SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Mechanisms and Consequences of Chronic Astrocytic and Neuronal Activation of Murine Mesopontine Regions

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Summery

Summarizing our findings in this project, chronic chemogenetic activation of astrocytes in the pedunculopontine nucleus and nearby mesopontine regions led to a decrease in the acoustic startle reflex amplitude and an increase in locomotion speed during the resting period. Additionally, gait alterations were observed, but spatial memory remained unaffected. These findings may be attributed to a significant reduction in neuronal populations: cholinergic neurons decreased to 54% of control levels, and non-cholinergic neurons dropped to 76% of control levels. Thus, chronic astrocytic overstimulated, with its subsequent neuronal loss, resulted in motor and circadian rhythm disturbances similar to brainstem-related symptoms that seen in progressive supranuclear palsy, suggesting a possible role for astrocytic overactivation in the development of this disease. Later histological analysis showed a significant loss of cholinergic neurons in the group with long-term artificial astrocyte activation compared to the control group, which received the same CNO treatment but did not have astrocyte overactivation.

The important role of potassium voltage-gated channel (KCNQ) in regulating startle reflex, we found KCNQ4 knockout mice exhibited a significantly exaggerated acoustic startle reflex and showed minimal habituation compared to wild-type mice. This heightened startle response, which differs from other forms of startle hyperexcitability, is attributed to both cochlear damage and altered neuronal excitability within startle networks resulting from the KCNQ4 subunit deletion.

New finding

Chronic astrocytic overstimulated, with its subsequent neuronal loss, resulted in motor and circadian rhythm disturbances similar to brainstem-related symptoms that are seen in progressive supranuclear palsy.

1. Introduction

The mesencephalic locomotor region (MLR) acts as a crucial conduit, that link the brain's higher motor control centers to the lower motor neurons that facilitate movement. The main excitatory elements within the MLR, include the pedunculopontine nucleus (PPN), which is the largest component of MLR, and extended nuclei to it, and their activation has been proposed to produce different forms of movement. In previous studies, scientists found the MLR by demonstrating that electrical activation of this brain region could make decorticated cats walk. Recent researches using optogenetic techniques has shown that the motor action of the MLR depends on excitatory signals from glutamatergic. MLR neurons regulate various aspects of movement. The cholinergic neurons of the PPN effect on muscle tone and modulate the acoustic startle response while the glutamatergic neurons of the PPN are linked to exploration movements; additionally, the activity of glutamatergic neurons in the CnF correlates with movement velocity. The PPN is an important part of the reticular activating system, which regulates activity cycles and overall brain states associated with REM sleep as well as consciousness by sending cholinergic and non-cholinergic projections to many subcortical regions. The changes observed can be related to the lesions of non-cholinergic neurons, as the targeted lesion of cholinergic neurons did not lead to learning impairments or modifications in the locomotor response to nicotine, instead developed of sensorimotor and gait deficits.

Our previous study indicated that neuromodulatory effects on astrocytes produce tonic excitability changes depending on metabotropic glutamate and N-methyl-D-aspartate receptors in the mesopontine region. Although these effects were significant in in vitro studies, but in vivo impact has not been examined.

Following to investigated the pathophysiological significance of regional astrocytic activation within mesopontine regions in vivo regarding to previous studies have shown that tau protein expression and a reduction in cholinergic neuronal number in the region result in reduced startle response amplitude and gait abnormalities. These findings effectively modeled the brainstem-related symptoms observed in progressive supranuclear palsy (PSP).

2. Literature background

2.1. Astrocytic diversity: Morphologies and their Functional in the CNS

There are many different glial cell types in the adult central nervous system (CNS), such as astrocytes, oligodendrocytes, and microglia. Astrocytes are one of the most common cell types in central nervous system. They play vital roles in maintaining the CNS and stability, including buffering ions, recycling neurotransmitters, maintaining the blood-brain barrier, and releasing gliotransmitters. However, different groups of astroglia perform these functions differently Astrocyte diversity and understanding functional specialization

2.1.1. Neuron-Astrocyte interactions of brain function and dysfunction

The functions of glial cells, especially astrocytes, have been reconsidered for several decades. An increasing amount of research suggests that astroglial cells are significant not only in trophic support, but also in the brain's information processing and cognitive processes. Gap junctions facilitate connections between astrocytes, forming astroglial networks that provide metabolic support to neurones. Studies conducted in the recent few years have demonstrated the role of disorders in astroglial connections in pathological nervous system lesions. It is of significant interest to study the morphological and functional modifications of astrocytic networks due to aging, diseases, or exposure to stress stimuli. First, Rudolf Virochow described glial cells as a homogenous connective tissue that supports neuronal components. Later studies on the cell composition of the brain by Cajal et al. revealed that different parts of the brain include morphologically unique types of glia and neurones.

In the last century, astrocytes have evolved into an important link between neurons and the external environment, supporting different physiological functions in the blood-brain barrier (BBB) formation, neuronal development, neural network function, neurotransmission, and metabolic assistance. Astrocytes maintain homeostasis in the brain by regulating the uptake of neurotransmitters, water, and amino acids. The end-feet structure of astrocytes forms the neurovascular unit between endothelial cells and neurons. Astrocytes have complex processes for making tripartite synapses and covering synapses and can monitor the ongoing synaptic circuit local activities and regulate homo/heterosynaptic in several ways.

2.1.2. Astrocyte-neuron interactions and its role in brain homeostasis

Astrocytes in the brain are the primary regulatory glial cells that play a variety of roles in maintaining brain homeostasis. These roles include preventing increase of extracellular potassium producing and storing gliotransmitters, providing growth factors, nutrients, and antioxidants to neurons, and regulating the immune response and iron homeostasis in the brain.

The production and upregulation of numerous gliotransmitters, including ATP, D-serine, and glutamate, by astrocytes modulate paracrine signalling between astrocytes and neurons, pericytes, microglial cells, and endothelial cells.

Astrocytes can control neuronal function in different ways, and the regulation of tonic inhibition might be particularly crucial. The gamma-aminobutyric acid (GABA)-related suppression of neuronal activity consists of both tonic and phasic components. When the vesicular is released from the presynaptic axon terminal, transient and discrete inhibitory postsynaptic currents (IPSCs) form the phasic component. Low-affinity GABAA receptors in synapses mediate these currents. In contrast, high-affinity, slowly desensitizing extrasynaptic GABAA receptors exposed to low ambient GABA concentrations mediate persistent tonic GABAergic inhibition. It has been shown that tonic inhibition is very crucial. Tonic inhibition significantly affects the excitability of both individual neurones and networks by modifying the membrane conductance of the postsynaptic neurons. Furthermore, tonic inhibition has become increasingly common in the treatment of pathophysiological disorders such as stroke.

2.1.3. The active role of astrocytes in synaptic communication

Astrocytes control the growth and plasticity of synapses by a range of secretory and contact-mediated signals. Astrocytes are believed to regulate the development of excitatory synapses and synaptic activities in the developing and adult brain as a component of tripartite synapses, including postsynaptic and presynaptic terminals. Many studies have demonstrated that astrocytes in different areas of the brain are not the same, and that astrocytes located next to each other in the same brain region could have differences.

Research has indicated that astrocytes can modulate firing synaptic structure and function, as well as synaptic transmission, by releasing gliotransmitters as a result of Ca^{2+} influx, such as D-serine and glutamate. Through both ionotropic and metabotropic signalling cascades, astrocyte receptors are connected to intracellular ionic signalling and astrocyte metabolism. Astrocytes receive neurotransmitter and neuromodulator signals from synapses through a variety of receptors. The expression of these receptors is likely regulated by the local neurotransmitter environment. The CNS contains a variety of cell types which express metabotropic glutamate receptors (mGluRs) in different ways. Three subgroups have been identified based on sequence homology and cellular signalling activation, and eight different mGluR subtypes have been identified.

As glutamate regulates both neuronal cell excitability and synaptic transmission via the second messenger signalling pathway, mGluRs are significantly associated with the control of neuronal firing and synaptic transmission throughout the CNS. They control the metabolism of energy to maintain neuronal activity, particularly those involved in memory formation. Additionally, memories can be categorised according to the kind of information that is transmitted and stored, which divides memories into two categories: implicit (non-declarative) and explicitly referred to as declarative in humans.

