnea and postictal respiratory suppression and repeatedly, seizures are related to respiratory alterations and serotonergic dysfunction in the brainstem (Manis et al., 2021, 2023). Moreover, repeated audiogenic seizures lead to increasing postictal respiratory suppression, which may result in death (Manis et al., 2023). In addition, there is evidence of inflammatory processes mediated by activated microglia and increase in cytokines in respiratory brain regions (Osmani et al., 2024). Altogether, these results show that the SS $^{Kcnj16-/-}$ model represents a good model of SUDEP, as sudden death is linked to repeated seizures. However, this model presents a significant bias due to its genetic background, as the SS strain is intrinsically related to

cardiovascular system alterations, including the renin-angiotensin system (Palygin et al., 2017; Staruschenko et al., 2022). Therefore, in this chapter we developed three independent yet interconnected approaches related to the *Kcnj16* gene, seizures, and respiration. In the first protocol, we characterized respiratory activity and seizure susceptibility in different *Kcnj16* mutations within a non-hypertensive genetic background (Sprague Dawley; SD). In the second protocol, we presented a conditional tissue-specific knockout model using a viral approach. In the third protocol, we evaluated the effects of serotonin depletion on postictal respiratory activity in the well-characterized SS $^{Kcnj16-/-}$ model.

4.2 OBJECTIVES

4.2.1 Objectives

1. To evaluate the susceptibility to audiogenic seizures and correlated respiratory alterations in SD *Kcnj16*$^{-/-}$ rats over 10 days.
2. To assess audiogenic seizure susceptibility and CO_2 chemosensitivity in conditional *Kcnj16* knockout rats (Sync rats) using a viral vector.
3. To evaluate the effect of serotonin depletion on audiogenic seizure susceptibility and correlated cardiorespiratory changes inSS $^{Kcnj16-/-}$ rats over 10 days.

4.3 MATERIALS AND METHODS

4.3.1 Animals

The experiments were performed at the Physiology department of Medical College of Wisconsin, United States. All procedures were performed in accordance with the Medical College of Wisconsin Institutional Animal Care and Use Committee prior to animal studies (AUA 1473). The animals were kept in the vivarium with a 12h:12h light:dark cycle (6am lights on/6pm lights off), with food and water *ad libitum*.

4.3.2 Genetically modified animals

Gene editing techniques are innovative tools that allow the creation of animals specifically designed to answer different hypotheses. In the present study, we focused on manipulation of the *Kcnj16* gene, located on chromosome 17q24.3, which transcribes the Kir5.1 protein, promoting mutation, deletion or insertion of gene bases, generating mutant rats. For the first protocol, mutations in the Kir5.1 protein of Sprague Dawley rats were generated, as illustrated in Figure 4.1, whereupon: i) substitution of T in position 64 for I (strain T64I -SD $^{Kcnj16-/-}$); ii) deletion of aminoacids 64-68 (strain M7 - SD $^{Kcnj16-/-}$); and iii) deletion of aminoacids 64-81 deletion (strain M8 - SD $^{Kcnj16-/-}$).

Homozygosity was confirmed by PCR and homozygous animals without the mutation were used as controls (wildtype, WT). Heterozygous animals did not show a phenotype of susceptibility to audiogenic seizures and their results will not be presented.

For the third protocol, we used strains already known and published by the group, the animals Dahl Salt Sensitive knockout for *Kcnj16* ($SS^{Kcnj16-/-}$) and the control group (SS WT) that were acquired from colonies of the Medical College of Wisconsin. The SS have dysfunction of the renin-angiotensin system and are prone to salt-induced hypertension.

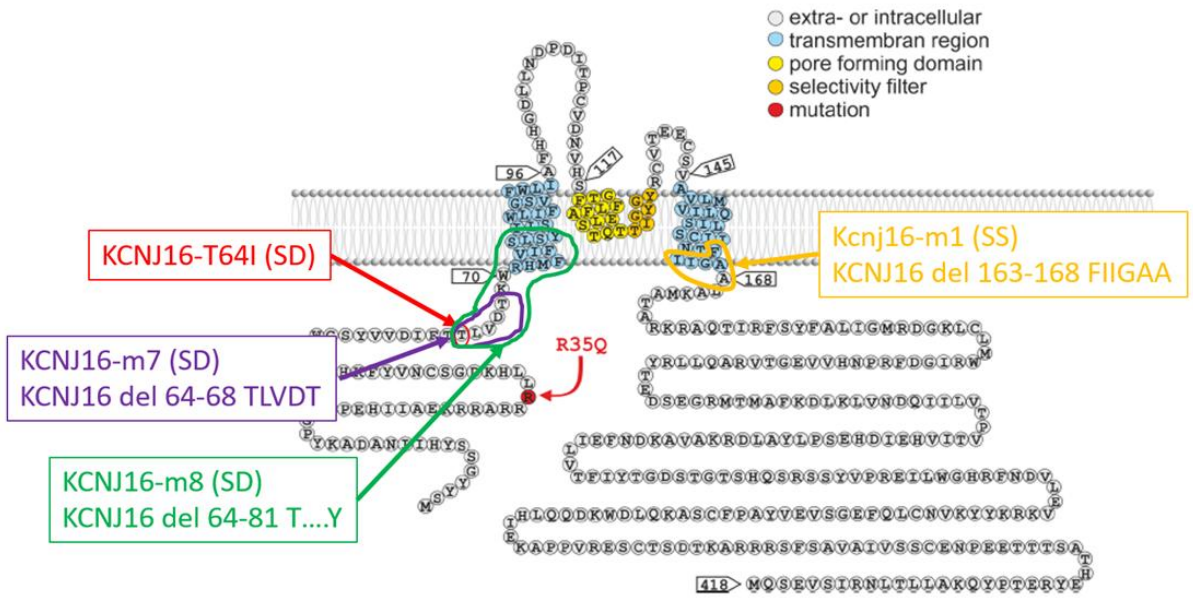


Figure 4.1. Structure of the Kir5.1 protein encoded by the *Kcnj16* gene. Highlighted are the locations of mutations or deletions: T64I SD $^{Kcnj16-/-}$ (in red), M7 SD $^{Kcnj16-/-}$(in purple), M8 SD $^{Kcnj16-/-}$ (in green) and $SS^{Kcnj16-/-}$ (in yellow).

Since mutations are present since the embryological period, they may cause various alterations that are not related to the main question of the project. For the second protocol, we formulated a rat lineage (SYNC SD), in which gene inactivation can be specific to tissues and even distinct cell groups. In this gene editing, a synthetic conditional intron (synthetic conditional intron = synchontron, SYNC) was added between an exon of *Kcnj16*. Since it is an intron, it is not transcribed by messenger RNA, but it contains loxP sites that are important for viral activation. Therefore, without the viral condition, there is no change in gene transcription and the artificial intron is edited out of the mature mRNA. However, a viral vector containing cre-recombinase recognizes the loxP sites and inserts the genetic code there, stabilizing the intron and

preventing the formation of the *Kcnj16* mRNA (figure 4.2). In the absence of the viral vector, the SYNC strain is similar to the wildtype, in which the Kir5.1 protein functions normally. In this study, conditional knockout was performed using a viral vector that infects all types of cells with a promotor that drives Cre expression in all cells (CMV - cytomegalovirus), with injections into the ventricular system (fourth ventricle and lateral ventricle). Thus, in the brain in general, cells no longer transcribe this protein functionally, while the kidney still produces the Kir5.1 protein and potassium regulation by the kidney is normal.

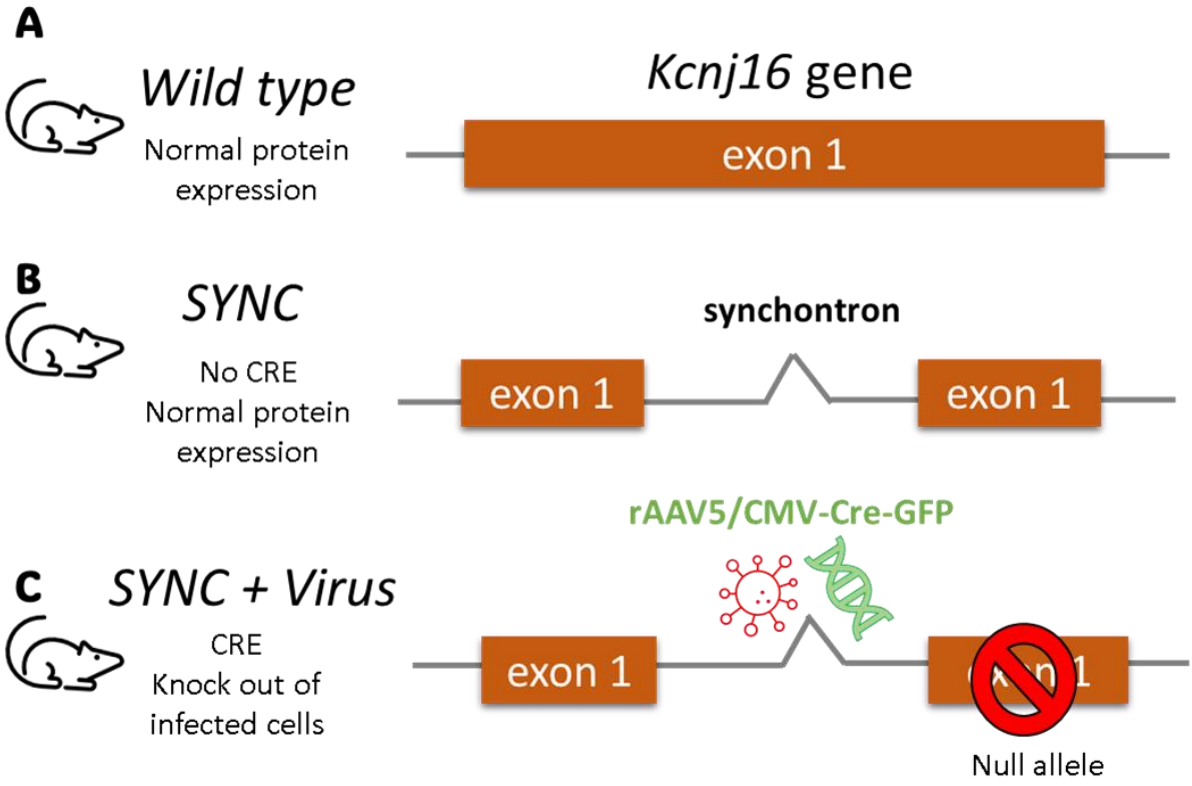


Figure 4.2. Schematic of the conditional knockout of the Kcnj16 gene using the viral vector (rAAV5/CMV-Cre-GFP). A) Wild type animal, normal expression of the protein from exon 1; B) SYNC animal, presents an insertion of an intron (syncontron) in the middle of the exon, expression of the normal protein in the absence of the virus; C) SYNC + Virus animal, virus drives Cre expression which recognizes the loxP sites and stabilized the intron preventing expression of a functional Kir5.1 protein.

4.3.3 Induction of seizures and evaluation of behavior

Audiogenic seizures were induced in the custom-built whole-body plethysmography chamber used for respiratory measurements during the seizure

protocol. The acoustic stimulus was either a 10 kHz audio frequency (produced by a function generator, model GW Instek: GFG-8020H) or white noise (produced by an untuned radio station) at 100 dB with a 50 Ω speaker (Visaton model: k50wp) positioned approximately 10 cm above the rat was used. Rats were placed in the chamber for 20 minutes for acclimation and acoustic stimulation lasted for 2 minutes. Seizure behaviors were video recorded, seizure severity was scored (0–4) and was determined by the final behavioral stage reached during acoustic stimulation based on a modified Racine scale, as in the table below (Manis et al. 2021).