In general, astrocyte Ca2+ dynamics stimulated and downstream effects on learning and memory can be investigated by activating Gi or Gq-GPCR signalling with hM4Di and hM3D(Gq) DREADDs or (Gq-coupled melanopsin). Using hM3D(Gq) DREADD like, indicates that improving astrocyte Ca signalling enhances spatial and T-maze, and fear conditioning tests show context-dependent memory formation. Furthermore, the role of hippocampal astrocytes in spatial memory has been demonstrated using a melanopsin-based method to temporally trigger Gq activation in astrocytes. Alternatively, in a learning by association test including initiated fear conditioning, triggering the Gq-GPCR pathway in the central amygdala through astrocytes using the same hM3Dq method decreases the firing of neurons by astrocyte-derived ATP release along with reduced fear reactions.

2.2. The pedunculopontine nucleus in health and disease

The pedunculopontine nucleus (PPN) and the nearby cuneiform nucleus (CnF) comprise the mesencephalic locomotor region (MLR), which is the first locomotor region to be identified and present in all classes of vertebrates. In addition, the PPN is also part of the reticular activating system regulating global brain states.

The PPN has been involved in motor function in many different of independent investigations conducted over the past few decades, both in humans and in animal models. It has been suggested that this nucleus could be a potential new target for treating motor symptoms in Parkinson's disease (PD) was first raised in 1989, specifically because of altered PPN activity in a parkinsonian model of non-human primates, which has crucial therapeutic implications.

The PPN is considered to regulate both waking and rapid eye movement (REM) sleep. It is recognized that the PPN is an important part of the reticular activating system (RAS). Pars compacta (PPNc) and pars dissipatus (PPNd) are the two subnuclei that comprise the human PPN. Small- and medium-sized neurons are spread across the superior cerebellar peduncle (SCP), and central tegmental tract, forming the PPNd, which consists of a compact cluster of massive neurons. Although cholinergic neurons also contain neuropeptides and a novel neuromodulator, the PPNc and PPNd also have GABAergic inhibitory neurons.

2.2.1. Astrocytes and neuromodulatory mechanisms in the PPN

As shown by Katona and Freund, endocannabinoids modulate multiple signalling pathways and synaptic connections, which have different effects on brain activity. Cannabinoid type 1 (CB1), receptor stimulation may affect sleep-wake cycles, among other factors. The prototypical endocannabinoid anandamide administered intracerebroventricularly or directly to the PPN, reduced wakefulness and increased both Slow-Wave Sleep and REM sleep, based on in vivo animal studies. Although anandamide's effect on sleep may be reversed by CB1 receptor antagonists, it is believed that these endocannabinoids affect sleep modulation by acting on CB1 receptors.

Our laboratory previously showed the presence of SICs after astrocytic activation in the PPN,

resulting in stimulation of a population of neurones, inhibition of another population, and no change in the third population. We have shown that both neurons and glial cells within the PPN respond to agonists that activate CB1 receptors. The response in neurons can be either depolarization or hyperpolarization, whereas astrocytes show a greater frequency of calcium waves, and none of these effects is observed in mice deficient in the CB1 gene. The hyperpolarization is prevented by blocking group I metabotropic glutamate receptors (mGluRs), whereas blocking group II mGluRs prevents depolarization.

The neuromodulatory agents, whether sleep- or wake-promoting, might change SICs based on the neural activity background when used for PPN function during sleep/wakefulness. This could potentially stabilisethe desynchronized state required for PPN neuron firing during wakefulness and sleep regulation. This balanced level of desynchronisation is important for wakefulness, attention, and associated learning and active memory, and possibly for other PPN functions such as sensory gating, reward, and locomotion. Therefore, what remains to be done is to observe behavioural changes in response to astrocyte overstimulation and relate them to known functions of the PPN. In conclusion, we found that PPN neurons react heterogeneously to CB1 receptor stimulation by activating astrocytic CB1 receptors, which change their membrane potential and action potential firing frequency. PPN neurons appear to be efficiently regulated by this indirect cannabinoid signalling pathway, which probably plays a significant role in the oscillatory activity of neurons.

2.2.2. The role of PPN pathological conditions

The PPN has been shown to be able to regulate motor initiation, duration, rhythm, and frequency in a variety of studies that have investigated its impact on locomotion using in vivo electrical monitoring, neuromodulation, and pharmacological interventions. The PPN that have extensive decline in neurons in the earliest stages of neurodegenerative diseases, including multiple system atrophy (MSA), dementia with Lewy bodies (DLB), Parkinson's disease (PD), and PSP, and express the neurotransmitter acetylcholine (ACh) in the PPNs of humans, non-human primates, and rodents, glutamate and gamma-aminobutyric acid (GABA), and glycine.

The most frequently studied cholinergic neurons are those that produce ACh; and recent research has revealed previously unidentified parcellation of cholinergic inputs to the dopamine containing midbrain neurons. Furthermore, to the striatum's direct cholinergic projections. The PPN has also been found to be involved in modulating multiple types of locomotion; dystonia may be the source of abnormal locomotion produced by PPN intervention.

The PPN and its connections with basal ganglia are complex networks containing forebrain nuclei which are important for motor control. It has been provided that a variety of neurodegenerative diseases, including Parkinson's disease (PD), may be directly related to any damage or instability of the BG.

Patients with Parkinson's who have reduced thalamic ACh metabolism and recapture are more likely to experience falls and FOG. where the main supply of ACh is the PPN. The GPi and SNr's synaptic inhibition and cell death are probably responsible for the decline in cholinergic tone. Additionally, in certain patients with PD, cholinesterase therapies reduce falls which block the breakdown of acetylcholine.

As a result of its importance in movement, deep brain stimulation (DBS) has been recommended as a possible therapy for postural instability, falls, and FOG in PD and atypical Parkinsonism. This method has produced a range of results. PPN-DBS have shown in numerous PPN-DBS improves general gait parameters and FOG in patients with PD. The PPN is a component of the reticular activating system (RAS), which consists of nuclei that control attention and consciousness. In order to regulate the tone of muscles during sleep and waking, the PPN receives SNr GABA and lateral hypothalamic orexin inputs, REM sleep is suppressed when GABAB receptors are attached.

Through thalamic projections that control cortical activity, PPN cholinergic neurons facilitate EEG desynchronization and alterations in consciousness. PPN cholinergic neurons demonstrate resting alpha activity and gamma oscillations associated with awake behaviour, respectively, during REM sleep and wakefulness. For Parkinsonian patients with severe gait disorders, postural dysfunction, and falls, including those with PSP and idiopathic

Parkinson's syndrome (IPS), the PPN has been suggested as a therapeutic target for DBS. It has been proposed that DBS excites the PPN and increasing the ratio of signal to noise may modify the defective movements associated with Parkinsonian illnesses. This leads to the design of neuronal processing in the basal ganglia, and particular processes could include the release of dopamine by the substantia nigra compacta in response to direct cholinergic input of the PPN to the cortex, thalamus, and basal ganglia.

In 1964, Drs. Steele, Richardson, and Olszewski first described the clinicopathological syndrome of PSP, describing a group of patients with postural instability, ocular motor abnormalities, facial and cervical dystonia, dementia, and other characteristics. Research suggests that some PSP symptoms, particularly those associated with posture, gait, and balance, may be caused by degeneration of the PPN. PPN dysfunction may be a factor in the movement coordination difficulties observed in patients with PSP, as the PPN is involved in the control of motor functions. It is believed that PSP and IPS reflect different disease entities even though they have similar clinical characteristics. Compared with IPS, PSP usually corresponds to a greater rate of progression of disease and more severe axial symptoms, such as gait disturbance.

Patients with both PSP and IPS have been reported to have degeneration of the PPN. Histopathological investigations have shown that patients with severe gait dysfunction have more declared degeneration of cholinergic neurons, and PSP patients have a higher neuronal loss than IPS patients. Additionally, recordings from two PSP patients had less recognized neuronal groups than recordings from five IPS patients, which is similarly consistent with a higher loss of PPN neurons in PSP as compared to IPS.

In 98% of patients with PD, sleep disruptions cause excessive sleepiness during the day. Although the frequency of sleep abnormalities associated with PD is worrisome in and of itself, REM sleep behaviour disorder (RBD) is associated with a higher risk of cognitive impairment in PD patients. RBD may play a pathophysiological role in PPN-ACh and Substance-P-expressing neurones. Cortex and thalamus cholinergic transmission are reduced in PD individuals with RBD compared to those without RBD, despite equal illness duration

and age of onset. Additionally, RBD symptoms are reduced by AChE inhibitors, suggesting that cholinergic tone loss may be the cause of RBD.

2.2.3. KCNQ4 contribution to neuronal excitability and synchronization in the PPN

In the central nervous system, multiple brain regions express KCNQ2, KCNQ3, and KCNQ5, while KCNQ4 is limited to specific nuclei within the auditory brainstem, including the cochlear nuclei, nuclei of the lateral lemniscus, and the inferior colliculus. Additional brainstem areas where KCNQ4 is expressed are the principal and spinal trigeminal nuclei, as well as components of the reticular activating system (RAS) such as the raphe nuclei and the ventral tegmental area (VTA). Previously, we found in our lab that cholinergic neurons, but not glutamatergic ones, in the PPN have the M-current. Activation of cholinergic inputs to the PPN can reduce this current. The M-current helps synchronise nearby neurons, uses different methods to show that a subgroup of cholinergic neurons express KCNQ4. Mice lacking KCNQ4 showed changes in their activity cycle. Our findings suggest new roles for KCNQ4 in the CNS, specifically in determining the makeup of KCNQ channels in the RAS. This, in turn, affects the RAS's electrical properties, which then influences the activity cycles. As KCNQ4 is mainly found in specific brainstem areas, drugs that target this subunit could be potential treatments for sleep-wake cycle problems.

KCNQ4 is also found in some places outside the brain, notably in the outer hair cells of the cochlea. When this subunit has a dominant negative mutation, it causes hereditary hearing loss that is not linked to other conditions (DFNA2). This condition involves the gradual breakdown of outer hair cells as people age. Interestingly, where KCNQ4 is expressed overlaps a lot with the brain network responsible for the startle reflex, which is a rapid motor response to strong, potentially dangerous stimuli. In mice, can distinguish between startles triggered by acoustic, vestibular, and tactile stimuli. Problems with the cochlea or the central nervous system can either make the acoustic startle reflex stronger or weaker, and how it's changed can be seen in various diseases or conditions.

2.3. Behavioral testing in neuroscience research: methods and applications

Behavioural tests play a crucial role in studying neurological disorders, as they provide

valuable insights into the functional effects of neurological changes. These tests are designed to assess various aspects of animal behaviour, allowing researchers to link the observed behavioural changes to specific neurological conditions.

The circadian rhythm and exercise motivation of rodents can be evaluated using voluntary wheel running. Sleep and psychiatric disorders are associated with deficiencies in these behaviours. Although running wheels require more energy from rodents, they generally increase voluntary activity. This can be beneficial for phenotypic analyses because it amplifies the activity differences between the control and mutant groups. This amplification is especially useful when examining the circadian rhythm of wheel-running activity, as rodents typically only engage in voluntary activity during their active phases. Rodents clearly perceive wheel running as a reward, and comparative psychologists have long viewed it as classic self-motivated behaviour. Sherwin's review of the evidence demonstrates that various species are often highly driven to run on wheels, even without any external reward is present Understanding the neural bases of cognition can be significantly enhanced by connecting behavioural changes that occur at specific times during development with simultaneous shifts in how the brain functions. Among effective test to evaluate spatial learning and memory deficits in rodents by the Barnes maze (BM) task. It operates on the principle that an animal, when placed in an unpleasant environment, will learns, and recall where an escape box is located under the platform's surface. Although the Morris Water Maze (MWM) is the primary method for evaluating spatial learning in rodents, the Barnes maze has several notable advantages. A major advantage is that the Barnes maze does not require swimming, thus avoiding potential complications associated with it. Swimming is known to be stressful, with studies showing that MWM training raises plasma corticosterone levels more significantly than the Barnes maze does.

Acoustic startle reflex (ASR), used for anatomical, physiological, and behavioral methods, researchers have identified the pathways responsible for mediating and modulating, which is referred to as the startle response, is an involuntary and natural response to a sudden and unexpected stimulus, like a loud noise or a quick movement. Startle reflex abnormalities have

been noted in several clinical applications and understanding these changes can have an impact on both diagnosis and treatment. The ASR is a useful tool for understanding the fundamental functions of the CNS. It is characterized by basic brain circuits, cross-species consistency, and sensitivity to behavioral experiments. Depending on what is categorized as olfactory, vestibular, tactile, visual, or auditory, the olfactory only exists in fish, and the visual is a characteristic of primates. Among these (ASR) also known as, has been the most widely studied clinical research experiment. This could be associated with differences in the stimulus processing times of the recognised sensory modalities, such as sound stimuli, which require less processing time than visual stimuli. This difference may be related to the auditory cortex's shorter distance from the ear and the ear's faster conduction time. Few studies have been conducted on the tactile startle reflex in contrast to the auditory startle reflex, which can frequently result in a local reaction which leads to a specific incubation period and an unexpected response.

Human studies involving physiological aging have indicated a reduction in ASR magnitude, an increase in ASR latency, an inverted U-shaped function of PPI with age (indicating peak PPI amplitude at intermediate ages), and no significant changes in startle habituation. Reduced habituation to repeated auditory stimuli has been observed in Alzheimer's. Negative stimuli stimulate the system of defence (aversive states), resulting in the potentiation of the ASR, while positive stimuli engage the appetitive system, leading to a reduction in ASR amplitude.

Most neurological conditions, including damage to the central and peripheral nervous systems, lead to gait changes. It is generally accepted that understanding the specifics of animal limb movements during walking—both after an injury and during recovery from spinal cord or peripheral nerve damage, when assessed using the right models—is crucial for evaluating these conditions. Gait analysis is an essential method for studying mouse models because it provides measurable behavioural data on how a particular disease, injury, or drug affects an animal's movement. Neurodegenerative diseases cause a gradual decline in brain cells, which eventually leads to death. This progressive loss of neurons impairs

neuromuscular control, resulting in issues with balance and walking (known as ataxia) or dementia. In addition, PSP is an uncommon neurological disorder that effects on movement, gait, and balance.

AIMS

- Chronic activation study:

i. Astrocytic overstimulation within the PPN using chemogenetic tools to precisely investigate the functional role of astrocytes within the PPN. The PPN plays a role in reward processing, sleep-wake regulation, arousal, and motor control. Because astrocytes behave differently in different parts of the brain, it is crucial to precisely target PPN astrocytes to investigate their contribution to these important physiological processes. This study employs a chemogenetic approach utilizing the hM3D(Gq) (human muscarinic M3 Designer Receptor Exclusively Activated by Designer Drugs) system. This system offers a powerful tool for selectively manipulating astrocyte activity without directly affecting neighbouring neuronal populations, thereby minimising confounding variables.

ii. To create an animal model of PSP, that causes damage to the PPN. The model will be a beneficial tool for examining the pathophysiology of PSP and identifying potential treatments.

- KCNQ4 study*:*

i. investigated whether KCNQ4 subunit loss is affected by the startle reflex, and whether these changes are caused by brainstem hyperexcitability.

3. Methods

3.1. Animals and preparation

All animal experiments followed the rules set by local, national, and international authorities, including EU Directive 2010/63/EU, and were approved by the Hungarian National Food Chain Safety Office (HB/06-ÉLB/129-1/2020; 19/2019/DEMÁB, HB/15-ÉLB/00136-42/2023; 3/2023/DEMÁB).