Table 4.1. Behavioral seizure score during acoustic stimulus.

Score 0	No distinctive seizure behavior.
Score 1	One episode of wild running.
Score 2	Two episodes of wild running.
Score 3	Wild running followed by clonic or tonic-clonic behaviors. Posture is regained immediately after the seizure.
Score 4	Running followed by tonic-clonic behaviors, ending with a sustained period of tonic extension and apparent loss of consciousness or ambulation.

4.3.4 Ventilatory Measurements

Ventilatory measurements were performed by continuous-flow whole-body plethysmography in a manner similar to that performed by Mannis et al. 2021. The chamber inlet flow was kept constant at 8 L/min, balancing it with the vacuum outlet. The oxygen (O_2) and carbon dioxide (CO_2) levels within the chamber, as well as the pressure, temperature, and relative humidity, were continuously monitored and recorded using ADInstruments' LabChart software. Ventilatory pressure signals were calibrated daily before each study, using 0.6 mL air injections at 100 cpm. All ventilatory measurements were processed and calculated according to previous methods.

4.3.5 Electrolyte quantification

At the end of the protocol, animals were deeply anesthetized with isoflurane (5%), the thorax was opened and blood (minimum 100 uL) was collected directly from the left ventricle. Electrolytes were measured with a PRIME CCS blood gas analyzer (Nova Biomedical, Waltham, MA, USA).

4.3.6 Immunofluorescence

At the end of the protocol, animals were deeply anesthetized with isoflurane (5%), then perfused transcardially with buffered saline (PB 0.1M; 100 mL) and fixed with 4% paraformaldehyde (PFA 4% 100 mL). After the brains were extracted, they were placed in a sucrose solution (30% sucrose + 0.1% sodium azide) until they were sectioned in series (25 µm thick). The tissue sections were kept in cryoprotective solution until the day of the immunofluorescence experiment. The immunofluorescence protocol consisted of washing selected tissues with 0.1M PBS and 0.1M PBST, incubation with the primary antibody overnight, and then the next day, after further washing, with the secondary antibody. The slides were mounted and stored at 4°C. The antibodies used are shown in the table below.

Table 4.2. Antibodies

Staining	Primary antibody	Anticorpo secundário	Fluorescência
NeuN	**Chicken anti-NeuN;** Millipore, #A3BN91 1:500	**Donkey anti-chicken** Alexa Fluor 488 Jackson, #703-545-155 1:500	Green
5-HT	**Goat anti-5HT;** Immunostar, #20079 1:1000	**Donkey anti-Goat** Alexa Fluor 594; Jackson, #705-585-147 1:500	Red
TPH	**Mouse anti-TPH** Sigma, #T0678 1:1000	**Donkey anti-mouse** Alexa Fluor 647 Jackson, #715-605-151 1:500	Cian

The images of the slides were obtained by illuminating them with light spectra of a specific wavelength that excites the fluorophore of the secondary antibody and then emits fluorescence (Table 4.2). In the case of SYNC animals, the fluorescence results from the viral expression of the GFP protein (green fluorescent protein), in the green wavelength spectrum. The emitted fluorescence was captured by the Keyence BZ-X810 microscope imaging system (Keyence), with 4X or 10X magnification. The subregions of the medullary raphe were defined with the help of the rat brain atlas (Paxinos & Watson, 2006). Relative fluorescence within images was quantified in gray scale using the Fiji ImageJ imaging program, as previously done (Manis et al. 2023).

4.3.7 Stereotaxic surgery

The SYNc male rats were deeply anesthetized (2% isofluorane + 100% O_2), placed in the stereotaxic equipment and fixed to the support bar, ensuring stability during surgery. A small incision was made to expose the skull. From the coordinates of bregma and lambda and using a rat stereotaxic atlas, the location of the 4th ventricle (AP: -12.6; LL: 0; DV: -7.5) or lateral ventricle (LV) (AP: -0.24; LL: -1.7; DV: -4.5) was determined. A small hole was made in the skull using a surgical drill where the cannula was gently inserted. The viral vector rAAV5/CMV-Cre-GFP (Vector Core, UNC) was used. This is an adenovirus (rAAV5) capable of replicating inside any cell included a broadly active promotor (CMV) that drives expression of the cre-recombinase protein (CRE) and green fluorescent protein (GFP). The injected volume was 200 nL, at a flow rate of 0.33 ul/min, with a concentration of 0.1 viral product/mL. After completion of the viral injection, the skin incision on the dorsum of the head was closed with sutures, and the rat recovered in a warm environment and monitored.

4.3.8 Telemetry implantation surgery

The $SS^{Kcnj16-/-}$ male rats were deeply anesthetized with isoflurane (2%, in 100% O_2), and then placed in the prone position on a surgical table. An incision (1.5 cm) was made between the scapulae, to which the telemetry probe (HD-S10, DSI) was attached. The animals were then placed in the supine position and an incision was made in the ventral region of the neck, approximately 1 cm long, parallel to the trachea.

The right carotid artery was isolated, and the catheter of the telemetry probe was carefully inserted into the artery. The animals received antibiotic and analgesic (Qais), and then monitored during the recovery period in a warm environment.

4.3.8 Statistical Analysis

Statistical analyses were performed using two-way repeated measures ANOVA. Appropriate post hoc tests were performed to examine significant differences between groups. Results were considered statistically significant when $p<0.05$.

4.4 PROTOCOLS

4.4.1 Protocol 1: Audiogenic seizures and respiratory changes in SD $^{Kcnj16-/-}$

For this protocol, adult Sprague Dawley $^{Kcnj16-/-}$ male rats were used. The experiment involved placing the animals in a plethysmography chamber to record ventilation and behavior during acoustic stimuli over a 10-day protocol. Body weight was measured once a day and rectal body temperature was measured before and after placing the animal in the chamber. The first day was intended to acclimate the animal to the chamber (day -1, figure 4.3). The acoustic stimulation protocol consisted of measuring basal ventilation for 20 minutes, followed by two minutes of acoustic stimulation, and then 20 minutes of post-stimulus/post-ictal recording. This procedure was performed once a day for 10 consecutive days. The chemoreflex was measured one day before and one day after the ten-day protocol using a hypercapnic mixture (7% CO_2, 21% O_2, balanced with N_2) for 15 minutes. Finally, the animals were euthanized, blood was collected and the brain was perfused and fixed.

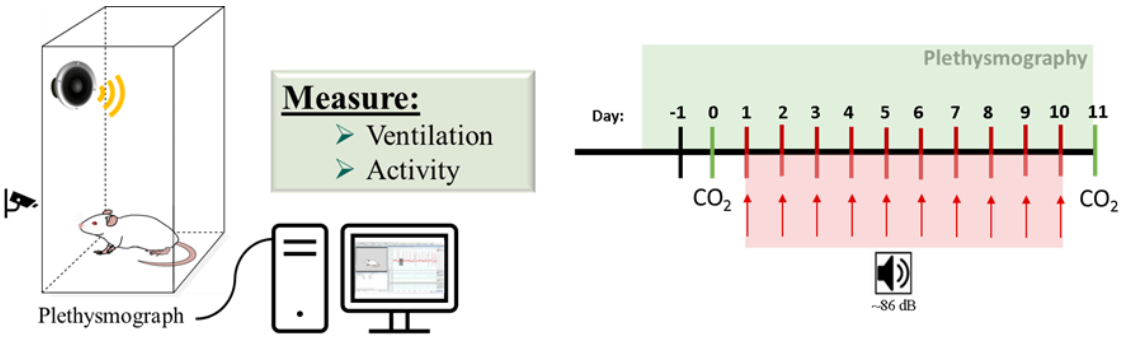


Figure 4.3. Schematic image of the first protocol experimental design. On the left, representation of the plethysmography box coupled with a sound system and camera connected to the Labchart recording program. On the right, timeline of the 10-day protocol. Figure made by Melissa Eilbes.

4.4.2 Protocol 2: Conditional Knockout of *Kcnj16*

For this protocol, we used adult SYNCs rats to evaluate the behavioral response to acoustic stimulation and chemosensitivity to CO_2 before and after conditional knockout of the *Kcnj16* gene in the brain (Figure 4.4). Initially, the animals were placed in the plethysmography chamber for acclimation (D0). Measurements were made in naïve condition. In this condition, ventilation was measured in room air for 15 minutes, followed by a hypercapnic challenge (7% CO_2 in 21% O_2 balanced with N_2) for 15 minutes. On the following day, the acoustic stimulation protocol was performed, which consisted of measuring basal ventilation for 20 minutes, followed by two minutes of acoustic stimulation and then 20 minutes of post-stimulus/post-ictal recording. Approximately two to three days later, stereotaxis surgery was performed for injection of the viral content. After 3 weeks of recovery for viral , the chemoreflex and acoustic stimulation tests were repeated as described. Finally, the animals were euthanized, blood was collected and the brain was perfused and fixed.

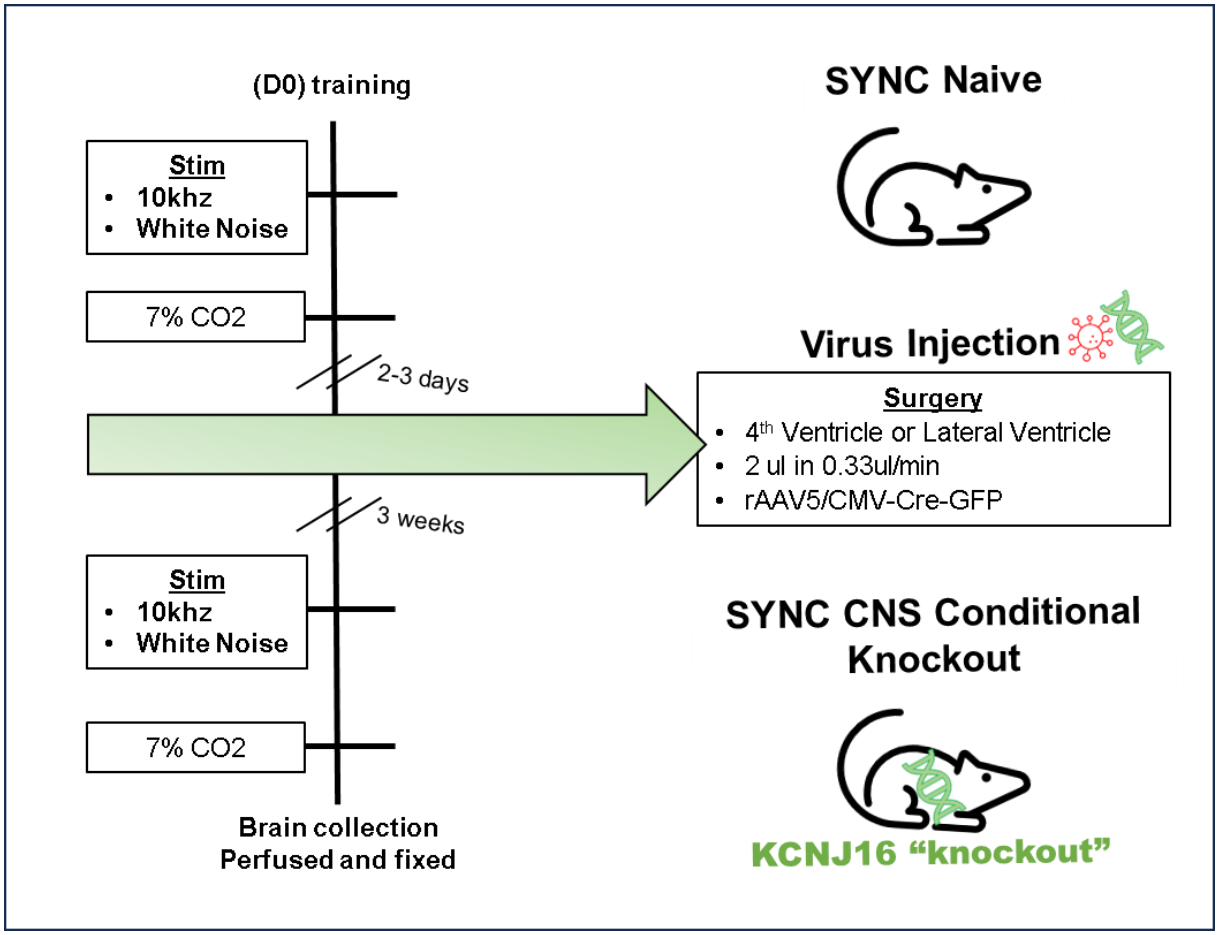


Figure 4.4. Experimental protocol for conditional knockout in SYNC rats. Acoustic stimulation (10kHz and White Noise) and hypercapnic challenge was performed before and after virus injection in the brain. The goal was to evaluate in the same animal CNS knockout of Kcj16 gene the susceptibility to seizures and chemoreflex alterations.