In this experiment, we used 20 young adult males (2-month-old) mice, of which 16 were wild-type C57Bl6 mice and four were ChAT-tdTomato mice. We labelled the latter mouse a cholinergic neuron by expressing the tdTomato fluorescent marker under the control of the ChAT promoter (Baksa et al., 2019). In the animal facility, the mice were produced in the animal facility by mating homozygous strains of ChAT-cre (B6;129S6-Chat tm2(cre)Lowl/J; Jax number: 006410) and floxed-stop-tdTomato (B6;129S6-Gt (ROSA)26Sor tm9(CAG-tdTomato) Hze/J; Jax mice the accession number: 007905) that were obtained from Jackson Laboratories (Bar-Harbour, ME, USA).

For the KCNQ experiments, Prof. Thomas Jentsch (MDC/FMP, Berlin, Germany) kindly provided the KCNQ4 knockout strain (Kcnq4−/−). In our facility, we bred heterozygous animals using 2-month-old knockout mice and their wild-type littermates (n = 8) and knockouts (n = 14). We used 12 more wild-type mice from the same breeding group for the viral injections. We stereotaxically injected a prepared retrogradely spread adeno-associated virus (AAV) into young adult mice (7–8 weeks old) of both sexes. The mice were housed with their littermates under standard animal facility conditions, which included regular cycles of light and dark. Water and food were accessible to them.

In the study focused on activating astrocytes, 20 mice underwent surgery; 13 of them were in the DREADD experimental group, which showed a specific marker called hM3D-(Gq) and an mCherry tag in their mesopontine astrocytes; the other 7 mice were in the control group, only showing the mCherry tag; and 16 mice were used for behavior tests, not including the two-bottle preference test. The last 4 mice in the DREADD group were used for the two-bottle preference. Some data were excluded from the analysis because of technical issues during the tests. The remaining 4 mice in the DREADD group were assigned to the two-bottle preference test. Certain measurements were removed from the analysis because of technical problems encountered during the tests.

3.2. Stereotaxic surgery

For anaesthesia, mice received intraperitoneal injections of xylazine (10 mg/kg) and ketamine (100 mg/kg). Complete anaesthesia, indicated by a lack of flexor and blinking reflexes, was achieved. All surgical procedures were performed using sterile instruments. Mice were placed on a 37 °C heating pad to monitor their body temperature. The head was secured within a stereotaxic frame (RWD Life Science Co., LTD, Shenzhen, China), and hyaluronic acid eye drops (Vizol S 0.21% eye drop, Penta Pharma Co., Budapest) were administered to the eyes to prevent corneal damage.

Following to the skin incision and trepanation by applying a microdrill (RWD Life Science Co., LTD, Shenzhen, China), a 200 nl viral volume for targeting the PPN in chronic activation of astrocytes (titer: 2 x 10^13 GC/ml for (AAV5)- pAAV-GFAP-hM3D(Gq)-mCherry and 1.7 x 10^13 GC/ml for pAAV-GFAP104-mCherry (AAV5)). For KCNQ study the same volume used (titer: 2 x 10^13 GC/ml for (AAVrg)-pAAV-hSyn-hM3D(Gq)-mCherry and 1.7 x 10^13 GC/ml for pAAV-hSyn-mCherry, (AAVrg) in the caudal pontine nucleus (PNc) was injected through a Hamilton syringe and microinjector (RWD Life Science Co., LTD, Shenzhen, China).

The surgical approach aimed to provide bilateral injections were performed using the following stereotaxic coordinates in PPN: 4.96 mm caudal from bregma, 1.25 mm from the midline, and 3.4 mm in depth. Mice was recived a pAAV-GFAP-hM3D(Gq)-mCherry (AAV5) virus vector containing a plasmid that encodes the hM3D(Gq) chemogenetic actuator and mCherry fluorescent tag, both expressed under the GFAP promoter. For the control group was administered a plasmid that only contains the fluorescent marker for mCherry (pAAV-GFAP104-mCherry (AAV5), provided by Edward-Boyden; Addgene plasmid # 58909; http://n2t.net/addgene:58909; RRID: Addgene_58909) for the chronic study.

While the stereotaxic coordinates of KCNQ group was performed bilaterally 6 mm from bregma and 0.6 mm from the midline at 5 mm depth of the caudal pontine reticular nucleus (PnC) and areas projecting to it (Maamrah et al, 2023). Mice recived the pAAV-hSyn-

hM3D(Gq)-mCherry virus, including plasmids that encode the mCherry tag and the human M3-muscarinic (hM3D-Gq) chemogenetic actuator (provided by Bryan Roth; Addgene plasmid # 50478; http://n2t.net/addgene:50478; RRID: Addgene_50478), prep #50474-AAVrg; http://n2t.net/addgene:50474; RRID: Addgene_50474. The control contained a pAAV-hSyn-mCherry, a gift from Karl Deisseroth (Addgene viral prep #114472-AAVrg; http://n2t.net/addgene:114472; RRID: Addgene_114472), that mCherry tag as control expressed in neurons. After surgery, bone wax was used to close the skull and the skin was sutured with 5-0 Vicryl (Ethicon). Postoperatively, mice received ibuprofen as a painkiller (30 mg/kg: Nurofen Baby, Reckitt Benckiser Ltd., Budapest, Hungary) at the end of anaesthesia and for the next 2 days after surgery.

They were placed in individual, ventilated cages for a duration of 7 days postoperative care. Clozapine-N-oxide (CNO) was administered in drinking water, and mice were administered to observe chronic actions of astrocytic overstimulation. After recovery, the mice underwent behavioural tests to observe the chronic actions of astrocytic overstimulation in the first group of experiments. While for the KCNQ study, only the acoustic startle reflex was used to assess the same outcome, examining its effect on the startle response in KCNQ4 knockout mice.

3.3. Behavioral tests

All behavioural tests were conducted in the sequential order illustrated in Fig 19, both before and after the 3-week consumption of CNO (Tocris Cookson Ltd., Bristol, UK; 1.04 mg/kg ingested; 4 μg/ml, 9.6 μM in drinking water). The dosage was calculated based on the average water consumption measured during the drinking experiments using a two-bottle preference test.

3.3.1. Two bottle preference tests

This test measured any bias related to low intake or overdose of CNO and compared the consumption of normal tap water with that of CNO water with respect to reward and motivation. In this experiment, mice were placed in cages, each containing a pair of drinking bottles. In the first 3 days of the experiment, both bottles were filled with the same solution,

especially tap water, and the total amount of liquid consumed, measured in millilitres, was recorded within these 3 days duration. Subsequently, for the next 3 days, the bottle labelled in red was filled with tap water with (CNO) at a concentration of (4 μg/ml; 9.6 μM). The mice used in this study were different from those used in the main experiment.

3.3.2. Activity wheel test

This test was used to assess voluntary physical activity and maximal velocity, and the mice were individually housed in running wheel-equipped cages for 10 days. Data from the initial three-day adaptation period were not included in the analysis. This test helps assess the circadian rhythm of the mice, and how it is influenced by various regions of the CNS that play a role in sleep-wakefulness regulation. As discussed previously, cholinergic, and glutamatergic neurons in the PPN have a vital role in initiating and promoting wakefulness. Astrocyte contribution to this mechanism is a strong possibility and the activity wheel test can be used to evaluate it. In this experiment, after a week of habituation without measurements, the mice were exposed to a 24-hour light-dark cycle (6 AM to 6 PM light) for one week. During this time, we measured the number of activity wheel rotations in 10-minute intervals, the distance travelled in centimetres, and the maximal running velocity in meters per minute

3.3.3. Barnes maze test

The Barnes Maze experiment evaluates spatial learning and memory by observing how mice transition from random to spatial search strategies, demonstrating their functional retention and spatial reference memory. In this setup, mice explore an elevated circular maze with 20 holes, one of which contains an escape box, under overhead illumination and behavioral camera recording. Each trial begins with a mouse placed at the maze's center, a covering box removed, and 60 dB white noise activated, with recording continuing until the mouse finds the shelter. The experiment has two phases: an acquisition phase where mice are trained twice daily for 5 days (with a 1-hour interval) to find the escape box within 3 minutes (or are guided to it for 15 seconds), and a probe trial 3 days later where the escape box is removed, and mice explore the maze for a single trial to test their memory of its location. To minimize

interference, the maze is disinfected between trials, and its orientation and environment remain consistent. Overall distance and duration spent to find the escape box are measured using ImageJ, with the experiment conducted both before and after chronic CNO treatment.

3.3.4 Startle reflex test

In our study, we used a setup developed by us to test the acoustic startle reflex. The setup involved a transducer connected to a perforated plexiglass box that limited the animal's lateral movement. The mice were habituated to the plexiglass box, which had been in their cage for one week, during which they often used it as a shelter. A constant 60 dB white noise performed as the background, while a camera recorded the mouse's movement. A 105 dB noise, administered 5 times consecutive and 1-minute intervals, induced the acoustic startle response, and measured the short-term habituation. The amplitude of initial startle response, average of five responses, and short-term habituation were measured. Short-term habituation can be identified by a decrease in amplitude across the five measured responses.

3.3.5. Footprint test

A footprint test was performed to measure muscle tone and gait. The setup involves a blank white piece of paper placed on a clean smooth surface, an escape box positioned 50 cm away from the starting point at the top of the paper, and two cardboard walls surrounding both sides of the paper. A separate piece of cardboard is physically positioned along the bottom edge of the paper during the experiment to prevent the mice from escaping. The result was a one-way path to the shelter with no exits from any other side, the path measured 45-50 cm in length, but only the middle 30 cm was used for analysis.

A test procedure involves taking a mouse from its cage and placing it on the paper surface, allowing them to move towards the vessel positioned on the upper section of the paper. Following this habituation phase, they are removed from the shelter, then labelling the front paws of the mouse with red food dye and the hind paws with blue food dye. The stride length, sway length, and stance length of the forepaws and hind paws were measured in cm.

3.4. Immunohistochemistry

Following behavioral measurements, mice underwent transcardial perfusion to assess injection sites and mCherry labeling in brain tissue. Brains were vibratome-sliced to 50 μm thickness for light microscopy. Free-floating slices were fixed in 4% PFA for histological analysis, then washed thrice for 10 minutes in Tris-Buffered Saline (TBS) to maintain pH. Non-specific binding sites were blocked by incubating slices for 60 minutes at room temperature in a solution of 10% donkey serum and 0.5% Triton-X 100 in TBS; Triton-X 100 permeabilized membranes for enhanced antibody penetration. Subsequently, a rabbit anti-NeuN primary antibody (1:1000 in 1% serum, 0.5% Triton-X 100 in TBS) was incubated with tissue slices for 48 hours at 4°C; negative controls omitted this step to confirm specific binding. After washing with TBS, a donkey anti-rabbit Alexa Fluor 488 secondary antibody (1:1000 in 1% serum, 0.5% Triton-X 100 in TBS) was applied to all slices, including controls, and incubated for 24 hours at 4°C; indirect labeling was used to improve signal. Samples were then stained with DAPI to visualize nuclei, crucial for later neuron counting. The immunohistochemical process concluded with visualization by confocal microscopy in a dark room, capturing images from two mice groups for neuron counting.

4. Results

4.1. 2Post-injection site evaluation

In KCNQ study, the injection site, and the regions of mCherry expression were evaluated post hoc for mice that underwent a viral injection. Firstly, Mice were transcardially perfused with 4% paraformaldehyde, and vibratome (Campden, Loughborough, UK) that used to cut 80-µm slices, then postfixed for 24 hours. Slices were scanned with a confocal microscope (Zeiss LSM 700 Live; Carl Zeiss AG, Oberkochen, Germany) were mounted on a coverslip using a mounting media containing 4',6 diamidino-phenylindol. While, the injection target was the pedunculopontine nucleus (PPN), which located 4.26-4.96 mm caudal to the bregma. However, the mCherry expression in astrocytes extended beyond the PPN, including neighboring regions as well.

The dorsal one-third of the oral pontine reticular nucleus, the middle region of the mesencephalic reticular formation, the retrorubral field and nucleus, the precuneiform nucleus (PrCnF), the lateral and ventrolateral periaqueductal grey, the lateral side of the dorsal raphe nucleus, the medial paralemniscal nucleus, the microcellular tegmental nucleus, and the ventral portion of the cuneiform nucleus.

4.1.1. Two bottle preference test

During a preliminary experiment used a two-bottle preference experiment to evaluate mouse water consumption and the effects of CNO dissolving. No significant variation in consumption was observed while using tap water in both leakage-free bottles (left bottle: 15.9 ± 8.8 ml/3 days; right bottle: 24.6 ± 10.1 ml/3 days; n.s.). The daily water consumption was 6.75 ml. Based on the average weight of the first 4 mice, which is 26.96 ± 2.93 g, a CNO concentration of 4 µg/ml was calculated for a daily dosage of 1 mg per body kg. the comparison between one bottle containing tap water and another with 9.6 µM (4 µg/ml) CNO dihydrochloride, no significant difference in consumption was observed (tap water: 18.66 ± 3.84 ml/3 days; CNO: 20 ± 6.93 ml/3 days).

The CNO concentration in drinking water was first determined to achieve a 1 mg/kg consumption based on this available data. However, the average body weight of the further

16 mice was 25.7 ± 3.33 g; thus, the calculated daily CNO exposure was slightly higher as planned (1.04 mg/kg). Therefore, no preference or avoidance of CNO consumption was found. followed a similar strategy for the chronic administration of CNO in drinking water.

4.1.2. Activity wheel test

In the DREADD group, mice showed increased restlessness during the lit resting period, while the control group was unaffected. Control mice exhibited no significant changes in inactivity levels during darkness (23 ± 5.7% before vs. 30.8 ± 4.9% after CNO), light (80.7 ± 3.8% before vs. 77.5 ± 5% after CNO), or overall (52 ± 2.3% vs. 54 ± 3%). Their daily travel distances (darkness: 6215 ± 1602 m/day before vs. 5540 ± 1255 m/day after CNO; light: 117 ± 107 m/day before vs. 81.5 ± 72 m/day after CNO; total: 6347 ± 1607 m/day before vs. 5522 ± 1228 m/day after CNO) and maximal running speeds (darkness: 25.6 ± 1.66 m/min before vs. 25.3 ± 3.1 m/min after CNO; light: 3.9 ± 2 m/min before vs. 4.3 ± 1.6 m/min after CNO) also remained unchanged. In contrast, the hM3D(Gq)-expressing DREADD group showed a significant decrease in resting time during the illuminated period (81.3 ± 1.4% before vs. 74.3 ± 2% after CNO; $p = 0.02$), though inactivity during darkness (24.1 ± 3% before vs. 31.3 ± 2% after CNO) and total activity (52.7 ± 1.6% vs. 52.8 ± 1.9%) were unaltered. Daily running distances in DREADD mice were also unchanged (darkness: 7713 ± 3076 m/day before vs. 5259 ± 369 m/day after CNO; light: 63.6 ± 21 m/day before vs. 169.1 ± 79.2 m/day after CNO; total: 7776.6 ± 3083 m/day before vs. 5428 ± 354 m/day after CNO). Notably, CNO significantly increased the maximal running speed in DREADD mice during the illuminated (resting) period (darkness: 24.7 ± 2.89 m/min before vs. 27.47 ± 2.05 m/min after CNO; light: 2.77 ± 1.61 m/min before vs. 8.45 ± 1.99 m/min after CNO).

4.1.3. Barnes maze test

In the Barnes maze task, it was examined how overstimulating astrocytes affects spatial memory and learning. They found no notable differences between the control group (expressing mCherry) and the DREADD group (expressing hM3D(Gq)). Before administering CNO, both groups showed similar spatial memory formation. Even after the 10th learning session, as well as following CNO treatment and a new round of 10 training

sessions, performance remained comparable between the two groups. Specifically: Before CNO, the control group traveled 1938.6 ± 706.2 cm, while the DREADD group covered 906 ± 189.3 cm by the end of training and after CNO administration, the control group moved 4978.7 ± 1409 cm, and the DREADD group moved 4463.6 ± 959 cm. After the second training series, the control group's distance was 1166.1 ± 240.3 cm, and the DREADD groups was 2136 ± 894 cm. These results suggest that astrocyte overstimulation did not significantly alter spatial memory or learning performance in this experiment.