4.4.3 Protocol 3: Serotonin depletion in SS $^{Kcnj16-/-}$ rats

For this protocol, the previously characterized SS$^{Kcnj16-/-}$ strain was used. As shown in figure 4.5 below, a telemetry device was implanted surgically. After the surgical intervention, the animals were allowed a one-week recovery period. Subsequently, the animals were placed in the plethysmography chamber, where the equipment's functioning was initially tested and physiological parameters such as respiration, blood pressure and body temperature were recorded. The following day, the animal was treated with PCPA (200mg/kg, i.p.) or saline, placed in the plethysmography chamber and physiological parameters were recorded. The following day, the animals were treated again and an acoustic stimulation protocol (to induce seizures) was initiated, which lasted for ten days, as previously explained. It is

important to note that the animals subjected to PCPA treatment did not survive until the end of the protocol.

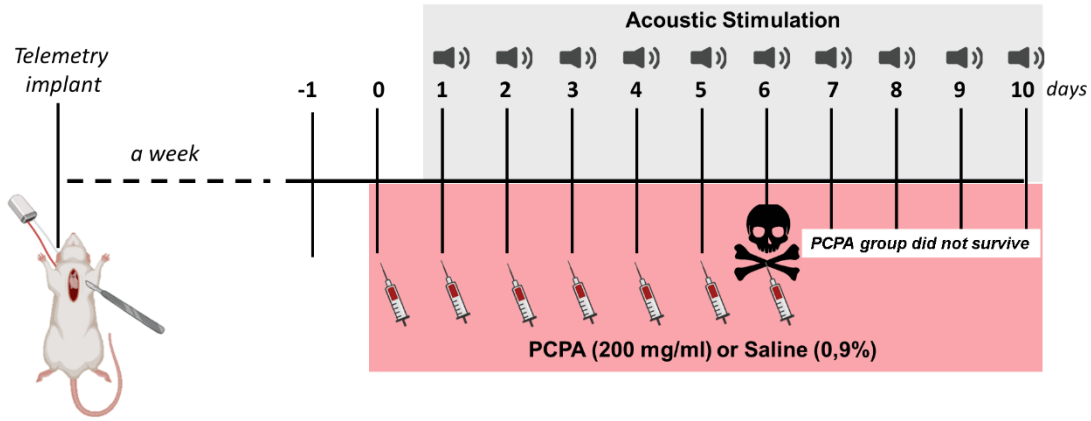


Figure 4.5. Experimental protocol for serotonin depletion in SS $^{Kcnj16-/-}$ animals.

4.5 RESULTS

4.5.1 Protocol 1: Audiogenic seizures and respiratory alterations in SD $^{Kcnj16-/-}$ rats in a non-hypertensive background

A) Confirmation of susceptibility to audiogenic seizures

The first test performed with the mutant SD animals was to confirm susceptibility to seizures. We measured the behavioral score at different frequencies of acoustic stimuli (0.1, 1, 10 kHz and white noise) in SD^{WT} and three different SD^{Kcnj16} mutant lines (M7, M8 and T64I). Generally, all SD$^{Kcnj16-/-}$ rats had seizures with acoustic stimulation of 10 kHz and White Noise, but not with 0.1 or 1 kHz. M7 SD$^{Kcnj16-/-}$ rats had a higher seizure score (more severe seizures) with 10 kHz and T64I rats with white noise (Figure 4.6). Similar to SD^{WT} rats, the response of heterozygous animals of all *Kcnj16* mutations was also studied and no seizure behavior was observed during acoustic stimuli.

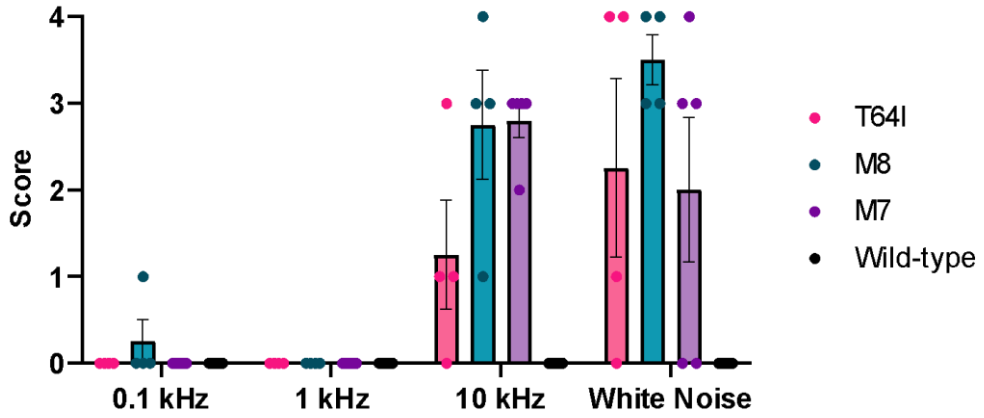


Figure 4.6. Behavioral score evaluation in several acoustic stimuli. In pink, T64I strain (n=4); in green, M8 (n=4); in purple, M7 (n=4) and finally in black, WT (n=5). Only homozygous animals presented seizures. Lower frequency stimuli (0.1 and 1kHz) did not induce convulsive behaviors. This result is the first confirmation that the KCJ16 knockout model in non-hypertensive background also presents audiogenic seizures.

B) Electrolytes

Blood collected at euthanasia suggests that SD$^{Kcnj16-/-}$ rats had lower levels of K^+, which is similar to that reported in SS$^{Kcnj16-/-}$ rats. However, there were no other obvious trends for differences in other electrolytes. The number of samples is low and more experiments need to be done to confirm these findings.

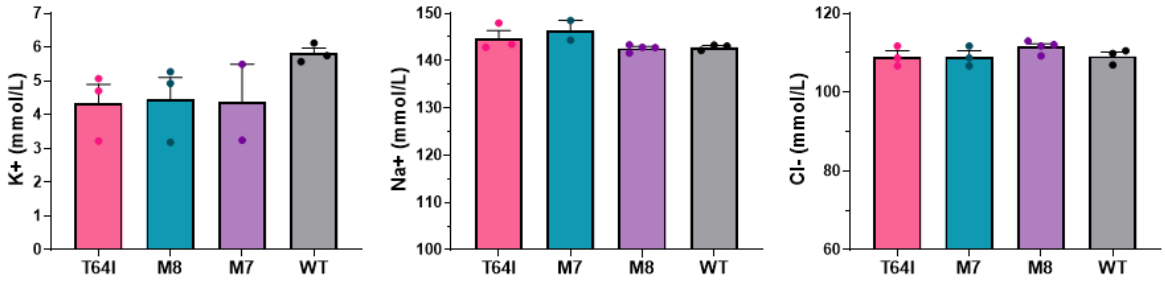


Figure 4.7. Blood concentration of Potassium (K+), Sodium (Na+) and Chloride (Cl-). In pink T64I strain (n=3); in green, M8 (n=3); in purple, M7 (n=2) and finally in black, WT (n=3).

C) Repeated seizures

Repeated acoustic stimulation did not promote changes in the average behavioral responses throughout the protocol, as shown in Figure 4.8. The average seizure score over 10 days of sound stimulation varied greatly. To quantify and

compare the score over time, the area under the curve of the group average was evaluated (Figure 4.8B). Our data suggest that the M7 strain has a higher score over the 10 days protocol, but further studies need to be conducted to confirm this suggestion (n=4). In addition, a very important result was the survival of the animals, because unlike the SS $^{Kcnj16-/-}$ animals, the SD$^{Kcnj16-/-}$ animals did not die throughout the audiogenic seizure induction protocol. Survival was 100% in all groups. This is a very important piece of data that shows that SUDEP-related mortality in the SS$^{Kcnj16-/-}$ model may in fact be related to components of the cardiovascular system, altered in the SS strain. This is a topic that would need a pronounced analysis, especially of the mechanisms involved.

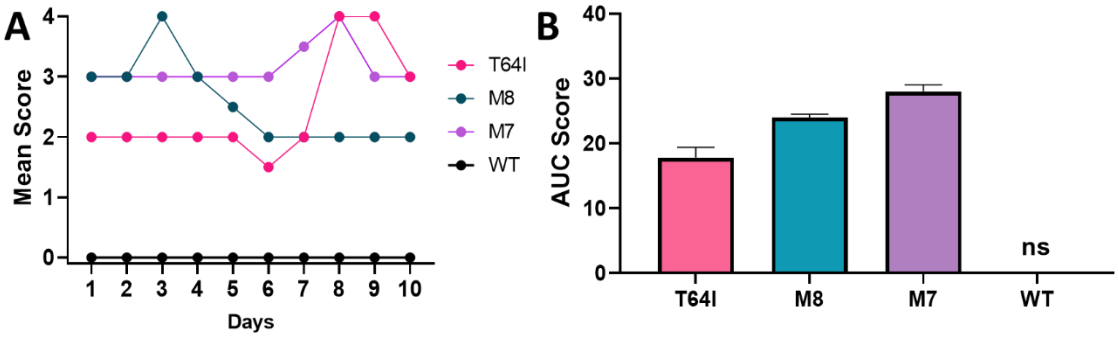


Figure 4.8. Seizure score over 10 days. Mean score (A) and area under the curve (AUC) of the score (B) over 10 days. In pink T64I strain (n=4); in green, M8 (n=4); in purple, M7 (n=4) and finally in black, WT (n=5).

D) Respiratory pattern during seizures

The figure below (Figure 4.9) shows a representative recording of ventilation during the first day of the audiogenic seizure protocol induced by acoustic stimulation. There are changes in the respiratory pattern during the acoustic stimuli, such as increase in fR and VT. Movement artifacts during sound stimulation prevent a more detailed respiratory analysis during the seizure. However, the results demonstrated that when SD$^{Kcnj16-/-}$ rats present generalized tonic-clonic seizures (GTCS), they also present ictal apnea. The post-ictal respiratory pattern also presents changes in fR and VT, quantitative analysis was explored in Figure 4.10. In the figure below, it is possible to observe the irregular post-ictal respiratory pattern. Interestingly, post-ictal apneas are not always observed and the different strains did not present exactly the same

responses.

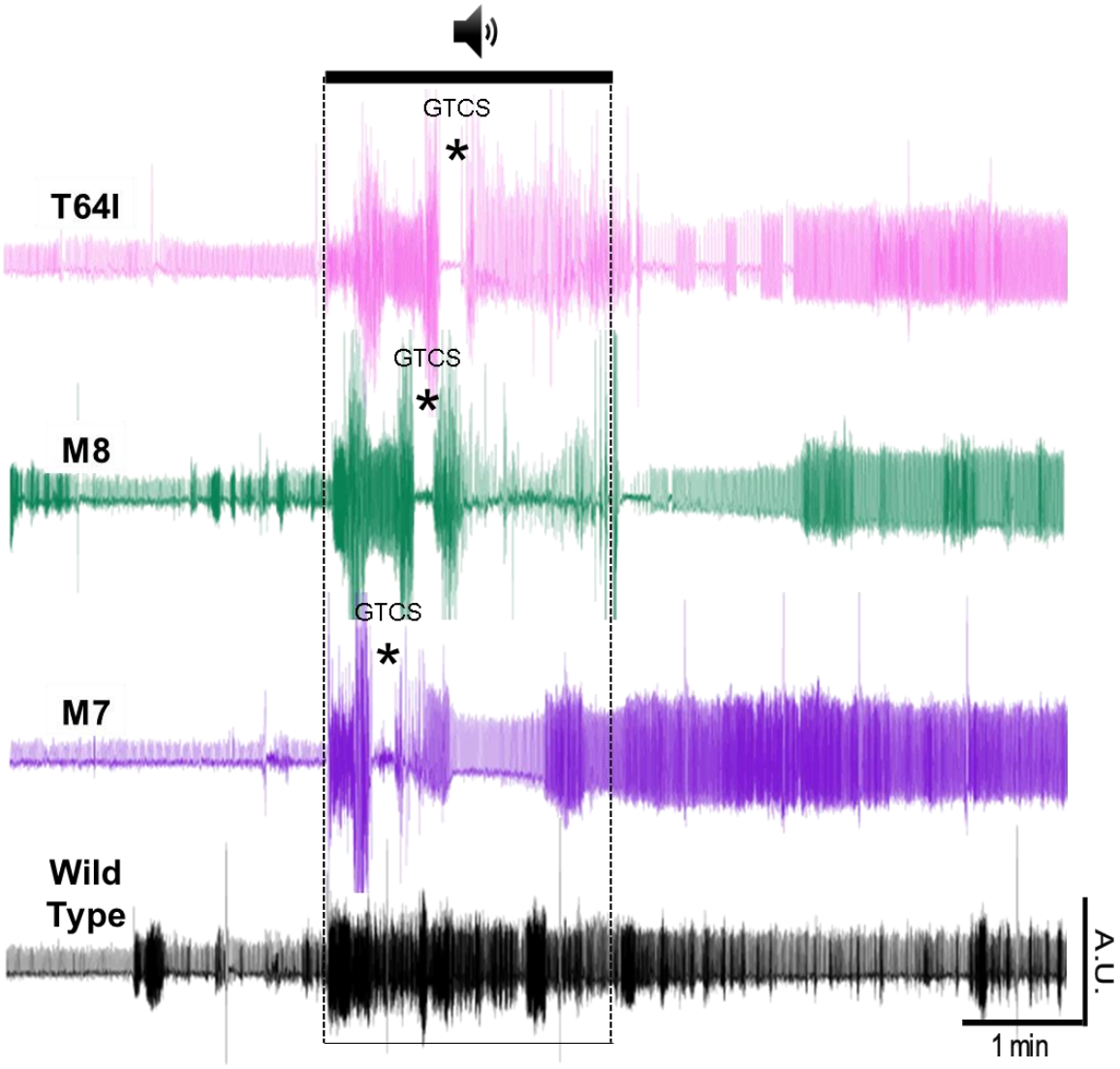


Figure 4.9. Representative ventilation recording of T64I (pink), M8 (green), M7 (purple) and Wild type (black) strains during an audiogenic seizure event induced by acoustic stimulus (dotted lines). During the GTSC seizure, marked by * symbol, the animals showed ictal apnea. Differences in signal amplitude before, during and after the seizure and also in frequency can be noted.

Respiratory variables were analyzed during the baseline period and at minutes 1, 2, 3, 4, 5, 10 and 15 after the end of the sound stimulus on day 1 and day 10 (Figure 4.10). On day 1, only M8 presented significant post-ictal respiratory depression. On day 10, all SD $^{Kcnj16-/-}$ presented a reduction in fR shortly after the seizure. VT increased in the ictal period after the seizure on the first day, but not on the tenth.

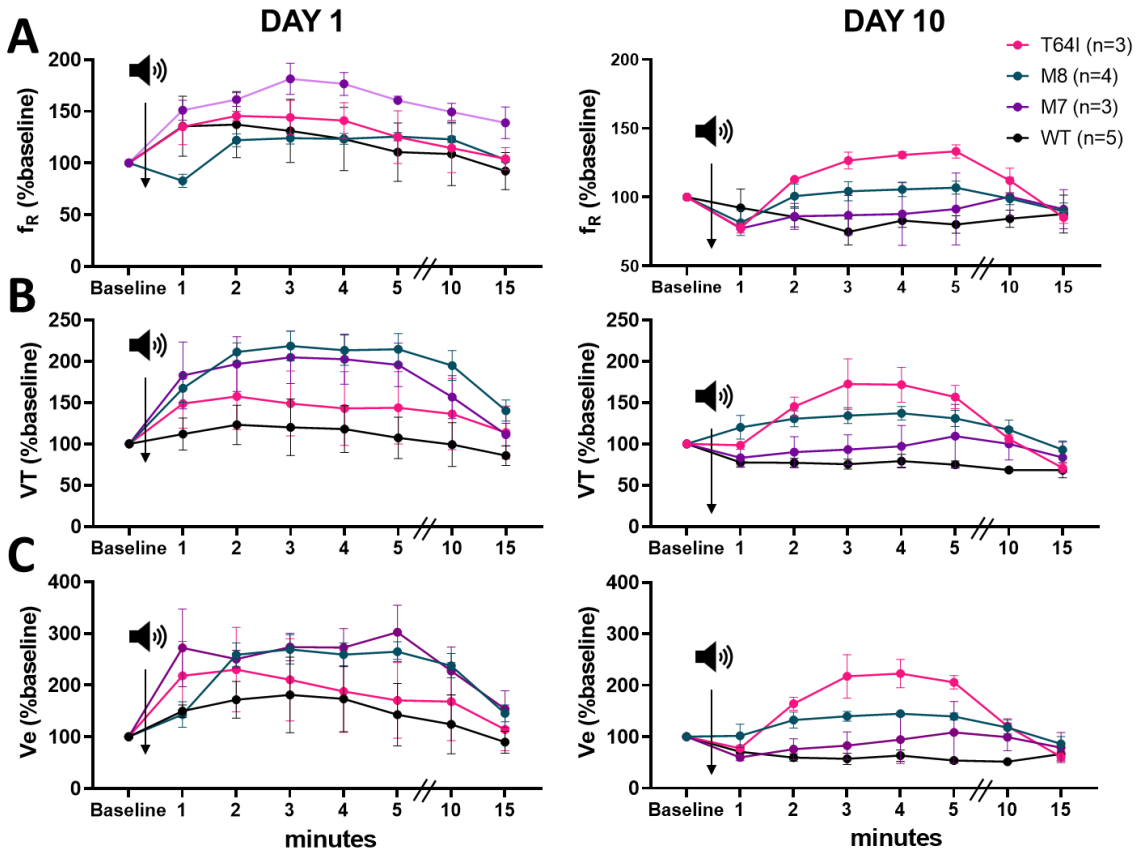


Figure 4.10. Ictal and post-ictal respiratory variables. Values in percentage related to the baseline value of day 1 or day 10. A) fR (respiratory rate); B) Tidal volume (VT) and C) Pulmonary ventilation (Ve). Values presented as mean + standard error of the mean.

E) Body temperature

Body temperature was measured before and after placing the animal in the plethysmograph at all times. Our results show that body temperature measured prio to seizure stimuli did not change throughout the protocol (figure 4.11A). However, in the post-ictal period, the SD$^{Kcn16\ -/-}$ rats presented an increase in body temperature that became more evident over time (figure 4.11B). For comparison between groups, the average delta temperature was evaluated over the 10 days (figure 4.11C), in which it became clearer that the animals presented hyperthermia after the seizure. Interestingly, we noticed that animals with lower scores had less variation in temperature, such as T64I animals, and animals with higher scores presented higher temperatures. Thus, we correlated the average delta temperature on the x-axis and the average score of each animal on the y-axis (figure 4.11D), and we observed a positive correlation ($p<0.05$).

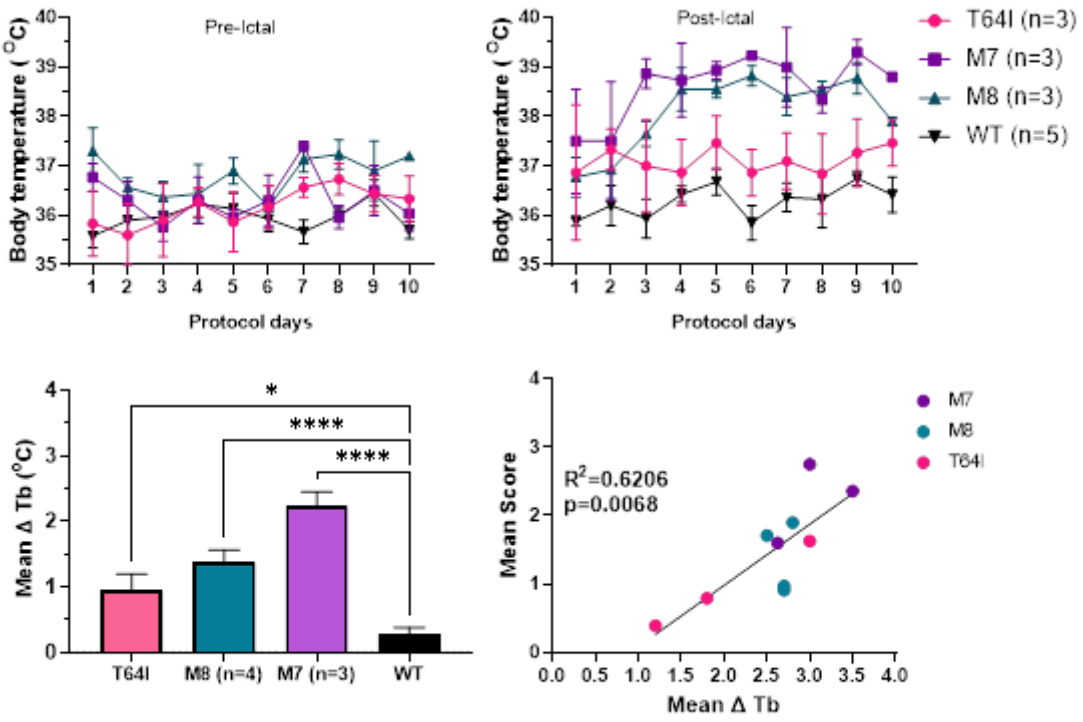


Figure 4.11. Body temperature and postictal hyperthermia. Body temperature before (pre-ictal, A) and after audiogenic seizure (postictal, B) over 10 days of protocol. C) Mean comparison of body temperature delta over ten days of protocol between groups (Mean Delta Tb). D) Correlation between the animal's mean score during the protocol and the mean temperature delta (Pearson's Correlation, $p<0.05$). Seizures induce postictal hyperthermia. Data presented as mean ± standard error of the mean, One way anova, *$p<0.05$, ***$p<0.01$.