4.1.4. Acoustic startle test

The acoustic startle reflex amplitude and its short-term habituation were examined. After normalizing the post-CNO responses to the pre-treatment condition, the DREADD group showed a significantly reduced first relative amplitude compared to the control group (first amplitude: control group with CNO treatment, 133 ± 23%; DREADD group with CNO treatment, 68 ± 12%; p = 0.012). Similarly, the mean normalized amplitude of the five simultaneously recorded startle responses was significantly decrease in the DREADD group than in the control group (average amplitude: control group with CNO treatment, 109.8 ± 8.5%; DREADD group with CNO treatment, 93.1 ± 19.4%; p = 0.006; Fig. 27C). In the control (mCherry) group, the first startle amplitude was 14.6 ± 2.7 mN under baseline conditions and increased to 18.2 ± 3.3 mN after CNO administration. In the DREADD group, the mean first amplitude was 26.2 ± 3.3 mN before decreasing to 16.5 ± 2.9 mN following CNO treatment (p = 0.021). However, subsequent amplitudes remained unchanged. In summary, chronic astrocytic stimulation reduced the initial acoustic startle reflex but did not significantly alter short-term habituation.

Interestingly, in the KCNQ4 knockout experiment the strength of their startle response was larger for all five stimuli tested. The difference was statistically significant for the first, fourth, and fifth stimuli (wild type: 1st: 23.22 ± 3.74, 2nd: 22.22 ± 6.47, 3rd: 21.86 ± 4.23, 4th: 18.3 ± 6.13, 5th: 12.52 ± 4.14 mN; knockout: 1st: 49.96 ± 7.15, 2nd: 42.02 ± 8.65, 3rd: 42.62 ± 9.26, 4th: 41.15 ± 7.75, 5th: 30.99 ± 6.19 mN; 1st: P = 0.007; 4th: P = 0.035; 5th: P = 0.028). The average startle response across all five stimuli was also significantly different between

the two groups (wild type: 19. 76 ± 2.14 mN; knockout: 41.27 ± 4.97 mN; P = 0.0025. The startle response during the repeated stimuli did not decrease over time in either the normal or the knockout mice (startle strength compared to the first response was: wild type: 2nd: 1.04 ± 0.24, 3rd: 1.12 ± 0.29, 4th: 0.954 ± 0.37, 5th: 0.84 ± 0.29; knockout: 2nd: 0.86 ± 0.09, 3rd: 1 ± 0.27, 4th: 1.03 ± 0.27, 5th: 0.84 ± 0.29; averages: wild type: 1.02 ± 0.18, knockout: 0.95 ± 0.14.

We investigated startle network hyperexcitability using two mouse groups: five DREADD mice with pAAV-GFAP-hM3D(Gq)-mCherry (AAV5) in the caudal pontine reticular nucleus (PnC) and its projecting areas (including ventral cochlear, principal sensory trigeminal, superior paraolivary, lateral and medial superior olive, laterodorsal tegmental, dorsal raphe, central inferior colliculus, reticulotegmental, latero- and medioventral pons, dorsal, intermediate, and ventral lateral lemniscus, and pedunculopontine nuclei, and five control mice with only the mCherry tag in the same regions. We measured startle reflex strength after saline or CNO injection. In mCherry-only mice, CNO caused no significant change in startle reflex strength (saline average: 18.47 ± 3.09 mN; CNO average: 25.59 ± 4.85 mN). However, hM3D(Gq)-expressing mice showed a significant increase in the first startle response after CNO (saline 1st: 14.85 ± 3.98 mN; CNO 1st: 42.05 ± 9.26 mN; p = 0.009), with an overall CNO average of 28.08 ± 7.83 mN (compared to saline average of 18.19 ± 3.13 mN). Comparing CNO treatment between groups, hM3D-expressing mice had a significantly stronger first startle response than mCherry-only mice. Furthermore, hM3D(Gq) mice treated with CNO exhibited rapid weakening of subsequent startle responses after the initial significant jump (1st: 5.17 ± 1.67; average: 1.82 ± 0.4), unlike the control mice (average: 1.55 ± 0.24). The initial startle strengths between mCherry-only and hM3D mice were also significantly different.

4.1.5. Gait alterations

In this experiment, we examined gait parameters such as stride length, sway length, and stance length. The control group, which expressed mCherry, showed no changes in these measurements after CNO administration compared to baseline (front limb: stride length—

5.39 ± 0.3 cm before vs. 5.27 ± 0.4 cm after; sway length—1.84 ± 0.19 cm before vs. 1.53 ± 0.15 cm after; stance length—3.67 ± 0.18 cm before vs. 3.99 ± 0.4 cm after; hind limb: stride length—5.27 ± 0.35 cm before vs. 5.33 ± 0.46 cm after; sway length—2.41 ± 0.15 cm before vs. 2.39 ± 0.17 cm after; stance length—3.61 ± 0.13 cm before vs. 3.83 ± 0.27 cm after). However, in the DREADD group—where mesopontine astrocytes expressed hM3D(Gq)—all measured parameters increased significantly after CNO treatment (front limb: stride length—4.3 ± 0.22 cm before vs. 5.88 ± 0.25 cm after, $p = 0.000116$; sway length—1.37 ± 0.1 cm before vs. 2.03 ± 0.18 cm after, $p = 0.0028$; stance length—3.13 ± 0.21 cm before vs. 3.86 ± 0.13 cm after, $p = 0.0053$; hind limb: stride length—4.4 ± 0.18 cm before vs. 6.22 ± 0.16 cm after, $p < 0.00001$; sway length—2.33 ± 0.12 cm before vs. 3.26 ± 0.18 cm after, $p = 0.00039$; stance length—3.39 ± 0.12 cm before vs. 5.13 ± 0.19 cm after, $p < 0.00001$).

4.2. Histological analysis

To assess the injection site and affected brain regions, we quantified cholinergic neurons, non-cholinergic neurons, and astrocytes using immunohistochemical markers. Cholinergic neurons were identified by ChAT immunopositivity combined with NeuN labeling, while non-cholinergic neurons showed NeuN positivity without ChAT expression. Astrocytes were defined by mCherry expression. Cell counts were performed bilaterally at the injection site within a standardized 0.43 × 0.43 mm square centered on the PPN. The analysis revealed comparable anatomical locations between groups: the counting region measured 4.5 ± 0.09 mm caudal to bregma in control mice (expressing only mCherry in astrocytes) versus 4.54 ± 0.08 mm in DREADD mice (co-expressing mCherry and chemogenetic actuators), with no statistically significant difference in positioning. This methodological consistency confirms our experimental groups were evaluated under equivalent neuroanatomical conditions.

Following chronic CNO administration, quantitative analysis revealed distinct cellular changes between groups. Control animals exhibited 58.62 ± 6.2 cholinergic neurons, 512.89 ± 28.72 non-cholinergic neurons, and 70.61 ± 10.02 astrocytes per mm^2. In contrast, the DREADD group showed significantly reduced numbers: 31.89 ± 7.03 cholinergic neurons (54.4% of controls, $p = 0.0054$) and 389.06 ± 31.9 non-cholinergic neurons (75.8% of

controls, $p = 0.0049$). Astrocyte counts in DREADD mice ($78.19 \pm 12.17/mm^2$) represented 110.7% of control values ($p = 0.32$), demonstrating no statistically significant change. These findings indicate that prolonged astrocytic activation preferentially affects cholinergic populations while sparing glial cells.

4.3. Statistics

The mean ± SEM has been applied for all data. Normality tests were used to evaluate the datasets normal distribution, and the student's t-test was used to determine statistical significance (the level of significance: $p < 0.05$).

5. Discussion

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5.1. Actions of astrocytic activation on the PPN

In earlier studies using in vitro slice electrophysiology and imaging, we identified an astrocyte-mediated mechanism shared across multiple neuromodulatory systems—including cholinergic, cannabinoid, serotonergic, and partly orexinergic pathways. We observed that astrocyte activation—whether triggered by neuromodulators or optogenetics—led to distinct neuronal responses: one subgroup of neurons hyperpolarized and reduced firing via group I mGluRs, while another subgroup depolarized and increased firing via group II mGluRs. A third group showed no response to astrocyte stimulation.

Another regulatory mechanism involved extrasynaptic GluN2B-containing NMDARs. When "slow inward currents" (SICs) had a low baseline frequency, neuromodulators consistently increased their occurrence. Conversely, if SICs were initially frequent, the same neuromodulators suppressed them. This effect resulted from NMDAR activation followed by desensitization and subsequent reactivation. While these findings were obtained in vitro, their potential physiological or pathological relevance remained unclear at the time. To explore this issue further, we carried out in vivo behavioral experiments to assess how artificial astrocyte overactivation might influence functions linked to the mesencephalic locomotor region (MLR) and nearby brain areas.

Previous studies using chemogenetic activation of astrocytes in different brain regions have shown varied behavioral effects. For example, acute Gq-DREADD stimulation of astrocytes in the hippocampus and medial prefrontal cortex enhanced memory and learning. Similarly, acute astrocyte activation in the nucleus accumbens altered reward processing and addictive behaviors, promoted goal-directed over habitual actions in the striatum, and reduced fear responses in the amygdala. Additionally, acute chemogenetic stimulation of pontine astrocytes was found to suppress REM sleep and decrease the number of REM time.

Chronic chemogenetic activation of hippocampal astrocytes—applied over periods ranging from 7 days to lifelong—produced effects that differed from neuronal activation, influencing fear memory differently. The outcomes on excitotoxicity were inconsistent: in some cases, it triggered astrocyte morphological changes and a neuroinflammatory state linked to cognitive impairment, while in others, it counteracted kainate-induced metabolic hyperactivity, protecting against excitotoxic damage and memory decline.

Progressive supranuclear palsy (PSP) is a neurodegenerative disorder with motor symptoms like Parkinson's disease. It involves gait and postural disturbances, along with cognitive and behavioral impairments such as dementia, REM sleep-related motor abnormalities, and the loss of the acoustic startle reflex. The disease is marked by the accumulation of tau protein in neurons, astrocytes, and oligodendrocytes. In early stages, tau pathology primarily affects brainstem and subcortical neurons, later spreading to cortical regions as the disease progresses.

5.2. Alterations of the acoustic startle reflex

The startle reflex changes relate to cochlear damage from aging, noise, or drugs. Minimal damage increases it, severe damage reduced this reflex. KCNQ4 knockout mice and DFNA2 patients have 60 dB progressive hearing loss due to outer (and lesser inner) hair cell degeneration These specific channels are noteworthy because they're activated by membrane potentials that are even more negative than the threshold required for an action potential. Additionally, neural KCNQ channels are crucial for epilepsy treatments and hold promise as therapeutic targets for various neurological conditions, including chronic and neuropathic pain, deafness, and mental disorders. The KCNQ channels are a family of voltage-gated potassium channels that play crucial roles in regulating cellular excitability across various tissues, including the nervous system, heart, and epithelial cells. They are often referred to as M-channels due to their inhibition by muscarinic acetylcholine receptor activation. Potassium channels activated by changes in voltage, known as Kv channels, are critical for controlling how excitable cells function. Problems with these channels can lead to various health issues like irregular heartbeats, seizures, and autism. The Kv7 family (Kv7.1-Kv7.5, also called KCNQ, from their respective genes KCNQ1-5), is a diverse group within Kv channels. which find them throughout excitable cells, where they help set the cell's baseline electrical charge, influence the shape and length of electrical impulses, and manage rhythmic cellular activities. Neuronal hyperexcitability in the auditory system or impaired glycinergic inhibition also increases startle amplitude. Previous studies suggested KCNQ4's role in startle, supported by its overlap with brainstem startle structures and presence in the pedunculopontine nucleus. Besides cochlear damage, KCNQ4 loss causes brainstem nuclei hyperexcitability. A chemogenetic model mimicking brainstem hyperexcitability (without cochlear damage) showed similar increased initial startle but stronger short-term habituation as KCNQ4 knockout animals.

Differences might be due to cochlear damage in knockout animals and more affected nuclei. CNO itself might also slightly inhibit startle. KCNQ4 deletion was long considered 'nonsyndromic' hearing loss, but elevated mechanosensitivity, and altered circadian rhythm adaptation. The exaggerated acoustic startle in KCNQ4-deleted animals, resulting from both cochlear damage and brainstem hyperexcitability.

5.3. Memory and gait alterations

The excitotoxic damage to the PPN (primarily affecting non-cholinergic neurons) has been shown to cause learning impairment and attention deficits. Similar cognitive disruptions, including memory and attention problems, are observed in progressive supranuclear palsy (PSP), while sleep and circadian rhythm disturbances may also contribute to memory dysfunction. Given these findings, we chose the Barnes maze test—which minimizes stress compared to fear conditioning or forced swim tests—to assess cognitive function. Our results revealed no differences between control and DREADD-treated groups, aligning with prior evidence that cholinergic neuron loss does not severely impair memory. Additionally, since the number of non-cholinergic neurons (which may influence cognitive performance) remained stable during our experiments, this further supports the absence of memory-related deficits in our model.

Analysis of footprint patterns revealed increases in all measured parameters, including stride, stance, and sway length for both front and hind paws. Previous studies have shown that loss of PPN cholinergic neurons leads to significant motor impairments, including rear paw slips and disrupted gait precision. Various neurological conditions alter stride and sway length—for example, hyperekplexia (characterized by heightened muscle tone) and Alzheimer's disease models both exhibit shorter strides and narrower step widths, with the latter also showing reduced walking speed. In contrast, progressive supranuclear palsy (PSP) presents with shorter strides but wider step widths. Our findings, which show an overall increase in footprint measures, do not align with these disease patterns. One possible explanation is that stride length correlates directly with movement speed, and our activity wheel tests confirmed faster locomotion. Thus, the observed changes may reflect a mix of PSP-like features (such as increased sway length) and the effects of heightened walking speed (leading to longer strides).

5.4. Alterations of neuronal numbers as the background of the behavioral changes

Previous research showed that excitotoxic damage to the posterior pedunculopontine nucleus (PPN) caused

learning impairments and changes in locomotor behavior. Later studies revealed that these effects were due to alterations in non-cholinergic neurons, since selective loss of cholinergic neurons did not produce the same deficits. Rodent models of brainstem-related PSP symptoms have been developed by targeting cholinergic brainstem regions. First, selective elimination of PPN cholinergic neurons replicated key PSP motor symptoms, including reduced acoustic startle response and movement impairments such as disrupted gait control. Second, introducing tau protein expression in the PPN induced tau pathology, leading to loss of cholinergic neurons in the PPN and dopaminergic cell death in the substantia nigra—producing symptoms consistent with the earlier findings.

Since tau pathology can trigger neuroinflammation and microglial activation—often alongside abnormal astrocyte reactivity—we addressed this gap by demonstrating that chronic astrocyte activation can induce symptoms similar to both PSP and PPN excitotoxicity. Chronic overactivation of astrocytes resulted in an almost 50% reduction in cholinergic neurons and a smaller but still significant decline in non-cholinergic neurons, while the number of astrocytes remained unchanged. This effect may be explained by chemogenetic astrocyte stimulation triggering glutamate release, which then induces tonic depolarization in PPN neurons expressing group II metabotropic glutamate receptors and phasic depolarization through extrasynaptic NMDA receptor activation.

However, since only some neurons have group I mGluRs—leading to hyperpolarization—and others show no tonic current response, not all neurons in the area are equally susceptible to excessive astrocyte activity. One of the key effects of chronic astrocytic activation in the MLR and surrounding areas was a reduction in the acoustic startle reflex, which is known to be regulated by the PPN. Optogenetic activation of cholinergic neurons increases this reflex, while non-cholinergic neurons likely help control it through prepulse inhibition. Supporting this, selective damage to PPN cholinergic neurons or tau protein buildup in these cells has been shown to reduce the startle reflex amplitude.