F) Changes in basal ventilation

In addition to the changes related to the ictal period, changes in basal ventilation were observed in SD$^{Kcnj16-/-}$ animals . The comparison of values in room air on the first day of acoustic stimulation (d1) and on the last (d10) can be seen in the figure below (figure 4.12). There was a significant increase in VT, in T64I and M8 strains SD$^{Kcnj16-/-}$, and a more discreet increase in fR, significant only in M7 strain, providing an increase in Ve in room air for the T64I and M8 strains. The VT/Ti variable gives a dimension of respiratory drive and in SD$^{Kcnj16-/-}$ animals, this variable presents a significant increase after repeated crises. Ve/VO_2 and Ve/VCO_2 were not statistically different. More experiments need to be performed to increase the sample size.

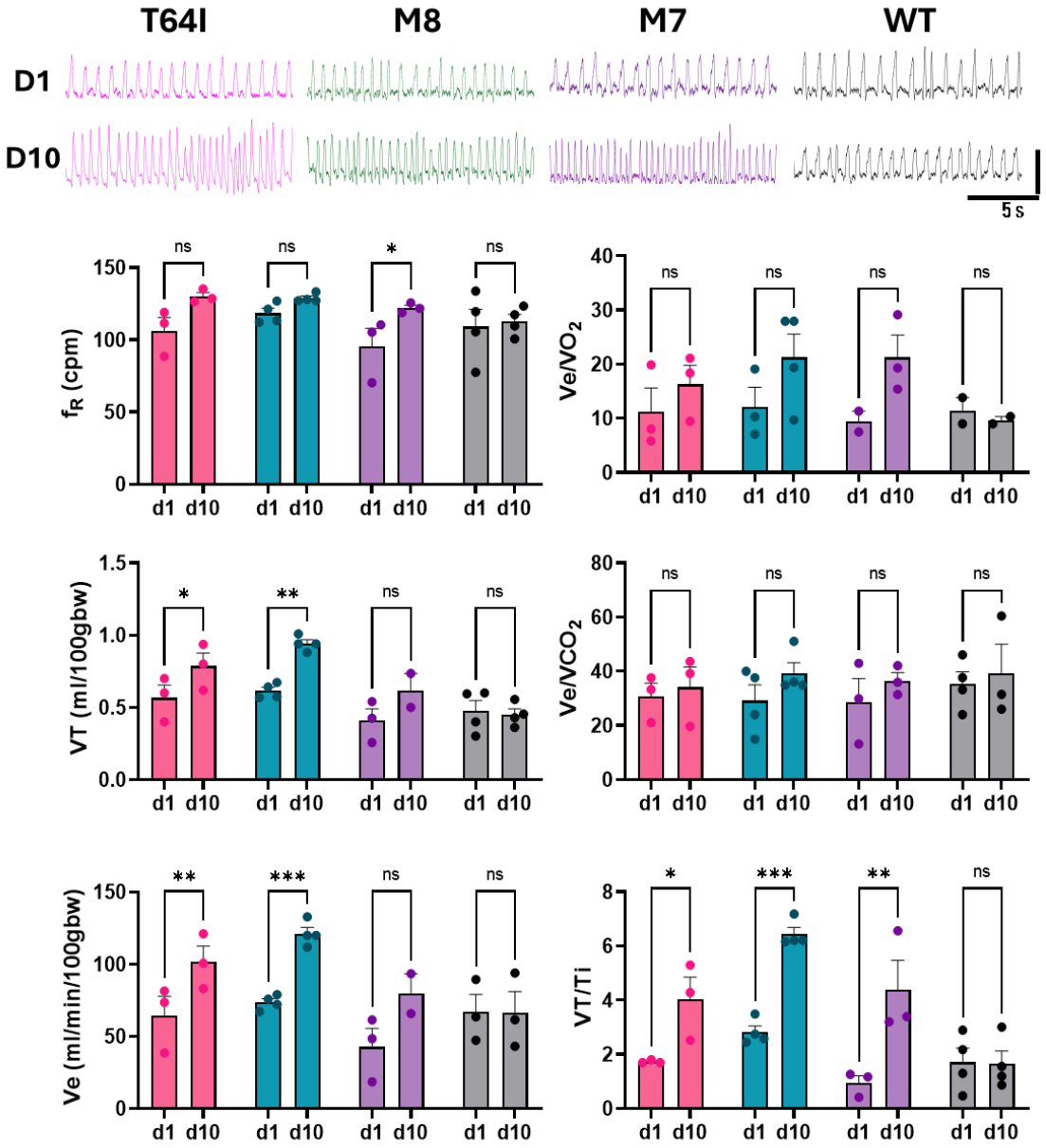


Figure 4.12. Altered basal ventilation after acoustic stimulation protocol. A) Representative ventilation records were obtained during the basal period on days 1 and 10 of the study. B) Minute ventilation (Ve), C) respiratory rate (fR), D) tidal volume (VT), E) respiratory equivalent (Ve/VO_2), F) carbon dioxide production (Ve/VCO_2) and G) respiratory drive (VT/Ti).

G) Response to hypercapnia does not change after repeated seizures

The ventilatory response to hypercapnia before the 10 day protocol was the same as after the protocol. Even though the graph of the hypercapnic ventilatory response (HCVR), which represents the ratio between pulmonary ventilation values during hypercapnia and those in room air, shows a tendency of altered chemoreflex.

The changes observed in the figure after repeated seizures are related to alterations in baseline values rather than the ventilatory response during the stimulus. In the figure below (Figure 4.13), values of respiratory rate (fR), tidal volume (VT), and minute ventilation (Ve) during exposure to 7% CO_2, both before (naive) and after 10 days of acoustic stimulation (repeated seizures), are presented as raw values. Due to significant changes in baseline values, expressing the response only as delta or HCVR could lead to a result that introduces interpretative bias.

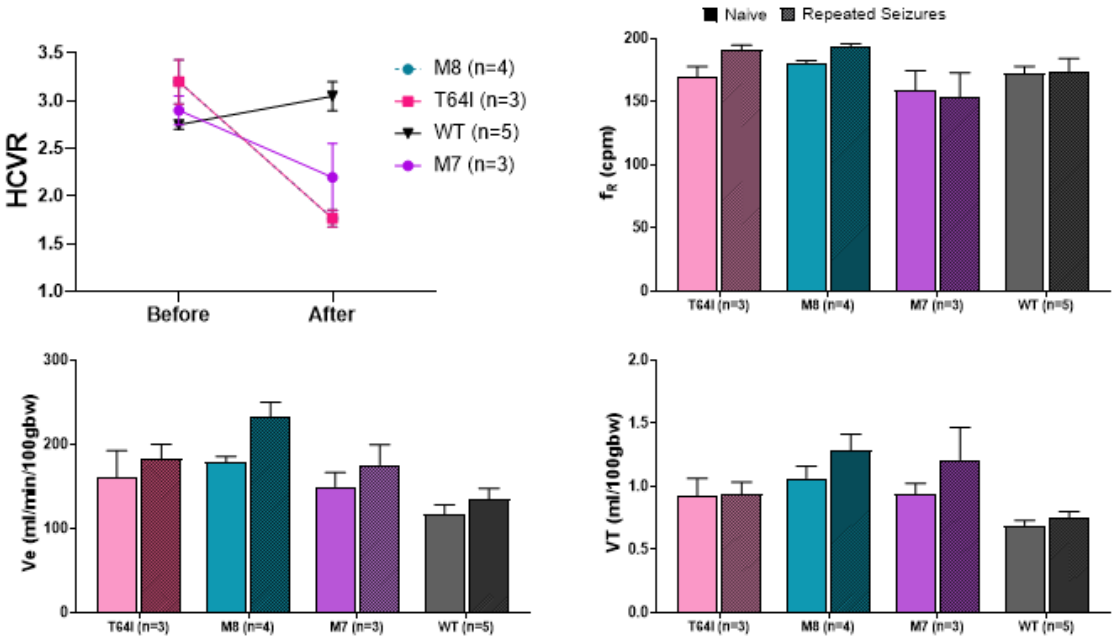


Figure 4.13. Response to hypercapnia after repeated seizures. Hypercapnic ventilatory response (HCVR) before and after 10 day protocol. VE, fR and VT expressed as raw values during the 7%CO_2 stimulus. Data presented as mean + SEM. Two Way ANOVA, p>0.05.

H) Serotonin expression after audiogenic seizures

The expression of serotonin (5-HT) and tryptophan hydroxylase (TPH) through immunohistochemistry is reduced after repeated seizures in the brainstem. In the medullary raphe region, the reduction in serotonin is most noticeable in the raphe pallidus in the three *Kcnj16* knockout lines. The M7 line shows a significant reduction in the raphe obscurus, pallidus and magnus.