Chronic activation of midbrain astrocytes led to disruptions in circadian activity patterns. Mice showed increased movement during their normal rest period, along with faster peak movement speeds. These changes could stem from disturbances in sleep regulation. Sudies found that acute chemogenetic stimulation of pontine astrocytes reduces REM sleep and shortens REM episodes, and since our primary target was the

PPN—a key area controlling REM sleep and wakefulness—this may explain our observations. Another possibility is that excitotoxic effects from excessive astrocyte activation boosted locomotor activity.

Astrocytes have been shown to affect movement in disease states—for instance, acute chemogenetic activation of astrocytes using Gi-coupled DREADDs improved movement impairments in a Parkinson's mouse model. Interestingly, chronic chemogenetic stimulation of serotonergic neurons in the dorsal raphe produced circadian motor changes similar to our findings: increased activity during the light (resting) phase and reduced movement in the dark (active) phase. These parallels could stem from the dorsal raphe's known role in regulating activity cycles, or they might result from indirect raphe neuron activation (via PPN-mediated effects) or direct stimulation (since DREADD expression partially overlapped with the dorsal raphe).

5.5. Limitations of the study

While our study clearly shows that astrocyte overactivation leads to significant motor changes, several limitations should be considered when interpreting the results. First, although we targeted astrocyte activation primarily in the MLR (centered on the PPN), the mCherry labeling was not confined strictly to this region—adjacent areas also showed expression. However, the fact that we observed a sharp reduction in ChAT+ neurons without significant changes in NeuN labeling suggests that cholinergic neuron loss likely drove the behavioral effects. That said, functional alterations in neurons or astrocytes (independent of cell counts) could also play a role.

Second, CNO administration relied on voluntary drinking, guided by preliminary water intake measurements. While less precise than forced methods like daily intraperitoneal injections or CNO depot formulations, this approach minimized stress and avoided confounding behavioral effects from handling-related anxiety or abdominal discomfort. While our voluntary CNO administration protocol may have introduced some variability, we recognize that precise dosing could yield more consistent results with reduced standard error—potentially meaning our reported effects are conservative estimates. Similar oral CNO delivery methods have been successfully employed in prior studies.

A third consideration is CNO's known off-target effects in naive animals. At our working concentration (1 mg/kg), CNO has been shown to diminish acoustic startle responses and modify sleep architecture by

altering NREM-REM transition latencies. Higher doses (5-10 mg/kg) reportedly suppress amphetamine-induced hyperactivity and disrupt NREM/REM sleep cycles without affecting baseline locomotion. To mitigate these confounds, our experimental design rigorously controlled for CNO/clozapine effects by comparing DREADD-expressing mice with operated controls receiving identical CNO treatment but lacking DREADDs. Importantly, control animals showed no startle response reduction or significant gait/circadian alterations, supporting that our observed phenotypes stem from targeted astrocyte activation rather than pharmacological artifacts. we potentially underestimated the actions of PnC and startle network hyperexcitability on startle reflex as CNO decreases startle amplitude.

Although the animal's cages, environmental instructions, and experiment time were maintained to reduce variables, it is crucial to remember that behavioural tests are never error-free and that results might be influenced by a wide variety of potential uncontrollable factors. It's probable that DREADD expression is not limited to the PPN area and that other regions have been affected as well, even though AAV viral injection of DREADDs is the greatest option for achieving a more localised effect. To solve this issue as effectively as possible, stereotaxic equipment, post-hoc histological examination, and the elimination of mice with limited expression were used. As was previously established, the acoustic startle reflex can be affected by CNO alone, regardless of the expression of DREADD. Comparing both the DREADD and control group before and after CNO administration can help differentiate the actual effect. Importantly, although the most probable cause behind cholinergic neuron loss in the background of the behavioral findings is an excitotoxic lesion related to gliotransmitters released from overstimulated astrocytes, an alternative explanation is cholinergic neuronal degradation due to loss of physical support from astrocytes.

5.6. Conclusions and clinical implications

This study successfully addressed its primary aims, providing critical insights into the functional role of astrocytes in the PPN and developing animal model for PSP.

1. Regarding to Astrocytic overstimulation within the PPN using chemogenetic tools to precisely investigate the functional role of astrocytes within the PPN. This aim was effectively achieved. The research employed a chemogenetic approach utilizing the hM3D(Gq) system to selectively overstimulate astrocytes within the PPN. The findings demonstrated that prolonged astrocyte overactivation in the PPN, likely through

excitotoxic mechanisms, lead to a reduction in cholinergic neuron numbers. Beyond cell loss, functional changes in neuronal activity were observed, contributing to behavioral shifts. Specifically, the study proposed that altered mGluR- and NMDAR-dependent signaling in mesopontine circuits triggered disruptions in circadian movement patterns, sensory processing, and muscle tone changes. This precise targeting of PPN astrocytes, as intended, allowed for the investigation of their contribution to physiological processes, revealing their previously underappreciated role in PSP progression.

2. To create an animal model of PSP, that causes damage to the PPN. This aim was successfully accomplished. our study developed an animal model of PSP by inducing astrocyte overactivation in the PPN. Specifically, this model demonstrated that astrocyte activation alone can reproduce comparable deficits to those seen in PSP, including cholinergic neuron loss and behavioral changes resembling brainstem-related symptoms of PSP (e.g., disruptions in movement, sensory processing, and muscle tone). This contrasts with previous PSP models focusing solely on cholinergic neuron loss or beta-amyloid accumulation, thereby establishing beneficial tool for examining the pathophysiology of PSP and identifying potential treatments. The observed decrease in the startle reflex, a characteristic clinical feature of PSP, further validates this model.
3. Regarding to KCNQ4 study: the KCNQ4 study investigated the impact of KCNQ4 subunit loss on the startle reflex and brainstem hyperexcitability, its conclusion directly informed the understanding of the PPN's role in the observed deficits and the relevance to PSP.
4. In conclusion, this project effectively utilized chemogenetic tools to illuminate the significant role of PPN astrocytes in neurodegeneration and behavioral alterations relevant to PSP. By demonstrating that astrocyte activation alone can induce PSP-like deficits, the study not only achieved its stated aims but also opened new avenues for therapeutic intervention targeting astrocytes to potentially slow disease progression. In future research, should invistagate pharmacological agents like such as the GluN2B NMDA receptor antagonist memantine to target the affected neural pathways to assist in identifying the cause. If the cause is truly excitotoxic in nature due to excessive glutamate release from astrocytes, the use of memantine will ameliorate symptoms and decreases the reduction in neuronal populations.

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List of publications related to the dissertation

1. **Maamrah, B.**, Pocsai, K., Hoang, B. M., Abdelhadi, A., Al-Khafaji, M. Q. M., Csemer, A., Sokvári, C., Szentesi, P., Pál, B.: Chronic Chemogenetic Activation of Astrocytes in the Murine Mesopontine Region Leads to Disturbances in Circadian Activity and Movement.
   Int. J. Mol. Sci. 26 (10), 1-21, 2025.
   DOI: http://dx.doi.org/10.3390/ijms26104793
   IF: 4.9 (2023)
2. **Maamrah, B.**, Pocsai, K., Bayasgalan, T., Csemer, A., Pál, B.: KCNQ4 potassium channel subunit deletion leads to exaggerated acoustic startle reflex in mice.
   Neuroreport. 34 (4), 232-237, 2023.
   DOI: http://dx.doi.org/10.1097/WNR.0000000000001883
   IF: 1.6

List of other publications

3. Csemer, A., Sokvári, C., **Maamrah, B.**, Szabó, L., Korpás, K. L., Pocsai, K., Pál, B.: Pharmacological Activation of Piezo1 Channels Enhances Astrocyte-Neuron Communication via NMDA Receptors in the Murine Neocortex.
   Int. J. Mol. Sci. 25 (7), 3994, 2024.
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   IF: 4.9 (2023)

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4. Csemer, A., Kovács, A., **Maamrah, B.**, Pocsai, K., Korpás, K. L., Klekner, Á., Szűcs, P., Nánási, P. P., Pál, B.: Astrocyte- and NMDA receptor-dependent slow inward currents differently contribute to synaptic plasticity in an age-dependent manner in mouse and human neocortex. *Aging Cell. 22* (9), e13939, 2023.
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