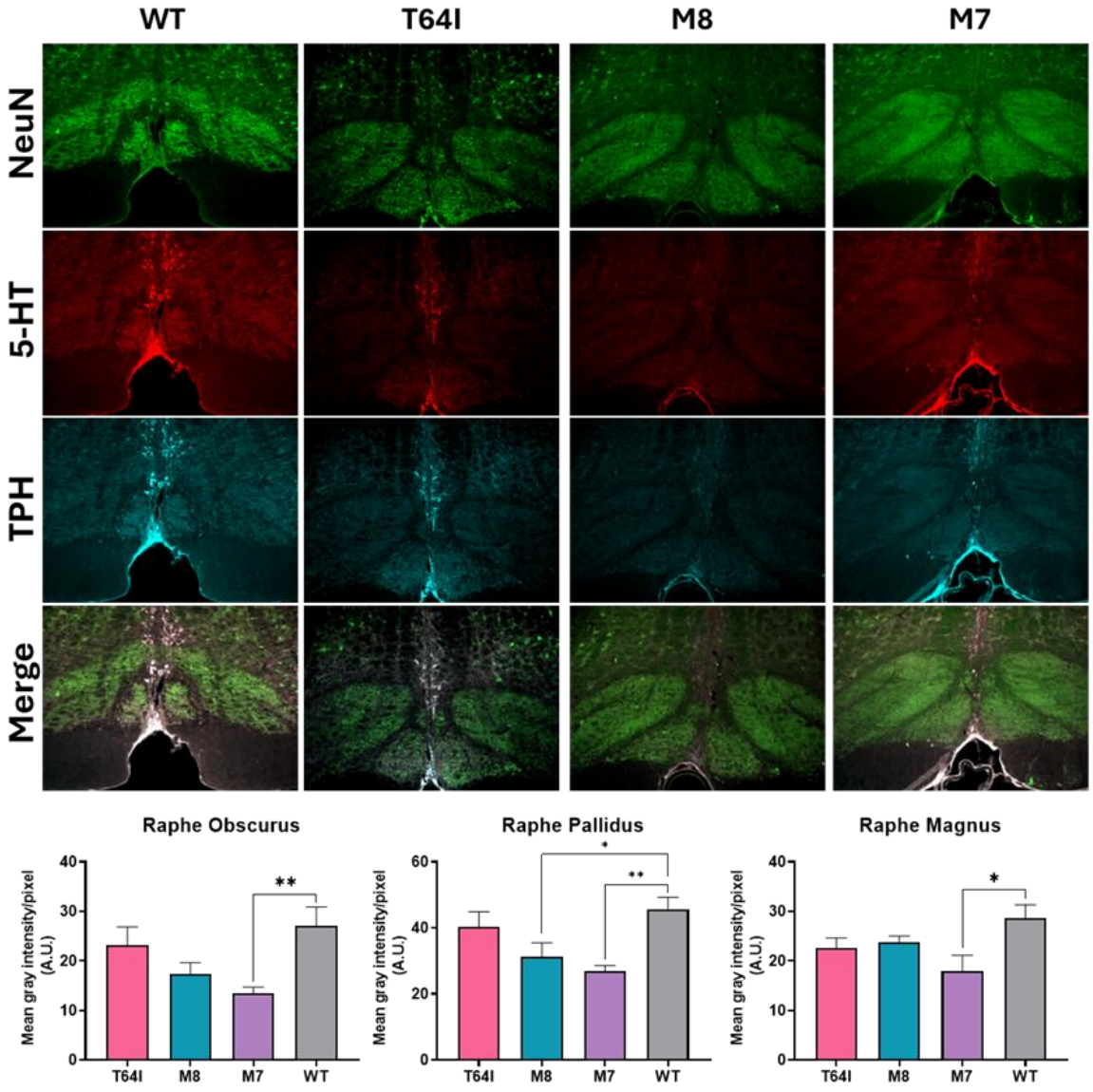


Figure 4.14. Serotonin labeling in the medullary raphe after 10 day protocol. Representative images of the SD$^{Kcnj16-/-}$ lines labeling neurons (NeuN, in green), serotonin (5-HT, in red) and TPH (tryptophan hydroxylase, in cyan). B) Mean gray intensity of the Nucleus of Rafe Pallidus, C) Rafe Obscurus and D) Rafe Magnus in the medullary raphe. Data presented as mean + standard error of the mean, *p<0.05.

4.5.2 Protocol 2: Conditional knockout of *Kcnj16* gene

A) Characterization of the viral transfection approach

To characterize the virus approach, a R26-LSLR rat strain was used to assess if viral-mediated Cre (marked with GFP) was sufficient to cause a recombination event in the brain. The R26-LSLR rat strain is a genetically modified animal in which a transgene containing loxP-stop-loxP upstream of a gene encoding a red fluorescent protein (Tdtomato). In the presence of Cre expression, the stop codon is removed and the cells express (TdTomato). Thus, viral infection and Cre (GFP) expression along with a Cre event (TdTomato) induces a yellow colocalization of both green and red fluorescence, as observed in the figure below (figure 4.15).

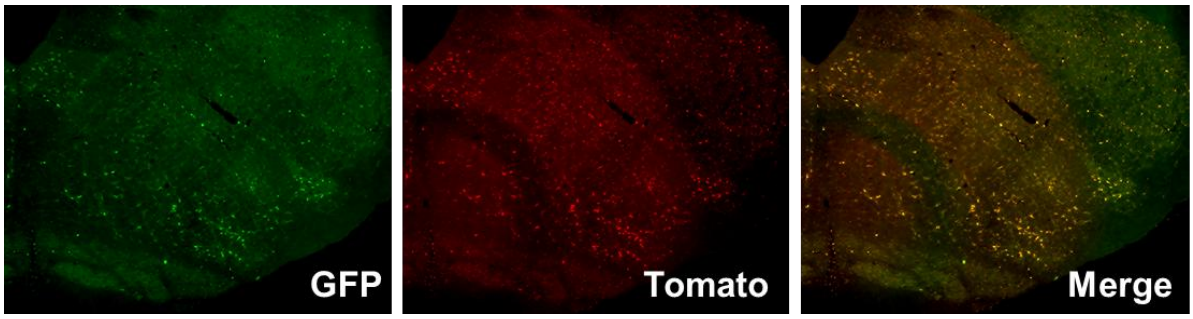


Figure 4.15. Virus efficacy in the TdTomato model. Image from Ambiguus Nuclei region of animal three weeks after viral injection in the 4V. GPF seen is related to virus replication. Tomato fluorescence is due to the special genome in R26-LSLR.

Importantly, the AAV does not have a cell specificity and transfects all cells in which Cre expression is ubiquitous. In Figure 4.16, the expression of GFP and NeuN (neuronal marker) in the cerebellum of a SYNC animal was evaluated. In the co-localization, not all infected cells in green show purple marking (NeuN). These cells are probably glial cells that were also infected. This shows that the virus was highly effective in infecting local cells and transducing GFP (Cre) expression.

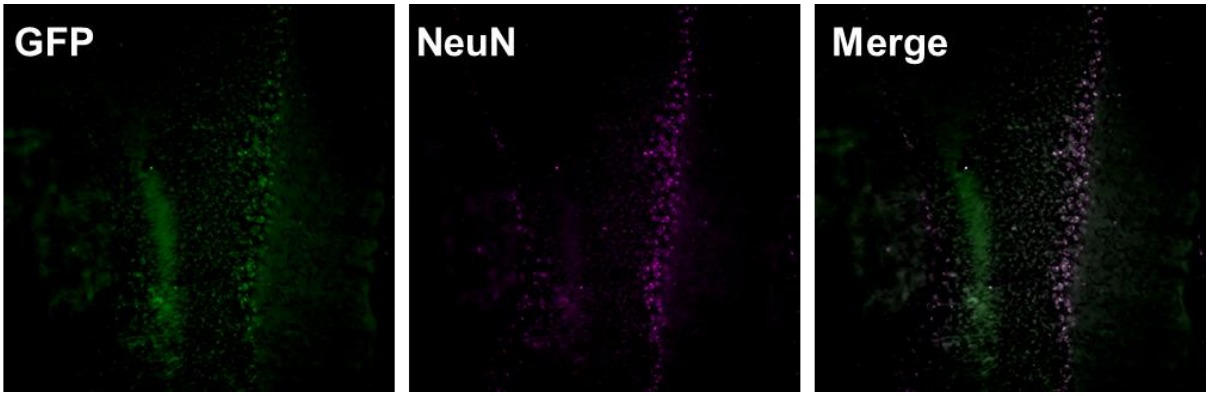
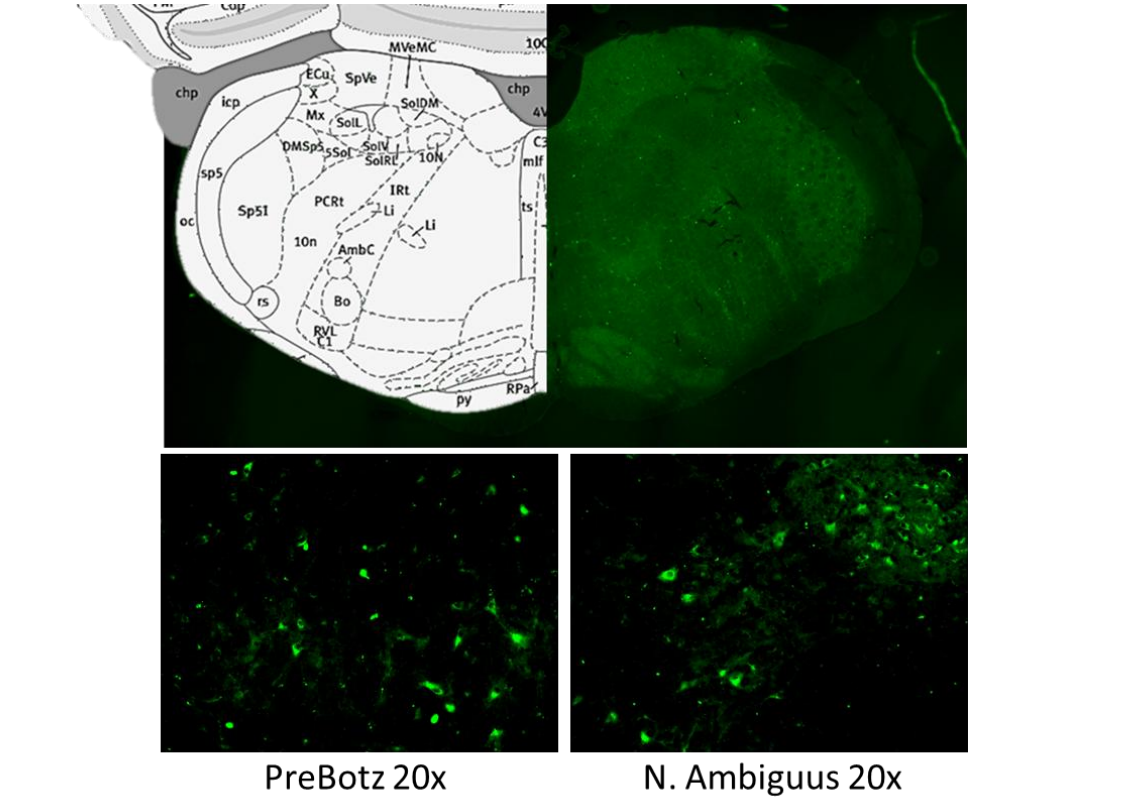


Figure 4.16. Viral expression in brain cells. Neuronal marking (NeuN) co-localized with GFP in the cerebellum (4x magnification) expressed by the viral vector AAV-CMV-CRE-GFP in Sync animals.

B) Hypercapnic ventilatory responses before and after knockout of gene *Kcnj16*

Initially, we injected the virus into the 4th ventricle (4V) in an attempt to infect regions of the brainstem that contain regions that participate in ventilation control as well as regions that perform the initial processing of hearing, such as the dorsal cochlear nucleus and the superior olivary nucleus (Browninga et al., 1989). Hearing is an important ictogenic factor in the *Kcnj16* knockout model, which is why the focus on this region of the brain (Fedotova et al., 2021; Garcia-cairasco et al., 2017). Infection in the 4V was successfully performed in 5 animals, in which the entire brainstem appeared to present generalized infection (Figure 4.17). The percentage of infected neurons was not evaluated. Our results showed that the test with 10kHz or white noise did not induce seizures in the animals before or after AAV-CMV-Cre-GFP infection in the 4V. The Ve, fR and VT also did not show statistical differences before or after infection (figure 4.17). Surgery on the lateral ventricle (LV) was also performed, in this way, pro-encephalic areas would also be infected. Interestingly, of the 2 animals performed, 1 had a seizure with the stimulus at the 10KHz tone frequency and white noise. This suggested that broad Cre-mediated Kcnj16 knockout in the higher brain centers may be sufficient to induce the seizure phenotype. However, further analyses and experiments are required. This preliminary result suggests that susceptibility to audiogenic seizure in this animal model requires involvement of prosencephalic areas as well as regions of the brainstem.



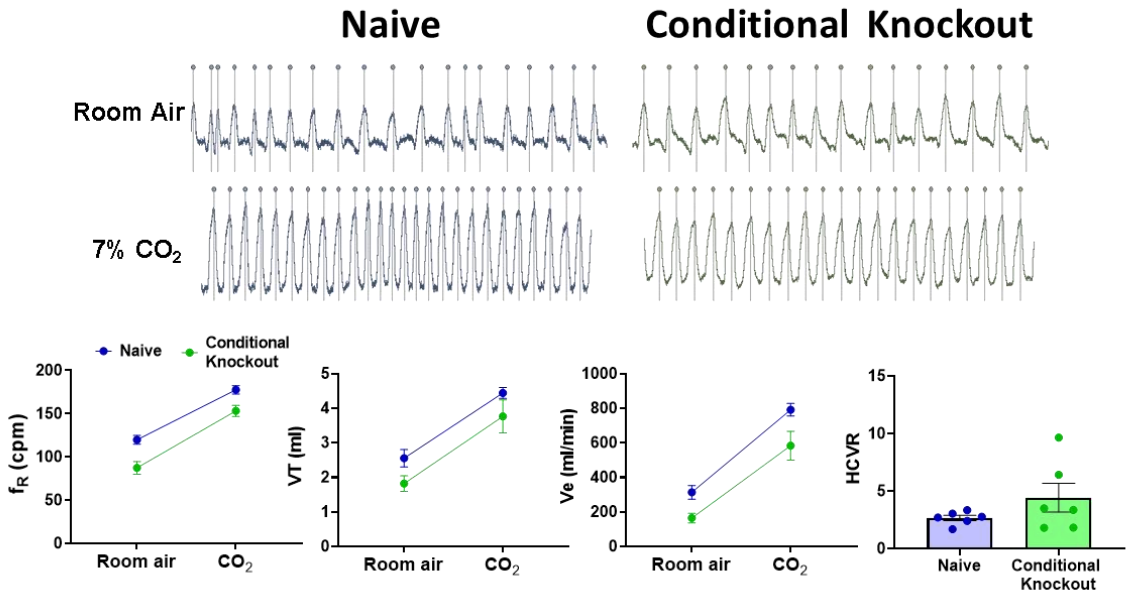


Figure 4.17. Viral infection of AAV-CMV-Cre-GFP in Sync animals. Representative image of the medulla oblongata region. In detail, the pre-botzinger region and nucleus ambiguus. Representative recordings of the same animal in room air or hypercapnia, before when naive or after conditional knockout. Data presented as mean + standard error of the mean. Two-way ANOVA with repeated measures, p>0.05.

4.5.3 Protocol 3: Serotonin depletion in SS$^{Kcnj16-/-}$ rats

A) Characterization of serotonin depletion

The drug PCPA is a potent irreversible inhibitor of TPH (Park et al. 1994). Given that SSkcnj16-/- rats show reduced 5-HT after repeated seizures and increased mortality, here we sought to test if PCPA could induce 5-HT reductions in the brain in our rat model. The dose of 200mg/kg was able to promote a significant reduction in serotonin and TPH in SS$^{Kcnj16-/-}$ animals (figure 4.18).

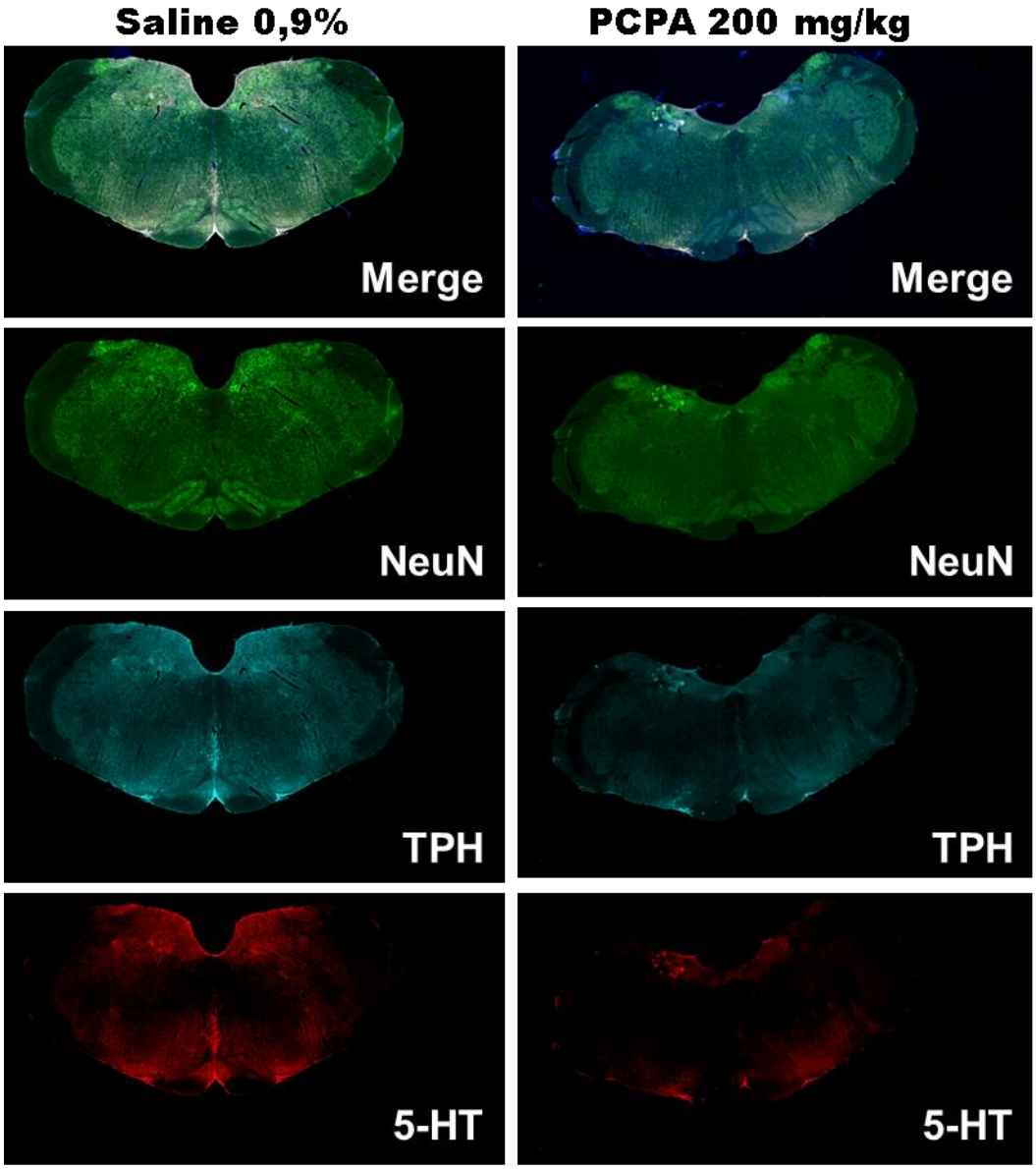


Figure 4.18. Immunofluorescence of serotonin in PCPA or saline treated SS$^{Kcnj16-/-}$ animals. Representative image of SS $^{Kcnj16\ -/-}$ animal treated with 0.9% saline or PCPA (200mg/kg) in NeuN (neuron marker), TPH, 5-HT and the merge.

B) Mortality

Mortality was evaluated in groups of SS $^{Kcnj16-/-}$ animals with telemetry system treated with vehicle (SS $^{Kcnj16-/-}$ + saline) or PCPA (SS $^{Kcnj16-/-}$ + PCPA). To evaluate if the telemetry system would alter mortality, a group without telemetry or treatment (SS $^{Kcnj16-/-}$) was added to the mortality comparison. And to evaluate if PCPA treatment would induce mortality without seizure, a SS^{WT} rat treated with PCPA (SS + PCPA) was also used in the comparison. The SS + PCPA animals were seizure-free and survived throughout the protocol. The groups SS $^{Kcnj16-/-}$ + saline and SS $^{Kcnj16-/-}$ (without telemetry) did not differ in mortality. The most significant finding was the high mortality observed in the SS$^{Kcnj16-/-}$ + PCPA, which did not survive the ten-day protocol, with many rats succumbing on the second day. This indicates the importance of serotonin in post-seizure physiological regulation.

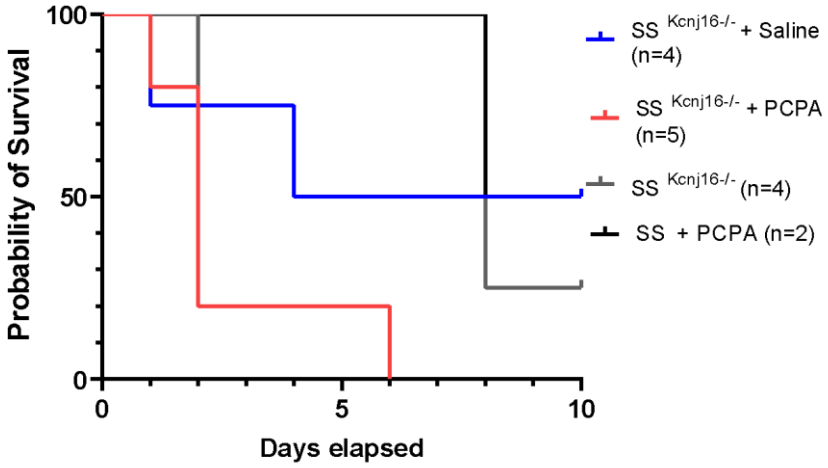


Figure 4.19. Survival under serotonin depletion and repeated seizures. SS $^{Kcnj16-/-}$ animals with telemetry system treated with vehicle (SS $^{Kcnj16-/-}$ + saline) or PCPA (SS $^{Kcnj16-/-}$ + PCPA), SS $^{Kcnj16-/-}$ animals without telemetry or treatment (SS $^{Kcnj16-/-}$) and SS wild type rat treated with PCPA (SS + PCPA).

C) Physiological parameters

Parameters such as body weight, seizure score, ictal and post-ictal apnea duration were similar between the SS$^{Kcnj16-/-}$ PCPA and saline treated groups (Figure 4.20). However, body temperature was significantly lower in the PCPA treated animals. This thermoregulatory dysfunction is associated with serotonin depletion (Hodges & Richerson, 2008). The latency (time required for seizures to occur) of the 5-HT

depleted animals showed a trend, but not statistically significant. The respiratory pattern at baseline was statistically similar between $SS^{Kcnj16-/-}$ saline and PCPA animals (Figure 4.21). During seizures, the animals presented bradycardia accompanied by ictal apnea (Figure 4.20), which was not quantified. The postictal ventilatory response was altered in PCPA treated rats, which show an increase in respiratory rate after seizures (Figure 22). This is a contrast to the pattern observed in the control saline group.

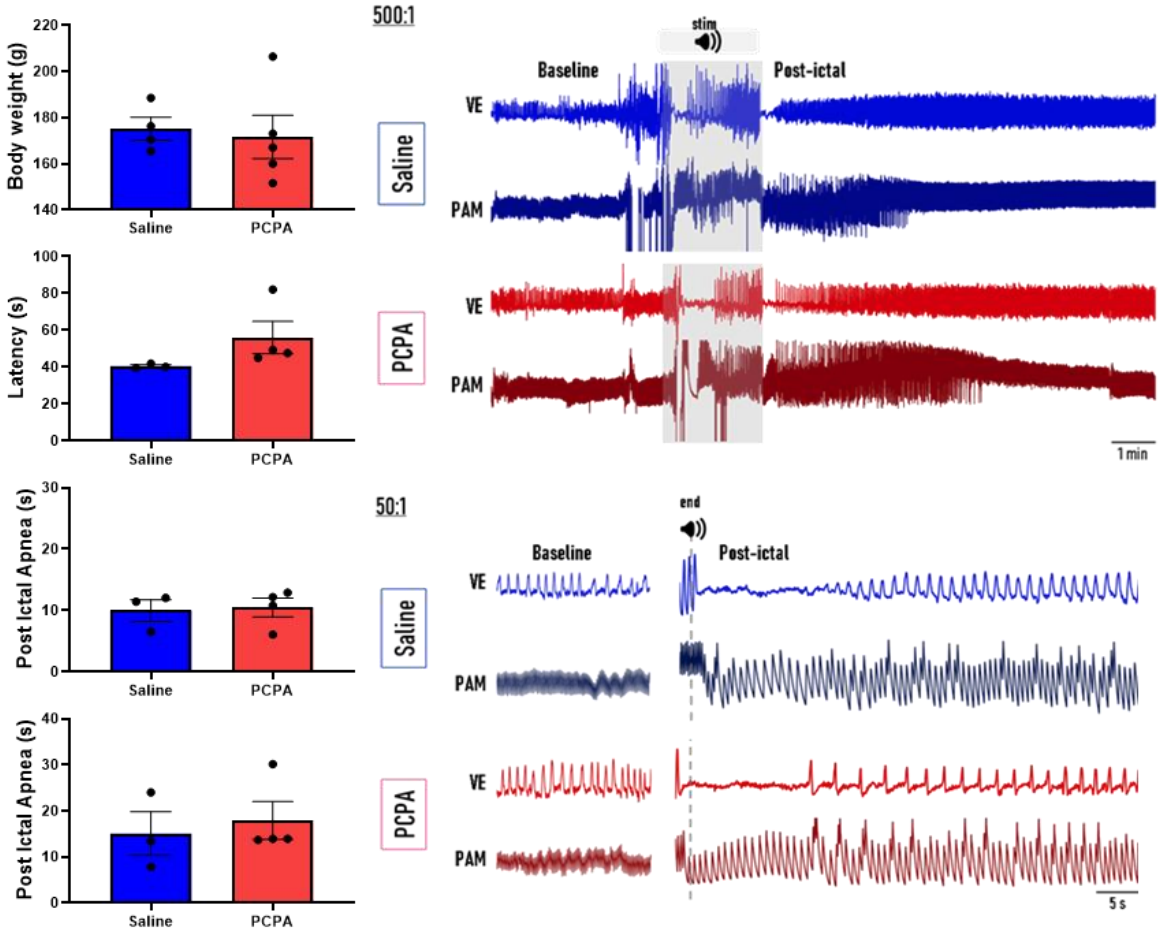


Figure 4.20. Behavioral and physiological parameters. Data of behavior presented as mean ± standard error of the mean. Student's t-test, p>0.05. Representative recordings of $SS^{KCNJ16-/-}$ saline (Blue) or PCPA treated (red) ventilation and arterial pressure in compressed (500:1) and expanded (50:1) air during room air, acoustic stimulation and postictal period. Blue cotrolNote changes in the pattern during seizure of breathing and cardiovascular arritmias.

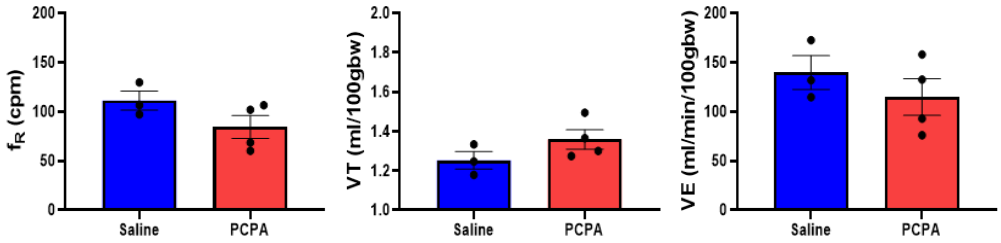


Figure 4.21. Ventilatory parameters in room air in animals with serotonin depletion. Data presented as mean + standard error of the mean. Student's t-test, p>0.05.

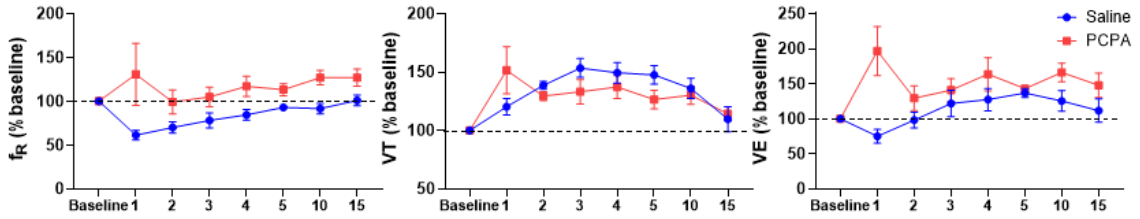


Figure 4.22. Ictal and postictal ventilation in animals with serotonin depletion. Data presented as mean + standard error of the mean.

4.6 DISCUSSION

4.6.1 SUDEP models and cardiovascular importance

Our findings in Sprague Dawley rats demonstrate that the knockout of the *Kcnj16* gene promotes susceptibility to audiogenic seizures. During these seizures, respiratory alterations are observed both ictally and postictally. Additionally, repeated seizures lead to changes in baseline breathing under room air conditions, as well as disruptions in serotonergic brainstem expression. Despite these physiological disturbances, $SD^{Kcnj16-/-}$ rats do not exhibit mortality. This observation suggests that Sprague Dawley rats with *Kcnj16* knockout may not serve as an appropriate model for studying SUDEP. In contrast, the same genetic knockout in SS rats is associated with mortality (Manis et al., 2021, 2023), suggesting that cardiovascular alterations, rather than respiratory factors, may play a more critical role in the pathophysiology of SUDEP in this context.

Seizures are strongly associated with alterations in cardiovascular function. During generalized tonic-clonic seizures (GTCS), bradycardia is commonly observed in conjunction with ictal apnea, as demonstrated in Figure 4.20. In the MORTEMUS study, which examined SUDEP cases, bradycardia was identified as a contributing factor, although cardiac arrest typically follows respiratory failure (Ryvlin et al., 2013). In the Wistar Audiogenic Rat (WAR) model, cardiovascular assessment revealed subtle autonomic dysfunction, affecting both sympathetic and parasympathetic activity (Fazan et al., 2011, 2015). Similar cardiovascular dysfunction have been noted in repeated seizures models, such as impairment in endothelial function in amygdala kindled Wistar rats (Ghazale et al., 2022). An important phenotype observed in SS rats is the alteration of the renin-angiotensin system (Hirawa et al., 1997; Simchon et al., 1989), which has been implicated in various pathophysiological conditions including hypertension and cardiac insufficiency (Watanabe et al., 2016). In this regard, evidence from epilepsy models suggests that disruptions in this system, such as hypertension, may be linked to SUDEP (Pereira et al., 2010; Szczurkowska et al., 2021), and explain the higher mortality in $SS^{Kcnj16-/-}$. Therefore, further studies are necessary to elucidate the role of the cardiovascular system in SUDEP, with a particular focus on integrating both respiratory and cardiovascular factors to better understand the underlying mechanisms.

4.2 Serotonin's Role in Seizures

Our results involving $SS^{Kcnj16-/-}$ rats have shown that serotonin depletion, through PCPA treatment, significantly increases post-seizure mortality. This comes in agreement with the hypothesis of serotonin neurons preventing seizure-induced death (Buchanan et al., 2014). The increase in mortality in our data might be related to postictal respiratory dysfunction, suggesting that serotonin plays a crucial role in maintaining respiratory stability and preventing fatal complications such as apnea and bradycardia during seizures. However, our data analysis only investigated breathing on the first day, not on the day of death, which represents a limitation of our study. Several studies suggest that post-ictal breathing impairment may be linked to 5-HT_2 receptors, specifically implicating 5-HT2A receptors in seizure-induced respiratory arrest. (Joyal et al., 2023; Pan et al., 2024). Moreover, not only genetic deletion of 5-HT2C receptors in mice cause audiogenic seizures, but also induce post-ictal respiratory arrest that leads to death (Brennan et al., 1997; Buchanan et al., 2014; Uteshev et al., 2010). This role of serotonin in both respiratory control and seizure susceptibility, highlights its function as a crucial regulator of neural stability and autonomic system, which reinforce the hypothesis that its dysfunction may exacerbate the risk of SUDEP (Richerson & Buchanan, 2011). Thus, therapeutic interventions that enhance serotonergic activity may offer a preventive strategy. Indeed, selective serotonin reuptake inhibitors (SSRIs) have been proposed to exert beneficial effects in improving seizure control, particularly in cases of pharmacoresistant epilepsy (Faingold et al., 2011; Joyal et al., 2023; Richerson & Buchanan, 2011).

4.7 CONCLUSION

Our preliminary results demonstrate that $SD^{Kcnj16-/-}$ rats, like $SS^{Kcnj16-/-}$ rats, present audiogenic generalized tonic-clonic (GTCS) seizures confirming that loss of Kir5.1 function leads to seizure susceptibility. In addition, seizures also lead to ictal apnea, as expected, along with ventilatory dysfunction and reduced TPH and 5-HT expression after repeated seizures. However, we did not observe seizure-related mortality in $SD^{Kcnj16-/-}$ rats with repeated seizures, which was unexpected.

Our preliminary results with the conditional knockout in Sync animals performed from 4V were not able to induce audiogenic seizures or respiratory alterations. However, from VL onwards, the effects seem promising and need to be further elucidated.

Finally, our preliminary results of serotonin depletion by PCPA increases mortality and causes significant alterations in the post-ictal ventilatory response, reinforcing the importance of serotonin in the regulation of physiological responses during seizures.

FINAL CONSIDERATIONS

The findings of this thesis underscore the significance of understanding how seizures impact respiratory patterns across different life stages. In this study, generalized tonic-clonic seizures (GTCS) were observed in both neonatal and adult subjects, providing insights into developmental differences in respiratory control. The animal models employed (WAR and *Kcnj16-/-*) enabled the identification of distinct vulnerabilities in respiratory regulation. For instance, neonatal WAR rats demonstrated increased susceptibility to apneas and a diminished ventilatory response to hypoxia even prior to any seizure induction at P1-3. Additionally, they exhibited heightened susceptibility to myoclonic seizures and impaired post-hypoxia ventilation at P10. In contrast, these respiratory alterations in adult WAR rats were only apparent after repeated seizure events. Notably, repeated seizures in adults led to an attenuation of CO2/pH chemosensitivity, reinforcing the notion that audiogenic seizures can induce long-term disruptions in chemoreflex function. This finding is consistent with existing evidence that chemoreflex sensitivity diminishes following recurrent seizures, highlighting the potential for chronic respiratory dysfunction (Sainju et al., 2019; Teran et al., 2023; Totola et al., 2019).

Audiogenic seizure models used in this study, WAR, SD$^{Kcnj16-/-}$, Sync, and SS$^{Kcnj16-/-}$, exhibited similar behavioral responses to acoustic stimulation. This consolidates that the underlying mechanisms of brain seizures in these models are comparable, even though the strain discrepancy. Studies have shown that in adult WAR and SS$^{Kcnj16-/-}$, repeated seizures leads to decrease in brainstem 5-HT (Manis et al., 2023; Totola et al., 2017). Herein, we showed that neonate WAR and SD$^{Kcnj16-/-}$ also present attenuation in this neurotransmission. Our work offers new insights into the relationship between seizures, respiratory control, and serotonin, which may contribute to the development of new therapeutic and preventive strategies for SUDEP and SIDS, and the importance of further investigating the neurotransmission mechanisms involved.

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